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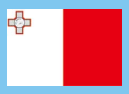

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FIELD OF VISION

Antidiabetic treatment, stroke severity and outcome

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Abstract

Ischemic stroke is a leading cause of mortality and long-term disability worldwide. Given the detrimental effects of acute stroke, several neuroprotective agents have been evaluated in these patients. However, the benefits of the evaluated agents appear to be limited and none is currently recommended for clinical use. On the other hand, prior treatment with agents that are used for the primary and secondary prevention of stroke, including statins and antiplatelets, has been associated with better outcome in patients who experience an acute stroke. In contrast, there are limited data as to whether prior treatment with antidiabetic agents is beneficial in diabetic patients who suffer a stroke. In this context, the findings of a recent study that showed reduced stroke size following pretreatment with linagliptin, a dipeptidyl peptidase-4 (DDP-4) inhibitor, compared with glimepiride, in both diabetic and non-diabetic mice, appear promising. Despite these preclinical findings suggesting neuroprotective effects of DPP-4 inhibitors in acute stroke, it is still unclear whether these actions will also be observed in humans. Of note, two recent large randomized, placebo-controlled studies did not show any effect of DPP-4 inhibitors on cardiovascular events, including stroke. Several other ongoing trials are evaluating the effects of DPP-4 inhibitors on cardiovascular morbidity and mortality. These studies also provide a major opportunity to assess whether patients treated with this class of antidiabetic agents will suffer from less severe strokes and whether their outcome after stroke will be more favorable.

Key words: Type 2 diabetes mellitus; Stroke; Dipeptidyl peptidase-4 inhibitors; Sulfonylureas; Neuroprotection

Core tip: A recent study showed reduced stroke size following pretreatment with linagliptin, a dipeptidyl peptidase-4 (DDP-4) inhibitor, compared with glimepiride, in both diabetic and non-diabetic mice. It remains to be shown whether these neuroprotective actions of DPP-4 inhibitors will also be observed in humans.

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INVITED COMMENTARY ON HOT ARTICLES

Ischemic stroke is a leading cause of mortality and long-term disability worldwide[1]. This often disabling and frequently fatal event puts a substantial burden on the family members and medical professionals who care for stroke victims[1].

The increasing prevalence of obesity results in an increased incidence of type 2 diabetes mellitus (T2DM) worldwide[2]. T2DM is a major risk factor for cardiovascular events, including stroke[3,4]. In addition, patients with T2DM appear to suffer more severe strokes and have a worse outcome than subjects without T2DM[3,5-7]. The increased incidence of cardiovascular events in patients with T2DM is not only due to hyperglycemia, but insulin resistance, low-grade inflammation and activation of the

Table 1 Major studies that have evaluated the effects of antidiabetic agents on stroke severity and outcome

Ref.	Design	*n*	Agent	Results
Weih *et al*[14]	Retrospective	146	Sulfonylureas	No effect on stroke severity or outcome
Kunte *et al*[15]	Retrospective	61	Sulfonylureas	Better neurological and functional outcome at discharge in patients who were on sulfonylureas prior to stroke
Favilla *et al*[16]	Prospective	1050	Sulfonylureas, metformin, insulin	Less severe stroke on admission in patients who were on sulfonylureas, metformin or insulin prior to stroke than in patients who were not receiving any antidiabetic agent, but no difference in functional outcome and mortality rates at 90 d between the 2 groups Similar stroke severity and outcome between patients treated with different antidiabetic agents prior to stroke (sulfonylureas, metformin and insulin)
Lee *et al*[17]	Case-control	60	Thiazolidinediones	Enhanced functional recovery in patients treated with thiazolidinediones

coagulation cascade are also involved[3,8].

Given the high morbidity and mortality rates associated with acute ischemic stroke, several neuroprotective agents have been evaluated in these patients[9]. However, the benefits of the evaluated agents appear to be limited and none is currently recommended for clinical use[9]. On the other hand, prior treatment with agents that are used for the primary and secondary prevention of stroke, including statins and antiplatelets, has been associated with less severe stroke, better functional outcome and reduced mortality in patients who experience an acute stroke[10-13]. In contrast, there are limited data whether prior treatment with antidiabetic agents is beneficial in diabetic patients who suffer a stroke. In an early study, prior treatment with sulfonylureas had no effect on stroke severity or outcome[14]. In contrast, a more recent study suggested that patients who were on sulfonylureas prior to stroke and continued to receive these agents during hospitalization were more likely to have a better neurological and functional outcome at discharge[15]. In another study, diabetic patients who were on sulfonylureas, metformin or insulin prior to stroke had a less severe stroke on admission than patients who were not receiving any antidiabetic agent. In contrast, functional outcome and mortality rates at 90 d after stroke were similar in patients who were on glucose-lowering treatment and in those who were not[16]. Stroke severity and outcome did not differ between patients who were on sulfonylureas, metformin or insulin prior to stroke[16]. A small retrospective study also suggested that thiazolidinediones enhance functional recovery in patients with stroke[17] (Table 1).

In this context, the findings of a recent study that compared the effects of pretreatment with glimepiride, a sulfonylurea, and linagliptin, a dipeptidyl peptidase-4 (DDP-4) inhibitor, on the outcome of stroke in diabetic and non-diabetic mice, appear promising[18]. It has been previously reported that administration of sulfonylureas after stroke reduces infarct size and mortality, primarily by preventing cerebral edema[19,20]. In this study, 44 male C57BL mice were divided into 2 groups. The first group (n = 21) was exposed to a high-fat diet for 32 wk, which resulted in substantial weight gain and development of insulin resistance and hyperglycemia[18]. At week 25, this group was assigned to oral administration of 10 mg/kg per body weight (bw) linagliptin daily, 2 mg/kg per body weight glimepiride daily or vehicle[18]. The second group (n = 23) was fed a normal diet and was also assigned to linagliptin, glimepiride or vehicle at the same doses with the first group[18]. After 4 wk of treatment, stroke was induced in all mice in both groups by transient occlusion of the middle cerebral artery[18]. Treatment with linagliptin, glimepiride or vehicle was continued for 3 wk following stroke, after which all mice in both groups were sacrificed[18]. The extent of ischemic stroke was assessed with measuring stroke volume and with stereological quantification of surviving neurons in the striatum/cortex[18].

In high-fat diet-fed mice, fed and fasting blood glucose levels decreased in both linagliptin- and glimepiride-treated mice[18]. This reduction was greater in mice treated with glimepiride. In contrast, in normal diet-fed mice, fed and fasting blood glucose levels decreased in glimepiride-treated animals but did not change in linagliptin-treated animals[18]. On the other hand, both high-fat- and normal diet-fed mice that were treated with linagliptin showed an increase in blood glucagon-like peptide-1 (GLP-1) levels due to a significant reduction in DPP-4 activity[18]. In contrast, GLP-1 levels and DPP-4 activity did not change in glimepiride- or vehicle-treated mice regardless of the diet they were fed[18].

Immunohistochemical staining of the cortex/striatum of high-fat diet-fed mice without stroke revealed GLP-1 receptor expression exclusively in the neurons[18]. Cortical pyramidal neurons showed the most pronounced expression of GLP-1 receptors[18].

In high-fat diet-fed mice, treatment with linagliptin resulted in a noticeable, albeit not statistically significant, trend towards reduction of stroke volume[18]. In contrast, glimepiride had no effect on stroke volume[18]. Moreover, stereological counting of surviving neurons revealed significantly more (approximately 30%) surviving neurons in linagliptin-treated mice than in either glimepiride- or vehicle-treated animals[18]. In contrast, in normal diet-fed mice, treatment with both linagliptin and glimepiride resulted in a comparable and non-significant trend for reduced stroke volume and was associated with a comparable and significantly higher number of surviving neurons compared with vehicle treatment[18].

Overall, this study[18] suggests that treatment with linagliptin prior to stroke increases the number of surviving neurons more than glimepiride in diabetic mice. This

neuroprotective effect of linagliptin appears to be glucose-lowering-independent since the reduction in blood glucose levels was smaller during treatment with linagliptin compared with glimepiride. In addition, linagliptin also prevented neuronal death in non-diabetic mice even although it did not affect glucose levels, further supporting a glucose-lowering-independent neuroprotective effect. Similar results have been reported very recently with another DPP-4 inhibitor, alogliptin[21]. Moreover, in humans, even although increased glucose levels at admission are associated with a worse outcome in patients with acute ischemic stroke[22-24], correction of hyperglycemia with administration of insulin does not reduce infarct size or neurological deficit[25-27].

Several alternative mechanisms besides glucose lowering may underpin the beneficial effects of linagliptin in the setting of acute stroke. First, treatment with linagliptin results in increased blood GLP-1 levels and pretreatment with exendin-4, a GLP-1 agonist, was shown to reduce stroke volume and neurological deficit in animal stroke models[28-30]. Antiapoptotic, anti-inflammatory and antioxidant actions, as well as stimulation of the proliferation of neural stem cells and attenuation of microglial activation, appear to contribute to these neuroprotective effects[29-31]. Interestingly, administration of exendin-4 in non-diabetic animals immediately after stroke also reduces stroke volume and improves outcome through similar mechanisms without affecting glucose levels[32]. These effects appear to be GLP-1 receptor-mediated since they are not observed in GLP-1 receptor knockout (-/-) mice[28]. Moreover, GLP-1 readily crosses the blood-brain barrier[33-35] and GLP-1 receptors are expressed in brain neurons in humans[36-39]. In addition, both ischemia and treatment with exendin-4 up-regulate the expression of GLP-1 receptors in pyramidal neurons[29]. Given the putative neuroprotective effects of GLP-1, this increased expression might be a defense mechanism against ischemic damage[29].

A second possible pathway through which linagliptin might exert its neuroprotective effects is the increased bioavailability of other bioactive DPP-4 substrates. Indeed, DPP-4 has many other substrates except GLP-1, some of which appear to exert neurotrophic or neuroprotective effects[40,41]. The latter include glucose-dependent insulinotropic polypeptide[42], pituitary adenylate cyclase-activating polypeptide[43] and stromal cell-derived factor 1a[44], which were reported in preclinical models to promote synaptic plasticity, neurogenesis and neuronal differentiation, to inhibit apoptosis and to reduce stroke size.

Another possible explanation of the different effects of linagliptin and glimepiride on stroke volume is that glimepiride exerts detrimental effects rather than that linagliptin is protective. Indeed, several recent studies suggested that patients treated with sulfonylureas have increased cardiovascular morbidity compared with patients treated with metformin[45-47]. Therefore, it would be of interest to compare the effects of prior treatment of DPP-4 inhibitors with prior treatment with metformin in experimental models of stroke or in patients who suffer a stroke.

Despite these promising preclinical findings suggesting neuroprotective effects of DPP-4 inhibitors in acute stroke, it is still unclear whether these actions will also be observed in humans. Interestingly, a recent randomized double-blind study showed that the addition of linagliptin to metformin reduces the risk of non-fatal stroke more than the addition of glimepiride, despite comparable decreases in HbA_{1c}[48]. Preliminary data also suggest similar reductions in stroke risk with other DPP-4 inhibitors[49]. However, these studies were neither planned nor powered to assess the effects of DPP-4 inhibitors on cardiovascular events[48,49]. On the other hand, two recent large randomized, placebo-controlled studies did not show any benefit of DPP-4 inhibitors on cardiovascular events, including stroke[50,51]. Several other ongoing trials are evaluating the effects of DPP-4 inhibitors on cardiovascular morbidity and mortality. These studies also provide a major opportunity to assess whether patients treated with this class of antidiabetic agents will suffer from less severe strokes and whether their outcome after stroke will be more favorable.

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TOPIC HIGHLIGHT

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New insights into insulin: The anti-inflammatory effect and its clinical relevance

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Abstract

Hyperglycemia, a commonly exhibited metabolic disorder in critically ill patients, activates the body's inflammatory defense mechanism, causing the waterfall release of numerous inflammatory mediators and cytokines, and eventually leads to organ damage. As the only glucose-lowering hormone in the body, insulin not only alleviates the detrimental effects of hyperglycemia through its metabolic regulation, but also directly modulates inflammatory mediators and acts upon immune cells to enhance immunocompetence. In this sense, hyperglycemia is pro-inflammatory whereas insulin is anti-inflammatory. Therefore, during the past 50 years, insulin has not only been used in the treatment of diabetes, but has also been put into practical use in dealing with cardiovascular diseases and critical illnesses. This review summarizes the recent advances regarding the anti-inflammatory effects of insulin in both basic research and clinical trials, with the hope of aiding in the design of further experimental research and promoting effective insulin administration in clinical practice.

Key words: Insulin; Inflammation; Hyperglycemia

Core tip: Hyperglycemia is closely correlated with poor outcomes of morbidity and mortality in critically ill patients. As the only glucose-lowering hormone in the body, insulin not only alleviates the detrimental effects of hyperglycemia through its metabolic regulation, but also directly modulates inflammatory mediators and acts upon immune cells to enhance immunocompetence. This review summarizes the recent advances regarding the anti-inflammatory effects of insulin from our laboratory as well as others, in the hope of leading to a better understanding of this old, classic and wonder hormone and its wider and effective applications in clinical practice.

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INTRODUCTION

Since its discovery in 1921, the importance of insulin in glucose homeostasis has been established, and it is universally used as a therapeutic agent for diabetes mellitus. Thousands of lives have been saved and many scientists were drawn into the study of this wonder drug. Under continuous intensive research, the mechanisms underlying the effect of insulin in its metabolic modulation, mainly glucose homeostasis, has become clearer, but there remains much interest in the elucidation of further

effects of insulin.

Glucose-insulin-potassium (GIK) has been used as an adjunctive therapy in patients with acute myocardial infarction (AMI) since its introduction in 1962. However, the mechanism underlying GIK's cardioprotection has remained largely speculative and controversial during the past 50 years. It was not until early in this century that we provided convincing *in vivo* evidence that insulin, rather than glucose or potassium, is the predominant protective component of GIK, and demonstrated for the first time that insulin exerted anti-apoptotic and pro-survival effects in the ischemic/reperfused (I/R) myocardium through the PI3K-Akt-eNOS-NO signaling pathway[1]. This prompted us to conceive the notion of the "survival signal", a new mechanism of cell protection which is totally independent of the metabolic effects of insulin, and explained its cardioprotective effects. In 2001, the classical landmark clinical trial by van den Berghe[2] revealed that maintaining blood glucose at or below 110 mg/dL with low-dose insulin infusion, significantly reduced mortality and morbidity resulting from multi-organ failure among critically ill patients in the surgical intensive care unit (ICU). A further study reported that markers of inflammation, such as intercellular cell adhesion molecular-1 (ICAM-1) and E-selectin were suppressed in the liver of these patients as was inducible NO synthase (iNOS) expression, which is mainly in monocyte/macrophage cells[3], suggesting an anti-inflammatory role for insulin. This article will summarize the relationship between insulin, glucose and inflammation, and discuss the implications for the management of patients with AMI and critical illness.

GLUCOSE, OXIDATIVE STRESS AND INFLAMMATION

Hyperglycemia is common in critical illness, and may lead to severe complications. It has been reported that pronounced hyperglycemia is associated with poor outcomes of morbidity and mortality in patients with AMI, stroke and coronary artery bypass grafting[4-6]. Glucose is pro-inflammatory, and hyperglycemia is even detrimental to these patients. A total of 75 g glucose intake causes acute oxidative and inflammatory stress, as reflected in increased superoxide radical O_2^- generation by polymorphonuclear leukocytes, mononuclear cells and the enzyme nicotinamide adenine dinucleotide phosphate[7]. Free radical O_2^- generation, on the one hand, reduces NO bioavailability, as it combines with NO to form peroxynitrite $ONOO^-$; on other hand, it activates a number of redox-sensitive major pro-inflammatory transcription factors such as nuclear factor kappa B (NFκB), activator protein-1 (AP-1), hypoxia induced factor-α (HIF-α) and early growth response-1 (Egr-1), leading to increased transcription of the pro-inflammatory genes and thus inflammation[8-10]. Meanwhile, glucose increases the expression of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in mononuclear cells. Moreover, it has led to increased TNF-α and IL-6 concentrations in plasma in a steady state of hyperglycemia with intravenous insulin secretion with somatostatin[11]. To sum up, glucose, oxidative stress and inflammation are inter-related, with reciprocal causation. As the only glucose-lowering hormone in the body, insulin therapy alleviates the detrimental effects of hyperglycemia through metabolic regulation, therefore hyperglycemia is pro-inflammatory whereas insulin is anti-inflammatory.

INSULIN MODULATES INFLAMMATORY MEDIATORS

The discovery of the anti-inflammatory effect of insulin can be traced back to the observation that insulin exerts a vasodilatory effect through endothelial NO release in arteries, veins and capillaries[12,13]. By inducing vasodilatation, it reduces leukocyte adhesion to the endothelium and subsequent infiltration. Furthermore, it has inhibitory effects on platelet adhesion and aggregation.

Studies have further confirmed that insulin suppressed three important inflammatory mediators: intercellular cell adhesion molecular-1 (ICAM-1), MCP-1 expression and NFκB binding in human aortic endothelial cells *in vitro*[14,15]. These suppressive effects can be blocked by the NOS inhibitor N(G)-nitro-L-arginine, indicating the effects are mediated by NO release. Among all the pro-inflammatory cytokines, TNF-α is the most active one in triggering the production of other cytokines such as IL-6 and other expression molecules[16]. We provided direct evidence in myocardial ischemia/reperfusion (I/R) rats that insulin inhibits TNF-α induction locally and systemically, and demonstrated for the first time that *in vitro* treatment with insulin attenuated I/R-induced TNF-α production in cardiomyocytes *via* the Akt-eNOS-NO signaling pathway[17]. Polymorphonuclear neutrophils (PMN) are the first defense line against infection and invasive microorganisms. Adherence of PMN to endothelial cells is an early requisite event in I/R-induced inflammatory injury. Thus we performed *in vivo* and *in vitro* experiments in a rabbit model to investigate whether insulin inhibits PMN-mediated adherence[18]. It was found that insulin reduced P-selectin and ICAM-1 expression in endothelium which mediates the initial interaction between PMNs and the endothelial cell surface, thus insulin attenuated PMN adherence and I/R-induced inflammatory injury. The Akt-eNOS-NO signaling pathway was involved in these effects. Moreover, insulin has been reported to ameliorate the endotoxin-induced systemic inflammatory response by decreasing IL-6, TNF-α expression and increasing the anti-inflammatory cascade in the context of normoglycemia in rat[19] and porcine models[20]. All these data indicate that insulin alleviates inflammation through suppression of pro-inflammatory cytokines and immune mediators, pointing strongly to its role as an anti-inflammatory agent.

INSULIN SUPPRESSES TOLL-LIKE RECEPTOR EXPRESSION

Toll-like receptors (TLRs) are a variety of conserved pattern recognition receptors that have been implicated in innate immune responses. Accumulating evidence suggests that TRLs play an essential role in tissue inflammation and damage such as cardiac I/R, post-ischemic remodeling and atherosclerosis[21-23]. TLR signaling and its critical roles in inflammatory cardiac conditions has been intensively studied, especially TRL2, TRL4' role with myocardial infarction and reperfusion injury. TRL2 aggravated myocardial tissue injury in I/R-based experimental animal models and its deletion was associated with a smaller MI size compared with control[24]. The TLR-deficient model, TLR2$^{-/-}$ mice, exhibited improved left ventricular dysfunction following I/R[25]. Besides, administration of anti-TLR2 antibody prior to reperfusion reduced MI sites and preserved cardiac function. TLR4 is the specific receptor of endotoxin, thus it mediates inflammatory changes induced by endotoxins. Oyama *et al*[26] first demonstrated that TLR4-deficient mice had more than 50% reduction in MI area, which was associated with attenuated myocardial inflammation, as evidenced by less neutrophil infiltration and fewer lipid peroxides. Inhibited by eritoran, a specific TLR4 antagonist, resulted in a 40% reduction in MI and decrease in TNF-α, IL-1β, IL-6 and MCP-1 expression[27,28]. Moreover, TRL4 has been found to act as a determinant of neutrophil infiltration after global MI through mediating KC and MCP-1 expression[29]. Suppression of TRL signaling is associated with smaller MI size and is beneficial in I/R-based animal models. It has been reported that insulin infusion (2 U/h) with type 2 diabetes (T2D) patients within 2 h has significantly suppressed TLR1, -2, -4, -7 and -9 mRNA expressions in MNCs, and this prompt suppression may be mediated by the suppression of PU.1 binding and subsequent activation of TLRs[30]. Thus, insulin suppresses the expression of several TLRs at the transcriptional level and alleviates TRL-mediated inflammatory injury.

INSULIN ACTS UPON IMMUNE CELLS

Peripheral blood mononuclear cells (PBMCs) is a critical component in the immune system, and mainly comprised of lymphocytes and monocytes. Investigations have been conducted to study the effects of insulin upon mononuclear cells in obese non-diabetic subjects[31]. The results showed that insulin reduced activation of the pro-inflammatory transcription factor NFκB, with downregulation of plasma soluble intercellular adhesion molecular-1, which facilitates the attachment of monocytes to endothelial cells and chemotactic factor MCP-1, which encourages monocyte migration into the subintimal space. This suppressive effect on NFκB in PBMC has also been reported in critically ill patients with intensive insulin therapy[32]. Similarly, Egr-1, another important pro-inflammatory transcription factor, was notably reduced in mononuclear cells with insulin treatment, resulting in decreased plasma concentrations of tissue factor and plasminogen activator inhibitor-1 (PAI-1)[33]. Taken together, insulin suppresses pro-inflammatory transcription factors in mononuclear cells and the subsequent inflammatory mediators regulated by them, thus ameliorating MNC-mediated inflammation.

Monocytes/macrophages (Mo/Mϕ) initiate immune and inflammatory responses. Insulin administration (10^{-7} mmol/L) retarded macrophage apoptosis and enhances BclxL mRNA expression by activating phosphatidylinositol 3'-kinase (PI3K) in a dose-dependent manner, thus improving macrophage survival[34]. Use of wortmannin, a specific inhibitor of PI-3K, has further confirmed its position in the anti-apoptotic effect of insulin in lipopolysaccharide-challenged THP-1 cells[35]. HLA-DR is a cluster of membrane molecules of Mo/Mϕ which are involved in the Mo antigen presentation to T cells. The intensity of HLA-DR expression is associated with immunocompetency of Mo/Mϕ[36]. Insulin treatment with blood glucose maintained between 4.4-6.1 mmol/L increased HLA-DR expressions of peripheral Mo cells. This upregulation means enhanced antigen presentation of Mo cells, indicative of improved immune function. Moreover, the phagocytosis, chemotaxis, and oxidative burst capacity of Mo have also been assessed in a burn-injured rabbit model, suggesting that insulin improved the capacity for phagocytosis and oxidative burst, but had no effect on chemotaxis[37].

T cell differentiation is important in the immune response. A single naïve T cell under cell differentiation is able to generate multiple subsets of memory T cells with different phenotypic and functional properties in response to infections, resulting in acquisition of immune functions required for pathogen clearance. Insulin was first confirmed to induce a shift in Th cell differentiation toward Th2 cells which is involved in secretion of inflammatory mediators (IL-4, IL-10, IL-13, *etc.*) and enhanced antibody-mediated responses[38]. Myocarditis is a severe disease of myocardial inflammation and often results from an autoimmune reaction. Significant T cell reduction was observed in cardiac myocarditis[39]. Thus, we investigated the effect of insulin on myocardial inflammation in experimental myocarditis in mice and its potential role in T cell regulation. The results showed that insulin promoted T cell recovery, particularly $CD3^+$ T cells without changing the naïve-to-memory T-cell ratio and had a direct effect on T cell proliferation, thus alleviating myocarditis[40]. It is possible that insulin may promote T cell recovery in myocarditis, especially in diabetic or hyperglycemic patients.

ANTI-INFLAMMATORY EFFECTS OF INSULIN IN HUMAN STUDIES

Cardiovascular disease (CVD) is the leading cause of death worldwide, and remains a great challenge in healthcare. Various risk factors of CVD, including hy-

pertension, diabetes and smoking, can initiate a chronic inflammatory reaction. Accumulating epidemiological and clinical studies have found strong and consistent relationships between markers of inflammation and the risk of future cardiovascular events[41]. Thus, inflammation is established as a definitive cardiovascular risk factor.

Hyperglycemia is pro-inflammatory and damaging, especially in critically ill patients. Pronounced hyperglycemia at hospital admission is associated with poor outcomes of morbidity and mortality in patients with AMI, thus effective glucose management is a necessary therapeutic intervention. It has been shown in large pilot studies, Diabetes and Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI)[42] and the Estudios Cardiologicos Latinoamerica (ECLA) study[43], that small doses of intravenously delivered insulin markedly improved clinical outcomes in patients with AMI. There was a marked 29% reduction in 1-year mortality in the insulin-glucose infusion group in the 1995 DIGAMI study, and a statistically significant reduction in mortality and a consistent trend toward fewer in-hospital events in the GIK group in the 1998 ECLA pilot trial, possibly as a result of rigorous glycemic control. The anti-inflammatory effect of insulin have been applied clinically. Plasma C-reactive protein (CRP) and serum amyloid A (SAA) concentrations are the two accepted markers of systemic inflammation which were impressively reduced to 40% in patients with AMI when treated with low-dose insulin infusion[44]. As the CRP concentration is correlated with the size of the infarct in AMI, a reduction is indicative of insulin's cardioprotective effects. Moreover, intensive insulin therapy has been given to critically ill patients in surgical and medical ICUs with improved outcomes[2,45]. In 1548 critically ill patients undergoing surgery, insulin infusion which maintained fasting blood glucose concentrations under 110 mg/dL dramatically improved the clinical outcomes with a reduction in total mortality by 48%, the incidence of bacteremia by 46%, acute renal failure requiring dialysis by 41%, ICU poly-neuropathy by 44%, and the need for red cell transfusion by 50% when compared with controls[2]. Mortality and morbidity in the surgical ICUs was dramatically reduced, as was morbidity in medical ICUs. No other agent has been shown to reduce mortality and morbidity by this magnitude in so many diverse ways in the ICU setting. Glucose control seems crucial, but several potential mechanisms may add to the benefits, including reduction of systemic inflammation[46], prevention of immune dysfunction[37], and protection of the endothelium[3,47]. The exact mechanisms underlying this simple and cost-effective intervention need further investigations.

INSULIN RESISTANCE AND INFLAMMATION

Insulin resistance (IR) is a pathological condition wherein insulin-stimulated glucose uptake and clearance in targeted organs are decreased. A few studies suggested that obesity, inflammation and IR are inextricably linked through the actions of specific inflammatory immune cells. The development of IR is thought to occur in response to increased production of pro-inflammatory cytokines by adipose tissue in obesity, that then have an inhibitory effect on insulin signaling pathways in multiple tissues. TNF-α was first found to be increased in adipose tissue of obese mice and able to induce IR[48]. In animal studies, administration of exogenous TNF-α induces IR, whereas neutralization of TNF-α improves insulin sensitivity. IL-1β, another key inflammatory cytokine, interferes with insulin signaling which leads to IR. TNF-α, and more generally, inflammation, activates and increases the expression of several proteins that suppress and impair specific pathways of insulin signaling, making the human body less responsive to insulin and increasing the risk of IR. In turn, IR states are pro-inflammatory. Increased levels of markers and mediators of inflammation such as fibrinogen, CRP, IL-6, PAI-1 and white cell count were shown to correlate with T2D[49-53]. These inflammatory mediators perpetuate and promote the progression of IR. Polycystic ovary syndrome, another IR state, was found to have chronic low-grade inflammation[54]. In other words, inflammation causes IR, and IR is inflammatory. Thus, anti-inflammatory treatment could be proposed as a therapeutic strategy in the treatment of IR.

ANTI-INFLAMMATION THERAPY FOR INSULIN RESISTANCE

Inflammation is hallmark of diabetes and a main cause of its long-term complications. Particularly in obese conditions in humans and animals, it contributes to the pathogenesis of T2D through IR. Therefore, anti-inflammation therapy may be proposed as a strategy for the improvement of IR.

TNF-α is a critical mediator of inflammation, and its increased expression was found to be associated with IR in the adipose tissue of obese mice[48]. *In vitro* studies demonstrated that TNF-α had a direct inhibitory effect on insulin signaling and impaired insulin-stimulated glucose uptake and metabolism in human subjects[55]. Clinically, neutralization of TNF-α with infliximab in patients with rheumatoid arthritis has significantly improved IR as reflected by the significant reduction in the Homeostasis Model Assessment Index[56]. Peroxisome proliferator-activated receptors (PPAR)γ inactivation leads to suppression of IRS-2, which is a signaling molecular in insulin pathways, thus further promotes IR. The anti-diabetic thiazolidinediones (TZDs), which include pioglitazone, rosiglitazone and troglitazone, are clinically used to improve insulin sensitivity in patients with T2D by lowering free fatty acids (FFA) in blood by activating PPARγ. Aspirin, another therapeutic agent, inhibits the activity of multiple kinases induced by TNF-α, and thus enhances insulin sensitivity by protecting proteins from serine phosphorylation[57]. Statins, as a class of anti-inflammatory drugs, have been shown to downregulate

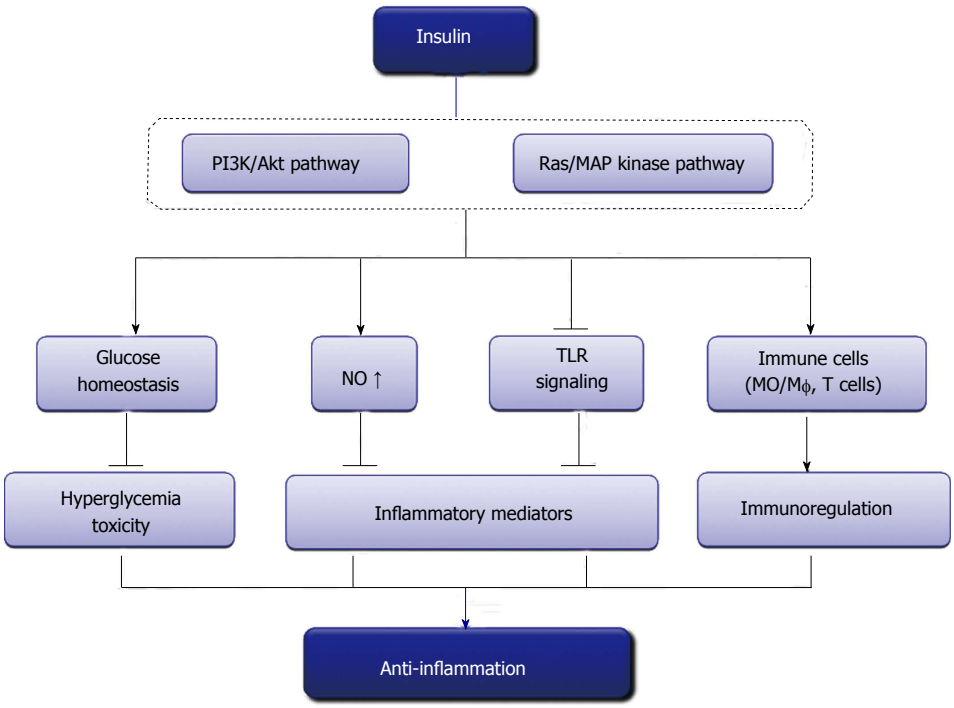

Figure 1 Anti-inflammatory effects of insulin. TLR: Toll-like receptor.

transcriptional activities of NFκB, AP-1 and HIF-1α, with reductions in inflammatory cytokines[58]. Despite these modest anti-inflammatory properties, the statins do not appear to significantly influence either IR or glycemic status. In contrast, high-dose salicylates directly suppress inflammation by targeting NFκB, which improves insulin sensitivity and reduces blood glucose in patients with diabetes[59-61]. The anti-inflammatory properties of TZDs and statins have associated side effects apart from their primary modes of action, thus they may not be safe in the long term. It is necessary to investigate new classes of drugs.

Histone deacetylases (HDACs) are key enzymes that regulate gene expression. Inhibition of histone deacetylase activity has been reported as a new approach to treat diabetes mellitus. Butyrate or trichostatin A, which are histone deacetylase inhibitors, prevented high fat-induced obesity and improved IR in mice[62]. The multiple beneficial effects included: reduced systemic chronic inflammation[63-66], reduced lipid toxicity[67,68], promotion of beta-cell development, proliferation, differentiation and function[69]. Thus HDAC inhibitors may represent a novel drug in the treatment of IR.

CONCLUSION

Hyperglycemia, a commonly exhibited metabolic disorder in critically ill patients, activates the body's inflammatory defense system, causing the cascade release of numerous inflammatory mediators and cytokines, and eventually leads to organ damage. Insulin inhibits hypermetabolism, such as hyperglycemia and lipid degradation, thus could attenuate glucose and FFA-mediated inflammation and improve immunocompetence. More importantly, insulin directly suppresses pro-inflammatory cytokines and induces anti-inflammatory mediators through non-metabolic pathways (Figure 1). Currently, the effects are dependent upon its suppression of innate immune mechanisms and the suppression of transcription factors such as NFκB and Egr-1. With further investigation, the discovery and understanding of the mechanisms underlying the anti-inflammatory effects of insulin opens up the possibility that insulin therapy could be used in multiple clinical practices.

Hyperglycemia, inflammation and IR are inter-related and of reciprocal causation. The relationships between the three entities are far from being elucidated. Hyperglycemia leads to oxidative stress, which further results in inflammation. IR, commonly as a manifestation of hyperglycemia, is pro-inflammatory. Reactive oxygen species is believed to be an important cause of many pathological conditions, including inflammation and IR. It has been established that hyperglycemia is inflammatory whereas insulin is anti-inflammatory. From simple glucose maintenance to the discovery of cardiovascular protection, the knowledge and understanding about insulin is increasing. The pleiotropic effects of insulin including glucose control, and reduction in apoptosis, oxidative/nitrative stress and inflammation, contribute to cardiovascular protection and are beneficial in critical illness. It is not a single effect that mediates the important role of insulin, but it is the whole scenario that promotes its myriad effects. With consistent research, we will gain a better understanding of these working mechanisms, and in doing so, are likely to find more therapeutic targets and wider applications for this wonder drug.

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TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Expression quantitative trait analyses to identify causal genetic variants for type 2 diabetes susceptibility

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Abstract

Type 2 diabetes (T2D) is a common metabolic disorder which is caused by multiple genetic perturbations affecting different biological pathways. Identifying genetic factors modulating the susceptibility of this complex heterogeneous metabolic phenotype in different ethnic and racial groups remains challenging. Despite recent success, the functional role of the T2D susceptibility variants implicated by genome-wide association studies (GWAS) remains largely unknown. Genetic dissection of transcript abundance or expression quantitative trait (eQTL) analysis unravels the genomic architecture of regulatory variants. Availability of eQTL information from tissues relevant for glucose homeostasis in humans opens a new avenue to prioritize GWAS-implicated variants that may be involved in triggering a causal chain of events leading to T2D. In this article, we review the progress made in the field of eQTL research and knowledge gained from those studies in understanding transcription regulatory mechanisms in human subjects. We highlight several novel approaches that can integrate eQTL analysis with multiple layers of biological information to identify ethnic-specific causal variants and gene-environment interactions relevant to T2D pathogenesis. Finally, we discuss how the eQTL analysis mediated search for "missing heritability" may lead us to novel biological and molecular mechanisms involved in susceptibility to T2D.

Key words: Type 2 diabetes; Single nucleotide polymorphisms; Expression quantitative trait locus; Expression regulatory SNPs; Gene-environment interaction; Genome-wide association study

Core tip: Identification of genetic variants that modulate the susceptibility to disease and elucidating their function at the molecular level is a major focus of type 2 diabetes (T2D) research. This article highlights the utility of expression quantitative trait analysis in discovering regulatory variants that increase susceptibility to T2D by modulating the expression of transcripts in tissues important for glucose homeostasis.

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GENETIC DISSECTION OF TYPE 2 DIABETES SUSCEPTIBILITY

Diabetes is one of the most prevalent metabolic disor-

ders, characterized by elevated levels of plasma glucose, and is responsible for significant mortality and morbidity in human populations worldwide[1]. The latest estimate from the International Diabetes Federation indicates a global prevalence rate of 8.4% in adults and 382 million cases of diabetes in 2013[2]. It is one of the common diseases with a well-accepted genetic contribution[3]. Type 2 diabetes (T2D), a late onset subtype of diabetes, results from a derangement in the complex interplay of multiple physiological processes known to be involved in systemic glucose homeostasis. These processes include peripheral glucose uptake in muscle, secretion of hormones and incretins form pancreas and intestine, secretion of cytokines/adipokines from adipose tissue, hepatic glucose production, and neuro-endocrine regulation by central nervous system[4,5]. However, the relative contribution of these processes to T2D pathogenesis is debated. Based on this knowledge on intertwined and complex physiological processes it can be anticipated that T2D is a heterogeneous conglomeration of phenotypes, caused by multiple genetic perturbations and affecting different biological pathways. Predictably, deciphering the genetic etiology of T2D has remained challenging.

Until the last decade, searching for an association between T2D and sequence variants of selected candidate genes was the mainstay of research for finding genetic susceptibility factors. Based on available technology in those studies, researchers selected candidate genes either from loci detected by genome-wide linkage analyses or based on known physiological functions. In our earlier reviews, we discussed the knowledge gained from such studies in detail[6,7]. Success from those endeavors was very limited. However, this approach has identified genetic variants in the *TCF7L2* gene, to date is the best replicated and strongest (relative risk approximately 1.4) genetic susceptibility factor for T2D[8], but its role is still controversial[9-11].

In the middle of the last decade, a transformative change took place in the field of genetics of complex disease research. Advances in high-throughput genotyping technology, availability of the complete human genome sequence, a dense catalogue of common genetic variants, and a population-specific linkage disequilibrium map of these variants lead to the implementation of genome-wide association studies (GWAS), which interrogate the entire genome to identify common genetic variants (minor allele frequency $\geqslant 0.05$) associated with a disease[12]. GWAS have yielded unprecedented success in identifying well-replicated susceptibility loci for T2D, glucose homeostasis traits, obesity, and related metabolic phenotypes[3,13-15]. Nevertheless, these successes come with significant caveats. Based on the most recent analyses, the 63 T2DM-associated loci discovered so far in Caucasian populations together account only for 5.7% of the liability-scale variance in disease susceptibility, and sibling relative risk (λ_s) attributed jointly by these variants is 1.104[13]. Moreover, few of the T2D loci identified primarily in European- or Asian-derived populations are convincingly replicated in African American, Native American, and Hispanic populations, all of whom have a higher prevalence of T2D than Caucasians[14,16]. These GWAS-identified loci do not appear to explain the well-established roles for adipose, muscle, and liver in diabetes pathogenesis[17], and few of these loci have been linked to a molecular mechanism. Several investigators have attempted to implicate function to T2D-associated loci based on their proximity to a gene, assuming that the associated single nucleotide polymorphisms (SNP) alters the function of a nearby gene[18]. Some have drawn enthusiastic conclusions about the role of these variants exclusively in insulin secretion[19]. However, proof of such an assumption is lacking. Given the small effect on T2D susceptibility and the statistical noise inherent in performing 10^6 or more tests, exclusive reliance on larger T2D GWAS alone is unlikely to identify the source of undefined T2D susceptibility (often referred to as "missing heritability"[20]).

EXPRESSION QUANTITATIVE TRAITS: MOLECULAR ENDOPHENOTYPES

One of the major findings from the T2D GWAS is that most of the trait-associated SNPs are located in intronic, intergenic, or other non-coding regions of the genome[3,21]. Further fine mapping analysis also failed to find any coding or other variants that would provide a molecular biological explanation of the elevated disease risk attributed by these loci.

The abundance of a transcript is a quantitative trait. Studies in human populations showed a wide, heritable variation of transcript levels among individuals, and thus lead to the concept of "expression quantitative trait loci" (eQTL)[22,23]. The heritability of eQTLs has been replicated in multiple human tissue or cell types, with approximately 30% of eQTLs having $h^2 > 0.3$, and an estimated 58%-85% being heritable[24-28]. The abundance of a transcript can be directly modified by polymorphisms in non-coding regulatory elements. Many SNPs are associated with quantitative transcript levels and are considered as expression regulatory SNPs (eSNPs). eSNPs close to the transcription start sites (TSS) of the eQTLs are named "*cis*" or "local" eSNPs , whereas eSNPs located $> \pm 500$ kb from the TSS or on a different chromosome are considered "*trans*" or "distal" eSNPs[22,29]. Similar to a published study[30], here we will refer to eQTLs as the transcripts rather than SNP-transcript pair, and eSNPs as the genetic variants (SNPs) associated with the expression profile of a transcript.

Based on this knowledge, many laboratories (including ours) hypothesized that GWAS-associated non-coding variants are eSNPs and can modulate T2D susceptibility by altering transcript levels (or splicing). This concept is based on the "central dogma" of gene expression and presents a causal model of genetic susceptibility (Figure 1). In this model, transcript abundance is considered as an intermediate phenotype between genetic loci (DNA sequence variants) and subclinical (*e.g.*, insulin resistance)

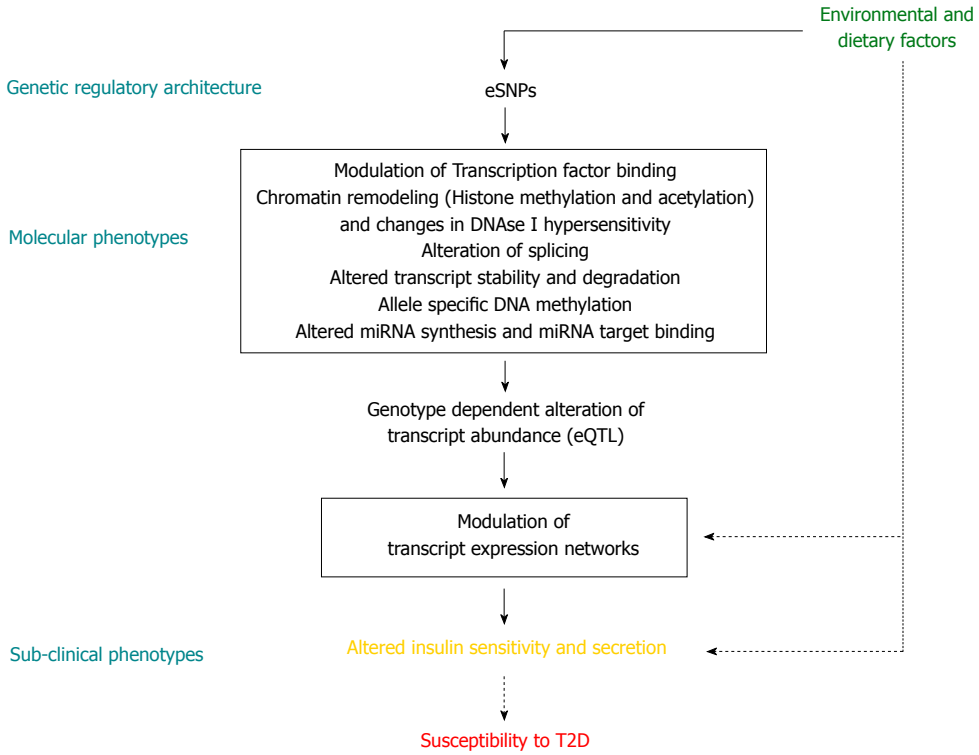

Figure 1 A causal model of genetic susceptibility. Genetic regulatory architecture modulates molecular phenotypes in interaction with environmental factors and alters disease susceptibility. eSNP: Expression regulatory SNP; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes.

or clinical (*e.g.*, T2D) phenotypes. Since transcript abundance is a proximal molecular endophenotype affected by genetic variants, it is likely to be a less heterogeneous phenotype (compared to complex clinical phenotypes like those of T2D), and thus more amenable to genetic mapping methods due to superior statistical power.

EQTL MAPPING

Study designs and analytical frameworks for eQTL mapping are similar to those for mapping any other quantitative traits [*e.g.*, body mass index (BMI), fastin glucose, glycosylated hemoglobin]. However, genetic analysis of human phenotypes including QTLs carries a unique set of problems[29]. In general, eQTL analyses integrate genome-wide expression (in tissues or cells) and genotype data in multiple individuals (related or unrelated). These analyses use linkage- or association-based statistical genetic methods to map regulatory regions and genetic variants that may explain individual variations in transcript expression. Microarray- or RNA-seq[31-33] based methods are used to generate large numbers of quantitative transcript phenotypes. Therefore, the number of statistical tests involved in eQTL mapping studies is significantly higher than in traditional QTL analysis[34]. A detailed discussion on methods used in eQTL analysis is beyond the scope of this article, and we refer our readers to other reviews on this specific subject[29,34-36].

Published eQTL studies have implemented linkage analysis by using 400-2000 microsatellite makers[24,26] to localize regulatory intervals, whereas other studies have genotyped large numbers of common SNPs (> 100000) to discover the eSNPs[25,28,37] associated with eQTLs. With the advancement of genomic technology, we can now simultaneously genotype more than 4.5 million SNPs or can have a whole genome sequence for each individual included in an eQTL study by highly multiplexed "next generation" sequencing methods[38]. These advances pose additional statistical and computational challenges, and will require appropriate correction and adjustment of significance thresholds for the massive number of independent tests performed (and hypotheses tested) to control false discovery. The power to detect eSNPs depends on their effects (average difference in the transcript abundance between genotypes, scaled by the standard deviation of the transcript abundance within genotype classes) and allele frequency[34]. Consequently, detection of eSNPs with a lower effect allele frequency and a lower effect size will require a larger sample size.

One interesting observation from published eQTL studies is that most of the strong eSNPs are located near the TSS with no discernable trend in the 5' or 3' direction[28,39,40]. As a result, most studies consider SNPs within close proximity of the TSS (± 500 kb window) as *cis*-eSNPs. Since the genomic context of most eQTL transcripts are known, statistical adjustment for the actual number of SNPs tested within 500 kb will be more appropriate for *cis*-eSNP discovery. Any SNP outside the *cis*-region is tested as a *trans*-eSNP for a transcript. The molecular biological basis of trans-regulation is less studied;

current information suggests that the variants that affect transcription factors, miRNAs, or long-range chromatin interaction may act as trans-eSNPs. To identify *trans*-eSNPs, the number of tests needed is far greater, and the tests require more stringent significance threshold criteria and a larger sample size. Thus, use of a false discovery rate based on a permutation analysis to correct for multiple testing[34], and considering the correlation among transcript levels and highly correlated SNP structures, are useful approaches to identify this biologically important class of regulatory SNPs.

Several heterogeneous sources of variability hidden in the data may lead to both spurious eSNPs and missed associations in eQTL analyses if not properly addressed. Statistical models that correct for hidden structures within the sample (such as race, admixture, and family relatedness), artifacts in expression data (including batch effects and probe bias), environmental influences, and other known and unknown factors are required to improve sensitivity and interpretability of eQTL analyses[41]. Methods that showed significant usefulness in tackling these confounding factors include Bayesian approaches developed by Stegle *et al*[42] (implemented in probabilistic estimation of expression residuals or probabilistic estimation of expression residuals software), linear mixed-effects model-based approaches developed by Listgarten *et al*[43] (implemented in LMM-EH-PS or Linear Mixed Model-Expression Heterogeneity-Population Structure software), surrogate variable analysis, and inter-sample correlation emended approaches[44,45].

The heavy computational burden involved in eQTL analyses sometimes forces researchers to restrict their analysis to a small subset of selected transcripts and SNPs. Improvement of computational algorithms, parallelization of programs by efficient scripting, and utilization of efficient processing hardware are among many approaches needed to improve scalability and computational efficiency required for eQTL analyses. Implementation of these approaches will enhance discovery by increasing the capacity to utilize the complete data set[46,47].

EQTLS AND DISEASE GENE MAPPING

Molecular and cell biological experiments in model organisms and cells have significantly advanced our understanding about the role of non-coding DNA sequences in genetic regulation, transcriptional circuitry, the transcriptional apparatus, and chromatin regulation. This work has led to new insights into the complex mechanisms involved in dysregulation of gene expression in various human diseases[48]. Recent genome-wide studies in human cells by different international consortia [including ENCyclopedia Of DNA elements (ENCODE)][49] further have improved our mechanistic understanding of the role of DNA sequence variants in quantitative modulation of gene expression[50-52]. eQTL studies have been extensively used to identify genetic regulators involved in natural variation of gene expression[28,37,39] and to understand tissue-specific architecture of genetic regulatory mechanisms[24,30,53-59].

However, an intriguing application of eQTL mapping is the use of eSNP data to interpret disease or disease-related phenotypic association signals, and thereby elucidate specific biological mechanisms underlying the increased genetic risk attributed by the DNA sequence variants. Identification of genetic variants simultaneously associated with disease and eQTLs (in relevant tissue) significantly facilitates identification of potential causal genes. Discovery of genetic variants in *ORMDL3* as a susceptibility factor for childhood-onset asthma[60] and *VNN1* variants that influence high-density lipoprotein cholesterol concentrations[26] are two early examples of the successful implementation of eQTL mapping in disease gene hunting. The review by Cookson *et al*[36] offer a more detailed discussion on those success stories.

Several recent studies have integrated GWAS and eQTL analyses (data generated in different sets of subjects) and have used the overlap of two signals as a tool to interpret GWAS findings. Although this work is a good starting point, we need to be cautious about using the overlap of two statistical signals (eSNP and the disease phenotype-associated SNP/phSNP). Careful thought is required before making a claim of identifying a disease-causing variant. Montgomery and Dermitzakis (2011) described three situations[41] when a coincidence of eQTL and disease phenotype GWA signal may distract from identification of causal variants: (1) eSNP and phSNP are in the same linkage disequilibrium (LD) block but are two different SNPs. This is not considered as exact overlap, and they may tag different causal variants; (2) eSNPs and phSNPs are the same but SNP density differs between the eQTL and GWAS data. Lack of proper resolution in one or both studies may be misleading and will not elucidate the correct functional SNP; and (3) eSNPs may have a pleiotropic effect and may regulate the expression of "gene Y" in "tissue 1", but the same eSNP may regulate the expression of "gene X" in "tissue 2". Thus, if the eQTL study is done in "tissue 1" (a "surrogate" tissue) but not in "tissue 2" (the "disease-relevant" tissue in which the true causal effect is manifested), then despite the overlap of eSNPs and phSNPs, we will incorrectly link "gene Y" to the disease phenotype.

In general, eSNPs that are universal have a stronger effect, but a significant proportion of eSNPs show tissue-specific effects[30,53,54]. However, it is difficult to select "relevant" tissue, or the relevant tissue may not be accessible from human subjects for analysis for many complex diseases. Ongoing efforts of international consortia, including GTEx, to develop multi-tissue eQTL databases (Table 1) is a significant step forward in addressing this limitation[61-64].

Many investigators have developed statistical approaches to formally test the overlap of GWAS and eQTL signals to distinguish accidental colocalization from true sharing of causal variants. The regulatory trait concordance method designed by Nica *et al*[65] accounts for local LD structure and integrates eQTL and GWAS results to reveal the subset of association signals due to

Table 1 Selected expression quantitative trait loci databases

Database	Website (URL)	Cell/tissue type	Project
eQTL Browser	http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/	LCL, liver, brain, fibroblast, T-cell	17 projects
Genvar	http://www.sanger.ac.uk/resources/software/genevar/	Adipose, LCL Skin fibroblast from healthy female twins	MuTHER
		LCL from 8 populations	Hapamap3
		Fibroblast, LCL and T-cell from umbilical cord	GenCord
GTEx eQTL Browser	http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi	Multiple tissues including liver, brain regions, LCL	GTEx
PACdb	http://www.pacdb.org/	Gene-drug or GXD eSNPs from LCL model	Dolan and Cox lab
SGR Database	http://systems.genetics.ucla.edu/	22 mouse and several human datasets. Data includes aortic endothelial and smooth muscle, adipose, brain, liver, macrophages and muscle tissue Includes GXE eSNP data from cell experiments	Lusis lab
SCAN	http://www.scandb.org/newinterface/about.html	CEU and YRI LCLs from HapMap	Cox Lab
seeQTL	http://www.bios.unc.edu/research/genomic_software/seeQTL/	HapMap LCLs	

SGR: Systems genetics resource; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes; eSNP: Expression regulatory SNP; LCL: Lymphoblastoid cell lines; GXD: Gene-by-drug interaction; GXE: Gene-by-environment interaction; CEU: HapMap caucasian from CEPH collection; YRI: HapMap African from Yuroba, Nigeria.

cis- or *trans*-eQTLs. He *et al*[66] (2013) developed an algorithm named "Sherlock" based on a Bayesian statistical framework to identify potential gene-disease associations by matching genetic signatures of expression (collective information of *cis*- and *trans*-eSNPs) of a gene to that of the disease phenotype by using GWAS data of the disease and the eQTL data of related tissue. These novel approaches are likely to expand our ability to harvest new insights from genetic association studies for disease phenotypes.

T2D-ASSOCIATED VARIANTS ARE ESNPS IN TISSUES IMPORTANT FOR GLUCOSE HOMEOSTASIS

Genome-wide eQTL analyses in transformed lymphocytes (lymphoblastoid cell lines, or LCLs) provided the first evidence that SNPs associated with complex diseases phenotypes are more likely to be eSNPs than minor allele frequency-matched SNPs randomly selected from high-throughput GWAS genotyping platforms. Nicolae *et al*[67] (2010) utilized an Affymetrix GeneChip Human exome 1.0 ST array to generate exon-level expression data of LCLs from 87 Caucasian (CEU) and 89 African (YRI) subjects from the HapMap project. They performed a quantitative-trait transmission disequilibrium test to identify eSNPs from 2 million genotyped SNPs. A study by Nica *et al*[65] (2010) utilized an Illumina Sentrix WG-6-V2 whole-genome expression array to generate total transcript-level expression data of LCLs from 109 unrelated CEU subjects (from the HapMap 3 project) and performed Spearman rank correlation analysis to identify eSNPs from 1186075 genotyped SNPs. Key findings from these studies[65] include: (1) SNPs reproducibly associated with complex human traits are likely to be eSNPs; (2) Enrichment of complex trait GWAS-implicated SNPs are more evident among *cis*-eSNPs but not among *trans*-eSNPs; and (3) eSNPs discovered in LCLs are more strongly enriched for SNPs associated with immunity-related conditions (*e.g.*, Crohn's disease, type 1 diabetes, rheumatoid arthritis), but such enrichment was not observed for metabolic disorders (*e.g.*, T2D and coronary artery disease). These studies indicate that eQTL studies using surrogate tissue samples may be helpful for some diseases. However, understanding the functional role of T2D-associated SNPs will probably require expansion of eQTL studies into tissues more relevant for T2D pathophysiology. These studies also had significantly lower power to identify *trans*-eQTLs due to comparatively small sample sizes, and will require reevaluation of the role of *trans*-eSNPs in larger sample sets.

Zhong *et al*[68] (2010) used genetics of gene expression (GGE) analysis in tissues from two cohorts of human subjects (Cohort 1: liver-specific GGE cohort with post mortem liver samples from 427 subjects; Cohort 2: liver, subcutaneous adipose and omental adipose from 922 subjects who had Roux-en-Y gastric bypass surgery). They identified 18785 unique eSNPs in the combined set of data. They found 2189, 2286, and 1999 eSNPs specific to liver, omental adipose, and subcutaneous adipose, respectively. However, they also noticed that 72% of *cis*-eSNPs identified in liver, 79% of those found in omental adipose and 80.5% from subcutaneous tissue were also found in the other two tissues. Given the metabolic relevance of these tissues, they further interrogated data from three large-scale T2D GWAS datasets to test whether the set of eSNPs were more likely to be associated with T2D compared to randomly selected SNPs. These tissue eSNPs showed a significant enrichment of T2D-associated SNPs. For example, in the DIAGRAM (DIAbetes Genetics Replication and Meta-analysis) GWAS data set, 7.34% of the eSNPs showed a significant association with T2D ($P < 0.05$) compared to an average of 6.12% SNPs in the random sets, representing a modest 1.20-fold enrichment for SNPs in the eSNP (or SNP in LD at $r^2 > 0.89$) set over the random sets (p-enrichment = 1.33×10^{-9})[68]. In that study, omental adipose tissue eSNPs also showed

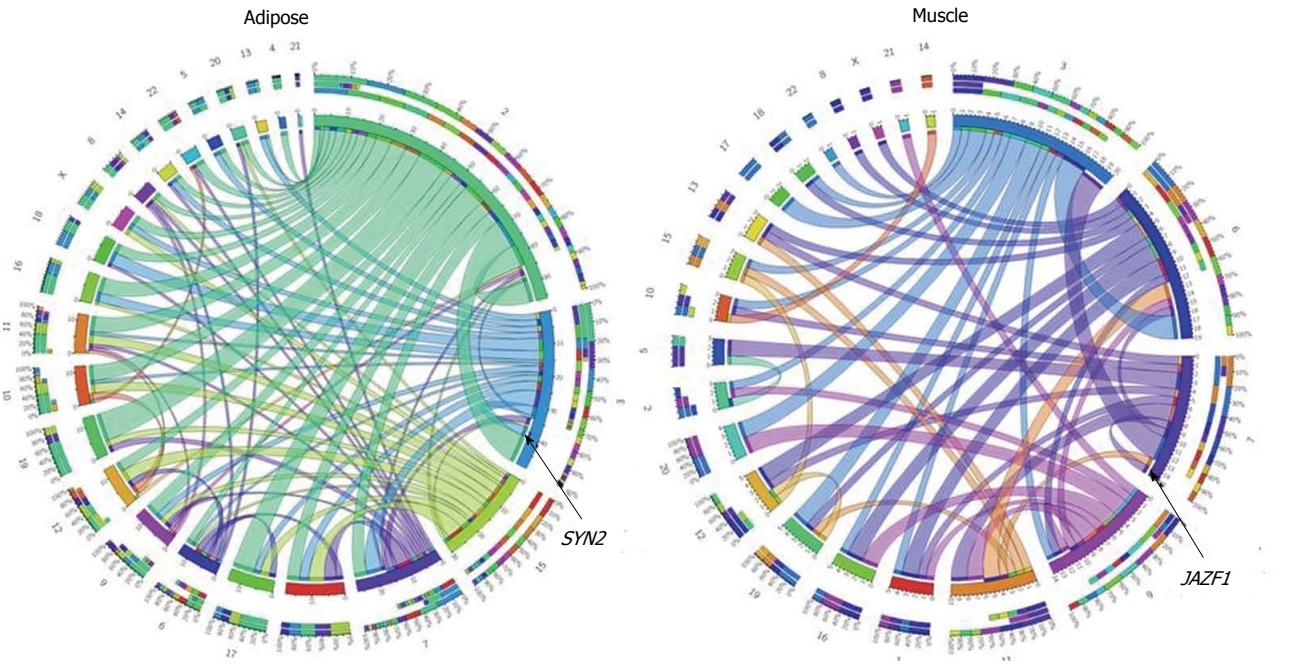

Figure 2 Type 2 diabetes or glucose homeostasis traits associated variants are expression regulatory SNP. We tested *Cis* and *Trans* regulatory role of 68 SNPs that showed reproducible associations with T2D or Glucose homeostasis traits[72]. At a threshold of $P < 0.0001$, 25 and 19 of these SNPs in adipose and muscle, respectively, showed association with expression of a *cis*- or trans-transcript. This figure represents a CIRCOS plot of eQTL and eSNP chromosomal location relationships, indicating the predominance of *trans*-regulation among 183 and 62 significant ($P < 0.0001$) eQTL-eSNPs associations in adipose and muscle respectively. Rare *cis*-regulation (*SYN2* in adipose and *JAZF1* in muscle) is marked. eSNP: Expression regulatory SNP; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes.

further significant enrichment when restricted to adipose expression network genes differentially expressed with T2D. Thus, these studies support the notion that T2D-associated SNPs may modulate expression of transcripts in tissues relevant for glucose homeostasis.

Fu *et al*[53] (2012) analyzed eQTLs in blood ($n = 1240$) and other tissues (liver, $n = 62$; muscle, $n = 62$; subcutaneous adipose, $n = 83$; and visceral adipose, $n = 77$); out of 1954 SNPs associated with complex disease traits from a GWAS catalogue, 907 were *cis*-eSNPs. However, 28.7% of these trait-associated *cis*-eSNPs showed a tissue-specific (in blood versus other tissue) and discordant effect on gene expression. The discordant effect includes tissue-specific regulation, alternative regulation by different eSNPs, different effect size and, in a few cases, opposite allelic direction. The study also showed that SNPs associated with complex traits are more likely ($P = 2.6 \times 10^{-10}$) to exert a tissue- specific effect on gene expression[53]. No comparisons were made between other tissues due to small sample size. This study indicates that use of tissues in eQTL analysis may have implications for inferring transcriptional effects of SNPs, especially for the complex disease susceptibility variants.

This work also emphasizes the importance of investigating disease-relevant tissue for characterizing functional effects of T2D and other disease-associated variants. However, it is difficult to determine "relevant tissue" even for diseases with known pathophysiology. T2D is clearly of polygenic etiology, and relevant tissue could be distinct for genes involved. Moreover, gene expression is regulated by environmental (*e.g.*, diet), epigenetic, and other unknown factors, and eQTL discovery from tissue samples may be affected by the physiological state of the donors[41]. For example, profound hyperglycemia and dyslipidemia observed in T2D subjects will modulate and even may mask primary causal changes in genetic regulatory networks. Thus, multi-tissue eQTL analysis in physiologically characterized individuals could be a safe option to scrutinize the circularity of cause and effect in genetic regulatory signals, and holds the promise to offer insights into the novel mechanisms driving genetic susceptibility to T2D.

Most initial eQTL studies seeking to identify a regulatory role for T2D-associated SNPs have focused on *cis*-eQTLs. However, studies by Voight *et al*[69] (in adipose, $n = 603$; and blood, $n = 745$ subjects) and our laboratory (in adipose and muscle of 168 non-diabetic subjects who were physiologically evaluated) showed that only a few top T2D GWAS-identified signals can be explained as *cis*-eQTLs, and T2D-associated non-coding SNPs are less likely to regulate expression of the closest gene[70]. Results were similar in an eQTL analysis that used human islet cells from 63 cadaver donors[71]. A genome- wide study by our laboratory[72] in adipose and muscle tissue of 62 subjects (31 insulin- resistant and 31 insulin-sensitive subjects matched for BMI) showed that at a less stringent threshold ($P < 0.0001$), among 68 well-replicated T2D/glucose homeostasis-associated SNPs, 25 and 19 of them were eSNPs in adipose and muscle, respectively (Figure 2). However, after stringent (Bonferroni) correction, only SNP rs13081389 was a *cis*-eSNP for the *SYN2* gene in adipose ($P < 4.7 \times 10^{-8}$, 15507 expressed transcripts were

tested in adipose). Interestingly, these 68 SNPs showed significant enrichment for *trans*-eSNPs in adipose and muscle, but not in LCLs[72]. Many of these *trans*-eSNPs show associations with expression of ⩾ 10 transcripts and may be a "master regulator". Expanding this search for the top 1000 T2D-associated SNPs from a Wellcome Trust Case Control study also confirmed the *trans*/distal regulatory SNPs[72]. We also showed that replicated T2D- and glucose homeostasis-associated SNPs are enriched for *trans*-eQTLs for transcripts differentially expressed between insulin-resistant and insulin-sensitive people[72]. A recent eQTL study using a large cohort of blood samples also supported the *trans*-regulatory role of 233 complex trait-associated SNPs[73]. Thus, the genetic regulatory architecture of T2D is complex, tissue-specific, and likely extends beyond the *cis*-regulatory mechanism.

EQTL ANALYSIS FOR PRIORITIZING T2D-ASSOCIATED VARIANTS TO IDENTIFY NOVEL CANDIDATE GENES

The multiple testing corrections utilized in genome-wide statistical analyses allow detection of only the strongest effects and penalize weaker associations that may be biologically meaningful[74]. Investigators have implemented several approaches to prioritize T2D association signals from large GWAS datasets to identify biological mechanisms responsible for genetic predisposition. One common approach includes selection of genes close to T2D GWAS-implicated SNPs and shows differential expression in T2D subjects compared to normoglycemic subjects (or in animal models of T2D). This approach is based on the idea that T2D-associated variants may modulate the expression of nearby genes in tissues important for glucose homeostasis. Parikh *et al*[75] used publicly available expression microarray data from different tissues (pancreas, adipose, muscle, and liver from T2D patients and rat models of T2D) to prioritize among the 275 genes located near 1170 T2D GWAS-implicated SNPs. A recent study by Taneera *et al*[71] used expression profiling of human pancreatic islet cells for functional prioritization of genes in the vicinity of 47 T2D-associated SNPs. However, available data from several human tissue eQTL analyses indicate that only a few T2D-associated SNP act as *cis*-eSNPs, and no enrichment of differentially expressed genes was observed around T2D GWAS-implicated variants[72]. Thus, a logical alternative for prioritizing T2D-associated variants is to utilize a reverse genetics approach and restrict the genetic search space to the subset of variants that are eSNPs in relevant tissues. These eSNPs are statistically associated with expression of transcript and thus have a strong possibility of being a "key driver" in perturbing gene-expression regulatory networks.

Selecting the genes based on eSNPs among those also associated with T2D in large GWAS datasets will prioritize genes with a significantly high chance of being causally involved with susceptibility to T2D, and thus may be helpful in identifying additional genetic susceptibility loci from GWAS datasets. A genome-wide analysis of adipose tissue transcriptomes from 62 insulin-resistant and -sensitive subjects identified 172 differentially expressed transcripts[76]. We checked adipose eQTL data from the MuTHER study[55] to find eSNPs of these differentially expressed transcripts. We further mined the DIAGRAM GWA meta-analysis results[13] for association of these eSNPs with T2D. This analysis[77] identified that the strongest *cis*-eSNP (rs11037579, $P = 4.21 \times 10^{-6}$) for the *HSD17B12* in adipose tissue was also associated with T2D [$P = 3.80 \times 10^{-4}$, OR = 1.06 (95%CI: 1.03-1.1)]. Individuals carrying the T2D risk allele T for the intronic SNP rs11037579 had lower expression of *HSD17B12* in adipose tissue. This result corroborates the finding that *HSD17B12* expression is downregulated in the adipose tissue of insulin-resistant subjects. The *HSD17B12* gene codes a bifunctional enzyme involved in the biosynthesis of estradiol and the elongation of very long chain fatty acids. Several variants within ± 500 kb of this gene are eSNPs (including a 3'UTR SNP rs1061810) in adipose, LCL, and other tissues, and show an association with T2D (although below the genome-wide threshold) (Figure 3). Further functional studies will be required to identify true causal SNPs. However, this integrative approach demonstrates the validity of such an approach in prioritizing novel T2D susceptibility loci. In fact, two recent integrative genomic studies showed that eSNPs for *PFKM* (SNP rs11168327) gene in muscle and *ARAP1* (SNP rs11603334) gene in pancreatic beta cell are associated with T2D[78,79].

EQTL AND BIOLOGICAL NETWORK ANALYSIS TO IDENTIFY ETHNIC-SPECIFIC GENES FOR T2D:

Age-standardized prevalence of T2D varies among ethnic and racial groups[14,80]. T2D is almost twice as prevalent in adult non-Hispanic African Americans (14.9%) in the United States compared to European Americans (7.6%)[81]. Yet only a few of the associated T2D-loci - identified primarily in European- or Asian-derived populations - are replicated in African American, Hispanics, and Native Americans[14,16,82-84]. Intriguingly, studies have identified distinctive physiologic features of glucose homeostasis in African Americans and Hispanics[85-87]. Compared to non-Hispanic Caucasians matched on age, gender, and BMI, African Americans are more insulin-resistant (lower S_I), but show a greater acute insulin response to intravenous glucose (AIR_G) and a higher disposition index ($DI = S_I \times AIR_G$). A genetic basis for these physiological differences seems likely, but remains unidentified.

Published studies of expression across ethnic groups (mostly restricted to lymphocytes or HapMap LCLs) showed distinct ethnic-specific expression[37,88-90]. Zhang *et al*[90] (2008) reported differential expression of up to 67% of transcripts between LCLs from subjects of

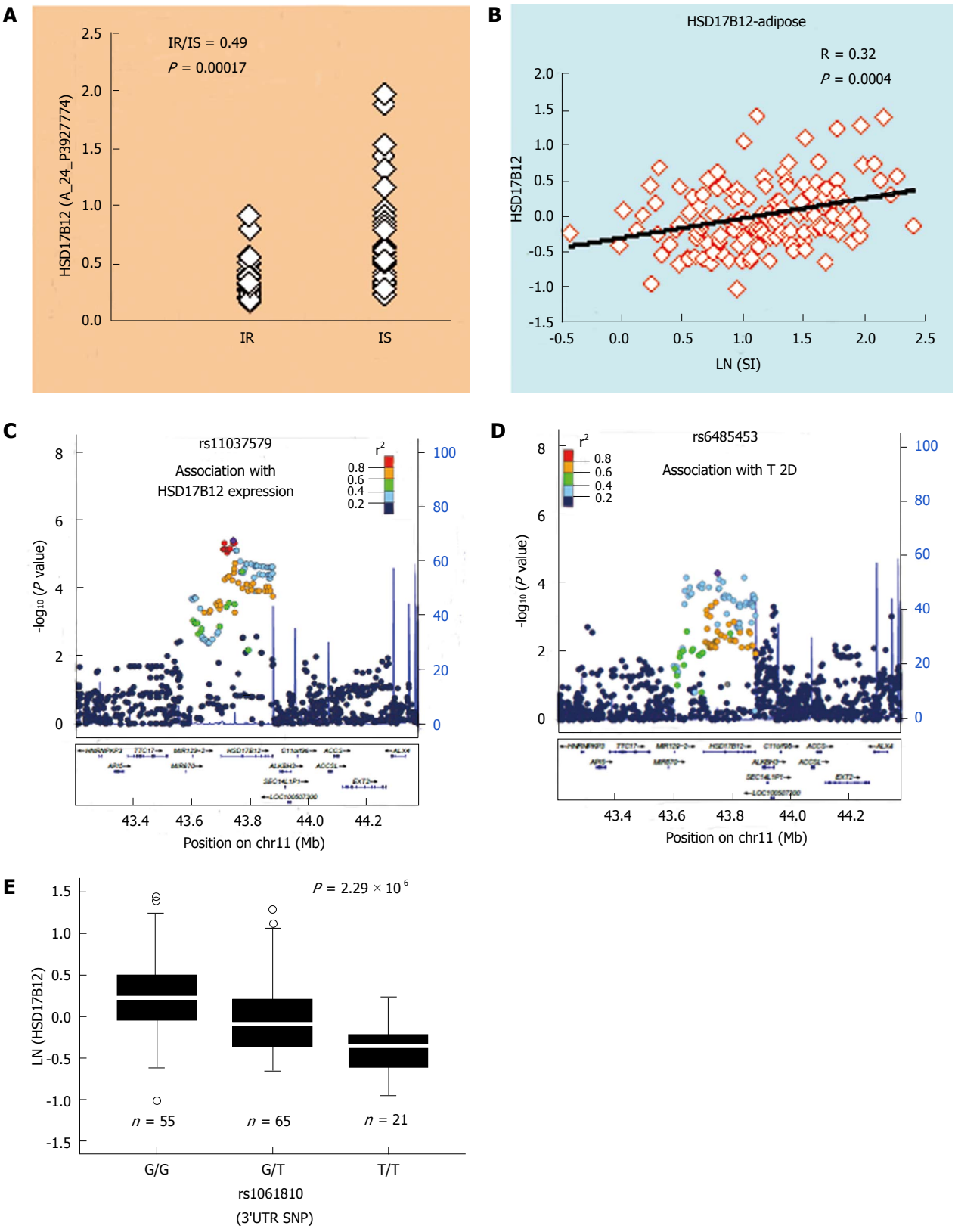

Figure 3 Prioritizing type 2 diabetes-associated variants by expression quantitative trait loci analysis: An example. *HSD17B12* is one of 172 genes differentially expressed in adipose tissue of insulin-resistant (IR, $n = 31$) *vs* insulin-sensitive (IS, $n = 31$) subjects in a genome-wide study (A) by Elbein *et al*[76]. Its expression in subcutaneous adipose of non-diabetic subjects ($n = 141$) also shows a significant correlation (B) with insulin sensitivity (SI). Strongest *cis*-eSNP for adipose tissue (C) expression of HSD17B12 (in adipose eQTL from the MuTHER project)[55] is also associated with T2D (D) in a large GWAS meta-analysis (in DIAGRAM.v3 data from 12171 T2D and 56862 controls)[13]. This locus also includes a 3'UTR SNP rs1061810 that shows association (E) with T2D and expression of HSD17B12 (in qRT-PCR analysis in adipose tissue from 141 non-diabetic subjects). eSNP: Expression regulatory SNP; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes.

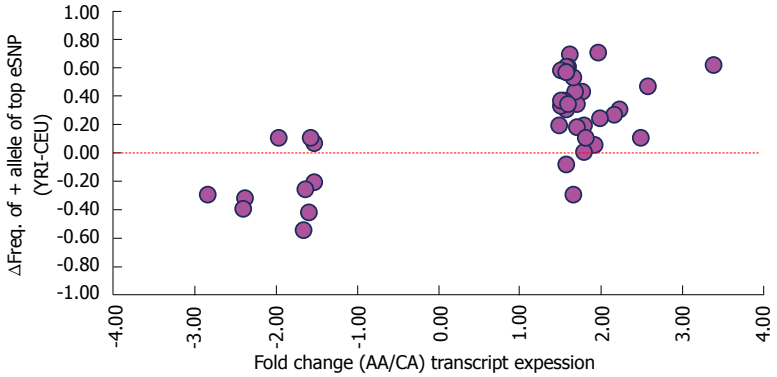

Figure 4 Population differences in expression of transcripts in adipose tissue is accounted for by the effect allele frequency difference of expression regulatory SNPs among racial groups. X axis: Fold change in average expression of 41 transcripts between African-American (AA, *n* = 37) and Caucasian (CA, *n* = 99) subjects. Y axis: Differences in strongest eSNP allele frequency of these transcripts between HapMap subjects of Caucasian (CEU) and African (YRI) ancestry for alleles associated with higher expression. *e*SNP: Expression regulatory SNP.

European (CEU) and African (YRI) descent, with enrichment of ribosome biogenesis, antimicrobial response and cell-cell adhesion. Spielman *et al*[37] (2007) attributed the 1097 genes that differed between CEU and Asian (CHB) LCL samples to eSNP frequency. Our comparison of genome-wide expression profiles (using an Agilent 44K expression array) from adipose and muscle tissue of non-diabetic Caucasians (n = 40) and African Americans (n = 22) identified transcripts associated with insulin sensitivity (S_I), many of which (*e.g.*, *CLIC6*, *HSD11B1*, *SERPINA3*, *THBS1*, *TMEM135* and *TNMD* in Adipose) show distinct ethnic-specific expression[76].

Comparison of adipose tissue expression data between Caucasians and African Americans in a larger cohort (using an Illumina –HT12.V4 array for 99 Caucasians and 37 African Americans) identified 117 differentially expressed (fold change $\geqslant$ 1.5 and false discovery rate $\leqslant$ 5%) transcripts[91]. By mining adipose tissue eQTL data from the MuTHER project[55], we found that about 35% of these differentially expressed transcripts are strongly modulated ($P < 1 \times 10^{-5}$) by *cis*-eSNPs in adipose tissue. In line with the findings by Spielman *et al*[37] (2007) in LCL, we also found that in adipose tissue, the degree of differential expression (fold change African Americans/Caucasians) shows strong concordance with the difference in the effect allele frequency of top *cis*-eSNPs (Figure 4) between HapMap African (YRI) and Caucasian (CEU) subjects.

These studies suggest that the distinct genetic architecture of eSNPs determines the ethnic-specific expression profile in tissues important for glucose homeostasis. Ethnic-specific derangements of gene expression networks in tissues involved in glucose homeostasis may explain distinctive physiologic effects, including differences in insulin action and secretion between ethnic and racial groups. Perturbation of gene expression networks associated with early pathophysiologic events (including insulin resistance) is driven by regulatory variants (eSNPs). The distinct genetic architecture of these variants (including linkage disequilibrium and allele frequency) may determine their ethnic-specific (or predominant) effect on expression and T2D susceptibility. Thus, integration of genome-wide expression analysis and eQTL analyses may be a useful approach to identify the primary genetic factors for ethnic-specific susceptibility to T2D.

Expression of transcripts involved in the same biological function tend to be co-regulated by similar factors (genetic or environmental) and can be identified as distinct network modules, where genes within a module are more highly interconnected (correlated) with each other than genes in other modules. Statistical approaches like weighted gene co-expression network analysis (WGCNA software package developed in "R" programming environment implements this analytical method) are useful for identifying modular structures of the co-expression networks[92,93] in tissues important for glucose homeostasis. Evaluation of the correlation of each module eigengene with the S_I and other T2D-related metabolic phenotypes, and determination of the preservation of these modules between ethnic groups based on observed network density and connectivity, will identify molecular processes or molecular interaction structures associated with phenotypes that undergo ethnic-specific reconfiguration by genetic or non-genetic causal regulators.

Several recently developed statistical metrics[94,95], including modular differential connectivity, offer powerful tools to identify the modules with significant ethnic-specific changes in interaction strength. The eSNPs are causal variants (or in linkage disequilibrium with causal variants) that regulate the expression level of neighboring (or distal) genes. Thus, eSNPs serve as a primary source of natural perturbation to infer causal relationships among and between genes in gene-expression networks[96]. The distinct allelic architecture of these SNPs may determine ethnic-specific modular differential connectivity. Genes with eSNPs can be considered as "parent nodes" in expression networks. This information is used as a "structure prior" in the network reconstruction analysis to orient the edges of the networks. Reconstructing ethnic-specific networks by utilizing different causality modeling methods, including Bayesian network reconstruction approaches, may identify key causal regulators of these networks[97,98]. Thus, a multiscale biological network analysis that utilizes eQTL information to distinguish causal from correlated disease effects is a novel approach to understand how causal regulators propagate their effects in mediating ethnic-specific susceptibility to disease.

A similar approach was used recently to identify genetic factors in animal models of diabetes and other complex human diseases, including Alzheimer's disease[95].

A study by Zhong *et al*[68] (2010) in adipose tissue of C57BL/6-ob/ob × BTBR-ob/ob mice F2 progeny identified a strong causal subnetwork for T2D traits (called the "purple" module, enriched for genes involved in plasma glucose and insulin levels). They found that 37 eSNPs of genes in this module showed significant association with T2D in a GWAS report. Through additional prioritization steps and subsequent function validation studies, they identified mallic enzyme (*ME1*) as a key causal gene in this T2D subnetwork. A strong *cis*-eSNP of *ME1* was associated with T2D. Future applications of such integrative genomic strategies in T2D or related disorders in human populations may prove insightful.

EQTL ANALYSES TO IDENTIFY GENE-ENVIRONMENT INTERACTIONS RELEVANT FOR T2D

As discussed above, GWAS have identified DNA sequence variants in the susceptibility to T2D, but these variants account for only a part of the estimated heritability[13,14]. Interactions between sequence variants and environmental stimuli are a logical step in better understanding the development of T2D. Thus, some of the missing heritability for T2D susceptibility may be explained by studies of the interaction between environmental factors and genetic variants or gene-environment (GXE) interactions[99]. Modeling GXE interactions in clinical or epidemiological settings is challenging and costly, due to few validated tools for assessing exposure (including dietary exposure), the need for large sample sizes, and the heterogeneity of exposures in populations[100-103]. Environmental factors usually influence insulin resistance and T2D risk over long periods of time; thus, accurate assessment of long-term exposure is needed to identify GXE interactions. A recent series of studies by Patel *et al*[104-106] utilized data resources from the National Health and Nutritional Examination Survey and integrated GWAS and environment-wide association studies to identify environmental factors, genetic factors, and GXE interactions involved in T2D susceptibility. However, they noted several significant limitations of such epidemiological approaches in adequately addressing influence of genetic variations on differences in environmental response in human populations.

Environmental factors, including diet and derived metabolites, can influence phenotypes by modulating gene expression in several ways. Variations in responses to environmental factors among individuals, and how these responses predispose to metabolic and other disorders, have been recognized[107]. Genetic variants modulate the environmental factor-mediated transcriptional response, which in turn dictates cellular response and may explain variability in metabolic responses to those factors[99]. Such dependency on external conditions or GXE interactions has been reported for genetic effects on gene expression in different organisms[108-110]. Transcripts responsive to environmental perturbation factors may manifest as eQTLs and are modulated by *cis*- and *trans*-eSNPs. A subset of these eSNPs associated with T2D, obesity, and/or glucose homeostasis traits may thus exhibit distinctive patterns of GXE eSNPs. Thus, identifying environmental factors that modulate insulin sensitivity and other early pathophysiological manifestations of T2D and its integration into eQTL analyses will further improve the power to construct causal gene regulatory networks involved in T2D susceptibility.

A few recent studies implemented a novel "cellular genomics" approach[111] to elucidate genetic controls on GXE interactions, critical to understanding the pathophysiology of complex diseases. In this novel paradigm, researchers analyzed the molecular consequences of genetic variants to assess interactions with environmental factors *via* quantification of processes (like gene expression) in cells from human subjects grown in uniform culture conditions. This concept is illustrated in Figure 5. Utilizing transformed lymphocytes, the studies examined genetic control in response to radiation, chemotherapeutic drugs, and hormones (glucocorticoids)[112-114]. Two similar studies in primary human cells mapped genetic regulators responding to growth factors (BMP-2), hormones (dexamethasone), cytokines (prostaglandin E2 in human osteoblasts), and oxidized low density lipoprotein (in human aortic endothelial cells)[115,116]. Despite the encouraging success of these studies, no studies so far have evaluated GXE interactions with a cellular genomic model relevant to T2D and related metabolic disorders. Although this model may miss some whole organism-level complexity[117] of T2D pathogenesis (which involves multiple tissues), it does represent an innovative approach by going from cellular to organismal phenotype analysis for identification of function of genetic variants involved in T2D susceptibility. Mapping GXE eSNPs for function-based prioritization of T2D and related metabolic disease-associated SNPs is a critical step towards designing efficacious strategies to reduce the public health burden of common metabolic disorders triggered by increased exposure to dietary and other environmental factors.

EQTL AND PHARMACOGENOMIC STUDIES FOR T2D

Several classes of anti-diabetic medications are used for the treatment of T2D[118]. Pharmacogenomic studies reviewing the role of genetic variants on drug responses (including adverse drug reactions) have yielded significant findings, including novel disease mechanisms for several complex diseases[119]. But a similar success for T2D has not been achieved[5,120]. Pharmacological interventions using peroxisomal proliferator activated receptor gamma (PPARγ) agonists like pioglitazone improve insulin sensitivity and can reduce the risk of progression to T2D[121]. However, approximately 25% of patients do not respond adequately to PPARγ agonists[122]. Genome-wide transcriptomic analysis by our laboratory showed significant

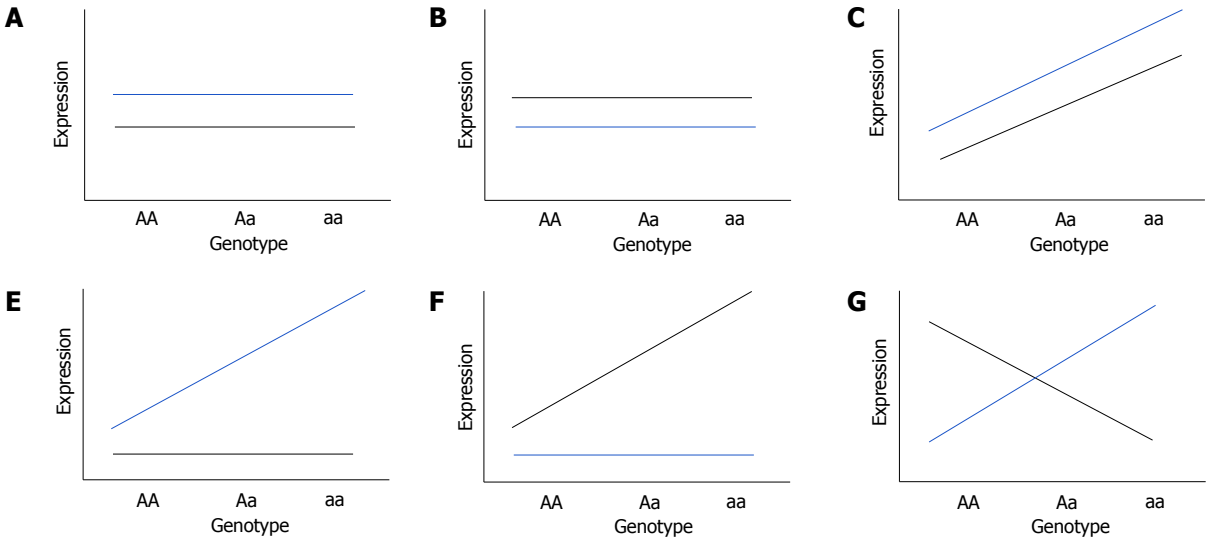

Figure 5 Types of gene-by-environment interactions in cellular genomic models to study gene-by-environment expression quantitative trait locis. Cells from a cohort of subjects are grown in pairs under uniform *in vitro* treated and untreated conditions to study environment-dependent or -independent effects of genotype on expression of transcripts (a quantitative trait). β1 and β2 are genotype effects on transcript expression under treated and control conditions, respectively. Different models of gene-by-environment (GXE) includes Null model: β1 = β2 = 0 (A and B); No-interaction eQTL model: β1 = β2 ≠ 0 (C); Treated-only expression quantitative trait loci model: β1 ≠ 0 and β2 = 0 (D); Control-only eQTL model: β1 = 0 and β2 ≠ 0 (E); and General interaction eQTL model: β1 ≠ 0 and β2 ≠ 0 but β1 ≠ β2 (F). Black line indicates expression in cells under control condition (untreated) while blue line indicates expression in environmental/dietary factor treated cells.

inter-individual variability in gene-expression response after pioglitazone treatment in people with impaired glucose tolerance[123]. However, little is known about the genetic architecture of variation in pioglitazone-mediated transcriptional response in human populations. Identifying the genetic variations that interact with pharmacological treatments like PPARγ agonists is of high clinical interest. eSNPs may modulate the expression of key transcripts in response to anti-diabetic drugs in target tissues and can explain the interindividual variability in treatment outcome[124,125]. Identifying genetic (and epigenetic) variants that modulate the pharmacological treatment-mediated transcriptional response, which in turn dictates the treatment outcome in T2D, is an open area of research. A novel approach that systematically characterizes the set of eSNPs involved in anti-diabetic medicine-mediated transcriptional modulation (gene-drug interaction eSNPs, or GXD eSNPs) in tissues relevant to glucose homeostasis will be useful in stratifying populations in efficacy studies, to improve the quality of clinical decision-making and treatment options for T2D.

FINDING EQTLS: END FOR A NEW BEGINNING

eQTL analyses provide statistical evidence for genotype-dependent variations in transcript abundance and should be considered a starting point for investigating the effects of DNA polymorphisms at the molecular level[34]. Transcript abundance depends on a dynamic relationship between transcript synthesis, stability, and degradation[48]. Thus, DNA polymorphisms may affect transcript abundance by several known and unknown mechanisms. Studies in human subjects have shown that sequence-specific regulation of mRNA expression is mediated by several molecular mechanisms, including allelic variability in transcription factor binding, chromatin remodeling, changes in DNase I hypersensitivity by histone methylation and acetylation, interaction between chromatin segments, alteration of splicing, sequence-dependent allele-specific DNA methylation, alteration of miRNA synthesis, and miRNA target binding[50-52,126-130]. GWAS-implicated variants for complex diseases are enriched in non-coding functional domains of the genome, including sequences involved in chromatin remodeling[131-133]. Many transcripts that show strong co-expression and *cis*-eSNPs for one transcript may appear as trans-eSNPs for a co-regulated transcript located in other chromosomes. Thus, a functional role of prioritized *cis*- and *trans*-eSNPs needs to be validated by appropriate molecular experiments to distinguish causal from correlative effects[134-136]. Studies have used allelic expression imbalance analysis, electrophoretic mobility shift assays, and transient transfection based luciferase reporter assays[56,137-141] to identify the molecular effects of genetic variants (*cis*-eSNPs) on gene expression; however, high-throughput methods are needed to validate in parallel the large number of findings from genomic studies[134,135,142]. Several novel high-throughput methods, including massively parallel reporter assays and massively parallel functional dissection, are now available to show evidence of causality for regulatory variants[143-146]. Functional relevance of the candidate eQTL transcripts in T2D pathophysiology also need to be validated by demonstrating their effects upon experimental up- or down-regulation in *in vitro* or *in vivo* experimental models[147,148].

In summary, many factors (including genetic, epigenetic and environmental factors) affect susceptibility to

T2D. Instead of investigating different sources of variation in isolation, an integrative functional omics paradigm that traces early molecular changes through layers of biological information, including eQTLs, promises to be a useful approach[136]. Such an approach will promote optimal understanding of the etiology of T2D and lead to the identification of ethnic-specific primary causal variants. Ultimately, the knowledge gained from studies using these approaches can be used to build better classifiers of T2D risk than those based on DNA sequence variants alone.

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TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Platelet thromboxane (11-dehydro-Thromboxane B_2) and aspirin response in patients with diabetes and coronary artery disease

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Abstract

Aspirin (ASA) irreversibly inhibits platelet cyclooxygenase-1 (COX-1) leading to decreased thromboxane-mediated platelet activation. The effect of ASA ingestion on thromboxane generation was evaluated in patients with diabetes (DM) and cardiovascular disease. Thromboxane inhibition was assessed by measuring the urinary excretion of 11-dehydro-thromboxane B_2 (11dhTxB_2), a stable metabolite of thromboxane A_2. The mean baseline urinary 11dhTxB_2 of DM was 69.6% higher than healthy controls (P = 0.024): female subjects (DM and controls) had 50.9% higher baseline 11dhTxB_2 than males (P = 0.0004), while age or disease duration had no influence. Daily ASA ingestion inhibited urinary 11dhTxB_2 in both DM (71.7%) and controls (75.1%, P < 0.0001). Using a pre-established cut-off of 1500 pg/mg of urinary 11dhTxB_2, there were twice as many ASA poor responders (ASA "resistant") in DM than in controls (14.8% and 8.4%, respectively). The rate of ASA poor responders in two populations of acute coronary syndrome (ACS) patients was 28.6 and 28.7%, in spite of a significant (81.6%) inhibition of urinary 11dhTxB_2 (P < 0.0001). Both baseline 11dhTxB_2 levels and rate of poor ASA responders were significantly higher in DM and ACS compared to controls. Underlying systemic oxidative inflammation may maintain platelet function in atherosclerotic cardiovascular disease irrespective of COX-1 pathway inhibition and/or increase systemic generation of thromboxane from non-platelet sources.

Key words: Diabetes; Cardiovascular disease; Platelets; Thromboxane; Aspirin

Core tip: The effect of aspirin (ASA) on platelet thromboxane (11dhTxB_2) generation in diabetes (DM) and symptomatic cardiovascular disease (CVD) was reviewed. Consistent with a heightened platelet hyperactive background, baseline 11dhTxB_2 was significantly higher in DM and acute coronary syndrome (ACS) than healthy individuals. ASA ingestion inhibited 11dhTxB_2 in all subjects, but there were more ASA poor-responders (ASA "resistant") in DM (14.8%) and ACS (28.7%) than controls (8.4%). Only post-ASA 11dhTxB_2 levels predicted adverse cardiovascular outcomes. ASA poor-

responders had higher isoprostane (8-isoPGF$_{2\alpha}$) levels suggesting an underlying systemic oxidative inflammatory process not affected by ASA that may maintain platelet hyperactivity in DM and atherothrombotic CVD.

Lopez LR, Guyer KE, Garcia De La Torre I, Pitts KR, Matsuura E, Ames PRJ. Platelet thromboxane (11-dehydro-Thromboxane B$_2$) and aspirin response in patients with diabetes and coronary artery disease. *World J Diabetes* 2014; 5(2): 115-127 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/115.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.115

INTRODUCTION

Thromboxane A2 (TxA$_2$) is a clinically important prostaglandin metabolite derived from arachidonic acid through the cyclo-oxygenase (COX) pathway with roles in hemostasis and cardiovascular disease (CVD)[1,2]. Platelet enzyme COX-1 converts arachidonic acid into prostaglandin G$_2$ (PGG$_2$), followed by the action of peroxidases into PGH$_2$ and into the biologically active TxA$_2$ by thromboxane synthases[3]. Mainly produced by stimulated platelets, TxA$_2$ behaves as a vasoactive agent that affects blood flow and pressure[4] as well as a pro-thrombotic agent capable of promoting the activation and subsequent aggregation of nearby platelets. The latter function is accomplished by TxA$_2$ binding to thromboxane platelet receptors (TPR), a typical G protein-coupled receptor system with trans-membrane segments. Once bound to TPR receptors, phospholipase C is activated to stimulate cytoplasmic Ca^{2+}-dependent Rho Kinases that activate phospholipase A$_2$ and the up-regulation and expression of glycoprotein complex GP Ⅱb/Ⅲa on the surface of platelets[5,6].

Because TxA$_2$ is the bioactive and clinically relevant pro-thrombotic thromboxane metabolite, it would be the logical choice for testing in the clinical laboratory. However, its high instability and very short half-life (20-30 s) makes the routine measurement technically difficult and impractical. Indeed TxA$_2$ is quickly hydrolyzed into a biologically inactive but more stable thromboxane B$_2$ (TxB$_2$) metabolite[7]. Serum TxB$_2$ may be measured in the laboratory but its concentration can be overestimated due to *ex vivo* platelet activation during blood collection and processing. Other serum factors may also interfere with TxB$_2$ measurements. TxB$_2$ is further metabolized by the liver primarily into an 11-dehydro-thromboxane B$_2$ (11dhTxB$_2$) form. This and other minor stable metabolites like 11dehydro-2,3-dinorTxB$_2$ and 2,3-dinorTxB$_2$ are excreted in the urine (Figure 1). Urinary 11dhTxB$_2$ directly reflects the platelet production of TxA$_2$[8,9], and represents a good and reliable biomarker for the laboratory assessment of platelet activity.

Aspirin (Acetylsalicylic acid, ASA) irreversibly acetylates platelet COX-1 for the entire life cycle of the platelet. Ingestion of low doses of ASA blocks over 95% of platelet COX-1 activity resulting in the inhibition of TxA$_2$ production. For these reasons, ASA is widely prescribed as an aid in the primary and secondary prevention of CVD. Despite its widespread use, not all individuals respond to ASA in the same way[10,11]. In addition, ASA effectiveness is limited because over 15%-25% of patients with arterial thrombosis may develop recurrent vascular events while on ASA treatment. This incomplete ASA response (or poor-responsiveness) to therapeutic doses has been referred to as "aspirin (ASA) resistance", a phenomenon described in healthy populations as well as in patients with diabetes (DM) and CVD. The exact mechanisms responsible for this clinical unresponsiveness remain unclear[12,13].

Currently, ASA is largely prescribed for the primary prevention of cardiovascular events in DM but the evidence supporting its efficacy is surprisingly scarce and controversial[14-16]. Recent observations demonstrate that healthy subjects and DM patients with poor ASA response not only seem to manifest an incomplete inhibition of COX-1, but also display a pro-inflammatory milieu and enhanced oxidative stress[17-19]. On the other hand, diet-induced weight loss in subjects with central obesity reduced platelet reactivity and restored platelet sensitivity to nitric oxide, prostacyclin, and physiologic anti-aggregating agents. High on-ASA Platelet Reactivity (HAPR) has been proposed as a more appropriate term than "ASA resistance" to describe a high platelet reactivity status despite ASA therapy in an individual patient. Further, HAPR has been associated with atherothrombotic events following major vascular procedures and may identify patients at high risk for re-occlusion following percutaneous intervention (PCI) with stenting[20].

11DHTXB$_2$ DETERMINATION AND ASA RESPONSE

There are two distinct groups of tests commonly used to measure platelet activity and response to ASA. The first group is blood-based and relies on platelet aggregation response to exogenous agonists or inhibitors by various means[21]. Because platelet activation or inhibition can be mediated by different receptors and pathways, it is not surprising to see a lack of correlation between the assays[22-24]. The second group of tests consists of serologic or urine-based immunoassays that measure both platelet (COX-1) and non-platelet (COX-2) production of thromboxanes. This discussion will focus on the measurement of urinary 11dhTxB$_2$ as a direct indicator of TxA$_2$ activity and platelet activation. One advantage of this type of assays is that thromboxane production is the primary target of ASA through an effective and irreversible COX-1 inhibition.

11dhTxB$_2$ is a biologically inactive down-stream metabolite of TxA$_2$ with a long (stable) circulating half-life that is readily excreted in the urine and relatively unaffected by *ex vivo* platelet activation and other pre-analytical variables[25,26], hence 11dhTxB$_2$ usefulness as a reliable biomarker to assess platelet activation. Due to its relative

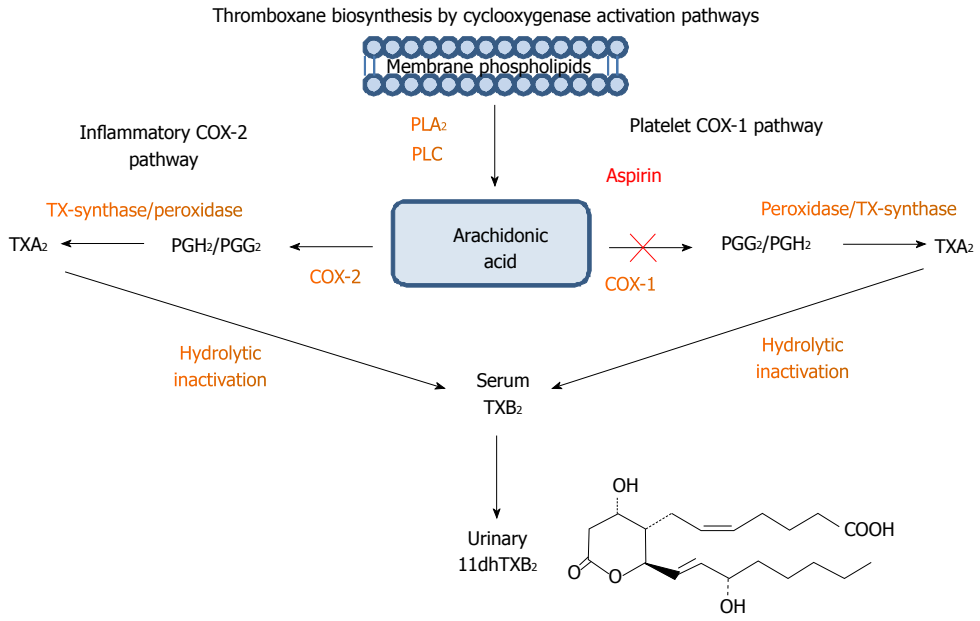


Figure 1 A schematic representation of the arachidonic/thromboxane metabolic pathway: Arachidonic acid generated from membrane phospholipids by phospholipase A2 and phospholipase C undergoes additional enzymatic transformation by cyclooxygenases (COX-1 and COX-2) into prostaglandin and thromboxane metabolites. In platelets, Arachidonic acid (AA) is metabolized by COX-1 into prostaglandins PGG_2, PGH_2 and by thromboxane synthase into the bioactive thromboxane A_2 (TXA_2), which is a potent activator of platelet aggregation with a short half-life. TXA_2 is quickly inactivated into a more stable thromboxane B_2 (TXB_2) and converted in the liver into an 11-dehydro-thromboxane B_2 ($11dhTXB_2$) metabolite excreted in the urine. Aspirin (ASA) irreversibly inhibits platelet COX-1 leading to decreased thromboxane-mediated platelet activation. TXA_2 and $11dhTXB_2$ can be generated by COX-2 present in various inflammatory cells, pathway not affected by ASA.

small size and low concentrations, urinary $11dhTxB_2$ levels are measured by a competitive enzyme-linked immunsorbent assay (ELISA) that uses a spot urine sample without time constraints. Spot urine $11dhTxB_2$ levels are normalized against urine creatinine concentration making the 24 h collection unnecessary. It is important to point out that this ELISA measures the systemic production of thromboxanes (COX-1 and COX-2-derived), and directly reflects COX-1 inhibition by ASA. $11dhTxB_2$ results are first calculated against a reference curve prepared from a reference solution and the final results are reported as pg/mg (pg $11dhTxB_2$ per mg creatinine) to normalize results for urine concentration.

To assess the demographic and clinical variables influencing urinary excretion of $11dhTxB_2$ we first studied apparently healthy adults before and after receiving controlled doses of ASA. Based on the resulting frequency of $11dhTxB_2$ levels, we established a cut-off value to assess an adequate ASA response at 1500 pg/mg of $11dhTxB_2$. This cut-off has been re-confirmed in subsequent studies using both healthy and diseased populations before and after ASA ingestion[27]. Those individuals with urinary $11dhTxB_2$ levels after ASA ingestion below the cut-off of 1500 pg/mg are considered good ASA responders while those with levels above 1500 pg/mg are poor ASA responders ("ASA resistance"). It is important to assess high platelet reactivity in spite of ASA ingestion because a series of actions may be undertaken to manage and reverse the incomplete effect of ASA. The rest of this discussion will focus on a series of clinical studies performed on DM and coronary artery disease (CAD) patients measuring $11dhTxB_2$ and using the quoted 1500 pg/mg cut-off to assess the significance of the ASA response in the development of CVD complications.

ASA poor response or "resistance": definition and clinical implications

ASA "resistance" has been referred to as the lack of a clinical and/or laboratory beneficial effect from ASA ingestion[28-30]. A true or complete ASA "resistance", defined as a lack of response to ASA ingestion due to pharmacologic and/or genetic deficiencies, has not been described to date. The great majority of individuals respond to ASA ingestion as defined by *ex vivo* measurements of platelet aggregation or thromboxane production. However, in most individuals the response seems to be only partial or incomplete. From the clinical point of view, the term ASA "resistance" has neither been fully described nor properly standardized, thus it lacks a nosological clinical definition. Furthermore, consensus guidelines for treatment or management of ASA resistance have not been put forward[31]. Most experts prefer the term ASA "poor or incomplete response" or ASA "insensitivity" to the term ASA "resistance". Throughout this discussion, we will occasionally use the term ASA "resistance" but with the clear understanding that we definitely prefer "poor or incomplete" ASA response as a more appropriate term.

Increased platelet turnover, platelet activation by alternative pathways, alternative/additional sources of TxA_2 production such as macrophage/monocyte COX-2, drug bioavailability, and genetic polymorphisms, have

been implicated in ASA poor responsiveness[13]. Recent reports suggested that CAD patients with high serum concentrations of cholesterol, triglyceride and C-reactive protein had reduced response to ASA measured by platelet aggregation and urinary 11dhTxB$_2$[29]. Compared to asymptomatic patients, those with full blown CAD had significantly higher levels of urinary 11dhTxB$_2$ following ASA ingestion. The HOPE[32] and CHARISMA[33] studies showed that urinary 11dhTxB$_2$ levels in ASA-treated patients predicted the future risk of stroke, myocardial infarction and cardiovascular death. These findings raised the possibility that elevated urinary 11dhTxB$_2$ excretion identifies patients on ASA treatment that are at elevated risk of adverse events and may benefit from additional anti-platelet agents or treatment modification.

Patients who experience a vascular ischemic event while taking ASA have been referred to as having a clinical ASA "resistance". Patients who show a limited inhibition of thromboxane levels, platelet activation, or aggregation after ASA ingestion assessed by biochemical or laboratory tests are referred to as having a laboratory ASA "resistance"[30]. This discussion focuses on the biochemical or laboratory ASA resistance, and more specifically on the clinical impact of the reduced inhibition of COX-1 thromboxane levels. As with any other drug, the dose, drug interference and poor patient compliance should be kept in mind when evaluating ASA responsiveness. The prevalence of laboratory ASA "resistance" ranges from 10% to 25% with occasional peaks up to 60%. However, this wide variability depends on the methods used to measure the ASA response and the patient population under study rather than on the individual response. Nonetheless, other important causes of a poor response to ASA are emerging amongst which is stress-induced inflammation/oxidation[34,35].

An overall poor response to ASA has been associated with up to 13-fold increase risk of atherothrombotic complications in patients with CVD[13,36,37]. A recent meta-analysis of over 20 clinical studies performed on a total of 2930 CVD patients taking ASA (75-325 mg) demonstrated a 4-fold increased risk for any cardiovascular (CV) event including CV death in those patients with poor ASA response[38]. About twenty-eight percent (28%) patients were classified as ASA poor responders ("resistance") suggesting an association with CVD risk. CV-related events were observed in 41%, death in 5.7%, and acute coronary syndrome (ACS) in 39.4% of patients with poor ASA response. It must be pointed out that the clinical studies included in the meta-analysis used different methods to measure platelet ASA inhibition and their own criteria to classify the response to ASA. An interesting observation of the meta-analysis is that ASA poor responders did not benefit from other anti-platelet therapy. The HOPE[32] study screened 5529 patients and measured urinary 11dhTxB$_2$ in 488 ASA-treated CVD patients. Age and sex matched controls also received ASA. CV outcomes including CV death were recorded during a 5-year follow-up. Poor ASA responders with urinary 11dhTxB$_2$ levels in the upper quartile had a 2-fold increasing risk of heart attacks and 3.5-fold risk of CV death. A sub-study of CHARISMA[33] that included 3261 ASA treated CVD patients confirmed the increased CVD risk in patients with 11dhTxB$_2$ in the upper quartile as previously reported by the HOPE study.

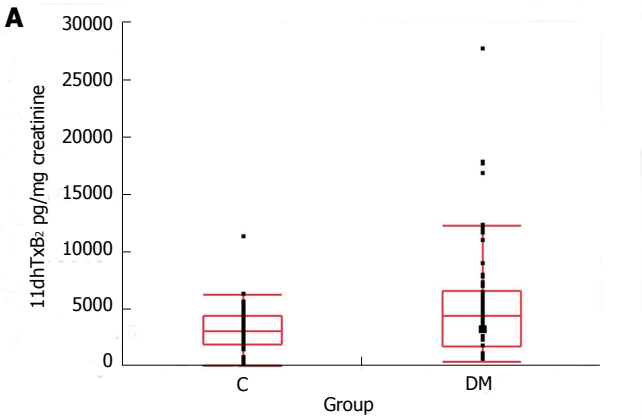
11DHTXB$_2$ AND ASA RESPONSE IN DM

CVD has been long recognized as a leading cause of morbidity and mortality in patients with type 1 and type 2 DM mainly by ischemic heart disease[39,40]. The use of ASA is known to reduce future secondary events in DM[41], however, a meta-analysis of randomized controlled trials failed to demonstrate a clear benefit of aspirin in the primary prevention of major cardiovascular events in patients with DM[42]. To further assess thromboxane levels and aspirin response in DM patients, two clinical studies were conducted on consecutive type 2 DM patients attending the endocrinology and diabetes outpatient clinics in Mexico. The diagnosis of DM was made by the attending physician following internationally accepted diagnostic criteria (World Health Organization DM criteria, 1985) that relied on the presence of abnormal fasting glucose (normal range 70-110 mg/dL), abnormal glucose tolerance test, chronic hyperglycemia and metabolic disturbances of lipid, carbohydrate and protein metabolism due to defects in insulin production or activity. Males and females between 18 and 79 years of age who had not taken ASA or other non-steroidal anti-inflammatory drugs for the previous 2 wk were included. Subjects with liver and kidney disease, symptomatic cardiovascular disease requiring ASA therapy (myocardial infarction, angina, stroke, peripheral artery disease), concomitant acute or chronic inflammatory diseases (bacterial or viral infections), autoimmune disorders, pregnancy, allergy or intolerance to ASA, and bleeding disorders were excluded. The use of ASA in Mexican patients with DM for primary prevention of CVD was significantly less common compared to the US, ensuring a good recruitment of DM patients not taking ASA while avoiding possible unethical discontinuation of the medication.

Baseline 11dhTxB$_2$ levels in DM

Baseline (ASA-free) urinary 11dhTxB$_2$ levels were measured in 100 subjects, 53 with DM and 47 healthy volunteers. None of the patients or controls in this group had received ASA for at least 2 wk prior to testing. The main objective of this study was to establish an average baseline urinary 11dhTxB$_2$ level in DM. The hypothesis was that patients with DM had increased baseline urinary 11dhTxB$_2$ levels hence a higher risk of developing cardiovascular atherothrombotic complication and would receive ASA therapy compared to healthy controls. The mean age of the population studied was 53.9 ± 12.6 years (54 females, 46 males) with mean disease duration of 9.1 ± 7.7 years.

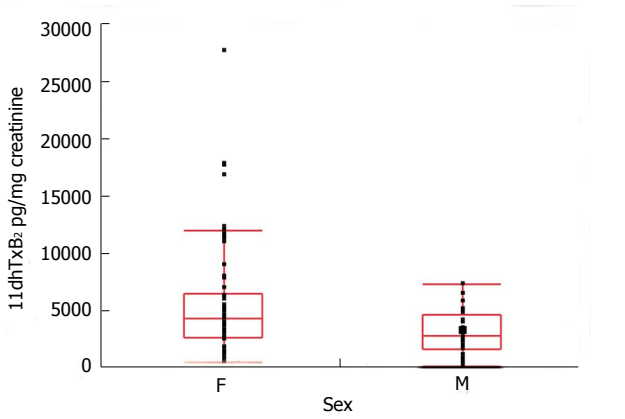
The distribution of baseline (ASA-free) 11dhTxB$_2$ levels of DM patients and healthy volunteers is shown in Figure 2A. DM patients presented with a baseline mean



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars.

Group	11dhTxB2 pg/mg			*P* value[1]
	Mean ± SD	Range	Median	
Diabetes (*n* = 53)	5656 ± 5257	524-27661	4511	0.024
Controls (*n* = 47)	3337 ± 1859	200-11323	3113	

[1]*P* value: Wilcoxon/Kruskal-Wallis test.



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars.

Gender	11dhTxB2 pg/mg			*P* value[1]
	Mean ± SD	Range	Median	
Females (*n* = 54)	5902 ± 5083	524-27661	4364	0.0004
Males (*n* = 46)	2998 ± 1833	200-7333	2891	

[1]*P* value: Wilcoxon/Kruskal-Wallis test.

Figure 3 Distribution of baseline (aspirin-free) urinary 111-dehydro-thromboxane B2 levels (pg/mg) measured in healthy individuals and diabetes patients according to gender. F: Females; M: Males.

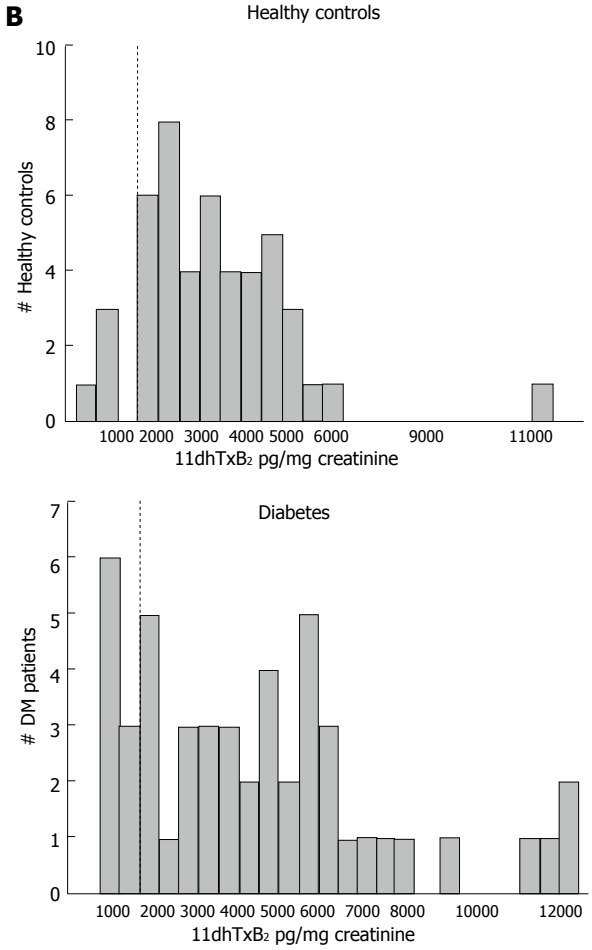


Figure 2 Distribution of baseline (aspirin-free) urinary 11-dehydro-thromboxane B2 levels (pg/mg) measured in healthy individuals and diabetes patients (A, top), and frequency distribution (histogram) of baseline urinary 11-dehydro-thromboxane B2 levels in the two groups studied (B, bottom). A: Comparison of baseline 11-dehydro-thromboxane B2 levels of diabetes and controls; B: Frequency (Histogram) of baseline 11-dehydro-thromboxane B2 levels in diabetes and controls.

urinary 11dhTxB2 excretion of 5656 pg/mg, a value 69.5% higher than the mean baseline 11dhTxB2 excretion of healthy controls at 3337 pg/mg, ($P = 0.024$). The highest 11dhTxB2 value seen in the DM group reached 27661 pg/mg while the highest value in healthy controls was 11323 pg/mg. Figure 2B shows the cumulative baseline frequency of urinary 11dhTxB2 excretion of healthy controls (up) and DM patients (down). The frequency of healthy controls followed a normal (Gaussian-like) distribution while DM patients had a distinctive flat distribution.

Influence of gender, age and disease duration on 11dhTxB2 levels

There were 34 females plus 19 males with DM, and 20 females plus 27 males in the healthy control group. Figure 3 depicts the urinary baseline (ASA-free) 11dhTxB2 levels according to gender. When evaluating all 100 subjects (DM patients and healthy controls), females exhibited a mean baseline urinary 11dhTxB2 excretion 50.9% higher than that of males (5902 *vs* 2998 pg/mg, $P = 0.0004$). When evaluating the influence of gender separately in DM patients and healthy controls, females consistently display significantly higher baseline 11dhTxB2 levels than males (DM $P = 0.01$, controls $P = 0.02$).

The mean age of DM patients was 56 years (range 29-80 years) and that of healthy controls 35 years (range 22-82 years). Figure 4 depicts the association of urinary baseline (ASA-free) 11dhTxB2 levels with the subject's age (in years). In this analysis all 100 subjects (DM patients and healthy controls) were included. The mean disease duration for the DM patients was 9.6 years (range 1-29

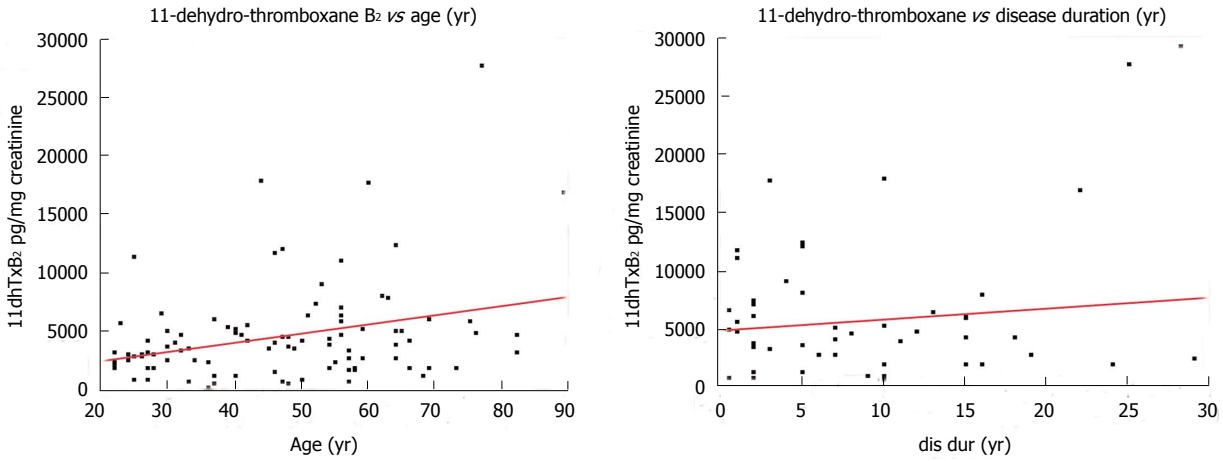


Figure 4 Correlation of baseline (aspirin-free) urinary 11-dehydro-thromboxane B2 levels (pg/mg) measured in healthy individuals and diabetes patients with age (left), and disease duration of diabetes patients (right). Red lines: Linear regression fit.

years). There was weakly positive correlation (r = 0.322, P = 0.001) between age (in years) and baseline urinary 11dhTxB2 levels (left), and a weak (but not statistically significant) positive correlation (r = 0.124, P = 0.3) between disease duration (in years) and baseline urinary 11dhTxB2 levels (right).

These variables were entered into a linear regression model to predict 11dhTxB2 levels (as a dependent variable). Only female gender remained as a significant (P = 0.02) predictor of 11dhTxB2 levels. Sex-related differences in platelet function and aspirin pharmacokinetics in rabbits and man have been previously described[43]. These results support previous findings that ASA reduces the risk of first heart attack in men but not in women suggesting that the ASA effect in women is different[42,44,45]. The results also support the relevance of measuring urinary 11dhTxB2 levels in DM patients to assist health care providers in assessing the risk for CVD and implementing an ASA preventive regimen.

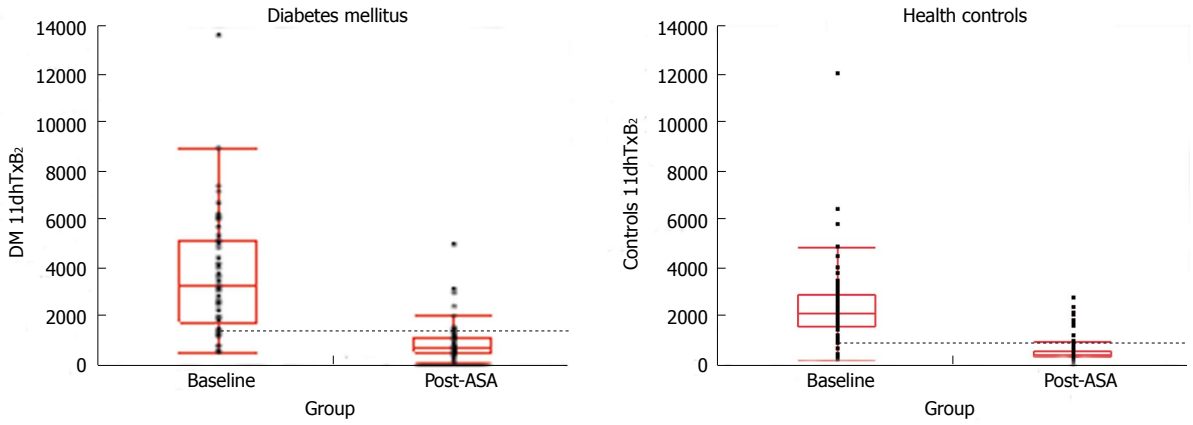
Effect of ASA on 11dhTxB2 levels in DM

The effect of ASA in DM was studied in 137 subjects, 54 patients with DM and 83 healthy volunteers. Each DM patient or control subject contributed two urine samples: one before receiving ASA (baseline) and a second sample after receiving 100 or 325 mg of ASA for 7 d. The main objective of the study was to corroborate that ASA ingestion reduces 11dhTxB2 levels in DM patients. The hypothesis was that ASA would inhibit urinary 11dhTxB2 excretion in DM patients but with more ASA non-responders (11dhTxB2 levels > 1500 pg/mg) compared to healthy controls. The mean age of the final population under study was 54.3 ± 13.1 years with 90 females and 47 males. The mean disease duration was 9.6 ± 7.6 years.

The baseline (ASA-free) and post-ASA ingestion values of DM patients and healthy controls are shown in Figure 5. ASA ingestion suppressed the mean baseline 11dhTxB2 excretion of DM patients by 71.5% (P < 0.0001) as well as the mean baseline of healthy controls (75.1%, P < 0.0001). The baseline 11dhTxB2 excretion of DM patients was greater than that of controls (3664 *vs* 2450 pg/mg, P = 0.001). Similarly, post-ASA 11dhTxB2 excretion of DM patients was greater than that of healthy controls (995 *vs* 624 pg/mg, P < 0.0001). Regarding the effect of the dose of ASA, the mean 11dhTxB2 excretion of subjects taking 100 mg of ASA was 708 pg/mg ± 507, whereas the mean of subjects taking 325 mg was 827 pg/mg ± 811 (P = 0.8). In this study, ASA dose used in DM and healthy controls had no significant influence of post-ASA 11dhTxB2 levels. Furthermore, a regression model to predict 11dhTxB2 levels (as a dependent variable) showed ASA (P = 0.0293) and obesity (P = 0.0467) as statistically significant predictors of 11dhTxB2 levels.

11dhTxB2 excretion shifted below the cut-off (1500 pg/mg) after ASA treatment in the majority of healthy controls, leaving 8.4% (7/83) of subjects classified as non-responders. In DM patients, 11dhTxB2 excretion shifted below the cut-off (1500 pg/mg) after ASA ingestion in the majority of patients, except for 14.8% (8/46) of subjects subsequently classified as ASA non-responders. These results confirm that ASA treatment significantly inhibits baseline urinary 11dhTxB2 levels in both healthy individuals and DM patients, However, there were twice as many ASA poor responders among the DM patients possibly implicating a high platelet reactive phenotype associated with DM [40,46,47].

Having established that DM patients express elevated baseline levels of 11dhTxB2 and twice as many ASA non-responders, we investigated the effect of oxidative stress and anti-oxidant biomarkers on 11dhTxB2 excretion in DM[35]. Urinary 8-iso-prostaglandin-F2α (8-isoPGF2α) and sP-Selectin, nitrite (NO_2^-), nitrate (NO_3^-) and paraoxonase 1 (PON1) activity were measured in baseline (ASA free) and post-ASA samples from these DM patients and controls. Compared to controls, DM expressed increased levels of 8-isoPGF2α (1457 *vs* 1009 pg/mg, P < 0.0001), NO_2^- (11.8 *vs* 4.8 μmol/L, P < 0.0001), NO_3^- (50.4 *vs* 20.9 μmol/L, P < 0.0001) and sP-Selectin (120.8 *vs* 93.0 ng/mL, P = 0.02). ASA demonstrated no effect on 8-isoPGF2α, NO_2^-, NO_3^-, sP-Selectin or PON1 activity in either DM or controls. Again, higher urinary 11dhTxB2 levels in DM suggest a state of heightened platelet acti-



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars. Horizontal broken line represents the 1500 pg/mg cut-off.

	11-dehydro-thromboxane B_2 pg/mg				
Group	Mean ± SD	Range	Median	*P* value[1]	% ASA poor resp
DM baseline (*n* = 54)	3665 ± 2465	508-13578	3255	< 0.0001	
DM post-ASA	996 ± 845	50-5016	693		14.8
Control baseline (*n* = 83)	2450 ± 1572	212-12082	2180	< 0.0001	
Control post-ASA	624 ± 509	37-2834	457		8.4

[1]*P* value: paired *t* test.

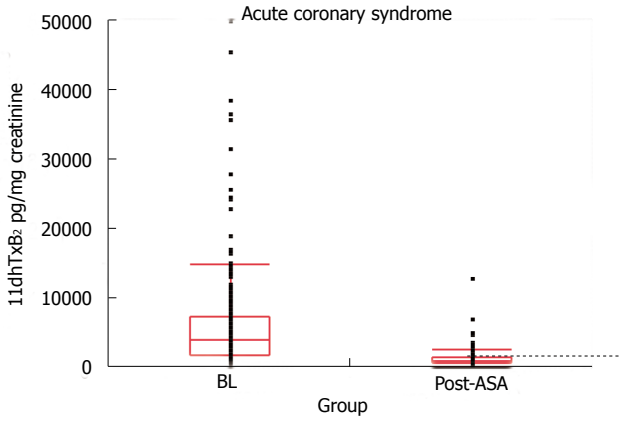
Figure 5 Distribution of baseline (aspirin-free) and post-aspirin urinary 11-dehydro-thromboxane B_2 levels (pg/mg) measured in healthy individuals (right) and diabetes patients (left). 14.8% of diabetes patients were classified as aspirin (ASA) poor responders compared to 8.4% of healthy controls (post-ASA 11-dehydro-thromboxane B_2 over the cutoff 1500 pg/mg).

vation. In addition to platelet hyperactivity, DM patients presented with an inflammatory/oxidative background not affected by ASA. In fact, among the biomarkers measured, only urinary 8-isoPGF$_{2\alpha}$ was significantly higher ($P < 0.009$) in DM patients with poor ASA response. These findings are in agreement with the hypothesis that an oxidative and inflammatory stress may maintain platelet activation irrespective of COX-1 pathway inhibition and/or increase the systemic generation of thromboxane from non-platelet sources *via* COX-2 pathway[34,48-50].

ASA treatment for CVD prevention is a widely accepted practice according to recommended guidelines, but evidence supporting its efficacy is somewhat conflictive and scarce, particularly for patients with DM[51]. The JPAD study (Japanese Primary Prevention of Atherosclerosis with ASA for Diabetes) involved 2539 type 2 DM patients between 40-85 years with no history of atherosclerosis randomized into ASA (81 or 100 mg/d) or non-ASA groups. ASA did not demonstrate a significant reduction in risk for any of the CVD-related endpoints. The POPADAD study (Prevention of Progression of Arterial Disease and Diabetes) included 1276 adults (> 40 years) with type 1 or 2 DM asymptomatic for CVD (ankle-brachial index less than 0.99). ASA (100 mg/d) also failed to demonstrate a significant reduction in risk for any CVD endpoint. Finally, the AAA study (Aspirin for Asymptomatic Atherosclerosis) included 3350 adults (50-75 years) asymptomatic for CVD (ankle-brachial index less than 0.95). ASA (100 mg/d) again did not demonstrate a significant reduction in risk for any endpoint. These studies suggest that DM somehow blunts the beneficial effect of ASA in CVD prevention. Additional mechanisms to explain these clinical findings are forthcoming and likely will help clarify the controversy surrounding the concept of clinical ASA "resistance".

11DHTXB$_2$ AND ASA RESPONSE IN ACS

Two clinical studies of ACS patients will be discussed. One study measured urinary 11dhTxB$_2$ levels after ASA ingestion on 77 consecutive patients attending acute care facilities. ACS patients over 18 years of age undergoing elective PCI at the participating institutions were enrolled. All patients were treated with 325 mg of ASA for at least one week. Each patient provided one urine sample while on ASA. The main objective of the study was to assess urinary 11dhTxB$_2$ excretion in response to 325 mg of ASA in relation to the manufacture's cut-off value of 1500 pg/mg established in apparently healthy individuals. The mean levels of urinary 11dhTxB$_2$ after 325 mg of ASA ingestion was 1550 pg/mg. The majority of ACS patients responded to ASA with 11dhTxB$_2$ levels below the cut-off. However, the percent of ASA non-responders in this ACS population was 28.6%. One common question ponders the dose of daily ASA necessary to inhibit COX-1 and overcome ASA poor response. Urinary 11dhTxB$_2$ levels were measured in 71 consecutive patients with stable CAD and randomized to receive 81 mg, 162 mg and 325 mg per day of ASA for 4 wk. The mean 11dhTxB$_2$ decreased from 931 to 763 pg/mg ($P = 0.046$) with increasing doses of ASA. In this study, the rate of ASA poor responders decreased with increasing ASA dosage. This ASA dose-dependent response is in agreement with previous reports by Gurbel *et al*[22]. Thus,



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars. Horizontal broken line represents the 1500 pg/mg cut-off.

	11-dehydro-thromboxane B2 pg/mg				
ACS (n = 287)	Mean ± SD	Range	Median	P value[1]	% ASA poor resp
BL (baseline)	7322 ± 13419	86-142691	4242	< 0.0001	
Post-ASA	1349 ± 1110	228-12797	1035		28.7

[1]P value: Paired t test.

Figure 6 Distribution of baseline (aspirin-free) and post-aspirin urinary 11-dehydro-thromboxane B2 levels (pg/mg) measured in acute coronary syndrome patients. 28.7% of acute coronary syndrome patients were classified as ASA poor responders (post-ASA 11-dehydro-thromboxane B2 over the cutoff 1500 pg/mg). ACS: Acute coronary syndrome; ASA: Aspirin.

ASA dose should be considered when evaluating ASA poor responses.

A second study included 287 consecutive aspirin-free ACS patients admitted to a hospital in Japan for PCI to evaluate a possible association between urinary 11dhTxB2 levels before and after aspirin ingestion with adverse events (AE)[52]. Inclusion criteria included ST elevation myocardial infarction (STEMI), non-STEMI or early onset (within 24 h) invasive revascularization procedure. Upon enrollment and prior to PCI, a baseline (ASA-free) urine sample was obtained, followed by a daily regimen of 100 mg of ASA. Urine samples from ASA-treated patients were collected at hospital discharge (7-14 d) and upon follow up at 6 and 12 mo. Adverse cardiovascular events (AE) were recorded during a 12 mo patient follow-up. Primary end-points included stent thrombosis, Q wave myocardial infarction (QMI), non-QMI, and death (cardiac and non-cardiac). Secondary end-points included stroke, transient ischemic attack (TIA), target lesion revascularization of PCI or CABG, or other vascular event.

The mean age of these ACS patients was 68.9 years. Age did not influence baseline 11dhTxB2 levels (r = 0.060, P = 0.310), but females had significantly higher mean baseline 11dhTxB2 (7675 pg/mg) compared to males (6949 pg/mg, P = 0.0171). The mean baseline ASA-free 11dhTxB2 was 7322 pg/mg for this cohort of ACS patients and was 2-3 times higher than healthy individuals (range 2450-3337 pg/mg). ASA significantly suppressed (81%, P < 0.0001) of baseline 11dhTxB2 levels to 1349 pg/mg at discharge and subsequent time points. The distribution of baseline (before ASA) 11dhTxB2 levels of the ACS patients is shown in Figure 6. In spite of a significant inhibition of 11dhTxB2 by ASA, 28.7% of ACS patients were classified as poor responders by failing to achieve levels below the 1500 pg/mg cut-off. The overall rate of AEs was 17.1%. The rate of AEs according to baseline (ASA-free) 11dhTxB2 levels decreased slightly from 19.4% in quartile 1 to 15.5% in quartile 4. In contrast, the rate of AEs in ASA treatment quartiles increased from 9.1% in quartile 1 to 24.2% in quartile 3 and 20% in quartile 4. The relative risk for AEs of quartile 3 was 2.7 (P = 0.019). When upper quartiles (3 and 4) were compared to lower quartiles (1 and 2), the relative risk was 2.1 (P = 0.011).

High baseline 11dhTxB2 levels were consistent with an underlying platelet hyperactivity that may contribute to the development of atherothrombosis. However, baseline ASA-free 11dhTxB2 levels did not predict 1-year AEs. High levels (> 1500 pg/mg) of 11dhTxB2 after ASA ingestion likely represent extra-platelet (*i.e.*, monocyte/macrophage-derived) COX-2 production of thromboxane. The increased relative risk (2.7) for AEs associated with high post-ASA 11dhTxB2 levels (upper quartiles) suggest that COX-2 production of thromboxane may be a factor associated with a cardiovascular inflammatory process. It is important to point out that ASA insensitive thromboxane generation has been associated with a pro-inflammatory milieu and enhanced oxidative stress in diabetes. Among several biomarkers tested, only baseline urinary 8-isoPGF2α discriminated between normal and poor thromboxane responders, suggesting that oxidative stress may maintain platelet function irrespective of COX-1 inhibition and/or increased systemic generation of thromboxane from non-platelet sources. Thromboxane alone may not be directly implicated in atherothrombosis. Nonetheless, these results confirm previous reports that post-ASA urinary 11dhTxB2 may be useful in predicting adverse outcomes in ACS patients.

Oxidative inflammation (stress) refers to prevailing levels of reactive oxygen species (ROS) in biological systems that overcome their removal by cellular or plasma repair (anti-oxidant) mechanisms[53]. The excess of superoxide anion ($O_2^{\bullet}$) produced by inflammatory cells may exert a free radical attack on cell membranes and/or lipoproteins in a process called lipid peroxidation. While the arachidonic acid metabolism mediated by enzymatic (COX) pathways has received most attention, a non-enzymatic free radical pathway is demonstrating relevance. The free radical oxidation of arachidonic acid generates biologically active F2-isoprostanes reflecting the oxidative status of the organism; is considered a reliable marker of oxidative stress *in vivo*; and has been shown to be an independent risk factor for CAD[54,55]. Some *in vitro* studies have demonstrated that 8-isoPGF2α is capable of stimulating platelet activation while other studies described pro-atherogenic properties through its interaction with the thromboxane platelet receptor (TPR). If 8-isoPGF2α binds to TPR, it may also be capable of competing with TxA2 and activating the Ca^{2+}/Rho kinase

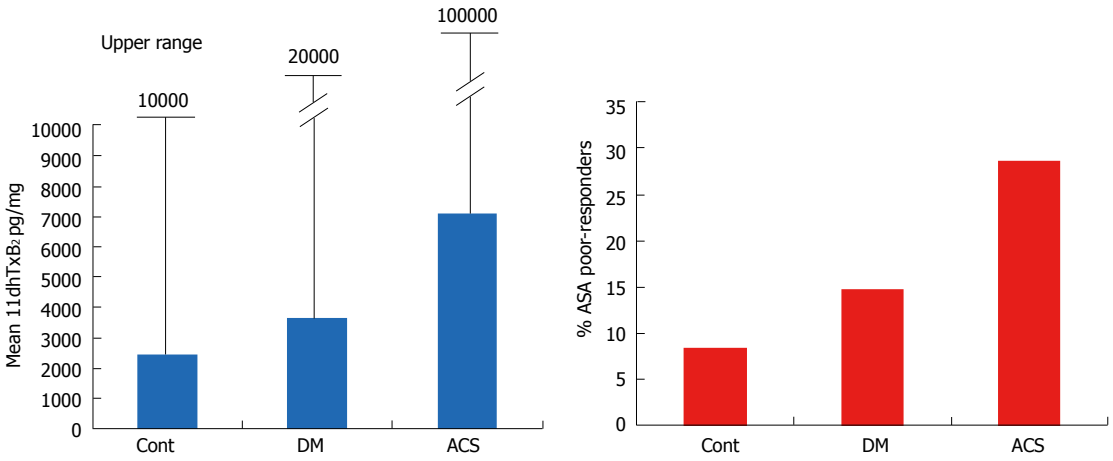


Figure 7 Mean and upper range of baseline (aspirin-free) urinary 11-dehydro-thromboxane B_2 levels (pg/mg) measured in healthy controls, diabetes and acute coronary patients (left), and percent (%) of aspirin poor responders (post-aspirin 11-dehydro-thromboxane B_2 over the cutoff 1500 pg/mg) in the populations studied (right). DM: Diabetes; ACS: Acute coronary syndromes.

pathway[56,57]. This may be particularly important because while TxA_2 enzymatic synthesis is inhibited by ASA, the non-enzymatic 8-isoPGF$_{2\alpha}$ production increases, perhaps as an alternative mechanism to maintain physiologic platelet activity.

Low dose ASA ingestion blocks COX-1 but has no effect on COX-2 or 8-isoPGF$_{2\alpha}$. During an oxidative inflammatory response, increased platelet hyperactivity would come from the combined COX-1, COX 2 and isoprostane (8-isoPGF$_{2\alpha}$) pathways. If ingesting ASA, platelet hyperactivity would be induced by COX-2 and 8-isoPGF$_{2\alpha}$ alone. Limited or no COX-1 TxA_2 production after ASA ingestion would leave unoccupied TPR available to bind 8-isoPGF$_{2\alpha}$ that has a longer half-life (1-10 min *vs* 20-30 s) and higher plasma concentration (351-1831 *vs* 1-66 pg/mL) than TxA_2[6]. Thus, blocking F_2-isoprostane derived from oxidative inflammatory pathways not affected by ASA may be considered in CVD management especially in those individuals with poor ASA response.

CLINICAL SIGNIFICANCE OF 11DHTXB2 MEASUREMENTS

The irreversible inhibition of platelet COX-1 and subsequent reduction of TxA_2 production by ASA has been recognized long ago, making ASA a cost-effective prevention regimen for atherothrombotic CVD. Low doses of ASA have been claimed to prevent over 150000 heart attacks annually. Furthermore, ASA ingestion has accounted for an overall 25% risk reduction of CV events, including a 34% reduction of non-fatal heart attacks, 25% of non-fatal strokes and 18% of all-cause mortality. However, between 25% to 50% of the patients with CAD and ACS did not fully benefit from ASA ingestion[12,13,58]. Thus, TxB_2 measurements to detect those individuals with poor ASA response and higher CVD risk is clinically relevant.

Our studies demonstrated that baseline (ASA-free) urinary 11dhTxB$_2$ excretion showed an upward trend across healthy controls, DM and ACS (Figure 7). The mean 11dhTxB$_2$ of the two control groups studied was 2893.5 pg/mg with an upper range up to 11702 pg/mg. The mean for DM groups was 4660.5 pg/mg with an upper range up to 20619 pg/mg and for ACS patients the mean was 7322 pg/mg with an upper range over 100000 pg/mg. The rate of ASA poor responders had a similar upward trend: controls with 8.4%, DM 14.8% and ACS over 28%.

Baseline 11dhTxB$_2$ levels in both the healthy and diseased populations clearly indicated a wide range of platelet reactivity with a considerable overlap among the groups. This wide range observed likely prevented the establishment of an ASA-free 11dhTxB$_2$ cut-off or even a normal range for clinical use. One relevant observation from the ACS study was that over 40% of ACS patients with high baseline (ASA-free) 11dhTxB$_2$ showed a poor response after ASA ingestion. This observation is in agreement with the concept that higher baseline levels in DM and ACS patients may predict higher rates of ASA poor responders. An explanation for these findings comes from reports that patients with metabolic syndrome (obesity, dyslipidemia, insulin resistance) have increased oxidative stress (oxLDL), higher CVD risk[59], platelet hyperactivity[60] and suboptimal inhibition of platelet COX-1 by aspirin[61], suggesting that higher TxB_2 places these patients at higher risk for thromboembolic events.

Russo *et al*[18] described that diet-induced weight loss in subject with central obesity reduces platelet activation restoring the sensitivity to anti-platelets agents. The Health Aging and Body Composition Study reported that the inflammatory marker interleukin-6 was a robust predictor for new negative health-related events and high urinary 8-isoPGF$_{2\alpha}$ and 11dhTxB$_2$ were associated with higher mortality risk[62]. More recently, Santilli *et al*[63] reported that high intensity physical exercise has broad beneficial effect on platelet activation biomarkers; urinary 11dhTxB$_2$ and 8-isoPGF$_{2\alpha}$ decreased 26% and 21% re-

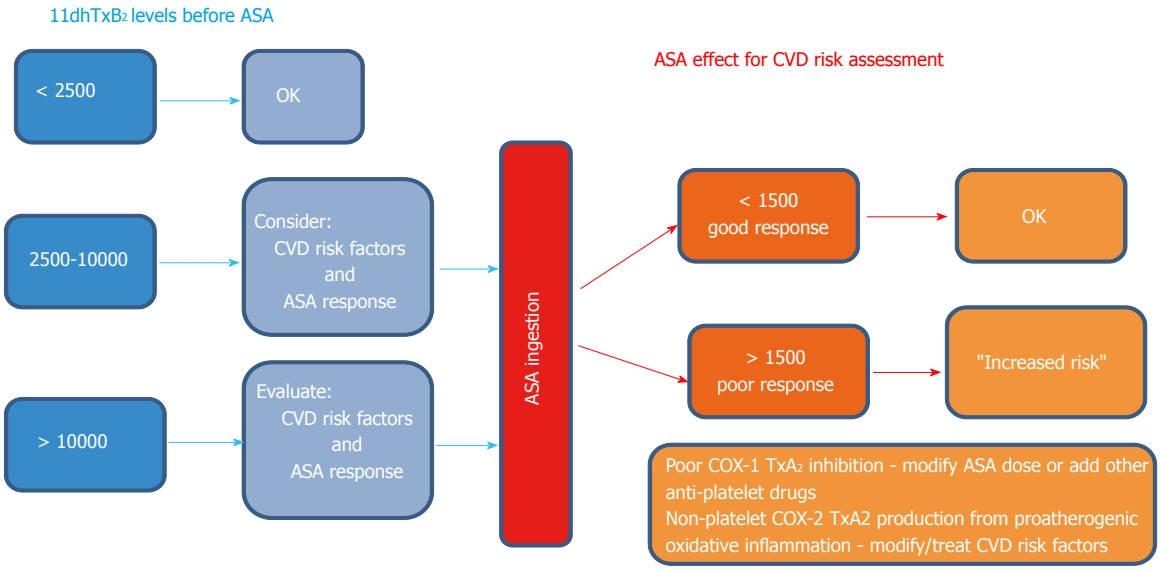


Figure 8 Proposed schematic representation (algorithm) to guide the clinical interpretation and decision making process for assessing CVD risk using baseline (aspirin-free) urinary 11-dehydro-thromboxane B2 (left), and post-aspirin 11-dehydro-thromboxane B2 levels (right). Baseline 11-dehydro-thromboxane B2 levels suggested were taken from the mean and upper range of healthy controls. The cut-off of 1500 pg/mg applies only for subjects on aspirin (ASA) therapy to be classified as good or poor responders.

spectively and esRAGE increased 61% compared to the sedentary control group and multiple regression analysis demonstrated that 8-isoPGF$_{2\alpha}$ and esRAGE were the only significant predictors of 11dhTxB$_2$ levels.

The suggestive algorithm discussed below (Figure 8) was developed taking into account the clinical studies discussed above and is proposed to interpret urinary 11dhTxB$_2$ results for CVD risk management.

If the subject is not taking aspirin and the 11dhTxB$_2$ level is

Below 2500 pg/mg: no action is necessary; Between 2500 and 10000 pg/mg: consider giving ASA to assess ASA response and/or consider other underlying CVD risk factors; Over 10000 pg/mg: give ASA to assess ASA response and/or look for other CVD risk factors.

If the subject is taking ASPIRIN and the 11dhTxB$_2$ level is

Below 1500 pg/mg: no action is necessary (good ASA response), continue monitoring CVD risk; Above 1500 pg/mg: the subject is a poor ASA responder ("resistance"). Consider patient compliance, adjusting ASA dosage, additional anti-platelet therapy, *etc*. And more importantly investigate and modify underlying CVD risk factors such as dyslipidemia and inflammatory/oxidative pro-atherogenic background likely responsible for the incomplete inhibition of thromboxanes.

The major impact of this algorithm is that consistently high baseline 11dhTxB$_2$ levels in subjects not taking ASA may justify further investigations for underlying CVD risks. However, only the presence of post-ASA high 11dhTxB$_2$ levels predicts increased risk of atherothrombotic disease. This highlights the need of a comprehensive (multimodal-approach) management that includes both anti-platelet as well as anti-atherogenic treatments.

CONCLUSION

Poor response to ASA frequently indicates an underlying incomplete COX-1 inhibition and increased CVD risk. Among several assays used to measure ASA effect on platelets, urinary 11dhTxB$_2$ reflects systemic production of thromboxanes and platelet reactivity directly affected by ASA. The incidence of ASA poor responders increases in DM and ACS patients, suggesting an active oxidative/inflammatory background likely responsible for both a continued platelet hyperactivity and a pro-atherogenic phenotype not affected by ASA.

Our studies of urinary 11dhTxB$_2$ levels in response to ASA ingestion in diseased populations indicate the following: (1) patients with DM and CAD have significantly higher mean baseline levels of urinary 11dhTxB$_2$ than healthy controls likely indicating a higher platelet activation and risk for CVD. Female gender seems to have a weak positive influence on 11dhTxB$_2$ and platelet reactivity; (2) ASA ingestion significantly inhibited urinary 11dhTxB$_2$ in DM, ACS and controls. However, the rate of DM ASA poor responders (14.8%) was about 2 times higher than controls (8.4%). This may also be a reflection of an increased platelet activation status in DM patients; (3) the rate of ACS ASA poor responders (28.7%) was about 3 times higher than controls; and (4) The results of the studies provide additional support to the laboratory measurement of urinary 11dhTxB$_2$ levels not only in apparently healthy individuals but also in patients with DM and CAD to assess their response to ASA ingestion.

ACKNOWLEDGMENTS

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TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Recent advances in the molecular genetics of type 2 diabetes mellitus

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Abstract

Type 2 diabetes mellitus (T2DM) is a complex disease in which both genetic and environmental factors interact in determining impaired β-cell insulin secretion and peripheral insulin resistance. Insulin resistance in muscle, liver and fat is a prominent feature of most patients with T2DM and obesity, resulting in a reduced response of these tissues to insulin. Considerable evidence has been accumulated to indicate that heredity is a major determinant of insulin resistance and T2DM. It is believed that, among individuals destined to develop T2DM, hyperinsulinemia is the mechanism by which the pancreatic β-cell initially compensates for deteriorating peripheral insulin sensitivity, thus ensuring normal glucose tolerance. Most of these people will develop T2DM when β-cells fail to compensate. Despite the progress achieved in this field in recent years, the genetic causes of insulin resistance and T2DM remain elusive. Candidate gene association, linkage and genome-wide association studies have highlighted the role of genetic factors in the development of T2DM. Using these strategies, a large number of variants have been identified in many of these genes, most of which may influence both hepatic and peripheral insulin resistance, adipogenesis and β-cell mass and function. Recently, a new gene has been identified by our research group, the *HMGA1* gene, whose loss of function can greatly raise the risk of developing T2DM in humans and mice. Functional genetic variants of the *HMGA1* gene have been associated with insulin resistance syndromes among white Europeans, Chinese individuals and Americans of Hispanic ancestry. These findings may represent new ways to improve or even prevent T2DM.

Key words: Genome-wide association study; Candidate gene; Genetic variants; High-mobility group A1; Insulin resistant diabetes

Core tip: Despite the progress in clinical and laboratory investigations, the fundamental cause of type 2 diabetes mellitus (T2DM) remains uncertain. Candidate gene, linkage and genome-wide association studies have highlighted the role of genetics in the development of T2DM. Using these strategies, a large number of variants have been identified in many genes, most of which may influence an individual's risk of developing T2DM. In this review, we compile information on genetic factors that influence the risk of T2DM. In addition, we discuss the results from recent studies on the role of HMGA1 on the issue, which might be important for future breakthroughs in this field.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic endocrine

and metabolic disease that is often associated with being overweight or frank obesity. It affects millions of people worldwide, with a rapidly increasing incidence and prevalence[1,2]. The latest estimate from the International Diabetes Federation (http://www.idf.org) is equivalent to a global prevalence rate of 8.4% of the adult population, while worldwide diabetes cases hit a new record at 382 million in 2013. Among the determinants of this steadily increasing trend is the combination of genetic and environmental factors responsible for either a positive energy balance resulting in body fat accumulation and weight gain and/or a reduced energy expenditure from a reduction in physical activity and a sedentary lifestyle. Despite extensive attempts at clinical management of T2DM, many diabetic patients will develop a wide variety of long-term complications, including retinopathy, nephropathy and cardiovascular diseases that are among the most frequent causes of morbidity and mortality in affected people, whose effective prevention and treatment require enormous efforts and funding[3]. Typically, T2DM is presented as a common, heterogeneous, complex disease in which both predisposing genetic factors and precipitating environmental factors interact together and cause hyperglycemia, which constitutes the primary hallmark of T2DM[4,5]. Although still poorly understood, the role of genetics in T2DM is well documented. This is supported by a series of evidence, including the strong familial aggregation of the disease, in which the risk of developing T2DM is 40% for those who have an affected parent (higher if the mother rather than the father) and 70% if both parents are diabetics[6]. The highest risk in first-degree relatives, compared to the general population, persists even after removal from the family of origin, for example, as a result of adoption. Furthermore, in identical monozygotic twins (with identical genetic makeup), the concordance rate for the disease approaches 100%, much higher than that seen in non-identical (dizygotic) twins or among siblings[7]. Genetic predisposition in T2DM is also supported by the observation that differences in disease prevalence rates exist among populations, even after migration of entire ethnic groups to another country, thus independent from the environmental influences[8].

On the other hand, the role of environmental factors in influencing susceptibility to T2DM is equally well known. Among these factors are increased caloric intake and a sedentary lifestyle, two conditions common in populations with a higher standard of living and a more westernized lifestyle, responsible for most of the excess weight and obesity in the modern adult's life[9]. The spread of the western way of life in developing countries also explains the epidemic explosion of the disease[1,2], whereas the existing epidemiological data show that the spatial and temporal distribution of T2DM in the geographical areas examined is comparable to the trend of being overweight and obesity[10]. The excess weight causes insulin resistance, which represents the initial step in the natural history of T2DM. Initially, in individuals destined to become diabetic, pancreatic β-cells compensate for the insulin resistance by secreting increased levels of insulin, thus ensuring post-prandial euglycemia[11]. Hyperglycemia in insulin resistant subjects develops later when the β-cells fail to compensate. Thus, from a pathophysiological standpoint, T2DM is characterized by a combination of peripheral insulin resistance and inadequate insulin secretion by the pancreatic β-cells. As supported by numerous studies in the literature[12,13], both defects are the result of a complex interaction between genetic and environmental factors (Figure 1), including chemical agents (calcium and zinc ions) and polluting organic substances that are suspected to play a role in amyloid fiber formation in pancreatic β-cells, thus contributing to the pathology of T2DM[14-17]. The involvement in the pathogenesis of T2DM of multiple genes that interact with each other in an epistatic manner may explain why, despite the enormous efforts made to date, the identification of genetic determinants responsible for an increased susceptibility to T2DM still remains unsolved[18,19].

The present review aims to give an overview of the recent findings in this context. We also discuss the results from some recent studies which might be important for future breakthroughs in this field.

GENETIC STUDIES

Over the past few years, various international research centers have been involved in the study and identification of genes predisposing to T2DM using various methods of investigation. Linkage analysis was used to identify potential genes associated with the disease, starting from the analysis of families and then studying a small number of individuals genetically related to each other. Genotyping for genetic markers in family members with and without T2DM has allowed the identification of DNA regions containing loci associated with disease risk. Thanks to this method, the association of T2DM with the calpain-10 (*CAPN10*) gene[20] was initially identified and later its association with the transcription factor 7-like 2 (*TCF7L2*) gene[21], whose genetic variants in affected individuals increase the risk of diabetes approximately 1.5 times[19].

Another approach used was to search for genetic variants within functional candidate genes encoding for protein(s) with important implications for glucose homeostasis and positional candidate genes that have a genetic association on the basis of a previous linkage study. This experimental strategy is applied to population studies rather than studies of families. Association studies of functional candidate genes represent one of the most powerful approaches as the pathogenetic mechanism of any genetic abnormality would be easily explained. The limit of this strategy, however, is constituted by the fact that it allows focused attention on a single gene at a time. Although many studies have reported associations of functional and positional candidate genes with T2DM, only some of these showed a significant and reproducible association with the disease (Table 1).

From 2007 onwards, the list of candidate genes has grown considerably, largely due to genome-wide associa-

Table 1 Type 2 diabetes mellitus susceptibility genes

Gene	Chr	Odds ratio	RAF	Study	Function and probable mechanism	Ref
ADAMTS9	3	1.09-1.05	0.68-0.81	MA	Metalloproteinase/Insulin action	[22–24]
ADCY5	3	1.12	0.78	MA	Adenylyl cyclases/Insulin action	[25]
ANK1	8	1.09	0.76	MA, CC	Cell stability/β-cell function	[26–28]
ANKRD55	5	1.08	0.7	MA, CC	Insulin action	[26,27]
ANKS1A	6	1.11	0.91	GWAS	Pathway regulator/Unknown	[29]
ARAP1	11	1.08-1.14	0.81-0.88	GWAS, MA	Actin cytoskeleton modulator/β-cell function	[22,24]
BCAR1	16	1.12	0.89	MA, CC	Docking protein/β-cell function	[26,27]
BCL2	18	1.09	0.64	GWAS	Cell death regulator/Unknown	[24]
BCL11A	2	1.08-1.09	0.46	MA	Zinc finger/β-cell function	[22]
CAMK1D	10	1.07-1.11	0.18	LA, MA	Protein kinase/β-cell function	[22–24]
CDC123					Mitotic protein/β-cell function	
CAPN10	2	1.09-1.18	0.73-0.96	MA	Calpain cysteine protease/Insulin action	[30–33]
CDKAL1	6	1.10-1.20	0.27-0.31	GWAS, MA	β-cell function	[24,34–36]
CDKN2A	9	1.19-1.20	0.82-0.83	GWAS	Cyclin-dependent kinase inhibitor/β-cell function	[24,34,35]
CDKN2B						
CENTD2	11	1.08-1.13	0.81-0.88	GWAS	β-cell function	[22,24]
CHCHD9	9	1.11-1.20	0.93	MA	Unknown	[22]
TLE4						
CILP2	19	1.13	0.08	MA, CC	Unknown	[26,27]
DGKB	7	1.04-1.06	0.47-0.54	MA	Diacylglycerol kinase/Insulin action	[24,25]
DUSP9	X	1.09-1.27	0.12-0.77	MA	Phosphatase	[22,24]
FOLH1	11	1.10	0.09	GWAS	Transmembrane glycoprotein/Unknown	[24]
FTO	16	1.06-1.27	0.38-0.41	GWAS, MA	Metabolic regulator/Insulin action	[24,37]
GATAD2A	19	1.12	0.08	GWAS	Transcriptional repressor/Unknown	[24]
GCK	7	1.07	0.20	MA	Glucokinase/Insulin action	[25]
GCKR	2	1.06-1.09	0.59-0.62	MA	Glucokinase regulator/Insulin action	[24,25]
GIPR	19	1.10	0.27	GWAS	G-protein coupled receptor/Unknown	[24]
GRB14	2	1.07	0.60	MA, GCS	Adapter protein/Insulin action	[26,27]
HFE	6	1.12	0.29	MA	Membrane protein/Unknown	[38]
HHEX	10	1.12-1.13	0.53-0.60	AL, MA	Transcriptional repressor/	[22,24,34,39]
IDE					Intracellular insulin degradation/	
KIF11					Motor protein	
HMG20A	15	1.08	0.68	MA, GCS	Chromatin-associated protein/Unknown	[26,27]
HMGA1	6	1.34-15.8	0.10	GCS	Transcriptional regulator/Insulin action	[40-42]
HMGA2	12	1.10-1.20	0.09-0.10	MA	Transcriptional regulator	[22,24]
HNF1A	12	1.07-1.14	0.77-0.85	MA	Pancreatic and liver transcriptional activator	[22,24]
HNF1B	17	1.08-1.17	0.47-0.51	GCS, MA	Transcription factor/β-cell function	[22,24]
IGF2BP2	3	1.14	0.29-0.32	GWAS, MA	Binding protein/β-cell function	[22,24,34,35]
IRS1	2	1.09-1.12	0.64-0.67	GCS, MA	Insulin signaling element/Insulin action	[22,24,43]
JAZF1	7	1.10	0.52	MA	Zinc finger/β-cell function	[22,23]
KCNJ11	11	1.09-1.14	0.37-0.47	GCS, MA	Potassium channel/β-cell function	[22,24,34,44]
KCNQ1	11	1.08-1.23	0.44	GWAS	Potassium channel/β-cell function	[22,45,46]
KLF14	7	1.07-1.10	0.55	MA	Transcription factor/Insulin action	[22]
KLHDC5	12	1.10	0.80	MA, CC	Mitotic progression and cytokinesis/Unknown	[26,27]
LAMA1	18	1.13	0.38	GWAS	Cellular migration mediator/Unknown	[29]
MC4R	18	1.08	0.27	MA, CC	G-protein–coupled receptor/Unknown	[26,27]
MTNR1B	11	1.05-1.08	0.28-0.30	GWAS, MA	Melatonin receptor/β-cell function	[24,47-49]
NOTCH2	1	1.06-1.13	0.10-0.11	MA	Membrane receptor	[22-24]
PPARG	3	1.11-1.17	0.85-0.88	GCS, MA	Nuclear receptor/Insulin action	[22,24,34,50]
PRC1	15	1.07-1.10	0.22	MA	Cytokinesis regulator	[22]
PROX1	1	1.07	0.50	MA	Homeobox transcription factor/Insulin action	[25]
PTPRD	9	1.57	0.10	GWAS	Protein tyrosine phosphatase	[51]
RBMS1	2	1.11-1.08	0.79-0.83	MA	DNA modulator/Insulin action	[24,52]
SLC2A2	3	1.06	0.74	GWAS	Glucose sensor/β-cell function	[24]
SLC30A8	8	1.11-1.18	0.65-0.70	GWAS, MA	Zinc efflux transporter/β-cell function	[22,24,25,34,53]
SREBF1	17	1.07	0.38	GWAS	Lipid transcriptional regulator/Unknown	[24]
SRR	17	1.28	0.69	GWAS	Serine racemase	[51]
TCF7L2	10	1.31-1.71	0.26-0.30	LA, MA,GWAS	Participates in the Wnt signaling pathway/β-cell function	[21,22,24,34]
THADA	2	1.15	0.90	MA	Thyroid adenoma-associated protein/β-cell function	[22-24]
TH/INS	11	1.14	0.39	GWAS	Catecholamine synthesis/Unknown	[24]
TLE1	9	1.07	0.57	MA, CC	Transcriptional corepressor/Unknown	[26,27]
TP53INP1	8	1.06-1.11	0.48	MA	Proapoptotic protein/Unknown	[22]
TSPAN8	12	1.06-1.09	0.27-0.71	MA	Cell surface glycoprotein/β-cell function	[22-24]
LGR5					G-protein coupled receptor/β-cell function	
WFS1	4	1.10-1.13	0.60-0.73	GCS	Transmembrane protein/β-cell function	[22,24,54,55]
ZBED3	5	1.08-1.16	0.26	MA	Zinc finger/b-cell function	[22]

ZFAND6	15	1.01-1.11	0.60-0.72	MA	Zinc finger/β-cell function	[22,24]
ZMIZ1	10	1.08	0.52	MA, CC	Transcriptional regulator/Unknown	[26,27]
Haplogroup B	mtDNA	1.52	0.25	GCS		[56]
OriB	mtDNA	1.10	0.30	MA		[57]

Chr: Chromosome; MA: Meta-analysis; LA: Linkage analysis; GWAS: Genome-wide association study; GCS: Gene candidate study.

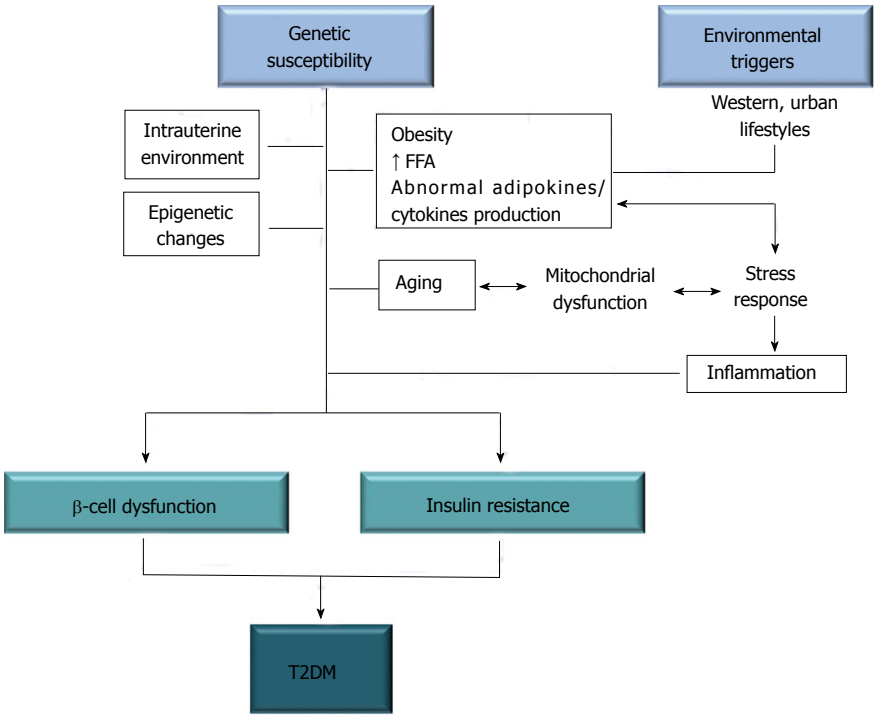

Figure 1 Overview of the pathogenic factors underlying development of type 2 diabetes mellitus. As a complex disease, T2DM is caused by a combination of genetic, environmental and lifestyle factors, all of which interact together to produce insulin resistance and β-cell dysfunction, leading to hyperglycemia, which is the clinical hallmark of diabetes. FFA: Free fatty acids. T2DM: Type 2 diabetes mellitus.

tion studies (GWAS), a technique commonly used to find links between genes and diseases across a substantial population. This strategy uses a database of over a million known genetic variants, which represent the majority of all common variants (minor allele frequency > 5%-10%), thus offering the possibility of simultaneously analyzing thousands of variations in a large number of patients and to perform meta-analysis of data from multiple studies. This methodology has helped to identify dozens of new associations between T2DM and genes with known or unknown functions (Table 1)[22-57], confirming some of the results from previous studies. However, despite the great potential of this approach, it is estimated that genetic variants identified through GWAS explain only 10% heritability for T2DM[58,59]. These relatively modest results can be explained taking into account some important limits of this strategy, such as the involvement of novel genetic variants not yet covered in the GWAS database, or the presence of variants with a frequency lower than the minimum threshold value. This means that the genes identified by GWAS so far are just the tip of the iceberg and that T2DM, far from being a condition limited to a few genetically and phenotypically prevalent forms, actually encompasses a heterogeneous group of genetically distinct disorders[18].

However, in many genetic studies carried out to date, the functional mechanism(s) by which the associated gene may increase susceptibility to T2DM is often poorly understood. In this respect, the intrinsic limitations of both the linkage analysis and GWAS are amplified by the fact that, in most cases, the genetic variants identified are located in non-coding regions of the DNA, whereby it becomes even more difficult to trace the role and influence of the associated gene in the development of the disease. In cases in which it was possible to ascertain the precise pathogenic mechanism, for example, through the study of association with the circulating levels of insulin or through the direct analysis of the gene's protein product, it has been seen that most of the genes identified are involved in pancreatic β-cell mass and/or function, thus with implications in insulin secretion defects (Table 1). This observation suggests that most of the risk associated with T2DM in the general population relates to genetic defects in β-cells, while peripheral insulin resistance predominantly suffers from the environmental component[18,19,60].

GENES INVOLVED IN β-CELL INSULIN SECRETION

Figure 2 depicts some of the genes whose alteration confers an elevated risk of T2DM. Using the analysis of functional or positional candidate genes, several variants have been identified, including polymorphisms of the gene insulin receptor substrate-1 (*IRS-1*)[22,24,43]. The Gly972Arg variant of IRS-1 determines a defect in the

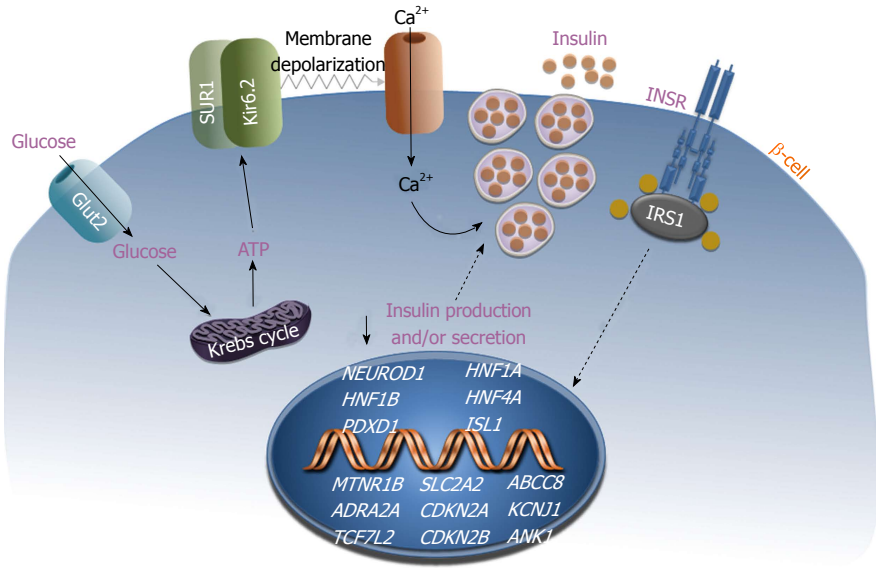

Figure 2 Schematic representation of the pancreatic β-cell. Reduced insulin secretion is shown in β-cells with gene variants linked to T2DM. Genes associated with defects in β-cell mass and/or function are indicated in white italic uppercase. T2DM: Type 2 diabetes mellitus.

binding of the p85 subunit of the phosphatidylinositol 3-kinase (PI3K) which in pancreatic β-cells causes a marked decrease in insulin secretion in response to glucose and sulfonylureas[61]. Other polymorphisms implicated in T2DM have been identified in the *ABCC8* (also known as *SUR1*) and *KCNJ11* genes, whose protein products take place in the formation of the Adenosine triphosphate (ATP)-sensitive potassium channel/sulfonylurea receptor of the pancreatic β-cell. The therapeutic response to sulfonylureas is compromised in patients with mutations in these genes. Other genes whose mutations were initially considered responsible for the less common forms of diabetes mellitus have subsequently been associated with an increased risk of T2DM[19]. Among these are the hepatocyte nuclear factor-1 homeobox A (A*HNF1A*) gene, whose mutations are responsible for the most common monogenic form of MODY (MODY3), a form of maturity onset diabetes of the young (also known as HNF1A-MODY), and the gene hepatocyte nuclear factor-1 homeobox B (*HNF1B*), which determines a less frequent but more severe monogenic form of diabetes, the MODY5. Both of these genes encode nuclear transcription factors involved in the development and function of pancreatic islets.

As already mentioned, the association between *TCF7L2* gene polymorphisms and susceptibility to T2DM was highlighted initially by linkage studies and confirmed thereafter by GWAS. However, only recently has the role played by the transcription factor TCF7L2 in the β-cell insulin secretion become evident[62]. Another gene that has recently been associated with T2DM is the melatonin receptor 1B (*MTNR1B*) gene which encodes for the receptor of the pineal hormone melatonin, MTNR1B, that is involved in the regulation and facilitation of sleep. Genetic variants of the *MTNR1B* gene, associated with gain-of-function of the MTNR1B receptor protein and a reduction in insulin secretion, have been reported in diabetic patients with abnormalities in melatonin secretion and circadian rhythm disorders of the sleep-wake cycle[63]. Another example of genetic abnormality associated with β-cell dysfunction and the risk of T2DM involves the *ADRA2A* gene that encodes for the alpha 2A-adrenergic receptor, which mediates the adrenergic suppression of insulin secretion[60]. Diabetic patients with polymorphisms of the *ADRA2A* gene may have overexpression of the alpha 2A receptor, resulting in insulin secretion deficiency. In pancreatic islets obtained from diabetic patients carrying this variant, pharmacological treatment with alpha (2A)-AR antagonists rescued insulin secretion[64].

Recently, large scale GWAS meta-analyses and imputation-based GWAS studies have demonstrated that the ankyrin 1 gene, a gene encoding for a protein of the ankyrin family, is associated with T2DM in different ethnicities[26-28]. Ankyrin 1 is typically expressed in the erythrocytes and functions as an adaptor molecule between membrane and skeleton proteins. Interestingly, mutations of this gene are known to determine hereditary spherocytosis. How this protein can be implicated in T2DM is not yet understood; however, ankyrin 1 is also expressed in β-cells, where a cognate protein, ankyrin B, plays a role in regulating ATP sensitivity by interacting with the sulphonylurea receptor isoform SUR1.

Another recent study has identified new loci and variants in a large-scale gene-centric meta-analysis that included the *SLC2A2* (solute carrier 2A2) gene[24]. This gene encodes the glucose transporter Glut2, which is expressed in pancreatic β-cells, liver and kidney, and functions as a glucose sensor to maintain glucose homeostasis. These findings support a previously postulated role of Glut2 in T2DM[65]. Also, variants of genes involved in the cell cycle, like the *CDKN2A* and *CDKN2B* (cyclin-dependent kinase inhibitor 2A and 2B) genes, have been associated with T2DM. Although not proved in humans, data from animal models support the idea that these genetic variants may affect β-cell mass later in life[66].

GENES INVOLVED IN INSULIN RESISTANCE

The first step in the mechanism of action of insulin is

the interaction of the hormone with its specific receptor, the insulin receptor (INSR), on the cell surface of insulin responsive cells and tissues (Figure 3). The functional activation of INSR is a key moment in the pathophysiology of insulin action, followed by the selective activation of specific intracellular signaling pathways which are necessary for proper hormonal signal transduction. Although defects in INSR have been reported in a large number of patients with T2DM, mutations in the *INSR* gene have been found only in a small percentage (3%-4%) of these patients in whom genetic defects leading to receptor protein abnormalities were identified as cause of disease. However, certain patients with apparently normal *INSR* genes have reduced expression of both the INSR protein and INSR mRNA levels[13,18,19]. In these patients, it is possible that there are mutations in genes encoding transacting factors which regulate the level of *INSR* gene expression[40].

The mechanisms by which gene variants may impair insulin action in insulin target tissues are schematized in Figure 3. Among the genes involved in insulin resistance are those encoding for the glucokinase regulatory protein, GKRP, and the insulin-like growth factor- I , IGF- I . Genetic variants of these genes that predispose a person to develop insulin resistance have been recently identified by GWAS[25]. In addition, T2DM risk alleles at three loci (at *FTO*, *KLF14* and *PPARG*) have been associated with higher fasting insulin (which is consistent with a primary defect on insulin action) and reduced insulin sensitivity[22]. In particular, variations in the fat mass and obesity-associated (*FTO*) gene appear to influence predisposition to T2DM through a positive effect on body mass index and obesity. Instead, the Krüppel-like factor 14 (*KLF14*) gene is considered a super gene with the ability to control other genes linked to body fat. The risk alleles at *KLF14*, along with those at peroxisome proliferator-activated receptor gamma (*PPARG*), appear to have a primary effect on insulin action which, unlike the alleles at *FTO,* is not driven by obesity[22].

A recently uncovered gene implicated in T2DM is the growth factor receptor-bound 14 (*GRB14*) gene[26,27], which codes for the Grb14 adaptor protein. Grb14 contains a C-terminal SH2 domain implicated in the interaction with a number of tyrosine kinase receptors and signaling proteins, and a domain called BPS (between pleckstrin homology), also required for binding to the INSR. This protein has been shown to specifically attenuate insulin action by inhibiting the catalytic activity of the INSR in insulin target tissues[67]. Many other recently identified diabetes-associated genes play still unknown roles in the pathophysiology of T2DM. Among them, the sterol regulatory element-binding transcription factor 1 (*SREBF1*) gene, which is involved in the transcriptional regulation of lipid homeostasis[24], and the high mobility group 20A (*HMG20A*) gene, which encodes a chromatin-associated protein and has previously been associated with a greater incidence of diabetes in obese subjects[26,27].

THE HIGH MOBILITY GROUP A1 GENE

Among the group of genes recently associated with insulin resistance and T2DM is the *HMGA1* gene, which encodes the architectural transcription factor, High Mobility Group A1 (HMGA1), a nonhistone basic protein that binds to AT-rich sequences of DNA *via* AT hooks, facilitating the assembly and stability of a multicomponent enhancer complex, the "enhanceosome", which drives gene transcription[68]. We previously found that HMGA1 is a key regulator of *INSR* gene expression[69-71] (Figure 4). Consistent with these findings, we identified two patients with insulin-resistant T2DM who had defects in HMGA1 expression and concomitant decreased INSR mRNA and protein in muscle, fat and circulating monocytes[72]. These individuals had normal *INSR* genes but had a novel genetic variant (*c.*369del*) in the 3' noncoding region of the HMGA1 mRNA that contributed to the reduction of mRNA half-life and subsequent decline in HMGA1 expression. Epstein-Barr virus (EBV)-transformed lymphoblasts from these patients demonstrated defects in HMGA1 and INSR expression, indicating that the defects observed *in vivo* were not due to the altered metabolic state of the patients. In addition, the *in vitro* restoration of HMGA1 RNA and protein expression in these cells normalized *INSR* gene expression and restored both cell-surface INSR protein expression and insulin binding capacity[72]. The pathogenetic role of HMGA1 in T2DM was confirmed in genetically modified mice, in which the loss of HMGA1 expression (induced by disrupting the *HMGA1* gene) considerably decreased INSR expression in the major target tissues of insulin action[72], thus supporting the concept that functional *HMGA1* gene variants decrease INSR expression in human and mice.

In the context of these investigations, we later showed that four functional variants of the *HMGA1* gene, leading to reduced INSR expression, were associated with insulin resistance and T2DM[40]. The most frequent functional *HMGA1* variant, c.136-14_136-13insC (also designated rs146052672), was detected in 7%-8% of patients with diabetes in individuals of white European ancestry[40]. Analysis of cultured EBV-transformed lymphoblasts from patients with T2DM and the rs146052672 variant revealed that these cells had lower levels of HMGA1 and INSR protein than cells from either patients with wild-type T2DM or controls. Once again, in transformed lymphoblasts from the patients with the *HMGA1* rs146052672 variant, restoration of HMGA1 protein expression by complementary DNA transfection (in the sense but not antisense direction) restored INSR protein expression and insulin binding to these cells[40]. Although not replicated in a heterogeneous French population[73], the *HMGA1* rs146052672 variant was significantly associated with T2DM among Chinese[41] and Hispanic-American[38] individuals. Further evidence, implicating the HMGA1 locus as one conferring a high cross-race risk for the development of insulin

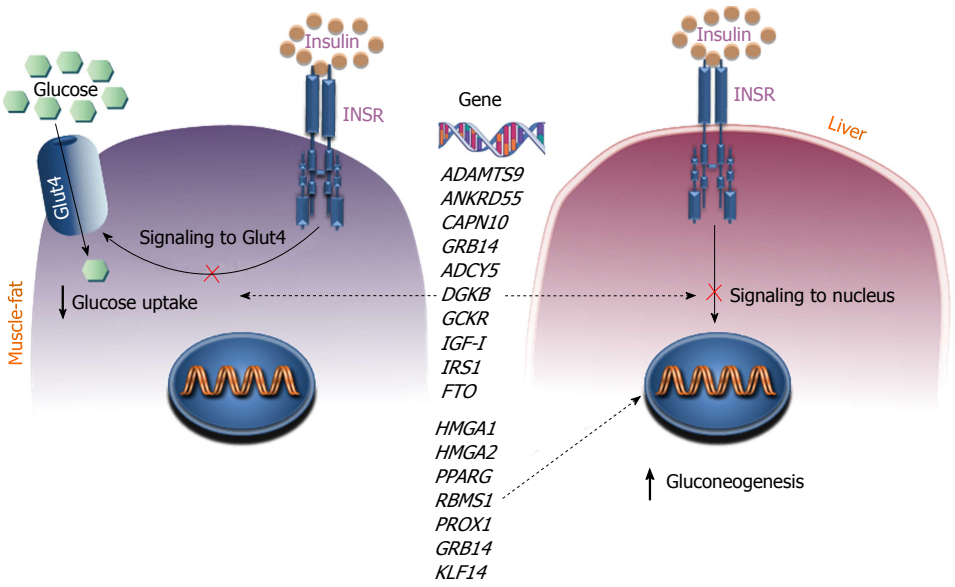

Figure 3 Mechanisms of insulin resistance. The figure shows the mechanisms by which gene variants may impair insulin action in the insulin target tissues muscle, fat and liver. Peripheral insulin resistance in muscle and fat reduces cellular glucose uptake, whereas insulin resistance in liver results in a failure to suppress glucose production and gluconeogenesis. Genes whose variations can influence the risk of developing insulin resistance and T2DM are indicated in black italic uppercase. T2DM: Type 2 diabetes mellitus.

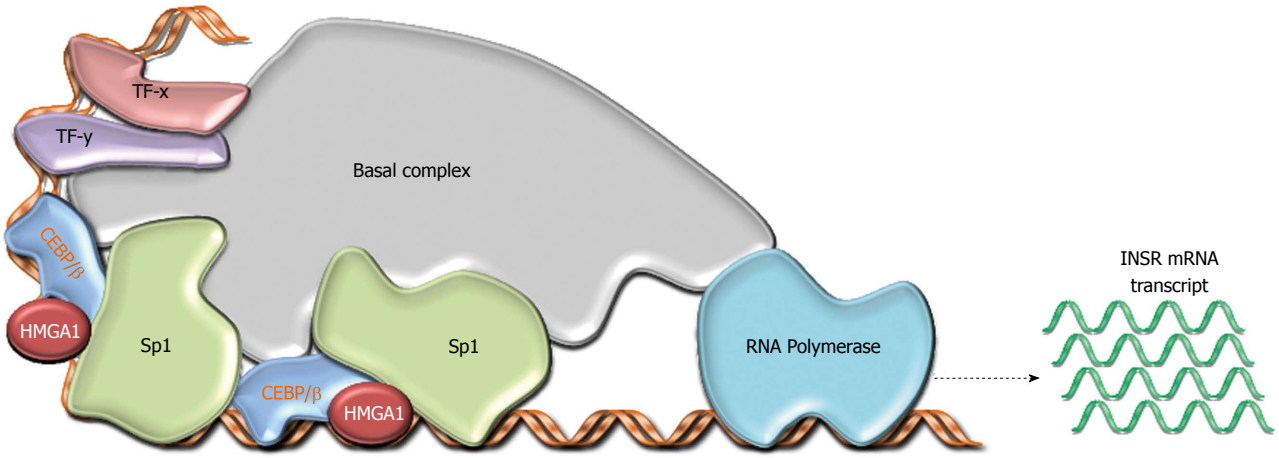

Figure 4 Model for the role of High Mobility Group A1 in type 2 diabetes mellitus. As a transcriptional regulator of the *INSR* gene, *HMGA1* gene variants may lead to decreased *INSR* gene transcription. This loss of insulin receptor (INSR) underlies the resultant insulin resistance and T2D in affected individuals. T2D: Type 2 diabetes.

resistant diseases, has been provided recently by showing that the *HMGA1* rs146052672 variant significantly associates with the metabolic syndrome in Italian and Turkish individuals and predisposes these (and other) populations to the unfavorable anthropometric and metabolic traits of the metabolic syndrome[74,42].

Overall, these data are consistent with the impression that the association of *HMGA1* gene variants with T2DM is accomplished through a pathogenetic mechanism related to peripheral insulin resistance. However, additional studies *in vitro* and *in vivo*, in normal and mutant mice, indicate that HMGA1, in addition to its role on *INSR* gene and protein expression, acts as a novel downstream target of the INSR signaling pathway[75], thus representing a critical nuclear mediator of insulin action and function. In this regard, evidence has been provided indicating that HMGA1 plays an essential role in the transcriptional regulation of a variety of insulin-target genes, such as the *IGFBP-1* gene, as well as the gluconeogenic genes *PEPCK* and *G6Pase*[76], contributing to the transcriptional regulation of glucose homeostasis.

PERSPECTIVES

Significant advances have been made in recent years in relation to the pathogenesis of T2DM. This has significantly improved our knowledge of one of the most serious health threats in the world, allowing identification of genes and pathways involved in the development and progression of the disease. It has recently become possible to acquire molecular and genetic level information from an individual (*i.e.*, DNA genotyping, gene expression, epigenomic profile, *etc.*). However, while such information is becoming increasingly available, how the identified genes and pathways impact on T2DM still remain largely unknown, due to the multifactorial nature of the disease. Understanding the pathogenesis of T2DM is necessary to enable the identification of prognostic and predictive biomarkers, as well as new therapeutic targets, which in turn should lead to improved outcomes in affected patients. Thus, once new therapeutic targets of

interest are identified, it is necessary to develop molecules that can rescue function to disease-associated genes or pathways and conduct studies that provide new strategies for the treatment of T2DM.

CONCLUSION

T2DM is a heterogeneous disease with a strong genetic component and familial inheritance. Considerable effort has been made in the last decades to identify genes that may explain all the diabetic phenotypes. Currently, however, genetic studies on T2DM can explain only a small percentage of its heritability. Until now, the *HMGA1* gene displays the strongest association with T2DM and its most frequent variant, rs146052672, confers the highest risk for human T2DM. Hence, from a strategic point of view, this finding suggests directing future research towards the identification of rare genetic variants with a stronger association, rather than common variants with a relatively small effect on the disease. It is evident that if a genetic variant confers a high susceptibility to T2DM it may become a useful biomarker to search for. For example, the genetic variants identified in the *HMGA1* gene may represent a predictive marker for early detection of T2DM, especially in those individuals with a family history of the disease. Moreover, variants in the human *HMGA1* gene may induce a different clinical course of disease compared to diabetic patients without the variant and may predict response to therapy, allowing identification of a priori patients who could most benefit from a specific pharmacological treatment[77]. Another important point in support of genetic studies in T2DM is the fact that they may integrate and improve our knowledge about the molecular mechanisms underpinning the pathophysiology of this disease.

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TOPIC HIGHLIGHT

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Role of P2X$_7$ receptors in the development of diabetic retinopathy

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Abstract

The P2X$_7$ receptor is one of the members of the family of purinoceptors which are ligand-gated membrane ion channels activated by extracellular adenosine 5′-triphosphate. A unique feature of the P2X$_7$ receptor is that its activation can result in the formation of large plasma membrane pores that allow not only the flux of ions but also of hydrophilic molecules of up to 900 Da. Recent studies indicate that P2X$_7$-mediated signaling can trigger apoptotic cell death after ischemia and during the course of certain neurodegenerative disorders. Expression of the P2X$_7$ receptor has been demonstrated in most types of cells in the retina. This purinoceptor mediates the contraction of pericytes and regulates the spatial and temporal dynamics of the vasomotor response through cell-to-cell electrotonic transmission within the microvascular networks. Of potential clinical significance, investigators have found that diabetes markedly boosts the vulnerability of retinal microvessels to the lethal effect of P2X$_7$ receptor activation. This purinergic vasotoxicity may result in reduced retinal blood flow and disrupted vascular function in the diabetic retina. With recent reports indicating an association between P2X$_7$ receptor activation and inflammatory cytokine expression in the retina, this receptor may also exacerbate the development of diabetic retinopathy by a mechanism involving inflammation.

Key words: P2X$_7$ receptor; Diabetic retinopathy; Vasotoxicity; Retinal microvessels; Interleukin-1β; Tumor necrosis factor-α

Core tip: This review summarizes the studies regarding the putative role of the P2X$_7$ receptor in triggering purinergic vasotoxicity in the retina and thereby contributing to the progression of diabetic retinopathy.

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INTRODUCTION

One of the most important characteristics of diabetic retinopathy (DR) is the death of microvascular pericytes and endothelial cells[1]. The loss of pericytes, contractile cells located on the abluminal wall of capillaries[2], appears to play a critical role in the development of microaneurysms and neovascular tufts[3]. Damage in the endothelial cells can result in a breakdown of the blood–retinal barrier and macular edema[4].

Currently, the mechanisms by which diabetes induces apoptosis in the retinal microvasculature remain uncertain, although oxidative stress, formation of advanced glycation end products, upregulation of protein kinase C, increased polyol pathway flux and focal leukostasis may be taken as important factors[5]. In fact, multiple lethal pathways may be activated during chronic hyperglycemia[6].

Extracellular adenosine 5'-triphosphate (ATP) is an excitatory transmitter both in the peripheral and central nervous systems. P2X receptors are a family of ligand-gated membrane ion channels activated by extracellular

ATP. P2X receptors consist of seven isoforms designated P2X1 to P2X7[7,8]. They are widely distributed in most types of cells in nearly every origin. They are involved in many actions, such as synaptic transmission in the peripheral and central nervous systems, contraction of smooth muscle, platelet aggregation, macrophage activation, cell death and immunomodulation[9,10].

In contrast to other ligand-gated channels in the purinoceptor family, the P2X7 receptor possesses unique features that are likely to be of both physiological and pathophysiological significance. Most importantly, not only does the initial activation of these receptors result in the opening of a non-selective plasma membrane channel, but with sustained activation there is in many types of cells the formation of trans-membrane pores that are permeable to hydrophilic molecules of up to 900 Da[11,12]. Indicative of P2X7 receptors having a role in cell pathology, this receptor has been found to be highly up-regulated in neurons and glial cells located in the ischemic cerebral cortex[13]. P2X7-mediated signaling is also implicated in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and multiple sclerosis[14].

P2X7 RECEPTOR IN THE RETINA

Expression of the P2X7 receptor has been demonstrated in most types of cells in the retina; these include neurons such as the ganglion cells[15,16], as well as glia[17,18] and vascular cells[19]. The P2X7 receptor was found to mediate the contraction of pericytes through an increase in intracellular calcium levels[19]. Interestingly, the spatial and temporal dynamics of this vasomotor response are established by the ability of P2X7 activation to potently inhibit cell-to-cell electrotonic transmission within the retinal microvascular network[19].

In the adult rat retina, immunolabeling for the P2X7 receptor is detected in a number of cells in the inner nuclear layer and ganglion cell layer, suggesting amacrine cells and ganglion cells[15]. This receptor was also found in processes presynaptic to rod bipolar cells, as well as other conventional synapses, suggesting that purines play a role in neurotransmission within the retina and may modulate both photoreceptor and rod bipolar cell responses[20].

In addition to the putative physiological roles of P2X7 receptors, it is reported that stimulation of these receptors can kill retinal ganglion cells *in vitro* and *in vivo* by a mechanism that appears to be dependent on a rise in intracellular Ca^{2+}[21,22]. One of those reports also suggested that the balance between extracellular ATP and its protective metabolite adenosine can influence ganglion cell survival in the living eye[22]. Another study suggested that an early up-regulation of neuronal P2X7 receptors may cause injury of retinal neurons and thereby contribute to the retinal damage[23]. Furthermore, data from our laboratory indicate that the activation of P2X7 receptors is involved in hypoxia-induced death of retinal neurons[24]. Other researchers have indicated mechanical strain triggers ATP release directly from retinal ganglion cells and that this released ATP autostimulates P2X7 receptors.

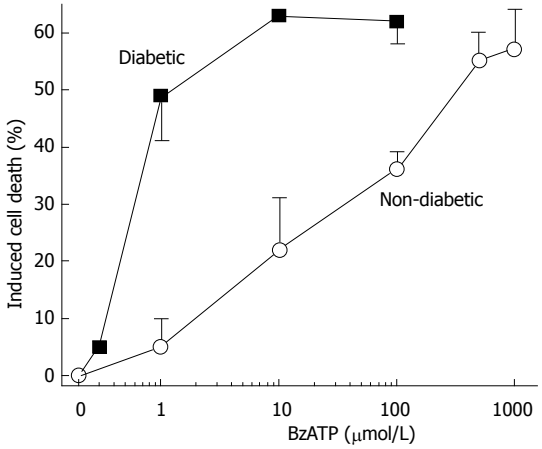

Figure 1 Cell death induced in non-diabetic and diabetic retinal microvessels by the P2X7 agonist, benzoylbenzoyl adenosine triphosphate. From Sugiyama *et al*[30] with permission from Investigative Ophthalmology and Visual Sciences. BzATP: Benzoylbenzoyl adenosine triphosphate.

Since extracellular ATP levels in the retina increase with elevated intraocular pressure and stimulation of P2X7 receptors on retinal ganglion cells can be lethal, this autocrine response may exert a deleterious effect on retinal ganglion cells in glaucomatous eyes[25].

P2X7 RECEPTOR AND DIABETIC RETINOPATHY

A study showed that human primary fibroblasts in a medium with a high glucose concentration underwent substantial ATP-mediated morphological changes and increased apoptosis. P2X7 was identified as the main purinergic receptor involved in these responses[26]. It has also been reported that fibroblasts from type 2 diabetes patients are characterized by a hyperactive purinergic loop based either on a higher level of ATP release or on increased P2X7 reactivity[27]. Another study revealed that changes in Müller cell membrane conductance in proliferative diabetic retinopathy (PDR), *i.e.*, the down-regulation of active Kir channels and the membrane depolarization, likely disturb voltage-dependent Müller cell functions, such as regulation of local ion concentrations and uptake of neurotransmitters[28]. The enhanced entry of calcium ions from the extracellular space and the subsequent stimulation of calcium-activated potassium channels may trigger Müller cell proliferation in PDR. Others reported that prolonged stimulation of the P2X7 receptor elicited permeabilization exclusively in microglial cells but not in neurons of the inner retina[29].

Our experiments, using pericyte-containing retinal microvessels, have shown a diabetes-induced increase in the vulnerability of retinal microvessels to the lethal effect of P2X7 receptor activation[30]. In other words, the agonist concentration needed to open large membrane pores and trigger apoptosis decreased markedly soon after the onset of streptozotocin-induced hyperglycemia in rats (Figure 1). It was also found that extracellular nicotinamide adenosine dinucleotide (NAD^+) caused cell death in the

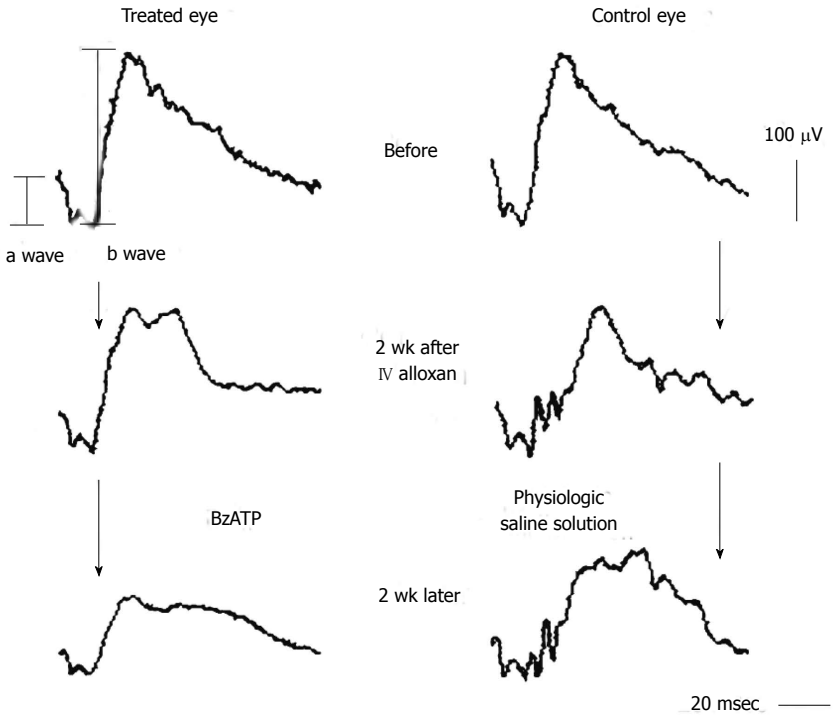

Figure 2 Typical changes of electroretinography after intravitreal injection (Ⅳ) of benzoylbenzoyl adenosine triphosphate (50 nmol) or physiological saline solution in an alloxan-induced diabetic rabbit. The amplitudes of a and b waves and oscillatory potentials were reduced in the BzATP-treated eye. From Sugiyama *et al*[32] with permission from Archives of Ophthalmology. BzATP: Benzoylbenzoyl adenosine triphosphate.

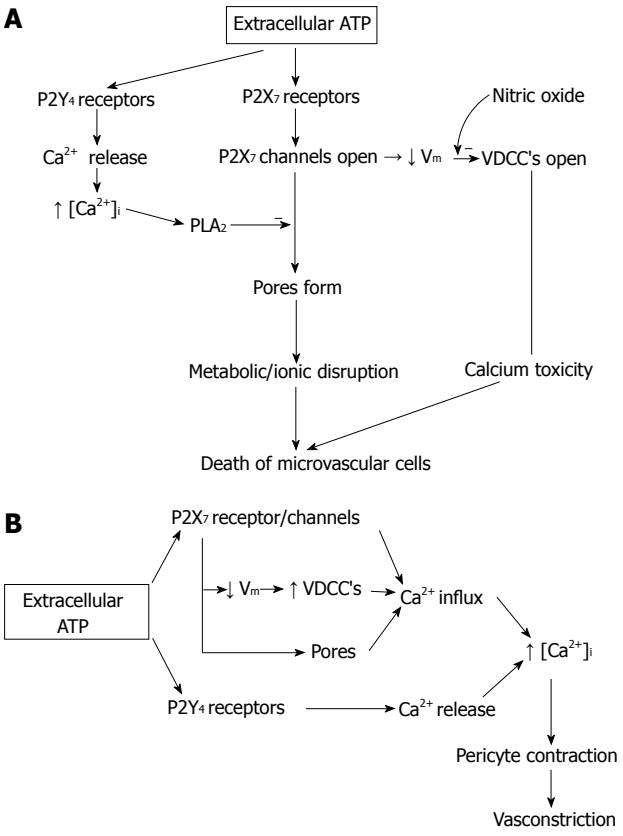

Figure 3 Models of the physiological and pathobiological effects of adenosine 5'-triphosphate in the retinal microvasculature. A: Putative mechanisms regulating purinergic vasotoxicity; B: Putative mechanisms by which extracellular adenosine 5'-triphosphate (ATP) causes pericyte Ca^{2+} levels to rise and thereby the contraction of these mural cells and the constriction of adjacent lumens. From Sugiyama *et al*[33].

retinal microvasculature by a mechanism involving the activation of the P2X7 purinoceptor and the formation of transmembrane pores. Soon after the onset of diabetes, the sensitivity of retinal microvessels to the vasotoxic effect of extracellular NAD^+ increased by approximately 100-fold[31]. In our *in vivo* study using the laser speckle circulation analyzer and electroretinography, soon after the onset of alloxan-induced diabetes, retinal blood velocity and function become more vulnerable to reduction initiated through the P2X7 receptor (Figure 2)[32]. Additional investigations indicate that, under physiological conditions, the formation of P2X7 pores is tightly regulated *via* a nitric oxide- and P2Y4-dependent pathway that limits the rise in pericyte calcium during the activation of these purinoceptors[33]. However, if this regulatory mechanism becomes dysfunctional, as appears to occur in the diabetic retina (Figure 3)[33], then purinergic vasotoxicity may contribute to the microvascular cell death that is a hallmark of DR.

Of additional interest, recent studies of DR in experimental models suggest the P2X7 receptors may have a role in mediating cytokine-induced vascular inflammatory reactions that can degrade the integrity of the blood-retinal barrier and thereby contribute to retinal vascular occlusion and ischemia[34]. More specifically, there are a number of reports linking P2X7 receptor activation in the retina with the expression of inflammatory cytokines[35]. For example, P2X7 agonists enhance the release of interleukin (IL)-1β and tumor necrosis factor (TNF)-α from hypoxia-activated retinal microglia[17]. In addition, our recent data suggest that the up-regulation of TNF-α, IL-1β and IL-6 may be involved in the retinal ganglion cell death that can occur with P2X7 receptors activated after an elevation in the intraocular pressure[36]. Although it is clear that more investigation is needed, these new findings further suggest that this purinoceptor may have a role in the progression of DR.

In conclusion, a variety of recent experimental studies are providing evidence that the P2X7 purinoceptor is a potential therapeutic target of a pharmacological strategy designed to diminish or prevent cell death in the diabetic retina.

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REVIEW

Knockout mouse models of insulin signaling: Relevance past and future

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Abstract

Insulin resistance is a hallmark of type 2 diabetes. In an effort to understand and treat this condition, researchers have used genetic manipulation of mice to uncover insulin signaling pathways and determine the effects of their perturbation. After decades of research, much has been learned, but the pathophysiology of insulin resistance in human diabetes remains controversial, and treating insulin resistance remains a challenge. This review will discuss limitations of mouse models lacking select insulin signaling molecule genes. In the most influential mouse models, glucose metabolism differs from that of humans at the cellular, organ, and whole-organism levels, and these differences limit the relevance and benefit of the mouse models both in terms of mechanistic investigations and therapeutic development. These differences are due partly to immutable differences in mouse and human biology, and partly to the failure of genetic modifications to produce an accurate model of human diabetes. Several factors often limit the mechanistic insights gained from experimental mice to the particular species and strain, including: developmental effects, unexpected metabolic adjustments, genetic background effects, and technical issues. We conclude that the limitations and weaknesses of genetically modified mouse models of insulin resistance underscore the need for redirection of research efforts toward methods that are more directly relevant to human physiology.

Key words: Insulin resistance; Mice; Knockout; Disease models, Animal; Glucose/metabolism; Signal transduction

Core tip: Insulin resistance is central to the pathophysiology of type 2 diabetes. The molecular origins of insulin resistance have been investigated using genetically modified mice. Much has been learned from this work, but new treatments for insulin resistance have not been forthcoming. Knockout mouse models of diabetes are limited by several factors including species differences in glucose metabolism. These are due partly to species differences in physiology, and partly to the failure of genetic modifications to produce an accurate model. Advancement may require a redirection of research efforts toward methods that are more directly relevant to human physiology.

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INTRODUCTION

Type 2 diabetes is a growing public health problem affecting approximately 26 million adults in the United States, with pre-diabetes affecting an additional 79 million[1]. The natural history of type 2 diabetes starts with insulin resistance, which develops over time and often

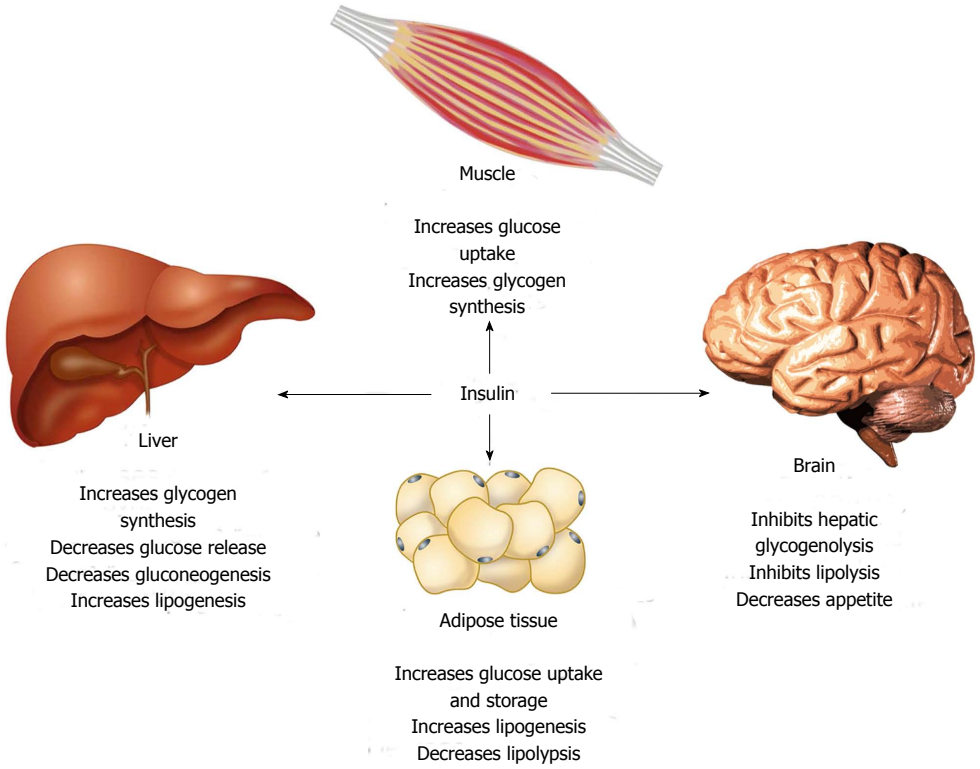

Figure 1 Insulin actions in main insulin-sensitive tissues. Insulin has different actions in each of the main insulin-sensitive tissues. In muscle, insulin promotes glucose uptake and glycogen synthesis. In liver, insulin promotes glycogen synthesis and lipogenesis and reduces gluconeogenesis and the release of stored glucose. In adipose tissue, insulin increases glucose uptake and lipogenesis and decreases lipolysis. In the brain, insulin Inhibits hepatic glycogenolysis and lipolysis and decreases appetite.

precedes a diagnosis by many years. The pancreas compensates for insulin resistance by increasing insulin secretion, often leading to hyperinsulinemia. For many insulin-resistant patients, the pancreas is unable to sustain a high level of insulin secretion. As the pancreas fails to meet the demand for insulin, plasma glucose rises. Patients are then at risk of morbidity and mortality associated with complications such as neuropathy, retinopathy, nephropathy, and increased risk of cardiovascular disease. Overall, type 2 diabetes decreases life expectancy at age 50 or older by about 8 years[2]. Aside from diabetes and the metabolic syndrome, insulin resistance is also associated with polycystic ovarian syndrome and other problems. Understanding the cellular and molecular causes of insulin resistance is an area of active research because of the need to discover new therapies to help patients.

Animal models are often used to investigate mechanisms of insulin resistance and develop therapeutic agents. In the field of type 1 diabetes, serious limitations of animal models have become apparent[3]; we therefore sought to assess the utility of select mouse models used in type 2 diabetes research, specifically insulin signaling and resistance. We begin with a brief summary of insulin signaling, followed by a closer look at general limitations of mouse models and specific limitations of knockouts lacking select insulin signaling molecule genes.

Insulin resistance is defined as the failure of cells to respond normally to insulin, and most importantly, to insulin's glucose-lowering effects. It can be measured by a number of approaches, including the Homeostatic Model Assessment of Insulin Resistance, which is based on fasting glucose and insulin levels, and the gold standard approach, a hyperinsulinemic-euglycemic clamp test[4]. On a cellular level, insulin resistance manifests differently in different tissues (Figure 1). Insulin-resistant muscle cells fail to uptake glucose and other nutrients in response to insulin, whereas in adipose tissue, insulin resistance leads to greater hydrolysis of stored triglycerides in addition to decreased nutrient uptake. In the liver, insulin promotes glycogen synthesis and prevents the release of stored glucose, thereby raising blood glucose levels. In the brain, insulin decreases appetite and hepatic glucose production[5].

The molecular mechanisms of insulin resistance in type 2 diabetes have not been fully characterized, although many important biochemical, metabolic, and genetic features have been identified. Accumulated findings have highlighted several pathways to insulin resistance, including lipid accumulation, oxidative stress, and inflammation[6]. An important common feature of these mechanisms is the activation of stress-sensitive kinases including protein kinase C ζ (PKCζ) that cause a dampening of insulin signaling[6,7].

Insulin is involved in a number of cellular processes apart from nutrient metabolism, including protein synthesis, mitochondrial biogenesis, growth, autophagy, proliferation, differentiation, and migration[8-10]. As illustrated in Figure 2, the binding of insulin to its receptor triggers a cascade of cellular events that leads to nutrient uptake

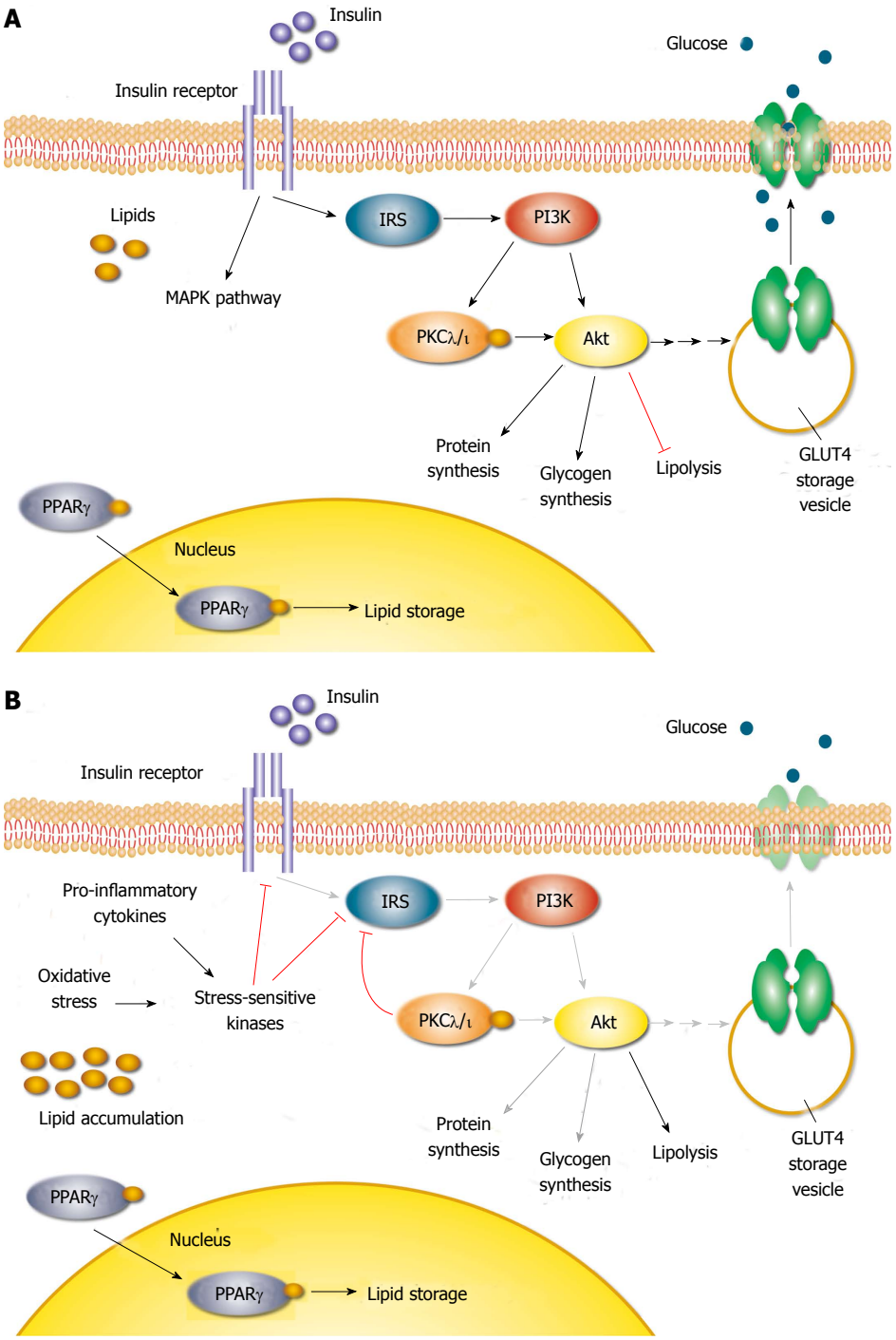

Figure 2 Insulin signaling in health and disease. Insulin signaling in health and disease. A: The binding of insulin to its receptor triggers a cascade of cellular events that lead to nutrient uptake and activation of various cellular programs. Insulin receptor substrate (IRS) activates phosphoinositide 3-kinase (PI3K) which produces a metabolite that activates protein kinase B (AKT) and protein kinase C λ/ι (PKCλ/ι). PKCλ/ι, which also depends on lipids for activation, can inhibit insulin signaling by a feedback mechanism. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), is important in lipid metabolism, and is the target of insulin sensitizing thiazolidinedione drugs. PPARγ becomes activated upon binding of lipids and promotes expression of genes involved in fat storage; B: Under insulin-resistant conditions, accumulation of lipids, oxidative stress, and pro-inflammatory cytokines cause activation of stress-sensitive kinases such as protein kinase C θ (PKCθ), inhibitor of nuclear factor kappa-B kinase subunit β (IKKβ) and c-Jun N-terminal kinase 1 (JNK1), which inhibit insulin signaling.

and activation of these various cellular programs[8]. Under insulin-sensitive conditions, as shown in Figure 2A, insulin receptor substrate (IRS) activates phosphoinositide 3-kinase (PI3K), which produces a metabolite that activates protein kinase B (AKT) and PKCλ/ι. PKC λ/ι, which also depends on lipids for activation, can inhibit insulin signaling by a feedback mechanism. The nuclear receptor peroxisome proliferator-activated receptor gamma, or peroxisome proliferator-activated receptor γ (PPARγ), is important in lipid metabolism, and is the target of insulin sensitizing thiazolidinedione drugs (TZDs). PPARγ becomes activated upon binding of lipids and promotes expression of genes involved in fat storage. As shown in Figure 2B, under insulin-resistant conditions,

accumulation of lipids, oxidative stress, and pro-inflammatory cytokines cause activation of stress-sensitive kinases such as PKCθ, inhibitor of nuclear factor kappa-B kinase subunit β (IKK-β) and c-Jun N-terminal kinase 1 (JNK1), which inhibit insulin signaling[6,7].

Evidence for insulin signaling pathways and mechanisms of insulin resistance comes from human and animal cell and tissue studies, clinical studies, and whole animal experiments. While data from various models have been useful in formulating and testing hypotheses, some approaches are more promising than others. Rodent models have been used in the study of type 2 diabetes and insulin resistance for decades. Conditions relevant to the study of insulin resistance and diabetes are induced in rodents using several approaches, including genetic, pharmacological, surgical, and dietary inductions. A number of these approaches and models have been reviewed elsewhere[11-14]. Many researchers favor targeted genetic manipulation because it allows specific and complete or near-complete removal of target gene function in a whole organism or specific tissues[15]. In combination with pharmacological, cell-based and molecular studies, these knockout mouse studies have mapped the insulin signaling pathway in mice to a high level of detail. Other authors have described how pathway connections tested in humans have been shown to be conserved (*i.e.*,[16]). Many would argue that knockout mouse studies have been especially important in defining the function of genes for which no pharmacological or other molecular-based functional ablation is available[17]. In this respect, the genetic approach has become a central component of preclinical research in diabetes and other fields.

Despite this progress in our understanding of insulin action, the causative molecular basis for acquired human insulin resistance remains unclear and controversial. Furthermore, improved understanding of rodent cell signaling has not translated into improved human therapeutics. To wit, it has been almost 20 years since the first insulin signaling knockout mouse studies were published[18,19], but no new drugs targeting the insulin signaling phosphorylation cascade have emerged to treat insulin resistance in type 2 diabetes[9]. While much of this research is conducted for the purpose of hypothesis testing rather than drug development *per se*, the identification of drug targets is often a primary or secondary goal[20]. In light of this, we discuss the limitations of research on insulin resistance using knockout mice of select proteins important in the insulin signaling cascade (Figure 2). The following sections will focus mainly on peripheral insulin resistance and extrapancreatic insulin-sensitive tissues, since many therapeutic and research efforts are in this area. We first address physiological, cellular, and molecular differences in glucose metabolism between mice and humans that limit translatability. We then review select knockout mouse models of insulin signaling dysfunction, identifying cases with contradictory or untranslatable results. Finally, we briefly discuss the limitations of genetic manipulations of these targets in mice in regard to the search for safe and effective drugs for type 2 diabetes.

GLUCOSE DISPOSAL IN MICE AND HUMANS

A central aspect of glucose homeostasis is glucose disposal, meaning the facilitated transport of glucose from blood into storage tissues and organs. Insulin resistance in humans with type 2 diabetes involves defects in glucose sensing and disposal in a number of tissues, but the most significant effects on glucose homeostasis result from insulin resistance in the major glucose-disposing tissues: skeletal muscle, liver and adipose tissue.

Glucose disposal and glycogen storage patterns differ in mice and humans. In healthy humans, about one-third of glucose is taken up by the liver[21]. Estimates of skeletal muscle glucose uptake vary widely, in part because they are often based on indirect measurements and assumptions regarding muscle mass and blood flow. One report that measured muscle glucose more directly using nuclear magnetic resonance demonstrated muscle absorbing 64%-91% of infused glucose in a single male volunteer[22]. A follow-up study of 11 subjects reported muscle glucose uptake of 90% in normal subjects and 67% in diabetic subjects[23]. In a separate study of 10 healthy volunteers, muscle accounted for 38.3% of systemic glucose disposal, based on data from blood sampled from a forearm vein[24]. Overall, the data show greater glucose uptake in skeletal muscle than liver in humans. Genetic evidence underscores the importance of skeletal muscle to whole-body glucose tolerance in humans. Polymorphisms in the gene for the primary glucose transporter in muscle, glucose transporter isoform 4 (GLUT4), have been linked to type 2 diabetes and insulin resistance[25]. Overall, defects in skeletal muscle glucose disposal are a major component of insulin resistance in humans[26].

By contrast, the liver is much more important for glucose disposal in mice. Interfering with glucose uptake in mouse liver causes whole-body insulin resistance and glucose intolerance, but similar manipulations in muscle usually do not. The muscle-specific insulin receptor knockout mouse has normal glucose tolerance, insulin sensitivity, and glucose and insulin levels, with only mild dyslipidemia[27]. Muscle-specific deletion of IRS1 and IRS2 also does not produce a diabetic phenotype, nor does a whole-body knockout of the major muscle glucose transporter, GLUT4[28,29]. One exception to this pattern may be a muscle-specific GLUT4 knockout strain that developed a diabetic phenotype in one study[30], a result that has not been replicated by others[31,32]. In contrast to the above strains deficient in muscle insulin signaling, a liver-specific insulin receptor knockout mouse strain was insulin resistant and severely hyperinsulinemic, and developed hyperglycemia and glucose intolerance at an early age (2 mo)[33]. Liver-specific deletion of IRS1 and IRS2 also cause insulin resistance under certain conditions[34]. Mice with a deletion of the primary glucose transporter in the liver, GLUT2, are hyperglycemic and die at 2-3 wk of age[35].

Glycogen storage is a major destination for glucose in mammals. In mice, approximately 8 times more glyco-

gen is stored in the liver than skeletal muscle[36], but the reverse is true in humans, where 3-8 times more glycogen is found in skeletal muscle[37]. These physiological differences in glucose disposal and storage have implications for modeling insulin resistance, since muscle and liver have different roles and different metabolic and signaling pathways.

There are two important differences in glucose transport between liver, the primary glucose disposal organ in mice, and skeletal muscle, the primary glucose disposal organ in humans. First, skeletal muscle cells have multiple pathways for glucose transport. Contraction-stimulated glucose transport in skeletal muscle is insulin-independent, mediated through 5' adenosine monophosphate activated protein kinase-mediated signaling mechanisms[38]. In contrast, liver has no such activity-stimulated transport method. Second, the transporters involved in glucose uptake are different in the two tissues. In liver, the low-affinity GLUT2 is present at high levels on cell membranes independent of insulin or other signaling[39], and glucose transport rates vary with the extracellular concentration of glucose[40]. In contrast, in skeletal muscle cells, the high-affinity glucose transporter GLUT4 is translocated from internal vesicles to the plasma membrane in response to glucose uptake signals[41]. In human skeletal muscle cells, this transport is facilitated by clathrin isoform CHC22, which is not present in the mouse[42]. The rate-limiting step in glucose metabolism in liver is phosphorylation, while in skeletal muscle it is transport through GLUT4[43]. The divergent features of cells in these organs, combined with the divergent physiology of rodents and humans, means that glucose disposal is affected very differently in the different species.

Because mice rely principally on the liver for glucose homeostasis, while humans rely on skeletal muscle where transport mechanisms and biochemical pathways differ, mice may not be expected to be analogous to type 2 diabetes patients in regards to mechanisms of glucose metabolism or its dysfunction.

Mice and humans have a number of other metabolic differences. The small size and fast metabolism of mice enables heart rates in the range of 350-550 beats per minute, while in humans, normal heart rate is about 70 beats per minute[44]. Mice are capable of the physiological state of torpor, a state of reduced metabolic rate, while humans are not[45]. Prolonged fasting in humans impairs insulin-stimulated glucose utilization, but causes enhancement in mice[46]. In regards to eating patterns, mice consume most of their food at night[45], and an overnight fast of 14-18 h, typical for laboratory experiments, induces a state akin to starvation[47]. In addition, circulating lipids have an inverted composition in mice, with high-density lipoprotein (HDL) being typically higher than low-density lipoprotein (LDL), while HDL is lower in humans[48]. The thermoneutrality point, that is, the temperature at which an organism expends minimal energy for temperature regulation, is higher in mice[49]. This last difference could be compensated for if mice were housed above room temperature, but that is not standard practice.

Finally, experiments investigating mouse metabolism present technical challenges. Insulin sensitivity is often measured using a hyperinsulinemic-euglycemic clamp test, which involves either implanted arterial catheters or repeated blood sampling. The results of this test are dependent on a number of experimental factors which are not standardized between laboratories, including fasting time, anesthesia use, and blood sampling site[46]. Fasting glucose, insulin, and lipid levels are often measured after 14-18 h overnight fasts, but this induces a catabolic state in mice, who normally eat mostly at night. Data shows that a 6 h fast is best to assess glucose tolerance in mice[50].

KNOCKOUT MODELS OF INSULIN SIGNALING

Mouse models of diabetes are often used to explore signaling pathways[13]. The following sections highlight cases relevant to insulin signaling dysfunction where similar or identical genetic manipulations produced disparate results. These cases are consistent with other results showing differences in insulin action, secretion, and responses to hypoglycemia in different inbred mouse strains[51]. Previous reviewers have also noted the strong effect of genetic background in knockout mouse experiments[52]. Other factors influencing disparate findings include compensatory metabolic adjustments and technical challenges associated with evaluating mouse metabolism. Later, we will focus on the challenges of translating mouse knockout results to humans.

INSULIN RECEPTOR AND INSULIN RECEPTOR SUBSTRATE

Binding of insulin to the insulin receptor is the first step in the insulin signaling pathway. Mice with complete deletion of the insulin receptor are about 10% underweight and suffer from chronic hyperglycemia[53,54]. They die within several days of birth due to diabetic ketoacidosis. In humans, donohue syndrome is a rare monogenic disease resulting from mutation of the insulin receptor. Individuals with this disease suffer from severe pre-natal and post-natal growth retardation, fasting hypoglycemia, and post-prandial hyperglycemia[55]. They generally die before adulthood. The difference between the glucose homeostasis in mice and humans with this mutation may be attributable to the fact that the human pancreas develops earlier in gestation, hence better enables the compensatory hyperinsulinemia[55].

The pancreatic beta-cell specific insulin receptor knockout mouse strain (called BIRKO) has impaired insulin response to glucose challenge and develops impaired glucose tolerance and high insulin levels[56]. In the initial description of this mutant strain, glucose levels and body weight were normal, however, a follow-up report from the same laboratory described consistent hyperglycemia and sporadic obesity[57]. In the same report, a muscle

Table 1 Knockout mouse reproducibility

Model	Ref.	Genetic background	Observed discrepancy
IRS1 knockout	Tamemoto *et al*[19]	C57BL/6 × CBA	Growth defect twice as severe in Araki 1994
	Araki *et al*[18]	C57BL/6	
IRS2 knockout	Withers *et al*[60]	C57BL6 × 129Sv	Growth defect observed only in Withers *et al*[60]. Much more severe glucose dysregulation in Withers *et al*[60]
	Kubota *et al*[61]	C57BL/6 × CBA mixed	
IR and IRS1 double heterozygous knockout	Kulkarni *et al*[62]	C57BL/6 129/Sv DBA/2	Diabetes not observed in 129/Sv mice, observed in 85% of C57BL/6 mice and 64% of DBA/2 mice. Glucose intolerance only in C57BL/6 strain
AKT2 knockout	Cho *et al*[64]	C57BL/6	More severe hyperglycemia and hyperinsulinemia in Garofalo *et al*[63]. Growth defect only in Garofalo *et al*[63]
	Garofalo *et al*[63]	DBA/1lacJ	
AKT1 knockout	Chen *et al*[65]	C57BL/6 × 129R1	High neonatal mortality only in Cho *et al*[64]. Improved glucose tolerance and insulin sensitivity only in Buzzi *et al*[68]
	Cho *et al*[66]	C57BL/6	
	Buzzi *et al*[68]	129/Ola, C57BL/6 mixed	
Pik3r1 heterozygote	Mauvais-Jarvis *et al*[72]	129Sv, C57BL/6 mixed	Improved glucose tolerance and insulin sensitivity and low glucose and insulin levels on normal diet only in Mauvais-Jarvis *et al*[72]
	McCurdy *et al*[73]	C57BL/6SVJ	
Liver-specific *Pik3ca*	Sopasakis *et al*[74]	129Sv, C57BL/6, FVB mixed	Insulin resistance and glucose intolerance on normal diet in Sopasakis *et al*[74] only
	Chattopadhyay *et al*[75]	129, C57BL/6J mixed	
GLUT4 heterozygous knockout	Stenbit *et al*[76]	CD1, C57BL/6 mixed	Unexpected more severe phenotype in heterozygous knockout than homozygous
PKCλ heterozygous knockout	Farese *et al*[79]	C57BL/6, 129P2/Sv, FVB mixed	Unexpected more severe hepatic steatosis in heterozygous knockout than homozygous
PKCδ knockout	Leitges *et al*[81]	129/SV × Ola	High neonatal mortality observed only in Bezy *et al*[82]
	Bezy *et al*[82]	C57BL6/J	
PPARγ	He *et al*[86]	C57BL/6J	Resistance to diet-induced insulin resistance only in Jones *et al*[85] study
	Jones *et al*[85]	C57BL/6J, FVB mixed	
Muscle-specific PPARγ	Norris *et al*[87]	129/sv, C57BL/6, FVB mixed	Insulin resistance and glucose intolerance on normal diet in Hevener *et al*[88] only. Improvement with rosiglitazone in Norris *et al*[87] only
	Hevener *et al*[88]	C57BL6/J	

Reproducibility problems in knockout mouse studies. Some variant results can be explained by differences in genetic background. IRS: Insulin receptor substrate 1; IR: Insulin receptor; AKT2: Protein kinase B isoform 2; GLUT4: Glucose transporter isoform 4; PKCλ: Protein kinase C λ; PPARγ: Peroxisome proliferator-activated receptor γ.

and beta-cell double insulin receptor knockout (BIRKO-MIRKO) mouse strain had an unexpectedly mild condition. This strain had impaired glucose tolerance, mild hyperglycemia, high triglycerides and free fatty acids, and extra fat pad mass. These findings would seem to indicate that muscle-mediated glucose disposal is dispensable for normal glucose homeostasis in mice, but 2-deoxyglucose uptake studies showed that both muscle-specific insulin receptor knockout (MIRKO) and BIRKO had normal muscle glucose uptake, suggesting most muscle glucose uptake under these conditions is insulin-independent[57]. Studies of liver glycogen synthesis and liver glycogen content confirm that mice with insulin insensitive muscle shifted glucose utilization away from muscle and towards liver[57].

Mouse strains lacking insulin receptor in other tissues have been developed. A knockout of insulin receptor in neuronal tissue (NIRKO) demonstrated elevated body weight, white adipose tissue, serum triglycerides, and circulating leptin, with most of these changes being more pronounced in the females[58]. In addition, both sexes of NIRKO mice had reduced fertility, demonstrating the importance of insulin in reproduction. A knockout of insulin receptor in adipose tissue (FIRKO) had low fat mass, and the normal relationship between leptin levels and fat mass was disrupted[59]. These mice were protected against age-related glucose intolerance.

The IRS proteins transmit signals from the insulin and IGF1 (insulin-like growth factor 1) receptors. Two groups independently showed a significant pre-natal and post-natal growth defect in IRS1 knockout mice[18,19] (Table 1). Despite having similar genetic backgrounds, only one of the strains exhibited glucose intolerance as measured by a glucose tolerance test[18]. In addition, the two strains had significantly different growth defect severities, with a 40%-60% decrease in weight at various life stages observed in one study[18], and a 20%-30% decrease in the other[19]. These differences could have been due to the genetic manipulation approaches or the genetic backgrounds.

Two independent groups described IRS2 knockout mouse models, and the phenotypes were different despite similar genetic backgrounds. Withers *et al*[60] observed a 10% decrease in body weight throughout all life stages for the IRS2 knockout mice in a C57BL6 × 129Sv background, while Kubota *et al*[61] observed the IRS knockouts to be of normal size in a C57BL/6 × CBA mixed background. Fasting hyperglycemia was observed at age 6 wk in Withers *et al*[60], but average glucose levels did not reach hyperglycemic levels in Kubota *et al*[61]. Hyperinsulinemia and glucose tolerance showed a similar pattern: more severe, earlier phenotypes observed in Withers *et al*[60] than in Kubota *et al*[61]. Reduced β-cell mass was observed by both groups.

Kubota *et al*[61] suggested that the difference in glucose and insulin levels between the two reports was likely due to low β-cell mass in their strain, caused either by β-cell death or by the failure of insulin-resistance induced hyperplasia, and acknowledge that genetic differences other than the intended manipulation may influence the results. The authors concluded based on their data and data from a related study that both β-cell dysfunction and reduced β-cell mass can contribute to the murine diabetic state, but only studies of human patients can validate whether one or both mechanisms are more important in the pathogenesis of type 2 diabetes in humans.

Double heterozygous knockout of IR and IRS1 were generated in three different genetic backgrounds: C57BL/6, 129/Sv and DBA/2[62]. While all three strains had mild growth retardation, the results in regards to glucose homeostasis were drastically different. In C57BL/6 mice, the double heterozygous knockout caused severe hyperglycemia and hyperinsulinemia in the vast majority of cases, whereas the glucose levels of 129Sv mice were not significantly different from control littermates. In DBA mice, more than half of the mice were hyperglycemic but maintained normal glucose tolerance. Triglycerides were significantly reduced in the double heterozygous knockouts of the B6 and DBA strains, and the wild type DBA strain had significantly elevated triglycerides as compared to the other wild type strains[62].

AKT/PROTEIN KINASE B

The metabolite phosphatidylinositol 3,4,5-trisphosphate (PIP3) activates AKT/protein kinase B and atypical protein kinase C. AKT has three isoforms in mammals, of which AKT1 and AKT2 are most important for metabolism. Two independently developed AKT2 knockout mouse strains in different backgrounds developed hyperglycemia, glucose intolerance, and insulin resistance[63,64]. Garofalo *et al*[63] observed hypoinsulinemia due to pancreatic β-cell death in a subset of male mice, and hyperinsulinemia with no pancreatic changes in the remainder, while Cho *et al*[64] observed hyperinsulinemia and associated pancreatic hyperplasia. In Garofalo *et al*[63], both hyperglycemia and hyperinsulinemia were more severe than in Cho *et al*[64], with average fed insulin measurements five times higher. Also, Cho *et al*[64] observed normal growth in the AKT2 knockout, but Garofalo *et al*[63] observed a mild growth deficiency evident at all life stages. Only Garofalo *et al*[63] observed lipoatrophy and high levels of serum triglycerides. The control mice in Garofalo *et al*[63] had near-diabetic random fed glucose levels that were almost as high as the knockout mice in Cho *et al*[64] Neither of these knockout strains were obese.

The characteristics of AKT1 knockout mouse strains are also sensitive to genetic background and environmental factors. Two labs independently reported that AKT1 knockout mice with different genetic backgrounds had a growth defect causing 15%-20% reduced body weight[65,66]. One of the studies observed high neonatal mortality among the knockout mice[66], while the other observed high mortality with γ-radiation[65]. Glucose tolerance in Chen *et al*[65] appeared normal, but the glucose tolerance test was performed using a longer fasting time and lower glucose dose than is optimal[50]. One study demonstrated a non-significant improvement in glucose tolerance and insulin sensitivity in males. A similar strain was later shown to be resistant to diet-induced obesity[67]. Later data on a third, independently developed AKT1 knockout strain showed dramatic improvement in glucose tolerance and insulin sensitivity[68].

Studies of spontaneous human genetic variants in AKT1 and AKT2 have confirmed the importance of these proteins in growth and glucose homeostasis, mostly respectively, although the manifestations of the mutations differ between humans and mice[16]. For example, the human patients with a specific AKT2 mutation display asymmetric hypertrophy[69], while the above-described AKT2 knockout mouse models have normal growth[64] or a growth deficiency[63].

PHOSPHOINOSITIDE 3-KINASE

PI3K, an enzyme complex composed of a regulatory subunit and a catalytic subunit that produces the metabolite PIP3. PI3K is activated by IRS proteins in the insulin signaling cascade (Figure 2). In humans, *PI3K* gene polymorphisms are associated with cancer risk[70] but not diabetes, to our knowledge.

Complete loss of the *Pik3r1* gene, which encodes isoforms of the regulatory subunit of PI3K, results in perinatal lethality in mice, perhaps due to impaired B cell development[71]. Mice heterozygous for *Pik3r1* deletion, having attenuated expression of all isoforms of the regulatory subunit, had improved glucose tolerance and insulin sensitivity and low glucose and insulin levels[72]. Lipid metabolism was unchanged except for a modest increase in serum free fatty acids, indicating that the observed insulin sensitivity was not due to indirect effects *via* changes in lipid metabolism. A minor increase in basal muscle glucose uptake was observed, but the authors note that changes in liver were likely most responsible for the increased insulin sensitivity[72]. A later, independent study observed that the heterozygous knockout mice were essentially indistinguishable from control mice on a normal diet[73]. On a high-fat diet, these mice showed lower fasting insulin levels, improved overall insulin sensitivity, and improved glucose uptake in fat and muscle[73]. Macrophage accumulation was reduced in the adipose tissue of these heterozygous knockout mice, but results from bone marrow transplant experiments suggested the improved insulin sensitivity did not occur solely *via* PI3K's role in inflammation.

The catalytic subunits of PI3K have also been studied using knockout mouse strains. Liver-specific deletion of *Pik3ca* caused mild obesity, insulin resistance, glucose intolerance, and high glucose and insulin levels[74]. The same genetic manipulation in a second laboratory produced a strain with normal glucose and insulin levels and body weight[75]. The *Pik3ca* knockout mice in the second

study were resistant to high-fat diet induced hepatic steatosis and somewhat resistant to diet-induced glucose intolerance as well[75]. For this gene, liver-specific deletion produced diabetes-like symptoms in one laboratory, but in another laboratory, glucose homeostasis was identical in control and knockout mice[74,75].

GLUT4

As described above, GLUT4 is the major glucose transporter in muscle, the most important tissue type for glucose disposal in humans. Unexpectedly, in GLUT4 knockout mice, glucose levels are normal except for mild fed hyperglycemia and fasted hypoglycemia observed only in males[29]. Consistent with results regarding insulin signaling and growth[18], these animals display significant growth retardation, shortened life spans, cardiac hypertrophy, and reduced adipose tissue[29]. Somewhat surprisingly, mice heterozygous for the GLUT4 knockout have a more severe phenotype. A diabetes-like condition developed at varying ages, with a majority of males both hyperinsulinemic and hyperglycemic by age 5-7 mo[76].

The authors pointed out that the unexpectedly mild condition of the homozygous GLUT4 knockout and more severe condition in the GLUT4 knockout heterozygote were likely due to compensatory metabolic adjustments that occur during development. These could include the transfer of glucose disposal from tissues that primarily use GLUT4 to tissues that primarily use GLUT2, as observed in the muscle-specific GLUT4 knockout[30], or the upregulation of alternative glucose transporters[52].

PROTEIN KINASE C

Protein kinase C enzymes (PKCs) are involved in regulating a variety of cellular functions in mammals, including insulin signaling[77]. Atypical PKCs include the isoforms PKCλ/ι and ζ (PKCλ refers to the mouse isoform of PKCι)[78]. Activated PKCs can inhibit insulin signaling by a feedback mechanism that prevents signal transduction between insulin receptor and IRS[7,78].

Atypical protein kinase C family member PKCλ was knocked out specifically in mouse muscle, resulting in diabetic symptoms including glucose intolerance, insulin resistance, hyperglycemia, and high insulin levels[79]. Altered fat metabolism was also observed: high triglycerides, and mildly elevated free fatty acids and liver triglycerides. While some symptoms were observed in both the heterozygous and homozygous muscle-specific knockout of PKCλ, the heterozygotes were as insulin resistant and glucose intolerant as the homozygous knockouts, and had more abdominal obesity and hepatic steatosis[79]. This is unexpected, since the heterozygous knockout had reduced, but not ablated, expression of PKCλ.

Differential expression of PKCδ has been identified as one factor in the different vulnerability of common laboratory mouse strains to diabetes[80]. One study of a PKCδ knockout mouse strain in a 129/Sv × Ola genetic background had normal growth and development[81]. Surprisingly, the same deletion in the C57BL6/J strain caused a high mortality rate, with survivors being 14% underweight[82]. The C57BL6/J PKCδ knockout mouse had better glucose tolerance than control mice[82], but glucose tolerance was not tested in the original knockout. The authors noted that improved glucose tolerance may have been due to decreased inflammation in adipose tissue[82]. In humans, PKCδ deficiency can cause B-cell deficiency with severe autoimmunity[83].

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ

The nuclear receptor PPARγ, becomes activated upon binding of lipids and is important for lipid metabolism and storage, adipogenesis, and insulin sensitivity. This nuclear receptor is the target of insulin-sensitizing TZDs[84].

Two independently generated adipose tissue-specific PPARγ knockout strains showed important differences in glucose homeostasis under high-fat diet conditions. On normal chow, both these strains had reduced adipose tissue mass, high blood lipid levels, and hepatic steatosis, but glucose tolerance was normal[85,86]. On high-fat diet with 40% of calories from fat, He *et al*[86] observed hyperinsulinemia and insulin resistance in both the knockout and control mice, although these traits were more severe in the knockout. The knockout strain studied by Jones *et al*[85] was resistant to diet-induced hyperinsulinemia and insulin resistance despite being subjected to a more extreme high-fat diet, with 60% of calories from fat. The knockout strains in both studies were more prone to high-fat diet induced hepatic steatosis.

Two studies on independently developed muscle-specific PPARγ knockout models have provided contradictory findings regarding the mechanism of action of TZDs. The first strain was more susceptible to diet-induced obesity, glucose intolerance, and insulin resistance but was indistinguishable from controls on a normal diet[87]. Rosiglitazone reduced the hyperinsulinemia and impaired glucose homeostasis observed in this strain on high-fat diet, therefore the authors suggested that muscle PPARγ is not required for the positive effects of this TZD[87]. In contrast, the second strain developed insulin resistance and glucose intolerance on a normal diet[88]. Glucose disposal in a hyperinsulinemic-euglycemic clamp experiment was not improved with rosiglitazone treatment, suggesting that the insulin sensitizing effect of TZDs is dependent on muscle PPARγ. In this case, two mouse models have provided conflicting data not just on the role of a gene, but also on a drug mechanism of action.

In conclusion, we above described several cases where genetic modification of insulin signaling genes produced significantly, sometimes dramatically, different results in separate studies or varied genetic backgrounds (Table 1). We also described two cases where heterozygous knockouts had unexpectedly severe phenotypes: GLUT4 and

PKCλ. Although the mechanisms behind the unexpected observations are unknown, it is known that organisms respond unpredictably to the absence of gene products during development. Compensatory metabolic adjustments that may occur during development constitute a general limitation of knockout mouse models. These concerns are mitigated by the use of conditional knockouts, however, those strains require injection or gavage of an inducing drug, which can produce artifacts[89]. These examples illustrate the challenges associated with producing reliable, reproducible, and translatable results in mice.

CLINICAL TRANSLATION

In the following section, we will address factors which limit the applicability of mouse models to human therapeutic treatment development. As described above, insulin signaling gene knockout mice often have phenotypes unrelated to type 2 diabetes including growth defects[18,33,60,63], neonatal mortality[66], and others, including resistance to tumor formation[90]. These phenotypes are a result of the loss of diverse non-metabolic insulin functions, and these studies have yielded information about those biological processes in mice. At this juncture, it is worth examining whether these mouse models of insulin resistance are contributing positively to the development of new, unique, safe, and effective type 2 diabetes treatments. Here we focus on select pharmaceuticals targeting the signaling proteins discussed above.

As might be predicted based on the importance of insulin to growth, several drugs targeting insulin signaling molecules PI3K and AKT are under investigation as therapeutics for cancer[91,92]. Unsurprisingly, some PI3K inhibitors have been shown to induce insulin resistance[93].

The nuclear receptor PPARγ is an important drug target, and is genetically linked to insulin sensitivity and type 2 diabetes risk[94,95]. However, PPARγ-activating TZD drugs are associated with a number of side effects and risks, including congestive heart failure[96]. Although some studies have been inconclusive in regards to certain risks associated with the TZD rosiglitazone[97], one meta-analysis of 42 studies found that the risk of cardiovascular death increased 64%[98]. Rodent studies did not predict these deaths, and in fact have provided conflicting evidence regarding cardioprotective and cardiotoxic effects of TZDs. The TZD pioglitazone was shown to limit myocardial infarct size after coronary occlusion in mice[99]. Similar results have been seen for rosiglitazone after ischemia/reperfusion injury[100]. TZDs have been shown to have both positive and negative effects on cardiac hypertrophy in rodents[101,102].

An inhibitor of PKCβ, LY333531, or ruboxistaurin, has been investigated as a potential treatment for diabetic microvascular complications[103]. Although initially promising results were observed in a trial for diabetic neuropathy, the drug was not shown to be effective in a larger, placebo-controlled study[104]. Promising results were also seen in a small trial for diabetic kidney disease[105], but these have not been replicated at a larger scale. Eli Lilly withdrew the marketing authorization application for ruboxistaurin as a treatment for diabetic retinopathy. Rather than diabetes or its complications, PKC inhibitors are now being investigated as potential treatments for cancer[106] and conditions requiring immunosuppressive therapy[107].

CONCLUSION

The limitations of these mouse models of insulin signaling dysfunction arise from a number of sources. Described above are physiological and molecular-level differences between mice and humans, reproducibility problems in mouse experiments, and complicating factors in drug discovery efforts that interfere with translating mouse results to human patients.

Researchers in a variety of fields have commented on the limitations of mouse models of human disease[108,109]. No single mouse model can accurately represent the spectrum of symptoms and complications associated with type 2 diabetes[11]. The translation of results from mice is further complicated by a plethora of immutable species differences at every level of glucose regulation from the molecular to the population level[110-113]. In addition, mice are not prone to hypertension, high LDL cholesterol, atherosclerosis, sedentary behavior, obesity, insulin resistance, or many other features common to human type 2 diabetes patients. Although all laboratory mice are more insulin resistant and have more fat tissue than their free-living counterparts[114], the risk for mice developing these symptoms varies widely depending on the specific inbred strain[62,80]. Genetic background, housing conditions, and diet can dramatically affect results. Examples highlighted here have shown that different studies even from the same laboratory often obtain different results with identical genetic modifications.

The idea that the limitations of genetically modified mouse models of human disease, and rodent models in general, are severe enough to warrant a shift in research approaches is controversial, and will likely continue to be for the next decade. Nonetheless, science in many medical fields has been progressing away from crude, animal-based experiments and towards more high-tech and human-based research methods, and that trend will continue. For example, one area of active research is additional uncharacterized insulin signaling cofactors, which could be identified using phosphoproteomics[115], protein array techniques, or protein interaction-based techniques[116] including yeast two-hybrid and computational approaches. Similar approaches could be used to identify gene products involved in acquired insulin resistance. In addition, insulin resistance can be investigated in human cells by gene silencing[117], metabolomics[118], and microarray technology. Remaining questions about the role of inflammation and accumulated intracellular lipids can be studied using tissue biopsy samples from various patient populations[119]. Many more *in vitro*[120], in silico[121], non-invasive[122], and minimally invasive[123] approaches are available and in development.

In the last 20 years, the use of genetically modified mice to investigate diabetes has become routine. While some findings have borne out in humans, investigations of insulin resistance using knockout mouse models are inherently limited by physiological, genetic, and metabolic differences between mice and humans. Researchers and patients would benefit from a transition towards human-based research methods.

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REVIEW

Chromium does not belong in the diabetes treatment arsenal: Current evidence and future perspectives

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Abstract

Chromium is considered to have positive effects on insulin sensitivity and is marketed as an adjunctive therapy for inducing glucose tolerance in cases of insulin resistance ("the glucose tolerance factor"). Case reports on patients who received prolonged parenteral nutrition indeed showed that the absence of trivalent chromium caused insulin resistance and diabetes. However, whether patients with type 2 diabetes can develop a clinically relevant chromium deficiency is unclear. This review summarizes the available evidence regarding the potential effectiveness of chromium supplementation on glycemic control (Hemoglobin A1c levels) in patients with type 2 diabetes. No studies investigating the long-term safety of chromium in humans were found. All clinical trials that have been performed had a relative short follow-up period. None of the trials investigated whether the patients had risk factors for chromium deficiency. The evidence from randomized trials in patients with type 2 diabetes demonstrated that chromium supplementation does not effectively improve glycemic control. The meta-analyses showed that chromium supplementation did not improve fasting plasma glucose levels. Moreover, there were no clinically relevant chromium effects on body weight in individuals with or without diabetes. Future studies should focus on reliable methods to estimate chromium status to identify patients at risk for pathological alterations in their metabolism associated with chromium deficiency. Given the present data, there is no evidence that supports advising patients with type 2 diabetes to take chromium supplements.

Key words: Chromium; Type 2 diabetes mellitus; Insulin resistance; Therapy; Supplements

Core tip: In some patients who received prolonged parenteral nutrition, absence of trivalent chromium caused insulin resistance and diabetes and supplementation with trivalent chromium "cleared" this metabolic disease. The question is, whether chromium deficiency is a relevant factor in the cause of type 2 diabetes in general and whether supplementation with trivalent chromium can have beneficial effects in type 2 diabetes. Unfortunately, no reliable methods to estimate chromium status exists and according to current evidence, chromium does not improve glycemic control in patients with type 2 diabetes and patients should be advised not to take chromium supplements.

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INTRODUCTION

Insulin resistance is an important target for pharmacological and non-pharmacological interventions in patients with type 2 diabetes. In addition to the well-established interventions, a multitude of suggested alternative solutions outside the field of regular conventional medicine is available. One of these suggested beneficial interventions is oral supplementation with chromium. Chromium is marketed as a substance that improves insulin sensitivity (being as part of the "Glucose Tolerance Factor" molecule), weight loss and improving glycemic control in patients with diabetes[1,2]. Chromium has become the second most popular dietary supplement after calcium in the United States, with sales amounting to approximately 100 million dollars annually[1,2].

Some studies have demonstrated that chromium supplementation in chromium deficient states indeed led to beneficial effects[3-6]. There are strong arguments supporting the hypothesis that chromium supplementation improves glycemic control in chromium deficient patients by improving insulin sensitivity[7]. In addition, patients with diabetes are thought to have a chromium deficient status that is induced by an altered chromium metabolism[4,8,9]. However, other studies have suggested that chromium metabolism is not altered in type 2 diabetes[10]. Unfortunately, cut-off points for chromium levels correlating with relevant changes in glucose metabolism and insulin resistance are lacking. There is no clinically defined chromium deficiency state, nor is there a validated method for estimating the total body chromium status[11-13]. A reliable assessment of the chromium status in biological tissues and fluids is difficult due to extremely low chromium levels[12]. Although some studies have demonstrated successful chromium level determination in hair, sweat, and blood, there is still no exact method for defining chromium deficiency[8]. In this theoretical framework the "diabetic state" is linked to chromium deficiency and chromium supplementation would amend glycemic control by improving insulin sensitivity. This review discusses chromium physiology and summarizes the current evidence that chromium supplementation improves glycemic control in patients with type 2 diabetes.

Several case reports demonstrated beneficial effects of chromium supplementation in patients requiring total parenteral nutrition for prolonged periods[3,5-7,14,15]. One case report, published in 1977, discussed a 40-year-old woman who had undergone a total enterectomy after mesenterial thrombosis and became dependent on total parenteral nutrition[14]. After three years, she started losing weight and developed diabetes mellitus. She was young, had a low body weight, and required 50 IE of insulin daily to reach a near-normoglycemic state. Chromium deficiency was considered as a possible cause. The chromium concentration in her serum and hair was measured and found to be low [154 ng/g (N > 500 ng/g) and 0.55 ng/g (N = 4.9-9.5 ng/g), respectively]. She was treated intravenously with 250 micrograms of chromium chloride daily for two weeks. This treatment decreased the amount of insulin needed, and after four months of chromium supplementation, she remained normoglycemic without insulin. After this and several other case reports[3,9,14], chromium was added to parenteral nutrition as a standard ingredient[6]. Nevertheless, the extent of chromium supplementation necessary during total parenteral nutrition is still debated[16,17].

CHROMIUM PHYSIOLOGY

The two most common forms of chromium are the trivalent (3+) and the hexavalent (6+) forms. Chromium 6+ is not present in nature and is toxic. The chromium found in food and in dietary supplements is the trivalent form. Whole grain products, such as whole grain bread, vegetables, nuts, and some spices contain low concentrations of trivalent chromium. Chromium supplements are available as chromium chloride, chromium nicotinate, chromium picolinate, high-chromium yeast, and chromium citrate. Chromium chloride appears to have a poor bioavailability, although there is limited data on chromium absorption in humans[12,15,18].

The role of trivalent chromium in glucose metabolism has been known since the 1950s[15]. Chromium can alter insulin sensitivity at the cellular level. The oligopeptide Apo-Low-Molecular-Weight-Chromium binding peptide (also known as Apo-chromodulin) plays an important role in potentiating the insulin response in insulin sensitive cells[18,19]. The Apo-chromodulin is loaded intracellularly with a maximum of four chromium ions. Chromium-loaded Apo-chromodulin is called Holo-chromodulin. The Holo-chromodulin molecule binds to the insulin receptor and potentiates the insulin response by activating the receptor. The degree of insulin receptor activation depends on the number of chromium ions bound to this peptide, with a minimum of 0 and a maximum of 4 ions. This chromium binding may lead to an 8-fold difference in insulin receptor activation (when 4 ions are bound compared to 0). Experiments using rat adipocyte cells with equal serum insulin concentrations confirmed that insulin receptor activation is eight times stronger in the presence of chromium than in the absence of chromium[18].

ADVERSE EFFECTS OF CHROMIUM

Several cell culture and animal studies using supraphysiological chromium doses yielded results suggesting that chromium may increase DNA damage[20-23]. Chromium is not unique in this respect; a number of other nutrients such as vitamins A and D, nicotinic acid, and selenium have also been implicated in causing toxicity when taken in excess[24]. Clinical trials of oral chromium supplementation did not demonstrate toxicity in patients on parenteral nutrition[24,25]. We could not find long-term chromium safety studies. The DNA damage identified in cases of supraphysiological trivalent chromium concentrations did not translate into potentially carcinogenic effects when a more physiological dose of oral trivalent chromium was

used in humans[24,26].

CLINICAL EVIDENCE FOR CHROMIUM USE

In 1997, the intervention trial by Anderson *et al*[27] was one of the first chromium-intervention studies in patients with type 2 diabetes. In this randomized controlled trial, chromium picolinate supplements or placebo were administered to 180 Chinese patients with type 2 diabetes. The patients were randomized into three groups: placebo, 200 mg chromium, and 1000 mg of chromium daily. After four months, the hemoglobin A1c (HbA1c) levels in the placebo group were unchanged (8.5%), while they decreased significantly in the 200 mg group, from 8.5% to 7.5%, and decreased in the 1000 mg group, from 8.5% to 6.6%.

In 2007, Balk *et al*[28] performed a systematic review of randomized controlled trials investigating chromium supplementation in patients with type 2 diabetes. At that time, 14 studies with 18 different chromium-based interventions had been performed using HbA1c levels as an endpoint. In 11 out of these 14 trials, there was no significant effect of chromium supplementation. The review by Balk *et al*[28] concluded that, due to the poor quality and heterogeneity of the data, additional studies addressing these limitations were needed before definitive claims could be made about the effect of chromium supplementation[28]. Nevertheless, the meta-analysis by Balk *et al*[28] reported an overall significant effect of chromium supplementation on HbA1c levels (-0.6%; 95%CI: -0.9% to -0.2%). This -0.6% mean benefit was largely due to the inclusion of the data reported by Anderson *et al*[27]. When the Anderson study was excluded, the effect of chromium on HbA1c levels was -0.3% (95%CI: -0.5% to -0.1%; NS)[28]. It should be noted however, that the Anderson study was inadequately blinded with concerns for detection bias and selection bias, and should be considered to be of poor-methodological quality[27,28].

Significant effects in the meta-analysis were only found in studies with poor methodological quality or in studies sponsored by chromium supplement producing companies. In addition, the effects of chromium supplementation were shown to be absent or non-relevant after stratifying the studies according to methodological quality, sponsor involvement, and a western *vs* non-western study location[6,29].

After the review written by Balk *et al*[28], a second Dutch double blind trial was performed in 2008 that studied the effects of chromium on HbA1c levels in patients with type 2 diabetes[29]. After 6 mo, the effect of chromium supplementation compared to placebo on HbA1c levels was 0.24% (95%CI: -0.06% to 0.54%). HbA1c levels were lower in the placebo group compared with the chromium group. All of the trials that have been performed had a relatively short follow-up period. No studies have been performed with sufficient follow-up and the ability to reliably investigate cardiovascular and/or microvascular end-points. All studies used surrogate end-points. None of the trials investigated whether patients had risk factors for chromium deficiency.

Although this review focuses on the most relevant method of estimating glycemic control (HbA1c levels)[30-32], several studies investigated the effect of chromium on other markers of glycemic control[11,13,33-35]. Meta-analyses showed that chromium supplementation did not improve fasting plasma glucose levels[33,36] and had no clinically relevant effect on body weight in individuals with or without diabetes[37-39].

DISCUSSION

Chromium plays a role in insulin physiology, and severe chromium deficiency can lead to insulin resistance. Chromium supplementation may be beneficial in rare cases of prolonged total parental nutrition when standard chromium supplementation is lacking[6]. Despite the lack of sufficient evidence that chromium supplementation improves glycemic control[28,29], chromium is still widely marketed as an effective supplement for improving glycemic control in patients with type 2 diabetes.

Do we need to worry that a low chromium status contributes to hyperglycemia in our patients?

For the average patient with type 2 diabetes, the answer is no. Trivalent chromium is sufficiently available in food, and the occurrence of severe chromium deficiency is highly unlikely. The sparse evidence that chromium supplementation might have effects on glycemic control in a broader population is derived from studies with important methodological flaws[27,28]. Well-performed trials and meta-analyses consistently show that there is no evidence for consistent beneficial effects on glycemic control (as assessed by HbA1c levels) that support prescribing chromium supplements to patients with type 2 diabetes[6,40]. Furthermore, the long-term safety of chromium supplementation has not been established.

Is all hope lost for chromium supplementation in patients with type 2 diabetes?

An important concern when interpreting the data from studies investigating chromium effects is the lack of a validated and precise estimate of chromium status. There is no reliable method for assessing the body's chromium status, and there is no information on the bioavailability of the different forms of chromium[41]. Performing randomized trials in patients with type 2 diabetes will become interesting only when we can properly assess the chromium status in patients at risk for chromium deficiency and when clinically relevant end points are defined.

Recommendations

Future research on chromium should focus on establishing a reliable method for assessing the body's chromium status. The bioavailability of different forms of chromium in Western and non-Western patients should be investigated in order to define a potential effective dose

and to identify patients at risk for chromium deficiency. New randomized trials should only be considered in type 2 diabetes patients with an established chromium deficiency. The long-term safety of chromium supplementation should be investigated in large population studies. Currently, chromium supplementation in patients with type 2 diabetes should not be recommended.

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REVIEW

Origin and therapy for hypertriglyceridaemia in type 2 diabetes

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Abstract

Hypertriglyceridaemia (HTG) is a risk factor for cardiovascular disease (CVD) in type 2 diabetes and is caused by the interaction of genes and non-genetic factors, specifically poor glycaemic control and obesity. In spite of statin treatment, residual risk of CVD remains high in type 2 diabetes, and this may relate to HTG and atherogenic dyslipidemia. Treatment of HTG emphasises correcting secondary factors and adverse lifestyles, in particular, diet and exercise. Pharmacotherapy is also required in most type 2 diabetic patients. Statins are the first-line therapy to achieve recommended therapeutic targets of plasma low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol. Fibrates, ezetimibe and n-3 fatty acids are adjunctive treatment options for residual and persistent HTG. Evidence for the use of niacin has been challenged by non-significant CVD outcomes in two recent large clinical trials. Further investigation is required to clarify the use of incretin-based therapies for HTG in type 2 diabetes. Extreme HTG, with risk of pancreatitis, may require insulin infusion therapy or apheresis. New therapies targeting HTG in diabetes need to be tested in clinical endpoint trials. The purpose of this review is to examine the current evidence and provide practical guidance on the management of HTG in type 2 diabetes.

Key words: Diabetes; Triglyceride; Therapy

Core tip: Diabetic dyslipidemia relates collectively to hyperglycaemia, insulin resistance, hyperinsulinaemia, abdominal visceral adipose disposition, increased liver fat content, and dysregulated fatty acid metabolism. Insulin resistance in diabetes induces hypertriglyceridaemia by increasing the enterocytic production of chylomicrons and an impaired clearance capacity is also involved. Usual care for diabetic dyslipidemia is statin treatment, but a significant proportion of patients have residual dyslipidemia, related to hypertriglyceridaemia and atherogenic dyslipidemia. Current evidence supports the use of fenofibrate in type 2 diabetics with high triglyceride levels.

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INTRODUCTION

Hypertriglyceridaemia (HTG) is an important risk factor for cardiovascular disease (CVD)[1] and is defined as a fasting plasma triglyceride concentration > 95th percentile for age and sex in a population. HTG may be as prevalent as 50% in type 2 diabetes and is often unresponsive to statin treatment[2,3]. We review recent evidence on the role of HTG in atherosclerotic CVD and provide practical guidance on the management of HTG in type 2 diabetes.

PATHOPHYSIOLOGY OF HYPERTRIGLYCERIDAEMIA IN TYPE 2 DIABETES

Triglycerides, which originate from the intestine postprandially or endogenously from the liver, are packaged into lipoprotein particles containing apolipoprotein B-48 (apoB-48; chylomicrons) and apolipoprotein B-100 (apoB-100; very-low density lipoprotein, VLDL), respectively. Abnormalities in triglyceride-rich lipoprotein (TRL) metabolism are cardinal features of type 2 diabetes. Metabolic dysregulation resulting in HTG include enhanced hepatic secretion of TRL due to insulin resistance and delayed clearance of TRL involving lipoprotein lipase (LPL)-mediated lipolysis. Several genes causing loss of function of LPL can result in severe HTG, such as *LPL*, *APOC2*, *APOA5*, *GPD1*, *CPIHBP1* and *LMF1*[4,5]. Very few patients will have a monogenic disorder. Individuals with severe HTG are likely to be homozygous or compound heterozygous for mutations which impair the TRL catabolic pathway. However, HTG in type 2 diabetes due to several genes with mild effects that interact with non-genetic factors is probably more likely. These non-genetic factors include hyperglycaemia, alcohol abuse, concomitant medication, sedentary lifestyle, chronic kidney disease and insulin resistance[6].

Insulin resistance activates *de novo* lipogenesis, resulting in oversecretion of hepatic TRLs. This is also evident in the postprandial state, with enterocytic oversecretion of TRLs in the form of chylomicrons. With both secretion pathways on overdrive, competition between the TRLs and their remnants for lipolytic and receptor-mediated clearance further induces HTG. Insulin resistance is also associated with increased rates of apolipoprotein C-Ⅲ (apoC-Ⅲ) secretion, which further impairs receptor-mediated uptake of hepatic chylomicron remnants[7]. Glucose has also found to activate apoC-Ⅲ transcription, which may be the link between hyperglycaemia, HTG and CVD in type 2 diabetics[8].

Both LPL and hepatic lipase (HL) control the clearance of triglycerides. HL plays a particularly important role in the delipidation cascade from VLDL to LDL. Triglyceride-rich VLDL derives small, dense LDL particles which are more susceptible to oxidation[9]. Additionally, increased TRL in postprandial diabetic dyslipidemia leads to the exchange of TRL-triglyceride for HDL-cholesteryl ester and hence, triglyceride enrichment of HDL *via* cholesteryl ester transfer protein (CETP). CETP progressively decreases postprandially and limits the efficient removal of cholesterol[10]. Triglycerides in HDL are good substrates for hepatic lipase which leads to the production of small dense HDL particles and enhanced apolipoprotein A-Ⅰ (apoA-Ⅰ) clearance[11].

Given that HTG is related to a plethora of risk factors, the lack of independent association between triglyceride and CVD is expected[12], although two recent Mendelian randomisation studies have shown a causal association between variations in two related genes (*LPL* and *APOA5*) and myocardial infarction[13]. This supports that TRL causes CVD, and this probably applies to diabetes.

Hence, diabetic dyslipidemia relates collectively to hyperglycaemia, insulin resistance, hyperinsulinaemia, abdominal visceral adipose disposition, increased liver fat content, and dysregulated fatty acid metabolism. Diabetic dyslipidemia may also be exacerbated by chronic kidney disease and by co-prescribed medications, such as thiazide diuretics, non-selective beta-blockers and steroids.

MANAGEMENT OF HYPERTRIGLYCERIDAEMIA IN TYPE 2 DIABETES

Measurement and assessment

Triglyceride concentration is commonly measured with a fasting lipid profile. The fasting triglyceride level facilitates the calculation of the LDL cholesterol by the Friedewald equation[14]. Non-fasting triglyceride concentrations are reflective of the postprandial state and can be useful as a simple and practical screening test for HTG. A second non-fasting measurement is recommended if the initial triglyceride is > 2.0 mmol/L. Two or more measurements of elevated triglyceride in both postabsorptive and postprandial states are clinically indicative of HTG. Categories of HTG are differentially defined in international guidelines (Table 1).

Non-HDL cholesterol is another appealing method of assessment as it does not attract additional costs. Non-HDL cholesterol (total cholesterol minus HDL-cholesterol) does not rely on a fasting triglyceride concentration and provides a simple amalgamated measure all the atherogenic lipoproteins[15]. ApoB, on the other hand, does not adequately reflect chylomicron remnants and involves additional laboratory expenses. Discordance between non-HDL cholesterol and apoB measures, particularly in patients with type 2 diabetes and HTG, questions its value in assessing risk and defining treatment targets[16]. In the context of statin-treated patients, a meta-analysis has shown that non-HDL cholesterol is superior in its association with risk of future major cardiovascular events compared with LDL cholesterol and apoB[17]. Other TRL markers such as remnant-like particle cholesterol, apoC-Ⅲ and apoB-48 are expensive and are yet to be clinically established.

The hypertriglyceridaemic waist (HTWC) phenotype has suggested to be useful in assessing glucometabolic risk[18-21], in particular, among patients with a family history of diabetes[22]. The HTWC phenotype is defined by a waist circumference of ⩾ 90 cm in men and ⩾ 85 cm in women and triglyceride concentration ⩾ 2.0 mmol/L. Men with the HTWC phenotype have been shown to have a four-fold risk of diabetes compared to those with waist circumference and triglyceride in the normal ranges[23]. There is also a two-fold risk for development of coronary artery disease (CAD) in women[24] and an overall deterioration of cardiometabolic risk[25] in relation to progression of type 2 diabetes[26].

Table 1 Clinical categorisation of hypertriglyceridaemia according to guidelines based on fasting triglyceride concentrations

Ref.	Year published	Triglyceride categories	Triglyceride concentration (mmol/L)
National institutes of Health[31]	2001	Normal	1.7
		Borderline high	1.7-2.3
		High	2.3-5.6
		Very high	> 5.6
Rydén *et al*[33]	2011	Desirable	< 1.7
		Elevated	1.7-5.5
		Very high	5.5-25.0
		Extremely high	> 25.0
Berglund *et al*[34]	2012	Normal	< 1.7
		Mild	1.7-2.3
		Moderately high	2.3-11.2
		Severely high	11.2-22.4
		Very severely high	> 22.4
Hegele *et al*[37]	2013	Normal	< 2.0
		Mild-to-moderate	2.0-10.0
		Severe	> 10.0

Table 2 Clinical guide for the assessment and treatment of hypertriglyceridaemia in type 2 diabetes

Steps	
1	Obtain fasting lipid profile
2	Classify LDL-cholesterol concentration (primary target of therapy) < 2.60 mmol/L – optimal 2.60-3.39 mmol/L – above optimal 3.40-4.14 mmol/L – borderline high 4.15-4.90 mmol/L – high > 4.90 mmol/L – very high Establish therapy: LDL-cholesterol > 2.60 mmol/L – initiate dietary and lifestyle modifications LDL-cholesterol > 3.40 mmol/L – consider pharmacotherapy simultaneously with dietary and lifestyle modifications
3	Identify presence of atherosclerotic disease Clinical coronary heart disease Symptomatic carotid artery disease Peripheral artery disease
4	Assess: Glycaemic control Obesity Dietary intake (*e.g.*, Fructose, simple sugars, caloric intake) Physical activity Determine presence of other risk factors: Smoking Hypertension Family history of premature coronary heart disease (*i.e,*. in first-degree relative, male < 55 years, female < 65 years) Low HDL-cholesterol, < 1.0 mmol/L
5	Order of treatment considerations: Improve glycaemia (dietary and lifestyle modifications) Treat secondary risk factors Statins Fibrates n-3 fatty acids/niacin
6	Treat elevated triglyceride if triglyceride concentrations are > 2.30 mmol/L after LDL-cholesterol concentration target of < 2.60 mmol/L is reached Target non-HDL cholesterol (< 3.40 mmol/L) Triglyceride > 2.30 mmol/L – intensify LDL-lowering therapy or add fibrate Triglyceride > 5.60 mmol/L – very low-fat diet (< 15% of calories from fat), weight management, physical activity and add fibrate

Adapted from the NCEP ATP Ⅲ guidelines[31]. LDL: Low density lipoprotein; HDL: High density lipoprotein.

Guidelines and recommendations

Guidelines for managing HTG in diabetes have been published, with lifestyle modifications being first-line therapy followed by statins, fibrates, n-3 fatty acids and/or niacin[27-30]. The national cholesterol education program (NCEP) adult treatment panel (ATP) Ⅲ guidelines recommend LDL cholesterol as the primary treatment target and non-HDL cholesterol as a secondary target, with the exception of a fasting triglyceride > 5.60 mmol/L, only then, triglyceride becomes the primary target owing to the risk of pancreatitis[31]. A simplification of the NCEP ATP Ⅲ guideline is presented in Table 2. Regardless of atherosclerotic disease and presence of other cardiovascular risk factors, type 2 diabetes is considered a coronary heart disease risk equivalent by the NCEP ATP Ⅲ.

The American Diabetes Association (ADA)/American College of Cardiology Foundation consensus statement recommends a non-HDL cholesterol target of 3.40 mmol/L in diabetic patients with no other cardiovascular risk factor and a target of 2.60 mmol/L if there is one or more cardiovascular risk factor such as hypertension, smoking, dyslipidemia and family history of CAD[32]. The LDL cholesterol target is 2.60 and 1.80 mmol/L, respectively[32] or alternatively a 30%-40% reduction from baseline levels[30]. The ADA position statement is the only guideline that provides desirable targets for triglyceride levels for patients with type 2 diabetes: less than 1.70 mmol/L[30]. Both the NCEP ATP and ADA guidelines place emphasis on weight loss and physical activity. A summary of recommended treatment targets is presented in Table 3.

The Scientific Statement from the American Heart Association (AHA) on triglycerides and CVD particularly emphasises the dietary and lifestyle modifications (weight loss, macronutrient distribution and aerobic exercise) for the treatment of elevated triglycerides, presenting a practical algorithm for screening and management[28]. The European Society of Cardiology (ESC) guidelines on diabetes and CVD developed in collaboration with the European Association for the Study of Diabetes (EASD) suggests targeting residual risk in patients with elevated TG (> 2.2 mmol/L), with dietary and lifestyle advice and improved glucose control[33], post first-line treatment. The Endocrine Society task force agrees with the NCEP ATP Ⅲ treatment goals and recommends fibrates as first-line treatment for lowering triglycerides in patients at-risk for pancreatitis[34].

The International Atherosclerosis Society position paper recognises the atherogenicity of VLDL and triglycerides and also favours non-HDL cholesterol as the main target for therapy, optimally at < 3.40 mmol/L[35]. The American College of Cardiology (ACC)/AHA published a new clinical practice guideline for the treatment of elevated blood cholesterol in people at high risk for CVD.

Table 3 Recommended treatment targets for diabetic dyslipidaemia

		NCEP ATP Ⅲ[31]	ADA[30]	NVDPA[128]	European Guidelines[33]
LDL-cholesterol (mmol/L)	Very high risk	< 1.8	< 1.8	< 2.0	< 1.8
	High risk	< 2.6	< 2.6	< 2.0	< 2.5
Triglycerides (mmol/L)			< 1.7	< 2.0	< 1.7
HDL-cholesterol (mmol/L)	Male		> 1.0	⩾ 1.0	> 1.0
	Female		> 1.3	⩾ 1.0	> 1.2
Non-HDL cholesterol (mmol/L)	Very high risk	< 2.6	< 2.6	< 2.5	< 2.6
	High risk	< 3.4	< 3.4	< 2.5	< 3.3
ApoB (g/L)	Very high risk		< 0.8		< 0.8
	High risk		< 0.9		< 1.0

NCEP ATP Ⅲ: Third Report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation and treatment of high blood cholesterol in adults (Adult treatment panel Ⅲ); ADA: American diabetes association; NVDPA: National vascular disease prevention alliance of australia; LDL: Low density lipoprotein; HDL: High density lipoprotein.

The guidelines do not provide recommendations for specific LDL-cholesterol or non-HDL targets and instead defines four major groups of primary and secondary prevention patients for whom LDL lowering is proven to be most beneficial[36]. Future guidelines to cover the treatment of HTG are proposed. A recent review by Hegele *et al*[37] recommended the simplification and redefinition of HTG: < 2.0 mmol/L as normal, 2.0-10.0 mmol/L as mild-to-moderate and > 10.0 mmol/L as severe; with desirable targets of < 1.7 mmol/L for triglycerides, < 2.6 mmol/L for non-HDL cholesterol and < 0.8 g/L for apoB in high-risk patients

Treatment of HTG depends on its severity, co-existing lipid abnormalities and overall cardiovascular risk. Severe HTG serves as increased risk of pancreatitis and warrants treatment to acutely reduce triglyceride levels. Current therapeutic strategies include diet and lifestyle modification, pharmacotherapy and in rare cases, continuous insulin infusion and apheresis.

Dietary and lifestyle modifications

Lifestyle interventions are central for controlling hyperglycaemia and HTG in patients with type 2 diabetic patients and impaired fasting glucose. These interventions include weight reduction, altered dietary composition, exercise and regulation of alcohol consumption. In type 2 diabetes, modest (5%-10%) weight loss can lower plasma triglyceride levels by up to 25%[38,39] and normalise postprandial triglyceride concentration[40]. Physical activity can aid the maintenance of weight loss achieved through caloric restrictions[41], although evidence for linking lifestyle modifications and sustained weight is limited[42].

The recently published look AHEAD trial, an intensive lifestyle intervention in type 2 diabetics, employing weight loss through caloric restriction and increased physical activity did not reduce the rate of cardiovascular events[43]. Whether alterations in dietary composition, such as with the Mediterranean diet, improves clinical outcome in diabetes warrants additional investigation[44], though the Mediterranean and low-carbohydrate diet can produce a greater reduction in triglyceride levels compared to the restricted-calorie diet in moderately obese individuals[45,46]. Plant sterols have been suggested for lowering TG in individuals with overt HTG[47]. Alcohol abstinence in patients with excessive alcohol intake can markedly lower plasma triglyceride levels[48,49]. Smoking cessation is also imperative[50].

Pharmacotherapy

Statin monotherapy: Statin therapy is the cornerstone of treatment of dyslipidemia in diabetes. Whilst reaching the LDL cholesterol target in most patients, only modest effects are exerted on triglyceride and HDL cholesterol. Hence, diabetics with HTG often have residual CVD risk[51] in spite of an optimal LDL cholesterol target. Statins may lower plasma triglyceride by increasing lipolysis and the clearance of TRLs, particularly with potent statins such as atorvastatin and rosuvastatin (up to 26% and 28% reduction in plasma triglyceride, respectively)[52-54]. Large statin outcome trials have supported its use in reducing coronary events and mortality[55-58]. All trials to-date have not specifically selected for HTG and in diabetics. However, sub-group analyses have been undertaken showing risk prevention with pravastatin[59], simvastatin[60] and rosuvastatin[61] in a subset of patients with high plasma triglyceride, recently reviewed by Maki *et al*[62], and supporting statins as first line of therapy. Whilst use of higher doses of statin has been linked to incidence of diabetes[63-65], the benefits of statin therapy for reducing CVD risk and events are outweighed for all diabetic patients with high CVD risk[57,63]. Aminotransferase, creatine kinase, creatinine and glucose should be monitored prior to initiating statins and before initiating a second agent, if required.

Fibrates and statin-fibrate combination: Fibrates (gemfibrozil, fenofibrate) act on peroxisome proliferator-activated receptor alpha. Fibrates decreases hepatic VLDL secretion and can confer an up to 30% reduction in plasma triglyceride, TRL remnants and apoB[66]. Five fibrate trials have undertaken secondary analyses in high triglyceride subgroups[67-79], two of these trials were in type 2 diabetic patients[70-72] and one had a subset of diabetics[73,74]. Collectively, these trials advocate the use of fibrates in reducing CVD events among patients with

a high triglyceride and low HDL cholesterol levels[75-78]. Of note, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study showed that fenofibrate decreased progression of diabetic retinopathy[79], though unrelated to dyslipidemia, and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study also showed a delay in the onset of eye complications[80]. Meta-analyses suggest that fibrates are useful for treatment of HTG[76] in diabetic patients[71,81,82]. Every 0.10 mmol/L reduction in triglyceride with fibrates confers a 5% reduction in CVD event, although no benefits were found on cardiovascular mortality[77,78].

Niacin and statin-niacin combination: Niacin can decrease plasma triglyceride by 30%[83] *via* the inhibition of hepatic diacylglycerol acyltransferase-2 (DGAT-2), a rate-limiting enzyme of triglyceride synthesis. Despite the earlier studies showing reduced mortality[84] and regression of subclinical atherosclerosis[85-87], the current use of niacin has been challenged by two large recent clinical trials which have failed to show significant benefits on CVD events[88,89] in spite of positive changes in lipid parameters. Both trials have limitations. The Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health (AIM-HIGH) study was underpowered and confounded by the higher statin and/or ezetimibe doses to match LDL cholesterol between groups[88]. The Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) study is the largest extend-release niacin trial to-date combined with laropiprant, a prostaglandin D2 inhibitor[89]. Despite no significant benefit on primary CVD endpoints, a recent sub-analysis in patients with both high triglyceride (> 2.24 mmol/L) and low HDL cholesterol (< 0.85 mmol/L) showed a trend towards benefit with niacin, although not reaching statistical significance (HR = 0.74, $P = 0.073$)[90]. Of note, the lack of potential benefit or harm in the HPS2-THRIVE study may not necessarily be due to niacin, but potentially to laropiprant. The safety of niacin use in type 2 diabetes has previously been questioned owing to impairment in glycaemic control and insulin sensitivity[91-93]. However, two prospective trials have showed that the effect of niacin on glycaemic control is minimal in a majority of patients with stable diabetes[94] and with no changes in low-dose (1 g/d) niacin[95].

Ezetimibe and statin-ezetimibe combination: Ezetimibe inhibits intestinal cholesterol absorption and primarily lowers LDL cholesterol *via* the Niemann-Pick C1-Like 1 protein. Ezetimibe has minimal effects in lowering plasma fasting triglyceride (8%)[96]. A more prominent effect is observed in ameliorating postprandial lipaemia and lowering TRL remnants in spite of background statin[97,98]. In a 6-wk trial of simvastatin-ezetimibe vs. simvastatin monotherapy, fasting and postprandial plasma triglyceride and apoB-48 concentrations were lowered in type 2 diabetic patients[99]. However, intensive lipid lowering with a statin plus ezetimibe may not consistently lower subclinical carotid atherosclerosis in type 2 diabetes, although progression of carotid artery intima-media thickness was inhibited with the combination[100,101]. The Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) study that is currently entering completion will endeavour to provide definitive evidence for the role of ezetimibe in high risk subjects on optimal statin therapy[102,103].

n-3 fatty acid and statin-n-3 fatty acid combination: Supplemental n-3 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are well known to improve HTG[104]. However, recent clinical outcome trials with have failed to show significant CVD benefits in high risk subjects including diabetics[105,106]. Both trials were undertaken against a background of optimal therapy, including statins. Also, patients were not selected for elevated plasma triglyceride levels. Pure EPA (1800 mg/d), added to statin therapy, showed promise in the Japan Eicosapentaenoic acid Lipid Intervention Study (JELIS) with major coronary events reduced by 19% ($P = 0.011$) in hypercholesterolaemic patients[107]. Two 12-wk EPA (AMR101) intervention trials in patients with very high[108] and persistent[109] baseline triglyceride observed significant reductions in triglyceride levels. The greatest decrease was seen in the highest triglyceride tertile where there was a 31% reduction compared to placebo on 4 g/d of AMR101. The Reduction of Cardiovascular Events with EPA-Intervention Trial (REDUCE-IT) is in progress and will endeavour obtain the CVD outcome data with AMR101 4 g/d in high-risk patients with HTG and at-target LDL cholesterol on statin therapy[110]. There are also recent data suggesting an increased risk of prostate cancer with high dietary intake of n-3 PUFAs[111]. Hence, caution is warranted when recommending long-term intake.

Incretin-based therapy: Incretins, such as glucagon-like peptide-1 (GLP-1), are insulinotropic, gut-derived hormones secreted in response to diet. GLP-1 receptor analogs such as liraglutide and exenatide, delay gastric emptying and this parallels the reduction in postprandial triglyceride response[112]. This mechanism may ameliorate impaired TRL metabolism in type 2 diabetes. By increasing plasma concentrations of GLP-1, Dipeptidyl peptidase-4 (DPP-4) inhibitors, such as sitagliptin, saxagliptin and alogliptin, can improve insulin sensitivity, β-cell function[113] and postprandial glycaemia[114] and lipaemia[115]. These agents could potentially prevent CVD events, independent of changes in glucose and lipid metabolism. A recent saxagliptin outcome trial failed to demonstrate significant changes in ischaemic events, though the rate of heart failure increased significantly[116]. Similarly, a trial in type 2 diabetic patients post-acute coronary syndrome with aloglipitin did not improve cardiovascular event rates compared with placebo[117]. Further investigation is required to clarify

their mechanism and use in type 2 diabetes.

MANAGEMENT OF SEVERE HYPERTRIGLYCERIDAEMIA IN TYPE 2 DIABETES

Insulin infusion, apheresis and gene replacement therapy

In severe cases of diabetic HTG and poorly controlled diabetes, continuous intravenous insulin infusion appears to be beneficial in restoring serum glucose and triglyceride[118]. Most of these patients will have an underlying genetic defect in TRL metabolism. A recent study in a group of 15 diabetics with a median triglyceride concentration of 26.23 mmol/L at admission had their triglyceride levels corrected to a median of 5.75 mmol/L at discharge with an average of 48 h of continuous insulin infusion[119]. For prevention of recurrent severe HTG in susceptible patients, counselling on medication adherence and long-term diet and lifestyle medications should be considered[120].

In extremely severe HTG and drug refractory HTG, plasma apheresis may be required[121,122], particularly with severe chylomicronaemia complicated by acute pancreatitis. A single session of apheresis can dramatically lower excessive triglyceride levels, 65.8% reduction in 2 h[123,124]. This method of triglyceride lowering is only indicated in medical emergencies owing to high costs and limited availability[125]. Further study is required to clarify the role of plasma exchange in the treatment of hyperlipidaemic pancreatitis.

In patients genetically diagnosed with familial LPL deficiency, Glybera® (alipogene tiparvovec; Amsterdam Molecular Therapeutics, Amsterdam, the Netherlands) is the first approved gene-replacement therapy[126,127]. Glybera® has only been studied in 27 patients, in whom the agent was well tolerated and with plasma triglyceride concentration significantly lowered with reduced rates of acute pancreatitis[126]. Long-term follow-up data and cost-effectiveness studies is warranted[126,127].

CONCLUSION

HTG is common in type 2 diabetes. HTG associates with a spectrum of cardiometabolic risk factors and increases CVD risk in type 2 diabetes. Dietary and lifestyle modification involving weight loss and exercise is fundamental to the management of HTG. Improved glycaemic control with use of metformin, DPP-4 inhibitors and insulin can also improve HTG. The expression of HTG in context of diabetes may depend on co-existing monogenic and/or multigenic disorders of lipid metabolism, such as familial combined hyperlipidaemia, familial hypertriglyceridaemia and type Ⅱ hyperlipoproteinaemia. Statins are the first-line of lipid-lowering therapy to target LDL cholesterol and triglycerides. Current evidence supports the use of fenofibrate in type 2 diabetics, with high triglyceride and low HDL, but also to prevent and treat diabetic retinopathy. More evidence is required from CVD outcome trials for the other add-on options, some of which are currently underway. Several new therapies with potential applications for treating HTG are DGAT inhibitors, microsomal triglyceride transfer protein inhibitors, and apoC-Ⅲ antisense oligonucleotides. These will agents will require to be tested for efficacy, safety and cost-effectiveness in future clinical trials.

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REVIEW

Phytotherapy in diabetes: Review on potential mechanistic perspectives

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Abstract

Diabetes mellitus (DM) is a widely spread epidemic disease that results from the absence of insulin, decreased secretion and/or impaired function. Since DM is a multifactorial disease, the available pharmaceuticals, despite their sensible treatment, target mostly one pathway to control hyperglycemia and encounter several side effects. Therefore, new therapeutic paradigms aim to hit several pathways using only one agent. Traditionally, antidiabetic plants and/or their active constituents may fulfill this need. More than 200 species of plants possess antidiabetic properties which were evaluated mostly by screening tests without digging far for the exact mode of action. Searching among the different literature resources and various database and in view of the above aspects, the present article provides a comprehensive review on the available antidiabetic plants that have been approved by pharmacological and clinical evaluations, and which their mechanism(s) of action is assured. These plants are categorized according to their proved mode of action and are classified into those that act by inhibiting glucose absorption from intestine, increasing insulin secretion from the pancreas, inhibiting glucose production from hepatocytes, or enhancing glucose uptake by adipose and muscle tissues. The current review also highlights those that mimic in their action the new peptide analogs, such as exenatide, liraglutide and dipeptidylpeptidase-4 inhibitors that increase glucagon-like peptide-1 serum concentration and slow down the gastric emptying.

Key words: Hypoglycaemic nutraceuticals; Antidiabetic phyto-constituents; Medicinal plants; Phytotherapy; Diabetes mellitus

Core tip: Diabetes is a serious metabolic disorder that is currently treated by different types of synthetic oral hypoglycemic agents, in addition to insulin. However, due to the unwanted side effects, the efficacies of these compounds are debatable and there is a demand for new compounds for the treatment of diabetes. Therefore, attention has been directed towards nutraceuticals originating from plants that are rich in antidiabetic phyto-constituents. Although the evidenced-based therapeutic usage of many plants is scarce, the plants cited in this review are those reputed traditionally for their antidiabetic effect and that were verified either experimentally or clinically.

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INTRODUCTION

Diabetes mellitus (DM) is a common disorder of car-

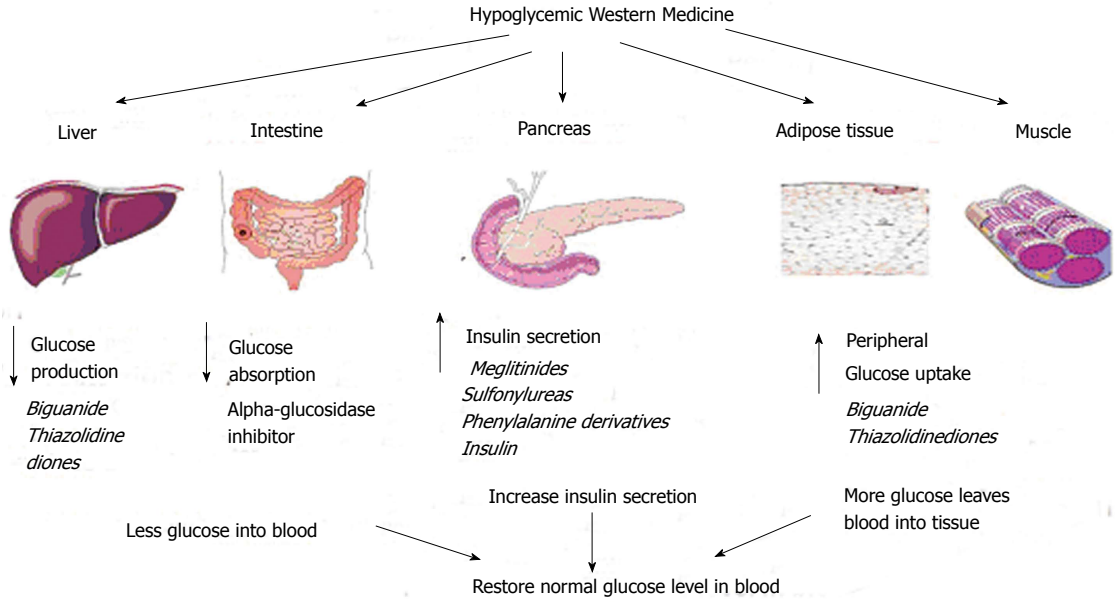

Figure 1 Pathophysiological mechanisms of hyperglycemia matched with the suitable pharmacotherapeutics (Data adapted from Hui *et al*[3]).

bohydrate, fat and protein metabolism reflected by an inappropriate fasting and postprandial high blood glucose levels (hyperglycaemia). This ailment results from the absence or scantiness of insulin secretion with or without concurrent impairment of insulin action. Consequently, the disease was classified into two types known as type I (insulin dependent, IDDM) and II (non-insulin dependent, NIDDM) according to the degree of the pancreatic defect. This classification has been even recognized since the time of Ibn Sinaa who mentioned it in his book "The Canon of Medicine".

DM is not confined to abnormal blood glucose level, but it progresses to affect other body systems. This fact was confirmed by several epidemiological studies and clinical trials that linked hyperglycemia to several complications at the macrovascular (coronary artery disease and cerebrovascular disease), as well as the microvascular levels (renal failure, blindness, limb amputation, neurological complications and pre-mature death)[1].

Based on the pathophysiology and severity of this disease, it can be controlled by dietary restriction, exercise, different types of synthetic oral hypoglycemic agents and/or insulin. Since DM is one of the multi-factorial based diseases, therefore, a balanced modulation of several targets can provide a superior therapeutic effect and a decrease in the side effects profile compared to the action of a single selective agent[2]. Hence, the current strategy used for the treatment of type II DM depends on combining an insulin secretagogue and an insulin sensitizer to provide a sensible therapeutic approach (Figure 1)[3]. Albeit reasonable management provided by these drugs, yet over time, some of the type II diabetic patients lose response towards conventional antidiabetics, leading to an inadequate control of their blood glucose level. Moreover, several side effects could hinder their capability in alleviating the symptoms of diabetes, such as severe hypoglycemia, lactic acidosis, idiosyncratic liver cell injury, permanent neurological deficit, digestive discomfort, headache, dizziness and even death. In addition, treatment of IDDM using insulin has also its complications, since continuous exposure to insulin causes a reduction in the number of receptors on the cell surface by promoting internalization, as well as degradation of hormone-occupied receptors[4].

In spite of the introduction and extensive utilization of hypoglycaemic agents, diabetes and its related complications continue to be a major health problem worldwide. Globally, around 150 millions of people are believed to be diabetic and the incidence rate is expected to double by 2025[5]. These expectations are "good to be true", especially for NIDDM, since the slothful lifestyle along with the high consumption of westernized diet, are considered to be the cornerstone for the development of this type of DM. Hence, it is a requisite to have safer and more effective oral hypoglycemic agents which can hit many targets to fulfill the new paradigm in drug discovery[6]. To achieve this objective, one may either employ a single compound to strike multiple targets; this can be termed as a one compound-multiple-target strategy[2], or use a combination of active compounds in one drug[7]. Therefore, attention has been directed towards nutraceuticals, since they can fulfill these criteria.

Herbal products may contain several active constituents or compounds that can act by several modes of action to influence multiple biological pathways and to alleviate the diabetic symptoms, providing thereby multifaceted benefits[8]. Nevertheless, this vision is not totally new, since prior to and after the discovery of insulin, herbs with hypoglycaemic effect have been used in folk medicine and are still prevalent[9-11]. As a support for this concept, metformin, which is notable for its substantial favorable impact on diabetes prevention, was purified from the French lilac *Galega officinalis* L.[12]. Moreover, the low cost of these compounds and the minimal side effects are

other reasons behind the hunt for effective natural agents to be used as complementary and/or alternative medicine.

Since restoring glucose homeostasis is influenced by several aspects, the current article classifies the hypoglycemic herbs that are available in literature resource from various database, into poroper categorization according to their potential mode of action to reduce blood glucose level. Different search engines were explored including Pubmed, Google, Asci database by using different keywords, as well as some of the traditional tertiary resources. Priority was given to research articles and information given by authentic organizations and federations. Plants cited in this review are those reputed traditionally for their antidiabetic effect and that were verified, either experimentally or clinically. The efficacy of hypoglycemic herbs is achieved by inhibiting glucose absorption from intestine, increasing insulin secretion from the pancreas, inhibiting glucose production from hepatocytes, or enhancing glucose uptake into the peripheral tissue *via* the glucose transporters (GLUT). Additionally, the plants that act by simulating the action of the new incretin peptide analogs were also mentioned in the present review.

INHIBITION OF GLUCOSE ABSORPTION

Postprandial hyperglycemia plays an important role in the incidence of type Ⅱ DM, since recent studies suggest that it could induce the non-enzymatic glycosylation of various proteins, resulting in the development of chronic complications. Therefore, controlling its level, *via* inhibiting the activities of α-glucosidase enzymes, is believed to be an important strategy to manage this disease. Alfa-glucosidase enzyme is a member of the glucosidases located in the brush-border surface membrane of the intestinal cells and is a rate-limiting step in the conversion of oligosaccharides and disaccharides into monosaccharides, necessary for gastrointestinal absorption[13]. In addition, α-amylase, which is present in both salivary and pancreatic secretions[14], is responsible for cleaving large malto-oligosaccharides to maltose which is a substrate for the intestinal α-glucosidase. Hence, the inhibition of α-glucosidase and/or α-amylase enzymes is currently in vogue, especially if these inhibitors stem from natural bases. The following are some examples of plants or their constituents that are proven to possess anti-enzymatic properties.

Methanolic extract of *Adhatoda vasica* Nees (*Acanthaceae*) was shown to have the highest sucrase inhibitory activity among forty species tested in an experimentally screening study by Gao *et al*[15]. This effect was attributed to its active constituents, *viz.,* vasicine and vasicinol, beside other constituents, offering thus, a possibility to develop successful α-glucosidase inhibitors. Previous studies by Gao *et al*[15] also reported the isolation of maltase inhibitory principles from the fruits of *Terminalia chebula*[16] and *Tussilago farfarae*[17].

Belonging to the same *Acanthaceae* family*, in vitro* studies on the ethanolic extract of *Andrographis paniculata* (*Burm. f.*) Nees and its principal active constituent, andrographolide (AG), seem to possess an antihyperglycemic activity[18]. They delay the quick digestion of starch, as well as sucrose, and prolong the absorption time of carbohydrates, pointing to an α-glucosidase inhibitory activity. Moreover, essential oils obtained from the woods of *Cedrus libani* A. Rich (*Pinaceae*), but not its leaves or cones, were able to inhibit the α-amylase activity[19].

Nigella sativa L. (*Ranunculaceae*), a plant commonly used in the Middle Eastern and North African traditional medicine was validated for its multi-factorial anti-diabetic actions. The crude aqueous extract tested in experimental rats was able to restore glucose homeostasis[20] and to improve glucose tolerance as efficiently as metformin. Apart from its effect to enhance insulin sensitivity in liver cells[21], and to possess an insulinotropic and insulin-like activities in cultured pancreatic β-cells, skeletal muscle cells and adipocytes[22], it is now documented[23] that the crude aqueous extract of *Nigella sativa* seeds directly inhibits the electrogenic intestinal absorption of glucose *in vitro*. This effect is mediated by reducing the intestinal sodium-dependent D-glucose cotransporter-1 (SGLT1) which is the major transporter of glucose in the intestine[24,25]. SGLT1 is also considered a key molecule in the sensing of glucose entry that is highly regulated by peptides and hormones[26].

Another plant that is widely used as an anti-diabetic in folk medicine in México is *Tournefortia hartwegiana,* where the decoction of its aerial parts controls the disease, when given orally for 10-14 d to alloxanized rats. The plant is thought to control the glucose level *via* several routes, including the inhibition of the intestinal α-glucosidase and other intestinal enzymes, as maltase and sucrase that are implicated in the digestion of polysaccharides and oligosaccharides[27,28]. The inhibitory effect of this decoction suppresses the absorption of carbohydrates from intestine and thereby reduces the post-prandial increase in the glucose level. On the other hand, Ortiz-Andrade *et al*[27] referred the anti-diabetic effect of the methanolic extract of the same plant to the enhancement of insulin secretion and/or action. Furthermore, other machineries , such as the modulation of the pancreatic and extrapancreatic effects[29-32], besides the enhancement of β-cell glucose metabolism or an activation of enzyme system generating cyclic adenosine mono phosphate (AMP) or phospholipid derived messenger[33], and/or blockage of glucose co-transporters from intestine to circulation[34], cannot be ruled out. These diverse mechanisms are attributed to the different components that were tested for their individual hypoglycemic action, where the "cocktail" of these constituents could trigger a synergic effect.

In 2004, Asano *et al*[34] in their search for an anti-glycosidase, succeeded to isolate new alkaloids from the bulbs of *Scilla peruviana* (*Hyacinthaceae*) that display an inhibitory action of bacterial β-glucosidase and bovine liver β-galactosidase to varying degrees. In addition, the methanolic extract of the rhizome of *Rheum emodi*, known as Himalayan rhubarb, inhibited the activity of both mild yeast and mammalian intestinal α-glucosidase as proven by Suresh Babu *et al*[35]. This action correlates with the

active components isolated from this rhizome such as chrysophanol-8-O-β-D-glucopyranoside, desoxyrhaponticin and torachrysone-8-O-β-D-glucopyranoside which showed a potent to moderate mammalian α-glucosidase inhibitory activity.

In a recent study, Loizzo *et al*[36] examined the influence of nine extracts of plant species collected in Lebanon, *viz*., Calamintha origanifolia, Satureja thymbra, Prangos asperula, Sideritis perfoliata, Asperula glomerata, Hyssopus officinalis, Erythraea centaurium, Marrubium radiatum and Salvia acetabulosa. The authors prepared different extractions with methanol, *n*-hexane and chloroform, yet the methanolic extracts of *Marrubium radiatum* and *Salvia acetabulosa* exerted the strongest activity against α-amylase and α-glucosidase. The leaf extract of the *Marrubium* related species, *viz., Marrubium vulgare*, is used in Brazilian and Mexican traditional medicine for its antidiabetic role, an effect that was documented clinically in patients with type Ⅱ non-controlled diabetes mellitus[37]. Several *Salvia* species have been reported for their hypoglycaemic effect in Iranian folk medicine[38,39] where they act by different mechanisms. For example, *Salvia lavandulaefolia* extract acts by decreasing the intestinal absorption of glucose, increasing the peripheral uptake of glucose, potentiating glucose-induced insulin release, and causing pancreatic islet cells hyperplasia[40].

The hypoglycemic mechanisms of another anti-diabetic plant, *Plantago ovata* husk, has also been studied and it was found that its aqueous extract hinders markedly the intestinal glucose absorption in rats; however, the extract failed to affect insulin secretion nor glucose transport in adipocytes[41].

Salacia species (*Celastraceae*) are widely distributed in East Asian countries and many plants from this genus (*e.g.*, *S. oblonga, S. reticulata* and *S. prinoides*) have been used for thousands of years in traditional medicines, particularly for the treatment of diabetes and obesity. Pharmacological studies have demonstrated that *Salacia* roots modulate multiple targets, including the inhibition of α-glucosidase, aldose reductase and pancreatic lipase, as well as the activation of peroxisome proliferator-activated receptor-alpha (PPAR-α)-mediated lipogenic gene transcription. All these mechanisms reinforce its usage in Ayurvedic medicine for diabetes and obesity. The methanolic extracts of *S. reticulata* and *S. oblonga* stems and roots reduced, dose-dependently, the postprandial hyperglycemia induced in rats by maltose, sucrose or starch, but not by glucose or lactose[42-44], pointing to their inhibitory effect on intestinal enzymes. Moreover, the aqueous extract of *S. reticulata* inhibited strongly the activities of α-glucosidase and α-amylase[42], while that of *S. chinensis* inhibited the α-glucosidase activity only[45]. These favorable effects are attributed to the identified components of the plant, *viz.,* mangiferin, salacinol, kotalanol and kotalagenin 16-acetate. Mangiferin causes concentration-dependent α-glucosidase inhibition *in vitro*[46], while salacinol, kotalanol and kotalagenin 16-acetate inhibited the increased serum glucose levels in maltose and sucrose loaded rats more than acarbose[43,44]. Thus, these findings suggest that the anti-diabetic property of *Salacia* is partially attributed to its intestinal α-glucosidase inhibitory activity.

Mangifera indica Linn. (*Anacardiaceae*) is a plant that possesses several properties, one of which is hypoglycemia that favors it to control type Ⅱ DM in some rural African communities[46]. Mangiferin is one of the active constituents of this plant, besides the polyphenolics, flavonoids, triterpenoids, and other chemical compounds. Therefore, the mangiferin-mediated inhibition of α-glucosidase activity[47], offers one mechanism for the hypoglycemic effect of this plant.

The potential antidiabetic activity of six pentacyclic triterpenes (oleanolic acid, arjunolic acid, asiatic acid, maslinic acid, corosolic acid and 23-hydroxyursolic acid) were isolated from the ethyl acetate extract of the leaves of *Lagerstroemia speciosa* (LSL) and were investigated by α-amylase and α-glucosidase inhibition assay[48]. However, the compounds showed week α-amylase inhibitory effect, while α-glucosidase was moderately inhibited, mainly by corosolic acid. In a search for an α-amylase inhibitory compound from plant origin, Ali *et al*[49] studied extracts of six selected Malaysian plants with a reputation of usefulness in treating diabetes using an *in vitro* model. Their work depicted that the hexane extract of *Phyllanthus amarus* had α-amylase inhibitory properties, an effect that was provoked by only three pure pentacyclic triterpenoids, namely, oleanolic acid, ursolic acid and lupeol.

The antidiabetic capacity of the standardized extract of maritime pine bark, derived from *Pinus pinaster*, Aiton. subs. Atlantica des Villar (Pycnogenol®), was documented clinically by Liu *et al*[50]. In their study a double-blind, placebo-controlled, randomized, multicenter study was performed with 77 type Ⅱ diabetic patients to investigate the potential antidiabetic effects of the French matitime pine bark extract, Pynogenol (100 mg) for 12 wk. Supplementation of Pycnogenol to conventional diabetes treatment loweres glucose levels and improves the endothelial functions, as evidenced by the significant reduction in HbA1c and endothelin-1. To characterize the possible mechanism of action, the authors attributed the effect of Pycnogenol to the suppression of α-glucosidase enzyme[50], rather than enhancing the insulin secretion, an effect that was more potent than green tea or acarbose[51]. The clinical antidiabetic effect was found also to be dose dependent and correlates positively with the procyanidins comprising of catechin and epicatechin subunits with varying chain lengths[52,53].

Another plant that is used extensively in folk medicine is the Fenugreek (*Trigonella foenum-graecum* L.), which is a member of the *Leguminosae* family, and is cultivated predominantly in Asia, the Mediterranean, and North African regions. Mainly the seeds are the part used for centuries for a wide range of diseases, as they were shown experimentally to possess significant hypoglycemic[54], antiathrosclerotic[55], anti-inflammatory[56], antinociceptive[57], antiulcerogenic[58], and antineoplastic effects[59]. Studies carried to elucidate its anti-diabetic mechanism(s) reveal that the plant works by inhibiting the intestinal glycosidase[60], in addition to its positive effect on glycolytic,

gluconeogenic, and lipogenic enzymes to restore glucose homeostasis in various animal models[60,61].

The *in vitro* α-glucosidase inhibitory model has been used by several research teams to verify the potential antidiabetic properties of different plant parts/extracts. In this context the antidiabetic effect of the Corni fructus (*Cornus officinalis Sieb. et* Zucc.) extract is mediated partly by inhibiting the α-glucosidase activity, an effect that reached to over 80% by one of the extract tested fractions[62]. Likewise, the alcohol extract of Alismatis Rhizoma-related hypoglycemic effect is mediated *via* the same mechanism, owing to its protostane-type triterpenes, besides promoting the glucose uptake *in vitro*[63]. Similarly, chemical components isolated from the safflower seed (*Carthamus tinctorius* L.)[64] and from the leaves of Ficus deltoidea, *viz.*, vitexin and isovitexin[65], as well as the methanolic extract of the aerial parts of Swertia corymbosa (used in Ayurveda herbal preparations in India)[66] exhibited *in vitro*/*in vivo* α-glucosidase inhibition.

Moreover, the same *in vitro* technique showed that the grape seed extract inhibits the intestinal α-glucosidases and α-pancreatic amylase that may delay carbohydrate digestion and absorption. Recently, this fact has been further documented, where grape seed extract has lowered the postprandial plasma glucose concentration in an acute, randomized, controlled crossover design study, in which healthy subjects received high carbohydrate meal with or without grape seed extract[67].

A prospective epidemiology links heavy coffee consumption to a substantial reduction in risk for type 2 diabetes, yet there is no evidence that coffee improves insulin sensitivity. Thus, it is reasonable to suspect that coffee influences the risk for beta cell "failure" that precipitates diabetes in subjects who are already insulin resistant. Indeed, coffee was proven to increase the production of the incretin hormone glucagon-like peptide-1 (GLP-1), possibly by its chlorogenic acid constituent (CGA-the chief polyphenol in coffee). The latter was also found to inhibit the intestinal glucose transport, as documented by the consumption of plants containing CGA, to be including coffee[68]. Further studies correlated the presence of CGA, the main polyphenolic compound in coffee, to the decreased diabetic risk where CGA slows carbohydrate absorption by its effect on the intestinal brush border membrane glucose transport, thus mimicking the effect of acarbose at the experimental level[69], as well as acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans[70]. CGA inhibits also the activity of glucose-6-phosphate translocase[71] which is now believed to play a role in glucose absorption[72,73]. In 2008, Andrade-Cetto *et al*[74] have tested the hypoglycemic effect of butanolic extracts of some Mexican plants and have found that *Malmea depressa* Baill R.E. and *Acosmium panamense* Benth. extracts resemble the effect of acarbose and decrease the plasma glucose level significantly by affecting the α-glucosidase enzyme. Nevertheless, the effect of the butanolic extract of *Cecropia obtusifolia* Bertol. was the most potent and it produced the highest reduction in the plasma glucose level that was even lower than the fasting level after 90 min, an effect that suggests an additive mechanism of action. This assumption could be true since this plant contains CGA which hits several targets in the diabetes metabolic pathways, besides its acarbose-like effect[75,76].

ENHANCEMENT OF GLUCOSE UPTAKE AND UPREGULATION OF GLUCOSE TRANSPORTERS

Stimulating the peripheral glucose uptake is one of the multiple mechanisms that control blood glucose level; hence, targeting this point is among the most promising goals for the treatment of type-Ⅱ DM. Basically, several factors assimilate to facilitate the glucose uptake process, including the activation of the GLUT in liver (GLUT-2), adipocytes and skeletal muscles (GLUT-4), the induction of the nuclear receptors, *viz.,* PPARs, especially the gamma subtype, as well as increasing the release of positive adipocytokines, such as adiponectin[77].

As illustrated in Figure 2, the cell membrane lipid bilayer is impermeable to carbohydrates, which necessitates the presence of specific transporters. These carriers are differentiated into two families, the first one is a sodium-linked GLUT that works actively and is limited to the intestine and kidney. The second family consists of eight homologous transmembrane proteins, GLUT-1-8, that are encoded by distinct genes, and they convey glucose by the facilitated diffusion down the glucose-concentration gradients[77]. However, the GLUT proteins have distinct substrate specificities, kinetic properties, and tissue distributions that dictate their functional roles. GLUT-1 is expressed in the brain, erythrocytes and endothelial cells, while GLUT-2 is found in the liver, kidney, small intestine, and pancreatic β-cells. This low-affinity GLUT (GLUT-2) has a role in sensing glucose concentrations in the islets of Langerhans. GLUT-3 is responsible for transporting glucose in neurons and placenta, while GLUT-4 is present in skeletal muscles, cardiac muscles and the adipose tissue. Of all the GLUT, only GLUT-4 is insulin-responsive. GLUT-5 has high affinity to transport fructose rather than glucose and it exists in the small intestine, sperm, kidney, brain, and adipose cells[77]. In 2003, Gorovits *et al*[78] found that GLUT-8, present in liver, plays a role in the regulation of glucose in case of diabetes. GLUT-4 is sequestered intracellularly and is translocated to the plasma membrane upon its stimulation by insulin. Thus, a decrease in the expression of GLUT-4 mRNA and protein reduced the insulin-mediated glucose uptake in diabetes[79]; in other words, imperfect GLUT-4 function could be a causative factor for insulin resistance[80].

From this point of view, herbs or their active constituents that can up-regulate GLUT-4 expression or that increase the translocation of this transporter could aid in the treatment of insulin resistance and hyperglycemia.

Cecropia obtusifolia Bertol (*Cecropiaceae*) is a plant extensively used for the empirical treatment of type Ⅱ diabe-

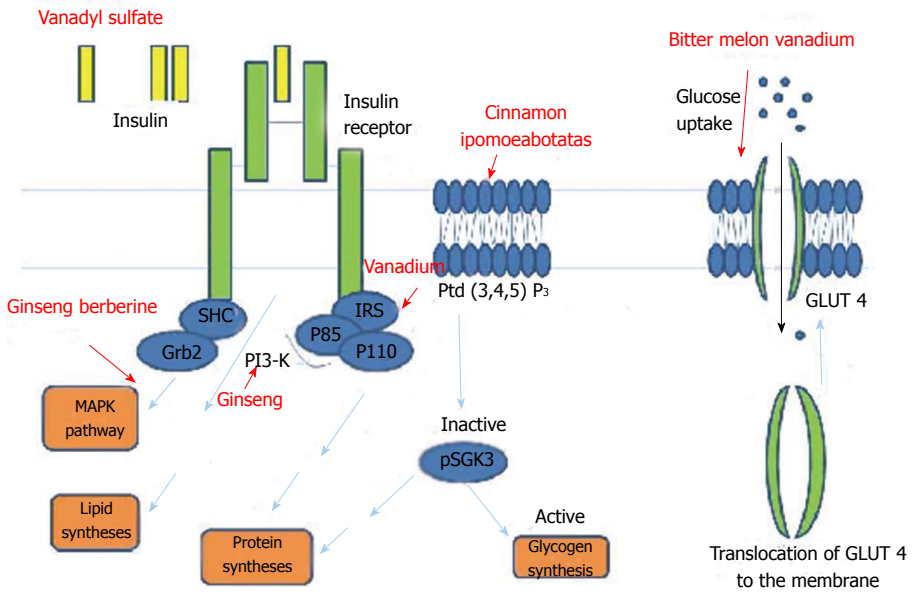

Figure 2 Insulin signaling pathway and insulin insensitive. The inner part of insulin receptor (IR) reveals a tyrosine kinase activity and coupled with multifunctional docking proteins IRS-1 and IRS-2. The in turn signaling leads to an activation of the MAPK cascade involved in mitogenesis and the open status of a hexose transporter protein (GLUTs) which is located in the cell membrane and is the only channel for glucose entery into cells. The decreased serine/threonine phosphorylation of IR, inactivates hexokinase and glycogen synthase, as well as defects in the phosphorylation of glucose transporter protein (GLUT4) and genetic primary defect in mitochondrial fatty acid oxidation, leading to insulin resistance and an increase of triglyceride synthesis contribute to this insulin insensitivity. The action sites of hypoglycemia herbs are indicated with red arrows[3].

tes in México[76]. The active hypoglycemic compounds found in this plant are CGA and isoorientin which are also found in other anti-diabetic plants as mentioned before[76]. In 2008, Alonso-Castro *et al*[76] studied the anti-diabetic mechanisms of *Cecropia obtusifolia* aqueous extract and its active compound CGA, and reported that the two preparations exert their anti-diabetic effects by stimulating glucose uptake in both insulin-sensitive and insulin-resistant adipocytes without appreciable pro-adipogenic effects. Thus, they could act by potentiating the insulin action or by activating a signaling pathway parallel to the insulin pathway.

Other anti-diabetic plants that act *via* increasing the glucose uptake in adipocytes, alone and in combination with insulin, include the ethanolic extract of *Amomum xanthioides* seeds[81], *Lagerstroemia speciosa*[82] and plants used by the Cree Nation in Canada, such as *Abies balsamea*, *Pinus banksiana* and *Rhododendron groenlandicum*[83]. Moreover, an aqueous extract from *Cinnamomum zeylanicum*[84], aqueous and ethanolic extracts of *Momordica charantia*[85], and aqueous extract of *Guazuma ulmifolia*[86], stimulated glucose uptake in 3T3-L1 adipocytes. However, none was evaluated on insulin-resistant adipocytes, except for *Guazuma ulmifolia*, which similar to *Cecropia obtusifolia,* mediated its action by stimulating glucose uptake in normal and diabetic adipocytes without inducing adipogenesis; nevertheless, its hypoglycemiant component(s) are not fully characterized.

Miura *et al*[87] validated the antidiabetic activity of *Lyophyllum decastes* (*Tricholomataceae*) in KK-Ay mice, an animal model of genetically type Ⅱ diabetes with hyperinsulinemia. The results of their work reported that mice receiving the aqueous extract showed an increase in the muscle content of GLUT-4 protein, which is responsible, at least in part, for decreasing insulin resistance. In 2004, Miura *et al*[88] again used the same model to test the hypoglycemic effect of corosolic acid, and found that it increased GLUT-4 translocation in muscle, without affecting the insulin level. This acid is one of the active constituents of *Lagerstroemia speciosa* L., banaba leaf. The plant is used traditionally in Philippines to treat diabetes and was studied by Takagi *et al*[89] who referred the antidiabetic effect to the inhibition of sucrose hydrolysis. However, the effect of corosolic acid on GLUT-4 can not be ruled out, although this requires further verification

In another study[90], the 3T3-L1 adipocytes were used to prove that the methanolic extract of *Liriope platyphylla Wang et Tang* (*LPWT*), *Liliaceae*, increased insulin-induced glucose uptake in adipocytes, by virtue of its homoisoflavone. This uptake was mediated through the translocation of GLUT-4 to the plasma membrane, *via* Insulin receptor Substrate - phosphatidyl inositol 3 kinase-Akt signaling mechanism. Aside from delaying the carbohydrate absorption *via* affecting α-glucosidase enzyme[19], *Andrographis paniculata* adopts another mechanism of action for its hypoglycemic effect through increasing the expression of GLUT-4. This was confirmed by the administration of its main constituent andrographolide in diabetic mice using streptozocin (STZ)[91].

Panax ginseng, also known as Korean red ginseng, appears to be a powerful anti-diabetic plant that has multi modes of action, due to its potent active constituents including ginsenoside Rh2. In a study by Lai *et al*[92], the authors reported that the ginsenoside Rh2 increases the gene expression of GLUT-4, at the mRNA and protein levels, in soleus muscle obtained from STZ-diabetic rats. They also suggest that the GLUT-4 expression is increased as a result of the increased β-endorphin secretion which will be detailed later in this review.

In an attempt to develop new substances for treating insulin resistance, obese Zucker rats were employed to screen the effect of myricetin, an active principle of *Abelmoschus moschatus* (*Malvaceae*), on insulin resistance[93]. The findings showed that myricetin increased insulin sensitivity by increasing the expression of GLUT-4 and by activating the phosphorylation of insulin receptor substrate-1. These results were also obtained from another study[94] using the methanolic extract of *Aegles marmelos*

and *Syzygium cumini* that are anti-diabetic medicinal plants used in Indian traditional medicine. The latter study reported an additive mechanism for lowering glucose level *via* the elevation of PPAR-γ, a nuclear receptor that will be discussed in the following section. *Azadirchata indica* Neem is among the Indian herbs that possess an antidiabetic effect. The hydroalcoholic extract of this herb exerted its antihyperglycemic activity by increasing glucose uptake, as well as glycogen deposition[95]. Furthermore, the anti-diabetic action of Tinospora cordifolia is mediated by increasing the expression of GLUT-4 by about 5 folds, as well as PPARα and γ, as tested in differentiated myocytes, L6 cells[96].

ACTIVATION OF THE NUCLEAR RECEPTOR PPAR-γ

The PPAR family belongs to type Ⅱ nuclear hormone receptors involved in the regulation of fatty acid, carbohydrate and glucose metabolism[97]. There are three isoforms of PPARs with specific tissue distribution and biological activity; they are identified as α, β or δ and γ with two subforms PPAR-γ1 and PPAR-γ2[98]. The receptors are ligand dependent, with the antidiabetic thiazolidinediones (TZDs) being the potent PPAR-γ agonist[97]. After their stimulation by their specific ligands, they regulate the transcriptional process *via* their heterodimerization with RXR, a retinoid X receptor, and then bind to peroxisome prolixferator-response element (PPRE)[97,98]. Clinical data demonstrated that the PPAR-γ agonists TZDs modulate glucose homeostasis by enhancing the peripheral glucose uptake through increasing GLUT-4 expression and translocation in adipocytes[99], as well as decreasing hepatic glucose output[100]. TZDs alleviate insulin sensitization by the redistribution of adipose deposits where these agents minimize visceral adipose content, responsible for the induction of insulin resistance, and redeposit it subcutaneously, in a phenomenon known as the "fatty acid steal" hypothesis[101]. In addition, activating PPAR-γ increased adipocyte fatty acid uptake, and decreased lipotoxic damage to insulin-sensitive tissues[102].

To date, the chief research interest in finding a nutraceutical compound(s) that mimics the PPAR-γ ligands constitute promising approaches for the treatment of diabetes, obesity and metabolic syndrome. Previously, multiple trials have shown conflicting results whether cinnamon lowers glucose or hemoglobin A1c (HbA1c). In 2009, Crawford[103] tested the cinnamon hypoglycemic activity in patients with type 2 diabetes through a randomized, controlled trial to evaluate whether daily cinnamon plus usual care versus usual care alone lowers HbA1c. Cinnamon lowered HbA1c (0.83%) compared with usual care alone lowering HbA1c (0.37%). Because one of the proposed mechanisms of cinnamon is increasing insulin sensitivity, hence, the treatment of patients with metabolic syndrome by adjunct cinnamon may yield weight loss, improved lipid profiles, and better glucose tolerance.

Park *et al*[104] used db/db mice, a typical non-insulin-dependent model, to study the anti-diabetic mechanism of Mulberry leaf water extract, Korean red ginseng and/or banaba leaf water extract. Herbs alone and their combination increased the expressions of liver PPAR-α mRNA and adipose tissue PPAR-γ mRNA in animals fed diets supplemented with the test herbs, in addition to restoring glucose and lipid homeostasis. Furthermore, the *Labiate* herbs rosemary and sage were documented in a recent study[105] as activators of the human PPAR-γ, possibly by their active constituents carnosol and carnosic acid.

What provides a potential validation for using traditional herbs as antidiabetics are the results of the screening study attained by Rau *et al*[106]. Among 52 ethanolic extracts, obtained from traditionally used herbs, the researchers found amazingly that nearly half the extracts activated PPAR-γ and 14 activated PPAR-α, while three of them were pan-PPAR activators, findings which were considered exceptionally high hit rate. The most active extracts were those of *Alisma plantago aquatica* (ze xie/European waterplantain), *Catharanthus roseus* (Madagascar periwinkle), *Acorus calamus* (sweet calamus), *Euphorbia balsamifera* (balsam spurge), *Jatropha curcas* (barbados nut), *Origanum majorana* (marjoram), *Zea mays* (corn silk), *Capsicum frutescens* (chilli) and *Urtica dioica* (stinging nettle).

The effect of the North American ginseng (*Panax quinquefolius*), a close relative to *Panax ginseng,* on glucose control was verified in a study by Banz *et al*[107], using male Zucker diabetic fatty rats. The findings showed that ginseng had marked effects on the expression of genes involved in PPAR actions and triglyceride metabolism. The authors encourage further research to determine the therapeutic value of this medicinal herb in treating human diabetes.

Green tea (*Camellia sinensis* L.) leaf extract on triglyceride and glucose homeostasis was evaluated in a fructose-fed insulin-resistant hamster model[108]. Supplementation of the green tea epigallocatechin gallate-enriched extract improves lipid and glucose homeostasis and increases the expression of PPAR-α and PPAR-γ proteins. These data suggest that intake of the green tea extract increases insulin-sensitivity, at least through boosting up PPAR.

Clematis species (*Ranunculaceae*) have been used continuously as anti-inflammatory agents by indigenous Australians. During examining the ethanol extract of *C. pickeringii*, *C. glycinoides* and *C. microphylla*, on COX-1, COX-2 and 5-lipoxygenase[109], the authors found that *Clematis pickeringii* has activated significantly the protein expression of both PPAR-α and PPAR-γ. These results merit the study of the potential antidiabetic mechanism(s) of these species. In a search for a natural PPAR-γ agonist, Atanasov *et al*[110] reported that the natural product honokiol from the traditional Chinese herbal drug Magnolia bark stimulates the basal glucose uptake in a comparable pattern to pioglitazone, but without inducing adipogenesis.

INCREASING ADIPONECTIN RELEASE

An additive role for PPAR-γ in the manipulation of glu-

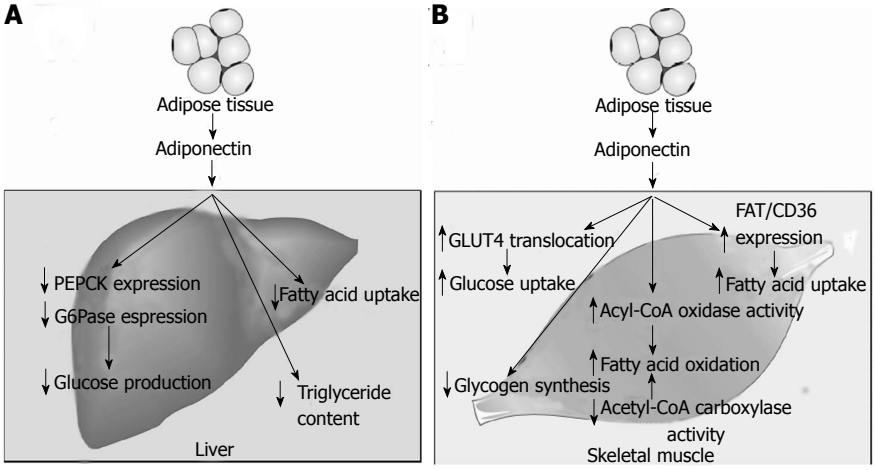

Figure 3 Effects of adiponectin on carbohydrate and lipid metabolism in liver and skeletal muscle (c.f. www.jpp.krakow.pl/.../articles/08_article.html[119]). FAT: Fatty acid translocase.

cose homeostasis is the modulation of adipocytokines. These bioactive substances are produced and secreted from adipose tissues which happened to be an endocrine organ[111]. Adipocytokines play central role in body insulin resistance, where the dysregulation of their production participates in the pathophysiology of the metabolic syndrome.

The plasma level of adiponectin is documented to be lower in patients with diabetes[112] and ischemic heart disease[113] than their age- and body mass index (BMI)-matched nondiabetic mates. This fact was further documented in a screening study on Japanese patients with type 2 diabetes and their age- and BMI-matched nondiabetic control subjects, and is attributed to the genetic mutation of the adiponectin gene associated with metabolic syndrome, including insulin resistant diabetes and atherosclerotic disease[114].

Consequently, adiponectin possesses antidiabetic and antiatherogenic properties[115]. The antidiabetic mechanism(s) involves enhancement of glucose uptake in skeletal muscles, activation of IRS-1-mediated phosphatidylinositol-3 kinase[115,116], acceleration of muscle β-oxidation *via* the activation of AMP-kinase[117], and suppression of hepatic glucose production[118,119]. These events are summarized in Figure 3.

Normal adiponectin plasma level is under the influence of PPAR-γ, where stimulation of this nuclear receptor potentiates its direct binding with the PPRE responsive element in the promoter region of the adiponectin gene, thus, enhancing the production and secretion of this cytokine.

Clinical studies now assure the beneficial effects of some plants in controlling glucose disorders. For instance, the extract of white-skinned sweet potato *Ipomoea batatas* (Caiapo) has been evaluated in type II diabetic patients, and was shown to control plasma glucose level through increasing insulin sensitivity along with the level of adiponectin[120]. Moreover, the mushroom *Agaricus blazei Murill* (ABM) extract was documented to improve insulin resistance and to elevate adiponectin level in subjects with type II diabetes receiving metformin and gliclazide; the latter cytokine provides at least one potential antidiabetic mechanism of this plant[121]. Concerning the antiatherogenic property of adiponectin, the extract of *Aronia melanocarpa* E. was administered to forty-four patients who survived myocardial infraction and have received statin therapy[121]. Compared to placebo, the chokeberry flavonoid extract increased adiponectin level, among other corrected parameters that nominate this extract as an adjunct therapy in patients with ischemic heart disease.

Momordica charantia owes its anti diabetic effect to its insulin-like action[122,123], antioxidant property[124,125], and glucose uptake enhancement[79]. The latter mechanism could be explained by the finding of Ryu *et al*[126], who stated that *Momordica*-induced glucose uptake is accompanied by, and may be the result of, increased adiponectin secretion, which is the communication between adipose tissue and skeletal muscle.

Adiponectin was also induced by the oral ingestion of *Plum ekisu*, tested on insulin- resistant obese Wistar fatty rats[127]. Dried plum is highly consumed in the West as a healthy food and is used in India as medicine to protect against geriatric related diseases, possibly by their phenolic compounds. Rats receiving plum concentrated juice showed better insulin sensitivity, increased PPAR-γ mRNA expression and marked elevation in adiponectin. These mechanisms are tightly correlated, where stimulation of PPAR-γ initiates the cycle, leading to increased production of adiponectin and alleviation of insulin sensitivity.

Apart from the multiple machineries by which *Salacia reticulate* extract mediates its antidiabetic effect[42], increasing the release of adiponectin adds also to these effects, which make it useful in the treatment of diabetes mellitus, insulin resistance and other metabolic diseases[128].

GLYCOGEM METABOLISM

Another cornerstone in controlling blood glucose level is the "hepatic output", which correlates with liver metabolic functions, including lipogenesis and glycogenesis. The latter process is precisely adjusted by adequate levels of insulin[129], which stimulates glycogen synthase and inhibits glycogen phosphorylase, resulting in the proper glycogen deposition in various tissues, especially skeletal

muscle. Since glycogen is the storable form of glucose, thus, insulin inadequacy initiates muscle protein breakdown to provide gluconeogenic precursors that could be the reason behind diabetes-induced weight loss. Consequently, compounds that enhance glycogen formation and/or increase its content in liver and muscles are considered beneficial anti-diabetic agents.

In an attempt to elucidate the anti-diabetic mechanism(s) of some plants used in the management of diabetes, it was found that *Caralluma sinaica* L. (*Asclepiadaceae*)[130] found in south Hejaz, west of Saudi Arabia, and Sinai region of Egypt, showed an anti-diabetic effect. This plant exerts its effect through opposing the STZ-induced glycogen depletion in liver and muscle, and by reversing weight loss in the diabetic rabbits, results that may be promoted by the realease of insulin.

Panax ginseng is suggested to induce glycemic control by sparing insulin and increasing glucose transport. Various preparations of *Panax ginseng* have been shown to upregulate insulin and non-insulin stimulated glucose transport in different animal models and cell lines[131-133]. Furthermore, *Momordica charantia* was able to renovate β-cells in the pancreas or partially destroyed ones[85] and to stimulate pancreatic insulin secretion[134]. These insulin-like properties[122,123] kindle glycogen storage by the liver and improve peripheral glucose uptake[126]. The anti-diabetic property of the aqueous extract of *Tamarindus indica* seed (*T. indica*) was also verified in a type I and II experimental models[135]. This action is mediated by restoring glycogen levels in liver and skeletal muscles, as well as inhibiting the glucose-6-P-ase activity. Increasing insulin level, however, was limited only to the type I model. Recently in 2012 the aqueous extract tested on STZ-induced diabetes showed that complex mechanisms stand behind its antidiabetic effect, such as β-cell neogenesis, calcium handling, as well as increasing GLUT-2 and GLUT-4. These findings show the scope for formulating a new herbal drug for diabetes therapy[136].

INSULINOMIMETIC AND INSULINOTROPIC EFFECT

In 2007, Eidi *et al*[137] studied again the hypoglycemic effect of the fenugreek seeds which was previously found to inhibit α-glucosidase, and they reported that the ethanolic extract significantly decreased serum glucose, triacylglycerol, cholesterol, urea, uric acid, AST, and ALT, whereas it increased serum insulin levels in treated STZ-induced diabetic rats. As a result, the authors concluded that fenugreek seeds extract encompasses antidiabetic activities similar to that observed for glibenclamide used as a standard drug. Eidi *et al*[138] have tested also the possible antidiabetic mechanism of Garlic (*Allium sativum*, *Liliaceae*) which is a common spice flavoring agent believed to lower plasma glucose level in diabetic patients. Therefore, using STZ-induced diabetic rats, they found that the alcoholic extract of garlic potentiates the insulin effect by increasing its pancreatic secretion from existing β-cells or its release from bound insulin. These effects are attributed mainly to the allicin-type compounds[139,140] which are disulphide compounds that can react with endogenous thiol containing molecules, such as cysteine, glutathione, and serum albumins to spare insulin from SH inactivation[141]. In another study, using STZ/high-fat diet Sprague Dawley rats, a comparison between the anti-diabetic effects of dietary freeze-dried ginger and garlic, was conducted. The experimental results revealed that ginger and garlic are insulinotropic rather than hypoglycemic, and that the anti-diabetic effects of ginger are better than those of garlic[142]. Using the same rat model, Islam *et al*[143] investigated the insulinotropic effect of dietary red chilli (*Capsicum frutescens* L.) in low and high concentrations and revealed that 2% dietary red chilli is insulinotropic rather than hypoglycemic at least in this experimental condition.

The effects of the ethanol extract and five partition fractions of the *Asparagus racemosus* root and *Ocimum sanctum* leaf were evaluated on insulin secretion together with exploration of their mechanisms of action. The ethanol extract and each of the hexane, chloroform and ethyl acetate partition fractions stimulated insulin secretion in isolated perfused rat pancreas, isolated rat islet cells and clonal β-cells. These findings reveal that constituents of both extracts have wide-ranging stimulatory effects on physiological insulinotropic pathways[144]. Similarly, the aqueous extract of *Asparagus adscendens* induced a significant increase in glucose-dependent insulinotropic actions in the clonal pancreatic β-cell line, enhanced glucose uptake in 3T3-L1 adipocytes and decreased starch digestion *in vitro*. These outcomes revealed that *Asparagus adscendens* possesses insulinotropic, insulin-enhancing activity and inhibitory effects on starch digestion[145].

The antihyperglycemic action of *Stevia rebaudiana (Asteraceae)* Bertoni leaves extracts were confirmed using type II diabetic Goto-Kakizaki rats[146]. The large quantities of the glycoside stevioside in the *Stevia rebaudiana* leaves are responsible for the anti-hyperglycaemic, insulinotropic, and glucagonostatic actions of the herb; results which support the traditional use of this herb in the treatment of diabetes in Paraguay and Brazil. Similar efficacy pattern was obtained by the crude extract of *Viscum album* (*V. album*) leaf which produced about 35.3% decrease in glucose concentration in STZ-induced diabetic rats and stimulated insulin secretion by about 81.5%. Although, only a subtle suppression in glucagon level was observed, yet it was significant. Thus, the *V. album* leaves extract may possess antihyperglycaemic, insulinotropic, and possibly, mild glucagonostatic agent(s) and may, therefore be a candidate for the anti-diabetic drugs[147].

Butanol extract of *Zizyphus spina-christi* L. (*Rhamnaceae*) leaves and its major saponin glycoside, christinin-A, were tested to evaluate their effect on serum glucose and insulin levels in non-diabetic control, type- I and type- II diabetic rats[148]. Both the extract and the saponin compound improved the oral glucose tolerance , potentiated glucose-induced insulin release, reduced the serum glucose level and increased the serum insulin level of non-diabetic control

and type-Ⅱ diabetic rats, but not those of type-Ⅰ diabetic rats. They also enhanced the glucose lowering and insulinotropic effects of glibenclamide. The data pointed to the insulinotropic capacity of the tested plant.

Furthermore, in traditional Nepalese folk medicine the leaf extract of the annual herb *Biophytum sensitivum* is used for the treatment of hyperglycemic patients. This property was documented by Puri[149] who ascribed the leaf extract hypoglycemic response to its insulinotropic effect, where he found that the tested extract induces the release and/or synthesis of insulin.

Similar insulinotropic effect was presented by pterostilbene, a flavonoid constituent derived from the wood of *Pterocarpus marsupium,* a herb used in the Indian folk medicine; the active compound causes pancreatic β-cell regranulation[150]. Marsupin, pterosupin and liquiritigenin obtained from the plant showed also antihyperlipidemic activity. Moreover, epicatechin, an active principle, has been found to be insulinogenic, enhancing the insulin release and the conversion of proinsulin to insulin *in vitro.* Like insulin, epicatechin stimulates oxygen uptake in fat cells and increases glycogen content of rat diaphragm. Aloe vera (Liliaceae) exerts its hypoglycemic effect in rats by its bitter principle through stimulating the release of insulin from the β-cells of Langerhans as documented after the use of single, as well as repeated doses of the bitter principle of the Aloe vera in diabetic rats[150]. Other insulinptropic Indian herbs include *Acacia Arabica* (Babhul), *Eugenia jambolana* (Indian gooseberry), *Annona squamosa* (sugar apple), *Caesalpinia bonducella* (Fevernut), *Hibiscus rosa-sinesis* (Gudhal), *Scoparia dulcis* (sweet broomweed) and *Tinospora crispa*[96].

Patel *et al*[151] presented a thorough review on 65 species of plants with insulinomimetic or insulin secretagogue. Most of these belong to the family Leguminoseae, Lamiaceae, Liliaceae, Curcubitaceae, Asteraceae, Moraceae, Rosaceae and Araliaceae. The most active plants are Allium sativum, Gymnema sylvestre, Citrullus colocynthis, Trigonella foenum greacum, Momordica charantia and Ficus bengalensis. *Citrullus colocynthis* (Cucurbitaceae) pulp ethanolic extract at 300 mg/kg, *p.o.* was found to increase insulin and decrease plasma glucose levels significantly in alloxan-induced diabetic rats. Moreover, the aqueous extract also showed a dose-dependent increase in the insulin release from isolated islets, as well as other different extracts, such as crude extract, aqueous, alcoholic, purified extract and beta-pyrazol-1-ylalanine, the major free amino acid derivative present in the seeds[151].

Trigonella foenum-graecum has been observed to cause glucose-induced insulin release *in vitro* and *in vivo*. 4-Hydroxyleucine, a novel amino acid from fenugreek seeds, increased glucose-stimulated insulin release from isolated islet cells in rats, mice and humans, and possibly hydroxyisoleucine which represents 80% of the free amino acids in *Trigonella foenum-graecum* seeds. The extracts, powder and gum of *Trigonella foenum-graecum* seeds may help to improve insulin sensitivity presumably due to the presence of fibers, which slow the metabolism of carbohydrates, resulting in reduced insulin levels and lowered blood glucose[151].

Alcoholic extract of *Gymnema sylvestre* (Asclepiadaceae) stimulated insulin secretion from the rat islets of Langerhans and several pancreatic β-cell lines. In another study, the oral administration of the water-soluble leaves extract (400 mg/d) to 27 IDDM patients on insulin therapy lowered their fasting blood glucose and their insulin requirements. In type II diabetic patients on *Gymnema sylvestre* supplementation the pancreatic β-cells is suggested to be regenerated or repaired as supported by the raised insulin levels in their serum. This assumption has been concluded also when the number of the pancreatic islet and β-cells, as well as insulin levels wre increase after oral administration of the aqueous extract to diabetic rats. Gymnemic acid molecules dihydroxy gymnemic triacetate had the ability to release the insulin by the stimulation of a regeneration process and revitalization of the remaining β-cells. The aqueous extract of *Gymnema sylvestre* leaves stimulated insulin secretion from mouse cells and isolated human islets *in vitro*, without compromising cell viability[151].

Among the glucagonostatic Indian herbs are *Caesalpinia bonducell, Coccinia indica, Boerhavia diffusa, Enicostema littorale and Murraya koenigii.* These herbal extracts increase glycogenesis, restore the activities of lipoprotein lipases and decrease the glucose-6-phosphatases, thereby inhibiting the glycogenolysis, and gluconeogenesis processes, as well as increasing the peripheral glucose utilization[150].

In a recent study, the ethanolic extract of ethanolic extract of Schizandra arisanensis and its isolated constituents provided some insulinotropic effects by ameliorating cytokine-mediated β-cell death and dysfunction *via* anti-apoptotic and insulinotropic actions[152].

ELEVATION OF D-CHIRO-INOSITOL

D-chiro-inositol (D-CI) is a rare inositol isomer present in inositol phosphoglycans (IPGs) which are putative insulin second messengers. These mediators are released from cell membranes, cells and human blood by insulin and other growth factors[153] and mediate some, but not all, of insulin actions[154]. D-CI acts as an insulin surrogate where it exhibited an anti-hyperglycaemic effect *in vivo*[155], and enhanced insulin-induced glucose incorporation into glycogen, *in vitro*[155]. Albeit, D-CI modulates favorably insulin's effect on peripheral glucose utilization under physiological conditions, Kennington *et al*[156], reported abnormal low or immeasurable levels of D-chiro-inositol in urine and muscle from type Ⅱ diabetic patients, suggesting that D-CI deficiency might be related to the insulin resistance. Accordingly, D-chiro-inositol when administered to STZ diabetic rats[157] and humans[158] decreased hyperglycemia and enhanced glucose disposal (Table 1).

Cucurbita ficifolia is traditionally used in Asia for the management of diabetes; however, its mechanism of action was not clarified. In 2006, Xia *et al*[159] found that *C. ficifolia* may be a natural source of D-CI which is present in fairly high levels in this plant and may be the cause for its anti-diabetic character. Using STZ diabetic rats,

Table 1 Following is a list of plants that are reported to have insulin mimetic or insulin secreatory action

1 Abies pindrow (Pinaceae)	34 Momordica charantia (Cucurbitaceae)
2 Aegle marmelos (Rutaceae)	35 Mucuna pruriens (Leguminosae)
3 Agrimony eupatoria (Rosaceae)	36 Nigella sativa oil (Ranunculaceae)
4 Aloe barbadensis (Liliacea)	37 Olea europia (Oleacea)
5 Annona squamosa (Annonacea)	38 Panax ginseng (Araliaceae)
6 Averrhoa bilimbi(Oxalidacea)	39 Pandanus odorus (Pandanaceae)
7 Bixa orellana (Bixaceae)	40 Parinari excelsa (Chrysobalanaceae)
8 Boerhaavia diffusa (Nyctaginaceae)	41 Prunella vulgaris (Labiatae)
9 Bougainvillea spectabilis (Nyctaginaceae)	42 Psidium guajava (Myrtaceae)
10 Brassica nigra (Cruciferae)	43 Pterocarpus marsupium (Fabaceae)
11 Camellia sinensis (Theaceae)	44 Radix glycyrrhizae (Fabaceae)
12 Capsicum frutescens (Solanacea)	45 Radix rehmanniae (Scrophulariaceae)
13 Catharanthus roseus (Apocyaceae)	46 Rehmania glutinosa (Scrophulariacea)
14 Cinnamon zeylaniucm (Lauraceae)	47 Ricinus communis (Euphorbiaceae)
15 Coccinia indica (Cucurbitaceae)	48 Salvia lavandifolia (Lamiacea)
16 Cornus officinalis (Cornaceae)	49 Sarcopoterium spinosum (Rosaceae)
17 Elephantopus scaber (Asteraceae)	50 Scoparia dulcis (Scrophulariaceae)
18 Enicostemma littorale (Gentianaceae)	51 Selaginella tamariscina (Selaginellaceae)
19 Ephedra distachya (Ephedraceae)	52 Semen coicis (Ggramineae)
20 Eriobotrya japonica (Rosaceae)	53 Smallanthus sonchifolius (Asteraceae)
21 Euccalyptus globulus (Myrtaceae)	54 Stevia rebaudiana (Asteraceae)
22 Fermented unsalted soybeans	55 Swertia chirayita (Gentianaceae)
23 Ficus bengalensis (Moraceae)	56 Swertia punicea (Gentianaceae)
24 Genistein	57 Syzygium cumini (Rutaceae)
25 Ginkgo biloba (Ginkgoaceae)	58 Tabernanthe iboga (Apocynaceae)
26 Helicteres isora (Sterculiaceae)	59 Teucrium polium (Lamiaceae)
27 Hibiscus rosa (Malvacea)	60 Tinospora crispa (Menispermaceae)
28 Hordeum vulgare (Gramineae)	61 Tribuluks terrestris (Zygophyllaceae)
29 Ipomoea batata (Convolvulaceae)	62 Urtifca dioica (Urticaceae)
30 Juniperus communis (Pinacea)	63 Vinca rosea (Apocyanacea)
31 Lausena anisata (Rutacea)	64 Zingiber officinale (Zingiberaceae)
32 Lepechinia caulescens (Lamiaceae)	65 Zizyphus spina-christi (Rhamnaceae)
33 Medicago sativa (Fabaceae)	

the fruit extract of *C. ficifolia* lowered the blood glucose level and increased the hepatic glycogen content, and the plasma insulin. Furthermore, the same extract improved the blood glucose tolerance when an oral glucose tolerance test was performed in fasted diabetic and normal rats. The results of this experimental animal study lend a pharmacological credence to the suggested folkloric uses of the plant in the management and control of diabetes mellitus, owing to its high content of the insulin-mimetic, D-CI. This compound is also the active constituent of *Fagopyrum tataricum* L. Gaench that possesses an insulin-like bioactivity. Yao *et al*[160] illustrated that the D-CI-enriched extract of *Fagopyrum tataricum* lowered plasma glucose, C-peptide, improved glucose tolerance, and enhanced insulin immunoreactivity in KK-Ay mice.

INCRETIN MIMETICS AND INCRETIN ENHANCERS

A new target for the management of type Ⅱ DM is the gut hormone, GLP-1 (incretin) which is secreted as a riposte to meal. This hormone maintains glucose balance by different routes where it stimulated glucose-dependent insulin secretion, delays gastric emptying, inhibits glucagon secretion, and protects or even exerts a trophic effect on β-cells, as illustrated in Figure 4. However, the hormone is rapidly degraded by dipeptidylpeptidase-4 (DPP-4), an enzyme that inactivates also glucose-dependent insulinotropic peptide (GIP)[161]. Thus, the aim in pharmaceutical research is either to inhibit DPP-4, to prolong GLP-1 duration of action, or to use compounds that can partially resist DPP-4. These compounds are either incretin-mimetic agents that simulate GLP-1 (exenatide) or a long-acting incretin analogue (liraglutide)[162]. Incretin, thus, challenged the pharmaceutical researchers to find a nutraceutical compound that could modulate this hormone.

In this regard, recent data reported that inulin-type fructans extracted from chicory roots regulated glucose and lipid homeostasis by enhancing colon production of GLP-1. Therefore, Urías-Silvas *et al*[163] evaluated the fructans extracted from *Agave tequilana* Gto. and *Dasylirion spp.* on glucose and lipid metabolism. The data showed a decrease in body weight of mice fed fructans-containing diet, besides the restoration of glucose and lipid levels. As a conclusion, the authors reported that fructans from any botanical origin initiates the production of GLP-1 from colon, and it is responsible for the amendment of glucose and lipid metabolism.

The potential antihyperglycemic activity of an ethanolic extract of *Artemisia dracunculus* L., called Tarralin, in diabetic mice was studied by Ribnicky *et al*[164]. This extract posed a positive antidiabetic action, *via* decreasing the mRNA expression of phospho-enolpyruvate carboxykinase (PEPCK), the main catalyzing enzyme in gluconeogenesis, and increasing the binding of incretin (GLP-1) to its receptor.

Impairment of β-cell function results from the improper insulin/IGF-1 signaling cascade through insulin receptor substrate-2 (IRS-2). Thus, induction of IRS-2 in β-cells can potentiate its function and mass, an effect that was attained by the GLP-1 receptor agonist, exendin-4, through elevation of intracellular cyclic Adenosine mono phosphate (cAMP)[165]. GLP-1/exendin-4 is known to enhance glucose-stimulated insulin secretion and to increase β-cell transcription factors, such as pancreas duodenum homeobox-1 (PDX-1), to promote β-cell growth and survival[165]. These promising actions of exendin-4 were associated with the induction of IRS-2, the pathways of

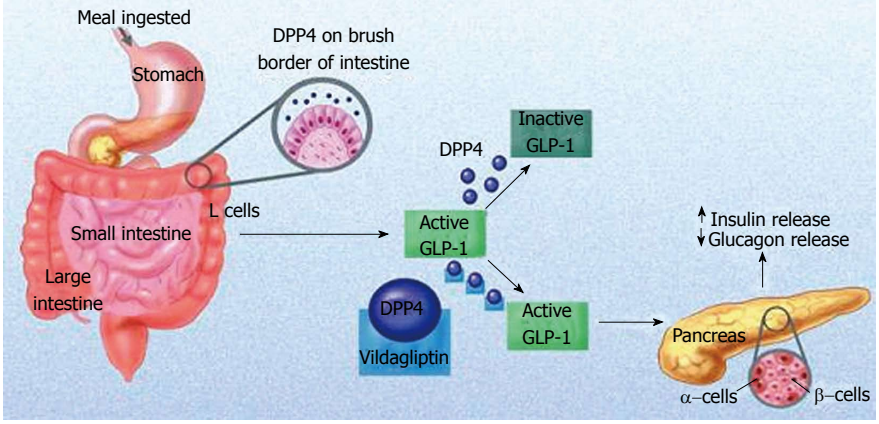

Figure 4 Effects of active glucagon-like peptide-1 and dipeptidylpeptidase-4 on glucose homeostasis [(c.f. www.medscape.com) Am J Health Syst Pharm, 2007]. GLP-1: Glucagon-like peptide-1; DPP-4: Dipeptidylpeptidase-4.

which play an important role in β-cell expansion, and augmentation of insulin secretion.

In a recent study, Park *et al*[165] examined the potential antidiabetic mechanism(s) of six herbs used in Chinese medicine to treat diabetes. These herbs were *Galla rhois*, *Rehmanniae* radix (*Rehmannia glutinosa* Liboschitz var. *purpurea* Making), *Machilus* bark (*Machilus thynbergii* Siebold et Zuccarini), *Polygonatum* radix (*Polygonatum odoratum* Miller Druce), *Ginseng* radix (*Panax ginseng* C.A. Meyer), and *Scutellariae* radix (*Scutellariae baicalensis* Georgi). The authors reported that these herbs induced IRS-2 in rat islets, improved glucose-stimulating insulin secretion and increased β-cell survival. In addition, *Rehmanniae* radix, *Ginseng* radix and *Scutellariae* radix were found to mediate insulin secretion through cAMP/PKA-dependent and/or -independent pathways. These herbs also induced PDX-1 and glucokinase, besides the increased expression of IRS-2. Activation of glucokinase could vindicate the enhancement of glucose stimulated insulin secretion, while induction of PDX-1 was associated with β-cell proliferation[165]. The promising effects of *Ginseng* radix and *Scutellariae* radix could be ascribed to the active constituents, ginsenosides and the flavonoid baicalein, respectively. The finding, hence, point to the presence of natural agents that possess incretin-like action and that mimic exendin-4.

ROLES OF ENDOGENOUS OPIOIDS ON GLUCOSE HOMEOSTASIS

Apart from the well known pharmacological actions of opiates, their binding to opioid receptors located in the pancreatic β-cells and their ability to manipulate diabetic disorders has been documented[166]. The opioid peptide β-endorphin, secreted from the adrenal gland[167] has been shown to induce insulin secretion also *via* activating the pancreatic opioid receptors[168]. Besides, this peptide also was found to regulate glucagon and somatostatin release from isolated islets of Langerhans[169,170]. Therefore, increased glucose utilization and decreased hepatic output may be a consequence to the increased release of β-endorphin and the activation of peripheral opioid μ-receptors (MOR). Activation of these receptors might enhance the expression of muscle GLUT and/or reduce hepatic gluconeogenesis at the gene level[171]. MOR-induced glucose uptake is accomplished by increased gene expression of GLUT-4 *via* a phospholipase C-protein kinase (PLC-PKC) dependent pathway[172]. It has also been observed that stimulation of α_1-adrenoceptors in the adrenal gland provokes the secretion of β-endorphin[173] depending also on the PLC-PKC pathway[174,175].

In STZ-diabetic rats, Hsu *et al*[176] stated that β-endorphin biosynthesis increases in the adrenal gland, along with the opioid μ-receptors gene expression[177]; events that may compensate for the glucose disturbed homeostasis. Therefore, development of pharmaceutical or nutraceutical agents that target β-endorphin secretion and/or stimulate peripheral MOR, *via* an insulin-independent action, donates a new hit that may have merit in glycemic control.

Since application of herbal plants or their products in the management of glucose metabolism is extensively searched, investigations were conducted to study their potential effect on β-endorphin and peripheral opioid μ-receptor. One of the early studies in this regard, is that carried out by Hsu *et al*[178] using caffeic acid, which is a phenolic compound contained in the fruit of *Xanthium strumarium*. After an intravenous injection of caffeic acid into diabetic rats of both STZ-induced and insulin-resistant models, a dose-dependent decrease in the plasma glucose was observed; moreover, it increased the glucose uptake in isolated adipocytes. This trial was followed by another study[179] to verify the mechanism of caffeic acid using STZ-induced diabetic rat. In this experiment, caffeic acid increased the release of β-endorphin from the adrenal gland through the activation of α_{1A} -adrenoceptors. These receptors were adopted as one of the antidiabetic mechanisms of andrographolide present in the leaves of *Andrographis paniculata* (*Burm. f.*) Nees. Using cultured myoblast C2C12 cells, andrographide was documented to activate these adrenoceptors *via* PLC-PKC dependent pathway to fascilitate glucose uptake[180]. Inhibiting α-glucosidase[19] and increasing GLUT-4 mRNA[91] were other mechanisms mediated by this active constituent. A recent study by Yu *et al*[181] validated the andrographolide-induced α_{1A}-adrenoceptors activation in type I diabetes-like animals, which enhance β-endorphin release that in turn stimulates the opioid micro-receptors. The authors reported also an increased expression of the GLUT-4 in

soleus muscle and a reduced expression of PEPCK in liver, effects that may explain the registered reduction in hepatic gluconeogenesis and enhancement of the glucose uptake. A similar pattern was recorded to rationalize the antidiabetic mechanisms of myricetin, the active principle of *Abelmoschus moschatus* (*Malvaceae*) using STZ-diabetic rats[182]. Myricetin, in insulin-deficient animals, activated peripheral MOR, in response to increased β-endorphin secretion. Opioid μ-receptor activation is held responsible for the enhancement of muscle *GLUT-4* gene expression and the attenuation of hepatic *PEPCK* gene expression observed in these myricetin-treated diabetic animals.

Another study was carried out to investigate the antihyperglycemic mechanisms of syringin, an active principle purified from the rhizome and root parts of *Eleutherococcus senticosus* (*Araliaceae*). STZ-diabetic rats showed an increased release of β-endorphin from the adrenal medulla after receiving a bolus intravenous injection of syringing[183]. Niu *et al*[183] concluded that the decreased plasma glucose, in the diabetic rats lacking insulin, is mediated by the effect of β-endorphin on peripheral micro-opioid receptors.

The antidiabetic potency of isoferulic acid, one of the active components in *Cimicifugae rhizoma*, is attained by lowering glucose level, improving glucose uptake in skeletal muscle along with inhibiting hepatic gluconeogenesis in rats with an insulin deficiency[184]. For precise clarification of its mode of action, Liu *et al*[185] tested its impact on the α_{1A}-adrenoceptor/β-endorphin system in a STZ diabetic rats. Formerly, Liu *et al*[186] showed that isoferulic acid can activate α_{1A}-adrenoceptor, leading to increased glucose uptake into cultured mouse myoblast C2C12 cells; however, the role of β-endorphin in the plasma glucose-lowering action of isoferulic acid is still unclear. In this work[187], the authors proved that isoferulic acid increased β-endorphin level *via* affecting α_{1A}-adrenoceptors, leading to stimulation of peripheral opioid receptors, resulting in increased expression of GLUT-4, and reduction of hepatic gluconeogenesis. Moreover, the same laboratory examined the mechanism(s) of plasma glucose lowering action of puerarin in STZ-induced diabetic rats and concluded that this isoflavone can act as a ligand to activate α_{1A}-adrenoceptors on the adrenal gland to initiate the aforementioned cascades[187].

ANTIOXIDANTS

In the course of normal aerobic metabolism, oxygen free radicals are produced during the reduction of oxygen into water. Since these radicals are inherently toxic, cells have built up defense systems to quench them. These defense systems are either enzymatic, including superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione reductase and glucose-6-phosphate dehydrogenase, or non-enzymatic, such as vitamins C and E as well as thiols, especially the reduced glutathione molecule[188]. If these oxygen free radicals, referred as reactive oxygen species (ROS), are excessively produced and are able to overwhelm the endogenous defense systems, then a state of oxidative stress originates. These ROS can bind with most normal cellular components to "pair up" its unpaired electrons; thus, they react with the unsaturated bonds of membrane lipids, denature proteins, and attack nucleic acids, resulting in cellular oxidative damage[189]. It has been suggested that oxidative stress plays an important role in many diseases, including DM, since hyperglycemia alone could not be exclusively responsible for the later complications associated with the disease[190]. ROS are considered an important independent risk factor that is developed in DM *via* what is known as "auto-oxidative glycosylation, a process which is relevant at elevated blood glucose level[191]. Hyperglycemia may also raise aldose reductase which depletes NADPH cell stores, thus perturbing defense system[192]. The elevated blood glucose level causes also non-enzymatic glycation of plasma proteins[193] leading to the production of more powerful oxidizing species[194]. Furthermore, it induces mitochondrial superoxide overproduction, which influences again the previous steps[195], creating what is known as "hyperglycemic memory"[196]. As oxidative stress plays a key role in insulin-resistance and β-cell dysfunction[197], ample of data allows the hypothesis that a viscous circle exists between hyper-insulinemia and free radicals that may be responsible for deterioration of insulin action[198], possibly *via* down-regulating insulin-mediated glucose uptake[199].

Given that antioxidants are favorably used as complementary agents in diabetic patients to reduce diabetic complications[200-203], attempts to discover antioxidants as useful drug candidates to combat diabetic complications are going on persistently.

Of the plants that exert their positive effects in experimental DM through their antioxidant characters are *Ficus carica via* restoring levels of fatty acids and vitamin E[204], as well as some Indian herbs, *viz*., *Allium sativum, Azadirachta indica, Momordica charantia,* and *Ocimum sanctum extracts,* which not only lowered the blood glucose level, but also inhibited the formation of lipid peroxides, reactivated the antioxidant enzymes, and restored levels of GSH and metals[124]. These results may authorize the use of the aforementioned herbs in the prevention of diabetes-associated complications. In addition, *Momordica grosvenori,* a traditional medicinal herb in China used as a substitute sugar for obese and diabetic patients, was tested in alloxan-induced diabetic mice[205]. The plant corrected the altered glucose level and effectively regulated the immune imbalance in diabetic mice. The authors assigned these effects to the plant-induced upregulation of heme oxygenase-1 (HO-1) protein, which has anti-inflammatory activities and antioxidant properties.

The ethanolic extract of *Scutellaria baicalensis*, as well, proves its antioxidant role in a STZ-induced diabetic model, and enhances the antidiabetic effect of metformin[206]. In addition, in a study on the antioxidant and antiglycation properties of some traditional Chinese medicine used to treat DM, *Aralia taibaiensis* outperformed other extracts in most of the assays except for the inhibition of early glycation products formation which was

mostly inhibited by *Acanthopanax senticosus* extract[207]. The antioxidant and antiglycation activities of these extracts were correlated with their saponin content[207]. The aqueous extract of *Albizzia lebbeck* was also verified for its antioxidant property using alloxan-induced diabetic rats[208]. The authors registered that the extract rescued all altered parameters caused by alloxan which confirmed the ability of the herb to resist the oxidative insult.

The hypoglycemic and hypolipidemic effects of *Lycium barbarum* fruit extract, its crude polysaccharides (LBP) extract and purified polysaccharide fractions (LBP-X), were documented in alloxan-induced diabetic rabbits[209]. Although the hypoglycemic effect of LBP-X surpassed the other extracts, yet the latter exhibited stronger antioxidant activity because crude extracts were identified to be rich in antioxidants (*e.g.*, carotenoids, riboflavin, ascorbic acid, thiamine, nicotinic acid). In Li[210] has isolated *Lycium barbarum* polysaccharides (LBP), which are identified as one of the active ingredients of the fruits, and tested its capacity to stand the oxidative insult using a STZ-induced hyperglycemic model. The author found again that the LBP reinstated the STZ-induced abnormal oxidative indices, results that are in line with another study by Wu *et al*[211], who also studied the antidiabetic effects of these polysaccharides, using rats with NIDDM. The authors found that LBP can control blood glucose and modulate the metabolism of glucose, leading to a significant improvement of oxidative stress markers (SOD, MDA), in addition to its ability to decrease DNA damage, possibly *via* leveling off oxidative stress. These findings point to the potential protective effect of LBP against deleterious oxidative stress, hence, preventing the development of diabetic complications.

Additionally, *Strobilanthes crispus* (*Acanthaceae*), which is used traditionally for the treatment of several ailments including DM, has shown antihyperglycemic and antilipidemic properties when tested in STZ-induced diabetic rats. The antioxidant effect of the herbal hot water extract (fermented and unfermented) contributed possibly to its and polyphenol contents[212].

Clinically, the valuable antioxidant effect of the herbal medicine, *Silybum marianum* seed extract (silymarin), was confirmed in a randomized, double-blind, placebo-controlled, clinical study of 51 type Ⅱ diabetic patients[213], where this extract induced a marked improvement in the glycemic profile of these patients.

In an attempt to study the effect of some herbal components against free radicals, Xiong *et al*[214] assessed the protective effect of puerarin, an isoflavone purified from Chinese herb radix of *Pueraria lobata*, on hydrogen peroxide (H_2O_2)-induced rat pancreatic islets damage. The results emphasize that puerarin can preserve islet cells from the ROS-induced damage. Likewise, the extract of *Plantago depressa var. montata*. was able to correct glucose and lipid homeostasis and to restore redox status in alloxan-induced diabetic mice, effects that are probably due to its antioxidant and free radical scavenging properties[215].

Another herbal drug evaluated for its hypoglycemic and anti-oxidant activities is the dried roots of *Morinda officinalis,* which was tested in STZ-treated rats and resulted in a decrease in fasting glucose and lipid peroxide levels, along with the restoration of the assessed redox indices. The study concluded that *Morinda officinalis* has anti-diabetic and antioxidant potentials[216]. Similarly, *Amaranthus esculantus* grain and oil fraction were found effective as both antioxidant and anti-diabetic, suggesting their beneficial effect in correcting hyperglycemia and preventing diabetic complications[217].

In the Turkish folkloric medicine *Gentiana olivieri* Griseb. (*Gentianaceae*) is used as a hypoglycemic plant, an effect that was verified by a recent study[218]. The hypoglycemic effect was attributed to its main active constituent, isoorientin, a compound that was documented for its favorable action on glucose homeostasis[219] partly *via* saving β-cells from oxidative damage by virtue of its potent antioxidant properties. Additionally, this compound may sensitize the insulin receptor to insulin or stimulate the stem cell of islets of Langerhans in pancreas of STZ-induced diabetic rats to restore plasma level of insulin[219]; however, these assumptions need to be tested.

Moreover, the ability of ginseng to scavenge free radicals is thought to add to its antidiabetic mechanisms[220]. Ginseng was found to decrease the rate of monosaccharide auto-oxidation, to elevate the activity of defence enzymes as SOD; and directly eliminate the superfluous free radicals. The same hold true for garlic (*Allium sativum* L., *Liliaceae*) which mediates its antidiabetic action by acts by its antioxidant character and by increasing insulin secretion[221].

The methanolic extract of *Phyllanthus amarus* (*Euphorbiaceae*), used traditionally in Indian herb medicine, was found to have a potent antioxidant activity added to its antihyperglycemic efficacy tested in alloxan-induced diabetic rats[222]. Other plants known for their antioxidant properties include *Capparis deciduas, Camellia sinensis, Emblica officinalis, Ficus bengalensis, Musa sapientum and Punica granatum*[151]. Additionally, the antidiabetic effects of fruit of *Vaccinium arctostaphylos* L. (Ericaceae), which is traditionally used in Iran for improving of health status of diabetic patients, was found to encounter several machinaries among which were the notable rising of the erythrocyte superoxide dismutase (57%), glutathione peroxidase (35%) and catalase (19%) activities of the alloxan-treated rats[223].

Hyperglycemia-induced aldose reductase activation results in the depletion of NADPH which is required for GSH reductase, hence, altering endogenous defense system. Therefore, inhibitors of aldose reductase could offer new approaches for the treatment of diabetes. Feng *et al*[224] reported in his study that some herbal active constituents, *viz*., flavonoid compounds and their derivates, have the ability to inhibit the activity of this enzyme, such as *quercetin, silymarin, puerarin,* and others. In addition, some *Salacia* root species possess this function, for example, the crude methanolic extract and ethyl acetate soluble fractions of *S. oblonga* showed inhibitory activity on rat lens-derived

aldose reductase[43]. In addition, the extract of *S. reticulata* stems, with its active constituent mangiferin, exhibited aldose reductase inhibitory activity[225], as well as the aqueous methanolic extract of *S. chinensis*[45].

CONCLUSION

From the previous data reviewed in the current article, it is obvious that herbs and/or their active constituents could attack several pathways of the hyperglycemic process. The multi-modes of their action allow them to outperform the conventional diabetic agents, besides the cost effectiveness and higher safety profile. These plants could be used as valuable therapeutic agents or as add-on conventional therapies for controlling glucose homeostasis. Although the evidenced-based therapeutic usage of many plants is scarce, the plants cited in this review are those reputed traditionally for their antidiabetic effect and that were verified, either experimentally or clinically.

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REVIEW

Glycemic control indicators in patients with neonatal diabetes mellitus

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Abstract

Neonatal diabetes mellitus (NDM) is a type of diabetes mellitus caused by genetic abnormality which develops in insulin dependent state within 6 mo after birth. HbA1c is widely used in clinical practice for diabetes mellitus as the gold standard glycemic control indicator; however, fetal hemoglobin (HbF) is the main hemoglobin in neonates and so HbA1c cannot be used as a glycemic control indicator in NDM. Glycated albumin (GA), another glycemic control indicator, is not affected by HbF. We reported that GA can be used as a glycemic control indicator in NDM. However, it was later found that because of increased metabolism of albumin, GA shows an apparently lower level in relation to plasma glucose in NDM; measures to solve this problem were needed. In this review, we outlined the most recent findings concerning glycemic control indicators in neonates or NDM.

Key words: Glycemic control; HbA1c; Glycated albumin; Fructosamine; 1,5-anhydroglucitol; Neonatal diabetes mellitus

Core tip: Neonatal diabetes mellitus (NDM) is a type of diabetes mellitus caused by genetic abnormality which develops in insulin dependent state within 6 mo after birth. Because fetal hemoglobin (HbF) is the main hemoglobin in neonates, HbA1c cannot be used as a glycemic control indicator in NDM. On the other hand, glycated albumin (GA), another glycemic control indicator, is not affected by HbF. We reported that GA can be used as a glycemic control indicator in NDM. In this review, we outlined the most recent findings concerning glycemic control indicators in neonates or NDM.

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INTRODUCTION

To prevent chronic diabetic complications, it is necessary to try to achieve normoglycemia as much as possible. Previously, glycemic control used to be evaluated by plasma glucose or urinary glucose. However, these indicators fluctuate continuously due to factors such as dietary intake, and it was difficult to evaluate glycemic control correctly by taking measurements at a particular time. Therefore, hemoglobin A1c (HbA1c), which reflects mean plasma glucose during the past 1 to 2 mo, was introduced as a glycemic control indicator[1], and is now widely used in clinical practice for diabetes mellitus. HbA1c can be used to evaluate glycemic control status; if poor glycemic control is observed, it is possible to make additions, changes, *etc.* to the treatment of diabetes mellitus[2].

Large-scale researches such as the Diabetes Control and Complications Trial revealed that HbA1c is related to the development and progression of diabetic microan-

giopathy[3]. That is, the development and progression of diabetic microangiopathy can be prevented by maintaining excellent glycemic control using HbA1c as an indicator. Recently, it also became possible to use HbA1c for the diagnosis of diabetes mellitus[4].

However, the following problems of HbA1c were pointed out: (1) abnormal HbA1c values may be observed because of variantl hemoglobin, hemolytic anemia, *etc.*; (2) HbA1c does not correctly reflect short-term glycemic control status; and (3) HbA1c does not correctly reflect postprandial plasma glucose/fluctuation of plasma glucose. Accordingly, new glycemic control indicators such as fructosamine, 1,5-anhydroglucitol (1,5-AG), and glycated albumin (GA) were introduced. Although these indicators compensate the disadvantages of HbA1c, they have their own disadvantages. For example, 1,5-AG is affected by the threshold of urinary glucose excretion in the kidney, and fructosamine and GA are affected by albumin metabolism[5].

Because fetal hemoglobin (HbF) is the main hemoglobin in neonates, HbA1c cannot be used as a glycemic control indicator in neonates. Therefore, glycemic control in neonatal diabetes mellitus (NDM) was traditionally performed using blood glucose measured by self-monitoring of blood glucose as an indicator, without using a glycemic control indicator. We demonstrated that GA, which is not affected by HbF, reflects glycemic control in NDM and can be used as a glycemic control indicator in NDM[6]. We also obtained various other findings about GA and HbA1c in neonates/infants or NDM. In this review, we outlined the most recent findings concerning glycemic control indicators in neonates or NDM.

NEONATAL DIABETES MELLITUS

NDM is a type of diabetes mellitus caused by single-gene abnormality which develops acutely in insulin dependent state; NDM accounts for the majority of cases of diabetes mellitus which develops within 6 mo after birth[7]. The frequency of NDM according to this definition is 1 in 89000 births, showing that NDM is a rare disease[8]. So far, more than 20 causative genes of NDM have been discovered; genetic mutations of some kind have been identified in not less than 70% of patients[8,9]. NDM is similar to type 1 diabetes mellitus in terms of the form of development (diabetes mellitus develops acutely); however, type 1 diabetes mellitus very rarely develops within 6 mo after birth, judging from studies on the frequency of human leukocyte antigen risk alleles and the presence of pancreatic autoantibodies[10,11]. Based on the clinical course, NDM is classified into two major categories: transient NDM (TNDM) and permanent NDM (PNDM)[12]. TNDM is a condition in which insulin secretion is restored spontaneously and normoglycemia is achieved without treatment; PNDM is a condition in which remission is not achieved and life-long treatment is required. The frequency of TNDM is about 60%, and that of PNDM is about 40%.

Although patients with TNDM require insulin therapy at the time of onset because of marked hyperglycemia, they can be weaned from insulin therapy at an average of 3 mo after the start of treatment[13-16]. This is called the remission period. However, in about half of patients, diabetes mellitus relapses from childhood to adolescence[14,17]. In 70% of patients with TNDM, the cause is overexpression of an imprinted gene *PLAGL1* which is located in the chromosome 6q24 region and is expressed from paternal allele (6q24-TNDM)[14,15,18,19]. In 25% of patients with TNDM, mutations of *KCNJ11* and *ABCC8* genes which encode the ATP-sensitive potassium channel (K_{ATP} channel) essential for glucose-stimulated insulin secretion have been identified (K_{ATP}-TNDM)[14,20,21]. 6q24-TNDM has the following characteristics: (1) it often develops within 1 wk after birth; (2) it is often diagnosed asymptomatically on routine blood collection; and (3) it is rarely accompanied by ketoacidosis[15,19]. On the other hand, the time of diagnosis of K_{ATP}-TNDM is 1 to 4 mo after birth, which is later than that of 6q24-TNDM[14].

The main causes of PNDM are K_{ATP} channel abnormality [*KCNJ11* gene (31%); *ABCC8* gene (10%)] and insulin gene mutations (12%); the median age at the time of diagnosis is 8 wk after birth and 10 wk after birth, respectively[9]. In contrast to TNDM, PNDM shows symptoms such as dehydration, poor sucking, and poor weight gain at the time of onset and is often accompanied by ketoacidosis[15,19]. A large proportion of other causative genes are expressed by autosomal recessive inheritance and account for about 10% of PNDM. In about 35% of patients with NDM, causative genes have not been identified[7].

Insulin therapy is required at the time of onset of NDM regardless of disease type in order to improve metabolic abnormality and weight increase[22]. It has been reported that because neonates have a small body and then receive a small dose of insulin, excellent glycemic control is achieved by an insulin pump which is capable of fine regulation[23-25]. As a treatment after withdrawal from the acute phase, a switch to high-dose administration of sulfonylurea (SU) drugs is an effective causal therapy for K_{ATP} channel abnormality; in not less than 90% of patients, a dramatic improvement of glycemic control is observed immediately without hypoglycemia and is maintained for a long period[26-28]. Therefore, when NDM is diagnosed, it is important to determine by gene analysis whether or not K_{ATP} channel abnormality is present. Early diagnosis makes it possible to switch to SU drugs during infancy, resulting in an extremely high quality-of-life[29-32].

1,5-ANHYDROGLUCITOL IN NEONATES

1,5-AG is a polyol with a structure in which hydroxyl at the 1st position of glucose is reduced; 1,5-AG is contained in a wide variety of food, but is hardly metabolized in the body[33]. Therefore, after being absorbed from the intestine, 1,5-AG contained in food is widely distributed in various organs to form an internal pool. The amount of 1,5-AG supplied from daily food intake is smaller than the internal pool, and so there is no change in serum

1,5-AG concentration before and after meal. Excessive intake of 1,5-AG is excreted in urine.

Usually, about 180 g of glucose is excreted daily from glomeruli; about 100% of the excreted glucose is reabsorbed by sodium glucose cotransporter 2 (SGLT2), which is located in proximal renal tubules and are specific to glucose[34], and SGTL1, which is located downstream of SGLT2. After the onset of diabetes mellitus, excretion of glucose will increase; when the increased excretion of glucose exceeds the reabsorption capacity of SGLT2 and SGLT1, reabsorption of glucose *via* 1,5-AG/mannose/fructose cotransporter (SGLT4), which is located downstream of SGLT2 and SGLT1, will start. Because glucose is usually not present, 99.9% of 1,5-AG is reabsorbed by SGLT4; however, this reabsorption mechanism is common to glucose; therefore, if inflow of glucose into tubules increases, reabsorption of 1,5-AG will be inhibited[35-37]. Therefore, in a hyperglycemic condition, excretion of 1,5-AG into urine will increase and serum 1,5-AG will decrease. Thus, serum 1,5-AG is a glycemic control indicator which reflects the degree of urinary glucose excretion.

Because serum 1,5-AG increases and decreases by excretion of urinary glucose, serum 1,5-AG reflects short-term changes in glycemic control more subtly than HbA1c. When glycemic control has worsened rapidly, serum 1,5-AG will decrease rapidly because the increased excretion of a large amount of glucose will inhibit reabsorption of 1,5-AG *via* SGLT4. In patients with marked hyperglycemia and a high excretion of urinary glucose, serum 1,5-AG will not increase in a short period even if glycemic control has improved rapidly because the internal pool of 1,5-AG has decreased.

Serum 1,5-AG is also affected by the threshold for urinary glucose excretion, and therefore shows a low level in renal glycosuria in which the threshold decreases. In addition, serum 1,5-AG shows an abnormally low level in conditions such as chronic renal failure in which reabsorption of 1,5-AG decreases[38-40], pregnancy[41], oxyhyperglycemia in which urinary glucose is observed transiently[42], patients receiving long-term hyperalimentation[43], and liver cirrhosis[44,45]. One of the causes of an abnormally high level of 1,5-AG is oral administration of a kind of Chinese medicines such as Ninjin-yoei-to and Kami-kihi-to which contain large amounts of 1,5-AG[46].

It is known that serum 1,5-AG during the neonatal period shows an apparently low level[47]. This is considered to be due to a small intake of 1,5-AG during the neonatal period. We reported that serum 1,5-AG is significantly lower in subjects with a habit of consuming dairy products than in subjects without such a habit[48]. The fact that breast milk or formula which contains galactose is the main source of nutrition during the neonatal period may be related to a low level of serum 1,5-AG in neonates.

FRUCTOSAMINE IN NEONATES

Protein undergoes glycation reaction in accordance with plasma glucose concentration, and ketoamine, an early Maillard reaction product, is produced *via* aldimine. Because the side chain binding of ketoamine takes a fructose structure, ketoamine is generically named fructosamine. Fructosamine is measured using the property that fructose-lysine (fructosamine), in which glucose is bound to the lysine residues of protein, has reducing ability under alkaline conditions. A large proportion of measurements are made by the chemical method; measurements are made by colorimetric determination by producing reduction color reaction using nitroblue tetrazolium (NBT) as a chromogen. Because 60% to 70% of serum protein is albumin, the main component of fructosamine is glycated albumin, but fructosamine contains glycated lipoprotein and glycated globulin as well. Fructosamine is not affected by anemia or variant hemoglobin. In addition, because the turnover of albumin, which accounts for the most part of serum protein, is faster than that of hemoglobin, it is possible to evaluate short-term glycemic control by measuring fructosamine[49]. A low fructosamine level is observed in hyperthyroidism[50,51] and nephrotic syndrome[52] in which protein (albumin) metabolism is accelerated; a high fructosamine level is observed in hypothyroidism[50,51] in which protein (albumin) metabolism is prolonged.

HbA1c and GA are glycation products of hemoglobin and albumin (single proteins), respectively, whereas fructosamine is the generic name of all glycated proteins and lacks specificity. Because albumin accounts for 60% to 70% of serum protein, fructosamine has similar properties to GA; however, there is a problem that because other glycated proteins are measured as well, a high fructosamine level is observed in myeloma[53]. Because HbA1c and GA are expressed as the ratio of hemoglobin and the ratio of albumin, respectively, they are not affected by dilution of serum; on the other hand, because fructosamine is expressed as reducing ability per 1 mL of serum, it is affected by serum protein concentration, and an apparently low level of fructosamine is observed in dilutional anemia. The level of fructosamine in young children is lower than that in adults[54], which is also partly due to low serum protein concentration. Because fructosamine is measured by colorimetric determination based on reduction color reaction, fructosamine is affected by bilirubin with reducing ability, *etc*. It is considered that the effects of ascorbic acid and vitamin E are mild; however, if a large amount of ascorbic acid or vitamin E is consumed, measurement of fructosamine may be affected.

GLYCEMIC CONTROL INDICATORS OF CORD BLOOD

The composition of hemoglobin in healthy adults is as follows: adult hemoglobin (HbA): 97%; HbA2: 2.5%; HbF: 0.5%[55]. On the other hand, HbF accounts for 80% to 90%, and HbA accounts for only 10% to 20% immediately after birth. After then, HbF decreases logarithmically and is replaced by HbA; by 6 mo after birth, the largest proportion of Hb is HbA; however, it is not until

1 year after birth when the proportion of HbF decreases to less than 1% (level of HbF in adults)[56,57]. Therefore, it is difficult to use the cation exchange high-performance liquid chromatography (HPLC) method, the immunological (latex immunoturbidimetry; LA) method, and the enzyme method which specifically measure HbA1c as glycemic control indicators in NDM.

We measured glycohemoglobin (GHb) in cord blood by various methods[58]. GHb measured by the HPLC method was less than the detection limit when Arkray's HA-8180 was used and was as low as 1.8% ± 0.2% when Tosoh's G8 was used. GHb measured by the LA method was less than the detection limit; HbA1c measured by the enzyme method was 1.1% ± 0.3%. Because these methods for measuring GHb measure HbA1c specifically and do not measure glycated HbF, the result is less than sensitivity or a very low level, and it was confirmed that these methods cannot be used as glycemic control indicators in NDM.

It is considered that measurement of GHb by the affinity method using boronic acid may be used as a glycemic control indicator during the neonatal period as well because it measures all glycated hemoglobins[59,60]. It has been reported that GHb in cord blood is higher in patients whose mother has diabetes mellitus than in patients whose mother does not have diabetes mellitus[61-63]. Our investigation revealed that GHb was 3.9% ± 0.2%, which was slightly lower than the reference value for adults (4.6% to 6.2%)[58]. Plasma glucose in cord blood was normal (94 ± 27 mg/dL); therefore, it is considered that the low GHb levels were due to shortened life span of red blood cells[64].

GA in cord blood was 9.4% ± 1.1%, which was slightly lower than the reference value for adults (11.6% to 16.2%)[58]. We demonstrated that low GA levels are observed in neonates because albumin metabolism in neonates is accelerated[65,66]. Low GA levels in cord blood are considered to be due to accelerated metabolism of albumin.

The level of 1,5-AG in cord blood measured in pregnant women including those with diabetes mellitus was similar to that in maternal blood at the time of delivery[67]. This finding was considered to be due to the fact that 1,5-AG in maternal blood was distributed in the fetus *via* the placenta.

The above results show that both GHb measured by the affinity method and GA were slightly lower than the reference value for adults, but could be used as glycemic control indicators in NDM. On the other hand, HbA1c measured by the HPLC method, the LA method, or the enzyme method and 1,5-AG cannot be used as glycemic control indicators.

GLYCEMIC CONTROL INDICATORS IN NDM: HBA1C AND GA

The etiologic diagnosis and treatment of NDM have been making rapid progress; however, there have been few studies on glycemic control indicators useful for evaluating the diagnosis of NDM and effects of therapy. Therefore, we hypothesized that GA is a useful glycemic control indicator in NDM[58] and conducted an investigation[6]. We found that GA, as a glycemic control indicator in NDM, has various advantages: (1) GA is not affected by HbF; (2) unlike fructosamine, GA is not affected by serum protein (albumin) because it is expressed as a ratio to albumin; (3) unlike fructosamine, GA is not affected by other proteins and has a high specificity because it reflects glycation products of a single protein (albumin); (4) GA reflects plasma glucose during a shorter period than HbA1c; and (5) HbA1c reflects mean plasma glucose, whereas GA reflects fluctuation of plasma glucose (postprandial hyperglycemia) in addition to mean plasma glucose[68-70]. HbA1c (%) is expressed as HbA1c/total Hb; therefore, if HbF is high, a relatively low HbA1c level will be observed. At the time of onset of NDM (mostly 1 to 2 mo after birth), a large amount of HbF remains in blood; therefore, a lower HbA1c level is observed in relation to plasma glucose level. In addition, it is estimated that during infancy, during which HbA increases, if plasma glucose level is constant, HbA1c will increase. In fact, in an investigation of five patients with NDM (age at the time of diagnosis: 38 ± 20 d), plasma glucose was markedly high [29.7 ± 13.1 mmol/L (535 ± 236 mg/dL)], whereas HbA1c measured by the HPLC method was within the normal range (5.4% ± 2.6%)[6]. As the course of treatment progressed, plasma glucose tended to decrease (Figure 1A), whereas HbA1c tended to increase (Figure 1B). A significant negative correlation was observed between HbA1c and HbF (Figure 2A), whereas no significant correlation was observed between HbA1c and plasma glucose level (Figure 2B). On the other hand, GA at the time of diagnosis was abnormally high (33.3% ± 6.9%)[6]. In contrast to HbA1c, GA decreased as treatment progressed (Figure 1C) and showed a strong positive correlation with plasma glucose level (Figure 2C). Thus, it was found that GA, but not HbA1c, is an appropriate glycemic control indicator in NDM.

From what age can HbA1c be used as a glycemic control indicator? Alternatively, if the effect of HbF is excluded or if a different principle of measurement is employed, might HbA1c be an appropriate indicator? And when using GA as a glycemic control indicator in NDM, what should be taken into account? In the following chapters, we will discuss these issues in relation to the current status and challenges in infants and NDM.

HBA1C IN NEONATES AND NDM

As mentioned above, when HbA1c is expressed as HbA1c/total Hb, it cannot be used as a glycemic control indicator in NDM. There are two ways to eliminate the effect of HbF. One way is to determine the HbA1c level corrected by HbF (HbF corrected HbA1c) by the formula: HbA1c/(total Hb-HbF), resulting in the correction of an apparently low HbA level. The other way is to determine GHb relative to all hemoglobins including HbF and to use this as a glycemic control indicator. For the latter, it

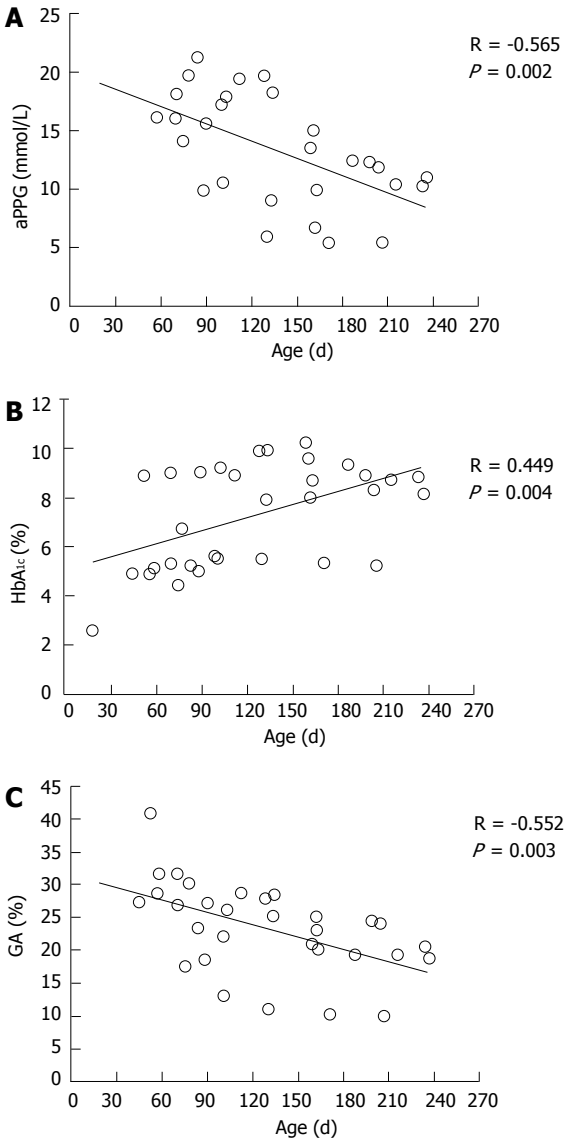

Figure 1 Time course of average preprandial plasma glucose for 1 mo (A), HbA1c (B), and glycated albumin (C) according to treatment in 5 patients with neonatal diabetes mellitus (modified from Ref[6], with permission from Copyright Clearance Center Inc.).

is possible to measure all GHb by the affinity method[71].

We measured HbA1c by the HPLC method and the LA method in 26 healthy infants (0 to 8 mo old), calculated HbA1c values corrected by HbF [Adj-HbA1c (HPLC) and Adj-HbA1c (LA), respectively], measured GHb by the affinity method [GHb (Affinity)], and evaluated correlations between these values and plasma glucose and between these values and GA[72]. As a result, only GHb (Affinity) had a significant correlation with both plasma glucose and GA (Figure 3A). Adj-HbA1c (LA) was correlated only with GA (Figure 3B); Adj-HbA1c (HPLC) was not correlated with either plasma glucose or GA (Figure 3C). These results suggest that GHb (Affinity) may be used as a glycemic control indicator in NDM. In this research, however, GHb (Affinity) within one month was lower than the reference range of HbA1c during 8 to 12 mo (4.8% to 6.0%)[73], and a large proportion of GHb values from 1 to 5 mo were lower than the reference range. The following three factors are thought to contribute together to this finding. The first factor is the effect of a low plasma glucose level during infancy, especially within one month after birth[65,74]. The second factor is the short half-life of red blood cells (about 90 d) during infancy[64]. The third factor is the glycation rate of HbF which is considered to be lower than that of HbA. In this regard, Little *et al*[75] reported that GHb measured by the affinity method is low when a sample which contains not less than 15% of HbF is used. In the LA method, HbA1c is measured using antibodies which specifically recognize peptides including glycated valine of hemoglobin β-chain N-terminal[76]. Theoretically, when interpreting Adj-HbA1c (LA) levels, it is necessary to consider a low plasma glucose level and shortened half-life of red blood cells of the infant; however, it is considered that Adj-HbA1c (LA) may be used as a glycemic control indicator; in fact, a correlation between Adj-HbA1c (LA) and GA was observed. However, the LA method is too complicated to be used in clinical settings because it is necessary to measure HbF using the HPLC method. In addition, our investigation revealed that Adj-HbA1c (HPLC) is not an appropriate indicator for the evaluation of HbA1c in infants. In the HPLC analysis, HbF and HbA1c migrate to adjacent locations. When a high HbF level is observed, separation of HbF and HbA1c becomes insufficient and so HbA1c cannot be measured correctly, which is considered to be one of the causes of the above-mentioned phenomenon. On the other hand, Little *et al*[75] and Rohlfing *et al*[77] reported on HbF-corrected HbA1c as follows: if HbF is not more than 30%, HbA1c measured by the HPLC method using Tosoh's G7 and G8 can be used as a glycemic control indicator. However, they did not use samples which contained 30% or more of HbF, and they did not state whether or not Hb in the samples used was derived from infants; therefore, these facts may be the reason for the difference from our data obtained from samples of infants.

So far, there have been no studies on the age at which HbA1c can be used for patients with NDM, and so research is needed to clarify the relationship between mean plasma glucose and HbA1c and between CGM and HbA1c. Regarding the reference value of HbA1c in healthy infants, there is only a report by Jansen *et al*[73] who investigated 100 healthy infants of 8 to 12 mo old. In that report, the reference value of HbA1c for infants was 4.8% to 6.0%, which was similar to the reference value of HbA1c for adults (4.6% to 6.2%). From our results, HbA1c levels in most infants of 6 mo of age or older were also within the reference range shown by Jansen *et al*[73] HbF decreases to less than 5% by 6 mo after birth[56,57]; therefore, it is considered possible to use HbA1c as a glycemic control indicator in patients with NDM of 6 mo of age or older.

GA IN NEONATES AND NDM

GA is a useful glycemic control indicator under conditions in which hemoglobin metabolism is affected. On

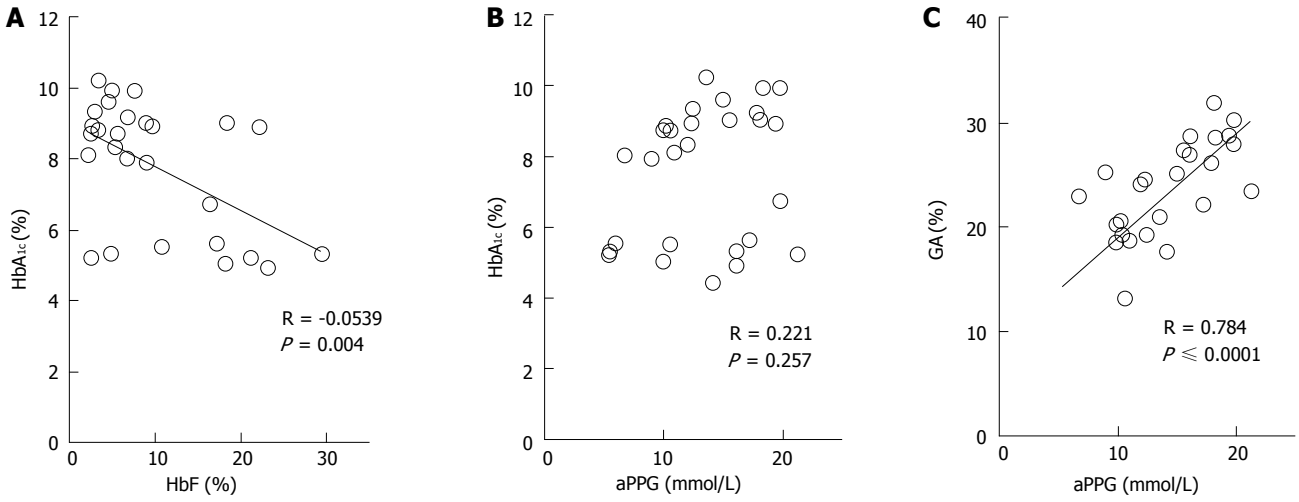

Figure 2 Correlations between HbA1c and HbF (A) and between HbA1c and average preprandial plasma glucose for 1 mo (B) and correlation between glycated albumin and average preprandial plasma glucose in 5 patients with neonatal diabetes mellitus (C) (modified from Ref[6], with permission from Copyright Clearance Center Inc.).

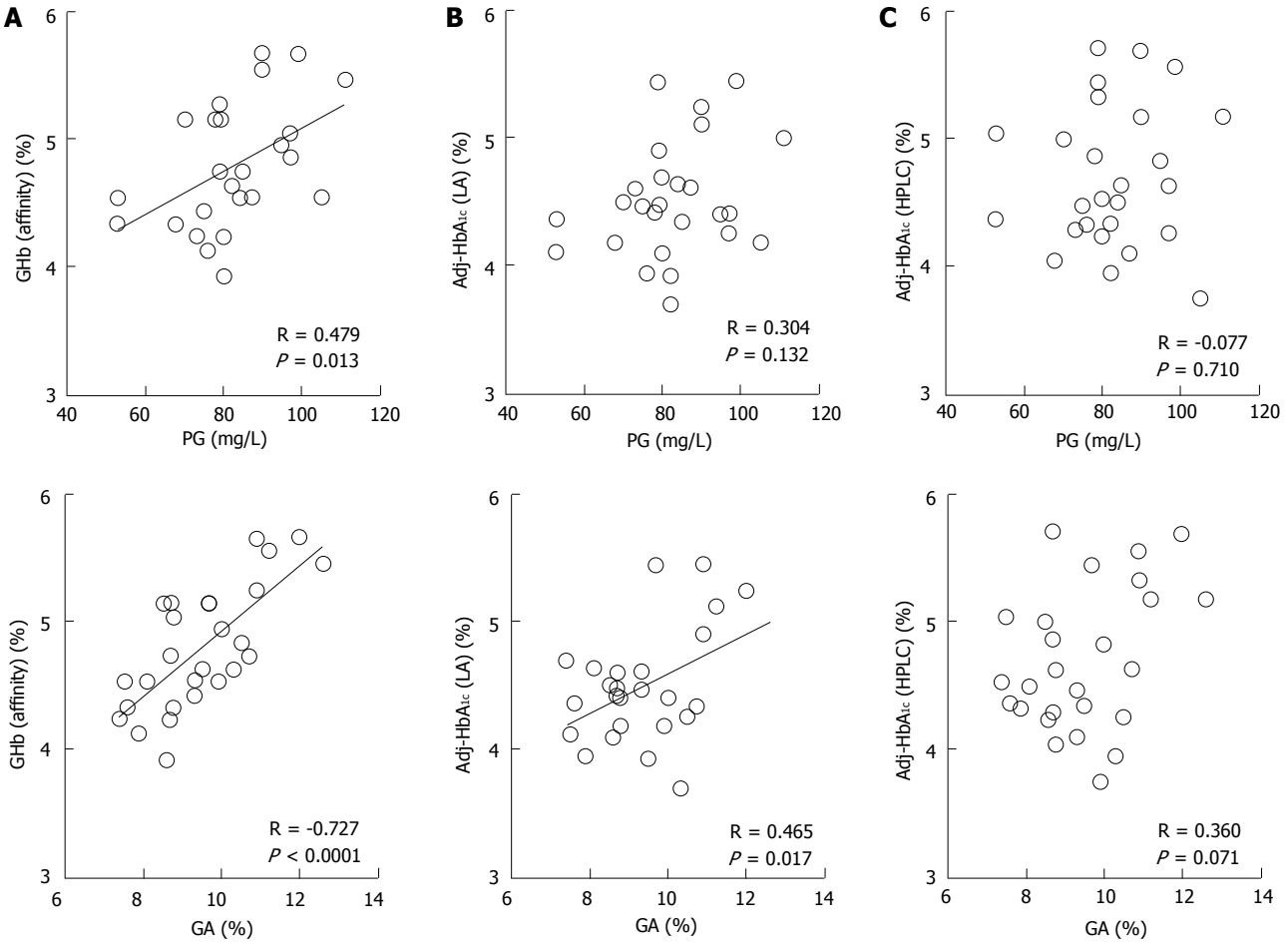

Figure 3 Correlations between glycated hemoglobin measure by various methods and plasma glucose or glycated albumin. Correlations between GHb measured by the affinity method [GHb (affinity)] (A), HbF-adjusted HbA1c measured by the immunological method [Adj-HbA1c (LA)] (B), and HbF-adjusted HbA1c measured by the HPLC method [Adj-HbA1c (HPLC)] (C), and PG or GA in 26 healthy infants were shown (modified from Ref[72], with permission from Copyright Clearance Center Inc.). GA: Glycated albumin; PG: Plasma glucose; GHb: Glycated hemoglobin; HbF: Fetal hemoglobin.

the other hand, abnormal albumin metabolism affects GA. It has been reported under various conditions that GA shows a low level when albumin metabolism is accelerated and shows a high level when albumin metabolism is suppressed[78].

While GA is a useful glycemic control indicator in

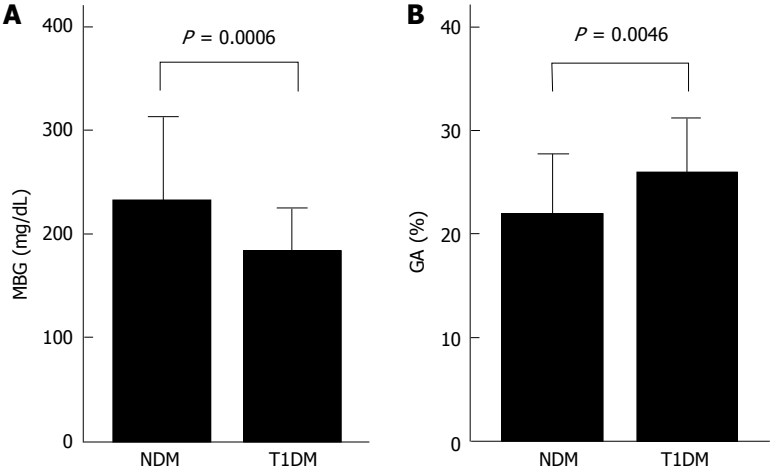

Figure 4 Comparisons of mean blood glucose for 1 mo (A) and GA (B) in 6 patients with neonatal diabetes mellitus and in 18 patients with type 1 diabetes mellitus (modified from Reference[65], with permission from Copyright Clearance Center Inc.).

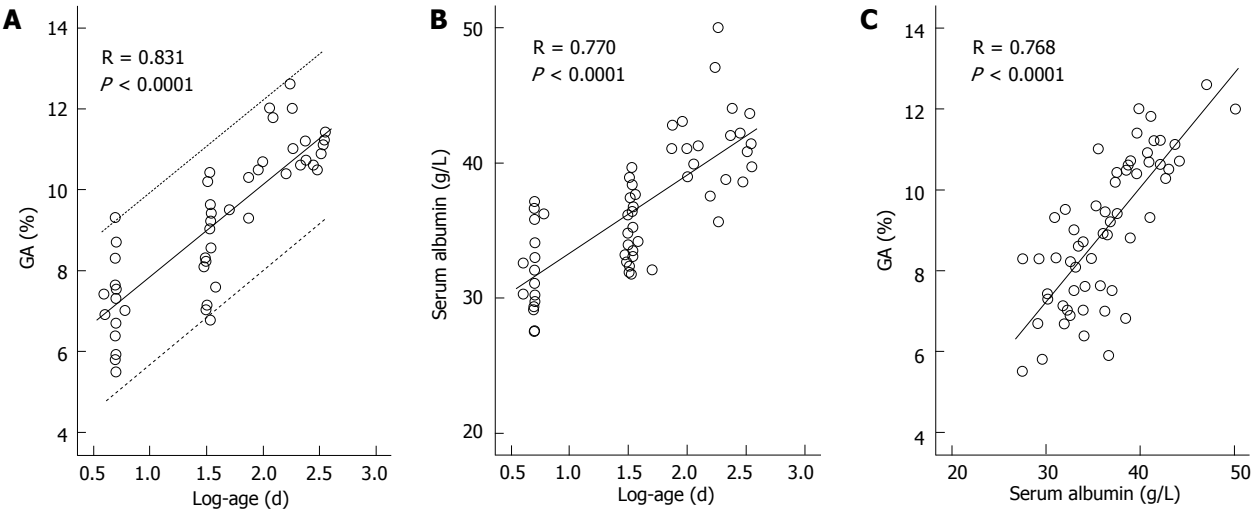

Figure 5 Correlations between glycated albumin and age and between serum albumin and age and correlation between glycated albumin and serum albumin in healthy infants. A: Correlation between glycated albumin (GA) and log-age. The dotted line shows the 95%CI; B: Correlation between serum albumin and log-age; C: Correlation between GA and serum albumin (modified from Ref[66], with permission from Copyright Clearance Center Inc.).

patients with NDM, it is necessary to keep in mind the following characteristics of GA during infancy: (1) it shows a lower level in relation to plasma glucose; and (2) it shows a positive correlation with logarithmically transformed age[65,66]. For GA in healthy infants, before the currently widely used enzyme method was developed[79], it had already been reported that GA measured by the HPLC method was lower than the reference value for adults[54]. It is known that protein metabolism is accelerated during infancy[80,81]. In addition, it has been reported that albumin synthesis is accelerated as well[82]. Therefore, acceleration of albumin metabolism may contribute to a low GA level during infancy. We compared the relationship between GA and plasma glucose level in patients with NDM and in patients with juvenile type 1 diabetes mellitus (T1DM), and found that patients with NDM had higher plasma glucose levels but lower GA levels than patients with T1DM (Figure 4); thus, we obtained a result which supports the phenomenon of accelerated metabolism of albumin during infancy[65]. In addition, we investigated in healthy infants the relationship between change in GA according to age and plasma glucose and between change in GA according to age and serum albumin. As a result, a strong positive correlation was observed between GA and logarithmically transformed age in days (Figure 5A), and multivariate analysis revealed that age and serum albumin affect GA levels more significantly than plasma glucose[66]. Because GA is expressed as a percentage relative to serum albumin, it is not affected by serum albumin, which is an advantage of GA over fructosamine[54]. However, an increase in serum albumin associated with aging is observed during infancy (Figure 5B) and there is a positive correlation between GA and serum albumin during this period (Figure 5C)[66]. Accordingly, we determined the reference value of GA in infants according to age in mo from the regression equation of GA and age, and proposed that a comparison between GA level and the reference value[65].

On the other hand, we found that regardless of age, GA can be evaluated based on the reference value for adults without using the reference value for infants by determining age adjusted GA (Aa-GA)[83]. We investigated

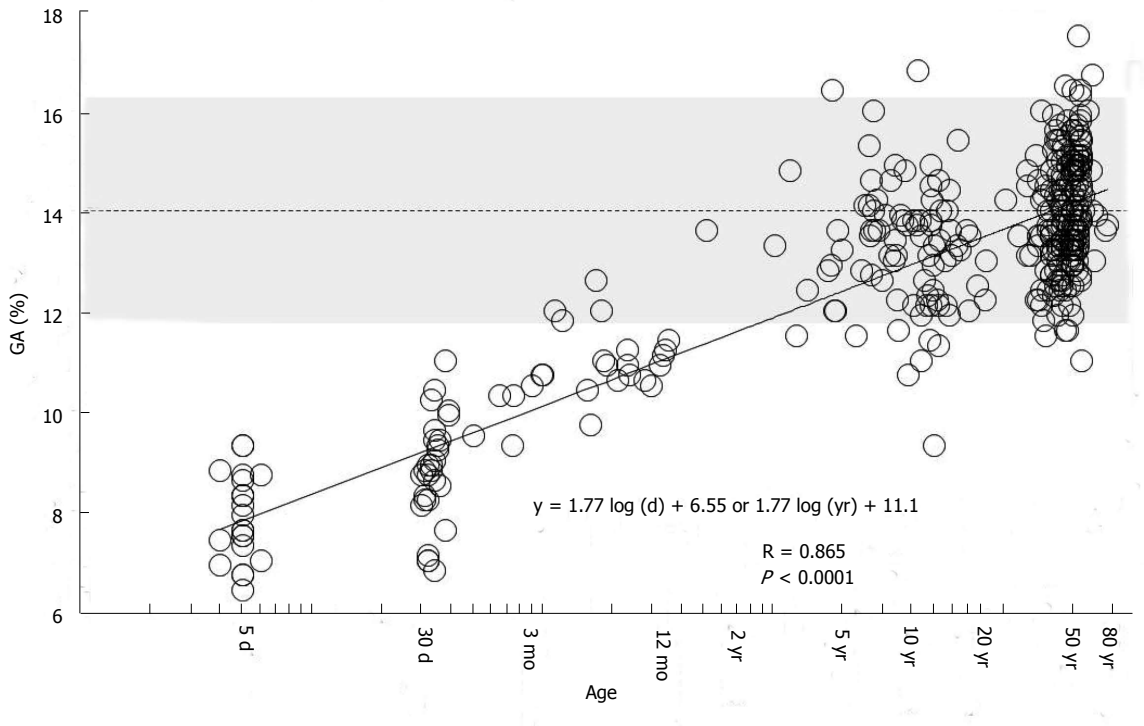

Figure 6 Correlation between glycated albumin and age in days (logarithmic transformation) in 376 healthy subjects (age: 4 d to 78 years). The dotted line indicates the mean reference value for adults (14%), and the shading indicates the range of reference values for adults (11.7% to 16.2%) (modified from Ref[83], with permission from Royal Society of Medicine).

GA in 376 subjects without diabetes mellitus of a wide range of age (neonates, children, and adults), and found that GA can be expressed as a primary regression equation of logarithmically transformed age (Figure 6). Based on this equation, the following formula for calculating Aa-GA was derived: Aa-GA = GA × 14.0 /[1.77 × log-age (d) + 6.55] or Aa-GA = GA × 14.0 /[1.77 × log-age (yr) + 11.1]. As mentioned above, GA in NDM shows an apparently low level; therefore, if GA in NDM is compared with the reference value for adults, the glycemic control status may be underestimated. By calculating Aa-GA and comparing it with the reference value for adults, it is possible to accurately evaluate the glycemic control status in NDM. The advantages of evaluating Aa-GA by the reference value for adults instead of evaluating GA by the reference value for infants according to age in month are as follows: (1) it is not necessary to consider the reference value according to age in month; and (2) regardless of age, it is possible to make comparisons of longitudinal changes in glycemic control status.

It is known that because the half-life of GA is shorter than that of HbA1c, GA reflects short-term plasma glucose correctly[84,85]. This characteristic also indicates the usefulness of GA as a glycemic control indicator in NDM. Because a large proportion of NDM develops within one month, the duration of the hyperglycemic status is short. This form of development is similar to that of fulminant type 1 diabetes mellitus[86]. In fulminant type 1 diabetes mellitus, pancreatic beta cells are destroyed in a very short period, and ketoacidosis develops shortly after the onset of diabetic symptoms. Therefore, at the time of onset, HbA1c is normal or only slightly high, but GA is already obviously high[87]. We reported that GA at the time of onset of NDM was abnormally high (33.6 ± 6.9%) in all patients[6], and an abnormally high GA level in NDM may be useful for differential diagnosis from transient hyperglycemia. In addition, when evaluating remission of patients with TNDM and when evaluating the effect of SU drugs administered to patients with PNDM, it will be possible to promptly evaluate an improvement of such glycemic control by using GA[5].

CONCLUSION

The usefulness of GA as a glycemic control indicator in NDM was demonstrated. However, it was found that GA is affected by albumin metabolism and shows an apparently low level. Therefore, it is necessary to compare GA with the reference value according to age or to calculate age-adjusted GA (Aa-GA). On the other hand, HbA1c measured by the HPLC method, the LA method, or the enzyme method does not correctly reflect the glycemic control status because it is affected by a high HbF level. GHb measured by the affinity method reflects the glycemic control status in NDM; however, this method is currently hardly used and cannot easily measure GHb routinely. In addition, it is unknown whether the kinetics of glycation reaction of HbF are similar to those of HbA. Taking into account such circumstances, it is desirable to select GA as a glycemic control indicator for patients with NDM and to evaluate the glycemic control status using Aa-GA.

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ORIGINAL ARTICLE

Exploration of natural enzyme inhibitors with hypoglycemic potentials amongst *Eucalyptus* Spp. by *in vitro* assays

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Abstract

AIM: To investigate the presence and potency of natural enzyme inhibitors with hypoglycemic potentials amongst *Eucalyptus* Spp. by *in vitro* assays.

METHODS: The leaf extracts of the three different *Eucalyptus* species [*E. globulus* (EG), *E. citriodora* (EC), *E. camaldulensis* (ECA)] were subjected to *in vitro* assay procedures to explore the prevalence of natural enzyme inhibitors (NEIs) after preliminary qualitative and quantitative phytochemical evaluations, to study their inhibitory actions against the enzymes like α-amylase, α-glucosidase, aldose reductase, angiotensin converting enzyme and dipeptidyl peptidase 4 playing pathogenic roles in type 2 diabetes. The antioxidant potential and total antioxidant capacity of the species were also evaluated.

RESULTS: Major bioactive compounds like polyphenols (341.75 ± 3.63 to 496.85 ± 3.98) and flavonoids (4.89 ± 0.01 to 7.15 ± 0.02) were found in appreciable quantity in three species. Based on the IC_{50} values of the extracts under investigation, in all assays the effectivity was in the order of EG > ECA > EC. The results of the ferric reducing antioxidant power assay showed that the reducing ability of the species was also in the order of EG > ECA > EC. A strong correlation (R^2 = 0.81-0.99) was found between the phenolic contents and the inhibitory potentials of the extracts against the targeted enzymes.

CONCLUSION: These results show immense hypoglycemic potentiality of the *Eucalyptus* Spp. and a remarkable source of NEIs for a future phytotherapeutic approach in Type 2 diabetes.

Key words: Natural enzyme inhibitors; Hypoglycemic; *Eucalyptus*; *In vitro* assays; Pathogenic; Polyphenols; Flavonoids

Core tip: Enzymes play an essential role in mediating important biochemical processes of life but hyper or hypo activity of such enzymes leads to malfunctions of the processes. Etiopathogenesis of diseases at molecular level has shown that enzyme inhibitors can serve as effective therapeutic bullets for several diseases. The plant kingdom is a giant hub of phytomolecules with variant pharmacology, largely unexplored. Volatile and non-volatile fractions of *Eucalyptus* include bioactive compounds like terpenes, triterpenoids, flavonoids, polyphenols, *etc.* The exploration of enzyme inhibitors amongst *Eucalyptus* species by *in vitro* assays will help in bioactivity guided isolations of such inhibitors to be targeted as natural hypoglycemics.

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INTRODUCTION

Diabetes mellitus (DM) is fast becoming the epidemic of the 21st century, becoming one of the major killers of the health of mankind after Acquired Immuno Deficiency Syndrome, cancer and cerebrovascular diseases[1]. The statistics of the global diabetic population is expected to show a steady growth to 366 million by 2030. The international diabetes federation has estimated the number of diabetics in India to be 40.9 million, which is expected to grow to 60.9 million by 2025[1,2]. Diabetes is a common metabolic disorder with abnormal elevations in the blood glucose lipid profile, leading to major complications like diabetic neuropathy, nephropathy leading to end stage renal disease, retinopathy leading to blindness and diabetic foot ulcers necessitating limb amputations[1,2]. But despite tremendous strides in modern medicines, the availability of insulin therapy and synthetic hypoglycemics, their failure to restore normoglycemia without adverse effects calls for phytotherapy and alternative medicine[3,4]. Enzymes play a vital role in mediating essential biochemical life processes like metabolism, cell cycling, signal transduction, *etc*. However, hyper or hypo activity of such enzymes leads to malfunctions of the respective biochemical processes which in many cases are the underlying causes of diseases like diabetes, Alzheimer's disease, myasthenia gravis and Parkinson's disease, as depicted by their etiopathogenesis at the molecular level. It is anticipated that enzyme inhibitors serve as important therapeutic targets for these diseases[5]. It has been found that enzymes like α-amylase, α-glucosidase, dipeptidyl peptidase 4 (DPP4), aldose reductase (AR), angiotensin converting enzyme (ACE) and peroxisome proliferator activated receptor-γ (PPAR-γ) contribute significantly to the pathogenesis of type 2DM. Reactive oxygen species (ROS) also play a pathogenic role in type 2DM.

Phytomolecules, as natural enzyme inhibitors (NEIs), can serve as successful therapeutic bullets in the control of this chronic disease[2,5-8]. The World Health Organization has recommended phytotherapy for diabetes due to safety, effectivity, availability and affordability. The NAPRALERT database (NAtural PRoducts ALERT) and the ethnobotanical literature have reported more than 800 anti-diabetic plant species[4,7-9].

Eucalyptus, also known as "gum tree", is taxonomically from the family Myrtaceae, indigenous to Tasmania, Australia and cultivated mostly in subtropical and warm temperate regions of the world. The bark and leaves of *Eucalyptus* Spp. have been used in folk medicine for the treatment of ailments such as colds, fever, toothache, diarrhea and snake bites. Uses of *Eucalyptus* leaf hot decoctions as "herbal tea" have been recorded in Aboriginal, European and British Pharmacopeias for the traditional remedy of type DM[10-21]. A rich literature exists, reporting over 500 *Eucalyptus* species with different pharmacological actions[11-22]. Hypoglycemic potentials of *Eucalyptuses* are documented, but the mechanistic actions need to be explored further[11-21].

Table 1 List of phytochemicals of *Eucalyptus* Spp. inhibiting the enzymes

Phytochemicals	Enzymes inhibited ↓
Flavonoids, like quercetin, kaempferol, myricetin; Phenolics-tannins, ellagic acid, and gallic acid; terpinoids-ursolic acid, oleanolic acid, p-cymene, and 1,8-cineole, 1-(S)-α-pinene	α-amylase
Polyphenols, proanthocyanidins, anthocyanins	α-glucosidase
Flavonoids, flavonols, terpenoids, mono-terpenes	Aldose reductase
Flavonoids, flavonols, terpenoids, mono-terpenes	Angiotensin converting enzyme
Terpenoids	Peroxisome proliferator activated receptor
Triterpenoids, phenolic compounds	Di-peptidyl peptidase 4

Inhibiting the actions of carbohydrate hydrolyzing enzymes like α-amylase and α-glucosidase helps to reduce post-prandial (PP) hyperglycemia. Inhibition of other enzymes like AR, DPP-4, ACE and PPAR-γ also presents an effective strategy to combat type 2 DM naturally[5,6,8,11]. Extensive literature surveys and our previous works have reported that *Eucalyptus* shows the presence of terpenoids, triterpenoids, flavonoids, polyphenols and tannins in its various volatile and non-volatile fractions[8,21,22]. Major phytomolecules isolated from the Eucalyptus *Spp* and their inhibitory activity against the enzymes are depicted in Table 1.

AR, a member of the aldo-keto reductase super family, is the first and rate-limiting enzyme in the polyol pathway and reduces glucose to sorbitol, utilizing reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. In type 2DM, due to increased availability of glucose in sensitive tissues like lens, nerves and retina, there is an increased formation of sorbitol through the polyol pathway. Intracellular accumulation of sorbitol leads to cataract, retinopathy and neuropathy. AR-inhibitors prevent the conversion of glucose to sorbitol and are capable of controlling diabetic complications[8,23-32]. Limited literature data and molecular docking analysis have shown that natural biomolecules with potent aldose reductase inhibitory actions include flavonoids like quercetin, quercitrin, myricitrin, coumarins, monoterpenes, stilbenes, *etc*. Flavonoids with binding energy (BE) ranging between -9.33 to -7.23 kcal/mol exhibited AR inhibitory properties, as evidenced by *in-silico* docking studies[8,32,33]. Five bioactive compounds, namely macrocarpals A-E detected in the ethanol extracts of the leaves of *E. globulus,* showed antibacterial actions, HIV-RTase (HIV-reverse transcriptase) inhibitory activity and also inhibited AR[8,32,33].

Attenuation in ROS level may be due to increased

production or diminished depletion of enzymes, like catalase, glutathione peroxidase and superoxide dismutase. Natural antioxidants which scavenge free radicals can provide a synergistic action to the overall antidiabetic potential of a plant[12,13]. Osawa and Namiki (1981, 1985) reported the presence of a powerful antioxidant, 4-hydroxytritriacontane-16,18 dione, in the leaf wax of different *Eucalyptus* species[24,25].

The enzyme ACE is associated with hypertension, a long term complication of diabetes. ACE activates histidyl leucine dipeptide called angiotensin-I into a potent vasoconstrictor called angiotensin-Ⅱ. Angiotensin-II influences aldosterone release which increases blood pressure by promoting sodium retention in distal tubules. Biomolecules like flavonoids, flavonols, anthocyanins and triterpenes are potent ACE inhibitors[8,34,35]. Molecular docking studies also recommend the use of herbal ACE inhibitors in the management of type 2 DM[8,34,35].

PPAR-γ is a key receptor in lipid and glucose homeostasis because of its ability to reduce the plasma free fatty acids and phytomolecules can exert their insulin sensitizing actions with their high affinity for the receptor PPAR-γ. Terpenoids act as PPAR modulators regulating carbohydrate and lipid metabolism. Several terpenoids have been isolated from the *Eucalyptus* species and PPAR antagonism is amongst the suggested modes of hypoglycemic action of *Eucalyptus*[8,36].

Glucagon-like peptide-1 (GLP-1) is a remarkable antidiabetic gut hormone with combinatorial actions of stimulating insulin secretion, inhibiting glucagon secretion, increasing beta cell mass, reducing the rate of gastric emptying and inducing satiety. DPP4 rapidly deactivates GLP-1. Phytomolecules, mostly triterpenoids, steroids and phenolic constituents with DPP4 inhibitory activity, help to increase the levels of endogenous active GLP-1 and act as an important therapeutic compound against type 2 DM, the fact being further supported by molecular docking studies[8,37].

The present report documents our studies aiming to explore the major phytochemicals amongst three *Eucalyptus* species, *E. globulus* (EG, blue gum or Tasmanian blue gum), *E. citriodora* (EC, lemon scented gum) and *E. camaldulensis* (ECA, river red gum or Murray red gum), along with the existence of NEIs of enzymes like α-amylase, α-glucosidase, AR, DPP4, ACE and antioxidant enzymes by *in vitro* assays, with the perspective to evaluate the potentiality of these three species to combat type 2 DM and its complications. Furthermore, such research will help in bioactivity guided isolation of potent NEIs.

MATERIALS AND METHODS

Plant materials

Fresh leaves of EG, EC and ECA were collected from natural and man-made forest areas of IIT Kharaghpur and adjoining areas, like Balarampur, Gopali and Arabari forest areas, and authenticated by Dr Shanta AK, Biotechnologist, Nirmala College of Pharmacy, Guntur, India.

Reagents and chemicals

Yeast α-glucosidase, bovine serum albumin, sodium azide, para-nitrophenyl α-D-glucopyranoside solution (pNPG), ACE (from rabbit lung, 3.5 units/mg of protein), starch azure, porcine pancreatic amylase, Tris-HCl buffer, hippuryl-L-histidyl-L-leucine (HHL) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals, United States. Other chemicals like diagnostic reagents, surfactants, polyphosphate, dextran sulphate, *etc.*, were purchased from Merck Co, India. Acarbose was a kind gift sample from Zota Pharmaceuticals Pvt. Ltd, Chennai, India.

Preparation of eucalyptus leaf extracts

A uniform methodology was followed for preparing the leaf extracts of the three different species of *Eucalyptus*. Typically, the leaves were washed first with tap water and then with distilled water to remove all dust, subjected to shade drying at 25 ± 3 ℃ temperature, and then finely powdered in an electrical grinder (Bajaj GX 11, India). The leaf powder was stored at room temperature in an airtight container until use and labeled separately as EG, EC and ECA. Extraction was carried out as described by Sugimoto *et al*[20,21] with few modifications. Briefly, 500 g of leaf powder of each species was extracted separately with ethanol-water (1:2 v/v) under reflux for 2 h, filtered through a Whatman filter paper no. 1, concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) and dried in a vacuum oven. The percentage yield of extracts was calculated with regard to the initial weight of dry powders and final weight of the extracts. These extracts were then subjected to preliminary and quantitative phytochemical evaluations and *in vitro* assay procedures.

Phytochemical investigations of the eucalyptus leaf extract

Phytochemical analysis of the major bioactive compounds of interest of the three different extracts (EG, EC and ECA) was performed using the methods of Harbone (1984), Trease and Evans (1989) and other literature methods[22,38]. The three extracts were analyzed for glycosides (Keller Killiani and Borntrager's tests), alkaloids (Mayer's, Dragendorff's reagents), saponins (Foam test), triterpenes (Salkowski and Libermann Burchard tests) and 1,8-cineole (Marquis reagent, Gallic acid reagent, conc. H_2SO_4 and phloroglucinol).

The total polyphenol content of the extracts was determined by ultra violet (UV) spectrophotometry (Perkin Elmer Lambda 25 UV-vis) at 760 nm using Folin-Ciocalteu reagent by the method of Othman *et al*[39] and Modnicki *et al*[40] (2009)[41,42]. The concentrations of the total polyphenols were determined in Gallic equivalents (GAE) per gram of the extract. The polyphenol content was calculated by the formula: $X = (5.6450 \times A)/m$. Where X is total phenolic compounds (%), A is absorbance of investigated extract and m is mass (g) of the investigated sample.

The total flavonoid content of the extracts was determined by the method of Djeridane *et al*[43], 2006, which

is based on the formation of a complex of flavonoid-aluminium, and the concentration of the flavonoids was expressed in terms of QE per gram extract.

The flavonol content of the extracts was determined according to Abdel-Hameed, 2009, which is based on the formation of a complex between the extract with $AlCl_3$ and sodium acetate and the total flavonol content was expressed in terms of quercetin equivalent (QE) per gram extract[42].

Tannins were measured according to the protocol of Hagerman and Butler, 1978, which is based on the obtention of a colored complex Fe^{2+}-phenol whose absorbance was measured spectrophotometrically at 510 nm. The tannin content was obtained in mg of tannic acid equivalent (TAE) per gram extract[44].

The three extracts were subjected to color reactions with Marquis Reagent, gallic acid reagent, concentrated H_2SO_4 and phloroglucinol reagent. Standard 1,8-cineole gives orange color with Marquis reagent, yellow color with gallic acid, dark yellow color with concentrated H_2SO_4, and no coloration with phloroglucinol reagent[22,38].

Gas chromatographic analysis of 1,8-cineole

Fresh leaves of the three *Eucalyptus* spp. (EG, EC and ECA) were air dried and 100 g leaves of each variety were subjected to hydrodistillation for 3-4 h to extract the essential oil, employing a Clevenger type apparatus[45]. Extracted oils were decanted from the water layer and dried over anhydrous sodium sulfate. The extracted oils of the three species were subjected to gas chromatographic (GC) analysis (perkin elmer clarus 500, with Flame Ionization Detector) as described by Quereshi *et al*[45]. The operating conditions were: nitrogen as carrier gas, injector and detector temperature of -250 ℃; column of 30 m (length) × 0.25 mm (inner diameter) and film thickness of 0.25 μm. The temperature was gradually increased at a rate of 15 ℃/min to 240 ℃ for 4 min.

In vitro assay procedures

After phytochemical investigations of the leaf extracts of EG, EC and ECA, *in vitro*-inhibitory assays of α-amylase, α-glucosidase, aldose reductase, ACE, DPP4, antioxidant assays like DPPH free radical scavenging activity, scavenging of hydrogen peroxide and total antioxidant activity in the ferric reducing antioxidant power (FRAP) assay were carried out following standard methods[46-57].

The enzymes mentioned above contribute to the pathogenesis of type 2 DM in one way or another. Inhibition of such enzymes helps to combat type 2 DM naturally. There are ample research works highlighting the hypoglycemic potentials of *Eucalyptus*. We explored such NEIs by the above mentioned *in vitro* assays and once again evaluated the hypoglycemic potentiality of *Eucalyptus*.

α-Amylase inhibitory assay: The study was carried out following standard literature methodologies with slight modifications[12,46,47]. Briefly, 2 mg of starch azure was suspended in a tube containing 0.2 mL of 0.5 mol tris-phosphate buffer (pH = 6.9) containing 0.01 mol calcium chloride as the substrate. After boiling the tube for 5 min, it was preincubated for 5 min at 37 ℃. Different concentrations (10, 20, 40, 60, 80 and 100 μg/mL) of the extracts of EC, EG and ECA were prepared by dissolving in 1 mL of 0.1% dimethyl sulfoxide. Then 0.2 mL of the extract of particular concentrations was put in the tube containing the substrate solution. Next, 0.1 mL of porcine pancreatic amylase in tris-HCL buffer (2 units/mL) was added to the tube containing extracts and substrate, at 37 ℃. After 10 min, the reaction was stopped by adding 0.5 mL of 50% acetic acid in each tube and the reaction mixture was centrifuged (Eppendorf-5804R) at 3000 *g* for 5 min at 4 ℃. The absorbance of the resulting supernatant was measured at 595 nm. Acarbose (A_{car}) in the concentration range of 1.25, 2.5, 5 and 10 μg/mL in distilled water was used to create the calibration curve. The assay was performed in triplicate. The concentration of the *Eucalyptus* extracts of three species (EG, EC and ECA) required to inhibit 50% of α amylase activity under the assay conditions is referred to as IC_{50} values. Absorbance was calculated using the formula: a amylase activity = [(Ac+) - (Ac-) - (As-Ab)]/[(Ac+) - (Ac-)] × 100.

α-glucosidase inhibitory assay: The assay procedure was developed as described by Basak *et al*[12] and Subramanian *et al*[47], with slight modifications. An aqueous ethanol extract of the three species (EG, EC and ECA) was used for the study. The yeast α-glucosidase enzyme solution was prepared by dissolving at a concentration of 0.1 U/mL in 100 mmol phosphate buffer, pH = 7.0, containing bovine serum albumin and sodium azide which was used as enzyme source. This enzyme solution was added to the aqueous-ethanolic extracts of EG, EC and ECA in increasing concentrations (1, 1.5, 2, 2.5, 3, 3.5 μL/mL). The reaction was initiated by adding 0.20 mL of para-nitrophenyl α-D-glucopyranoside solution (pNPG); 2 mmol pNPG in 50 mmol sodium phosphate buffer pH = 6.9) which acted as the substrate. The reaction was terminated by adding 1 mL 0.1 mol/L Na_2HPO_4. The test tubes were cooled under tap water and α-glucosidase inhibitory activity was determined at 405 nm by measuring the quantity of *P*-nitrophenol released from pNPG. The assay was performed in triplicate for each extract and the data presented as mean ± SD. The concentration of the *Eucalyptus* extracts (EG, EC and ECA) required inhibiting 50% of α-glucosidase activity under experimental conditions is defined as the IC_{50} value. Acarbose (A_{car}) was dissolved in distilled water to prepare a series of dilutions (1.25, 2.5, 5, 10 mg/mL) and was used as the positive control. The percentage inhibition was calculated according to the formula: %inhibition = (Abs400 control - Abs400 extarct)/Abs400 control.

IC_{50} values were determined from the plots of percentage inhibition *vs* log inhibitor concentration and were calculated by nonlinear regression analysis from the mean inhibitory values.

Aldose reductase inhibitory assay: The assay was carried out following reported literature methods and the experimental protocol approved by the Institutional Ethical Committee[48-50]. Two to three mo old healthy adult Wistar albino rats weighing about 150-200 g were acclimatized to laboratory conditions (12 h light and 12 h dark cycle, 25 ± 5 ℃, 30%-60% relative humidity) with free access to pelleted food and water *ad libitum*. Immediately after sacrifice, lenses were removed from the eyes, washed with saline and the fresh weights of a lens were measured. Next, a 10% homogenate was prepared from the rat lens in 0.1 mol/L phosphate-buffered saline (PBS) at pH 7.4, centrifuged at 5000 *g* for 10 min in the cold and the supernatant collected. The protein content of the supernatant was determined by literature methods[48-50].

For the determination of the aldose reductase (AR) inhibitory activity, 0.7 mL of phosphate buffer (0.067 mol), 0.1 mL of NADPH (25×10^{-5} mol), 0.1 mL of DL-glyceraldehyde (substrate, 5×10^{-4} mol) and 0.1 mL of lens supernatant were mixed in the sample cuvette. Absorbance was taken against a reference cuvette containing all other components except the substrate, DL-glyceraldehyde. The final pH of the reaction mixture was adjusted to pH = 6.2. On adding substrate to the solution mixture, the enzymatic reaction starts and absorbance (OD) was recorded at 340 nm for 3 min at 30 s intervals. AR activity was calculated and expressed as OD/min per milligram protein.

For the determination of the AR inhibitory activity of the *Eucalyptus* extracts, a stock solution was prepared by dissolving the *Eucalyptus* extracts (EG, EC and ECA) in PBS and different concentrations prepared from stock solutions were added to both the reference and standard cuvettes. The reaction was initiated by the addition of 0.1 mL DL-glyceraldehyde and the reaction rate measured as mentioned above. Percentage inhibitions of AR activity of the extracts were calculated with reference to normal rat lens to have 100% activity. The concentrations of the extracts required to inhibit 50% of AR activity under assay conditions is defined as the IC_{50} values which were calculated for each sample by plotting a graph between log dose concentrations *vs* percentage inhibition. Quercetin, a known AR inhibitor, was used as the positive control.

ACE inhibitory assay: The assay method was based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalyzed by the ACE. The assay procedure was carried as described [51,52] and other methods with slight modifications. Briefly, 50 μL of sample solutions (extracts of EC, EG and ECA) in the concentration range of 0.1-2.5 mg/mL were preincubated with 50 μL of ACE (25 mU/mL) at 37 ℃ for 10 min. Next, 150 μL of substrate solution (8.3 mmol HHL in 50mmol sodium borate buffer containing 0.5 mol NaCl at pH 8.3) was added and incubated for 30 min at 37 ℃. The reaction was terminated by addition of 250 μL 1.0 mol HCl. To the resulting solution, 0.5 mL of ethyl acetate was added and centrifuged (Eppendorf-5804R) for 15 min. Then, 0.2 mL of the upper layer was transferred to a test tube, evaporated under room temperature in vacuum and the liberated hippuric acid was dissolved in 1 mL distilled water and the absorbance was measured at 228 nm. Experiments were performed in triplicates. Captopril was used as standard (3.5 μg/mL) in the assay. The percentage of inhibition (ACEI) was calculated using the formula: %inhibition = (A-B)/(A - C) × 100. Where A is the OD at 228 nm with ACE but without inhibitor, B is the OD in presence of both ACE and inhibitor, C is the OD without ACE and inhibitor.

DPP4 inhibitory assay: The assay was carried out following reported literature methods using GPN-Tos (Gly-Pro p-nitroanilide toluenesulfonate salt) as the substrate[53-55]. Briefly, 0.5 mL of the assay mixture contained 40 mmol K-Na-phosphate buffer, pH 7.5, an enzyme sample. The reaction was initiated by adding a substrate to a concentration of 0.24 mmol and stopped by adding 0.2 mol acetic buffer at pH 5.5. The differential absorption at 390 nm was recorded against an identical mixture without the enzyme and the amount of p-nitroaniline depleted was evaluated from its extinction coefficient at the wavelength of 9.9 mmol/L/cm^{-1}.

Evaluation of antioxidant activity

Dpph free radical scavenging activity: The antioxidant activity of the *Eucalyptus* extracts (EC, EG and ECA) was determined on the basis of the scavenging effect on the stable DPPH free radical activity[12,39,51,56]. A stock solution of DPPH in methanol (33 mg in 1 L) was freshly prepared and kept in the dark at 4 ℃; after checking its initial absorbance, 5 mL of this stock solution was added to 1 mL of the solution of the extracts prepared in concentrations of 50-500 μg/mL. Next, 2.8 mL of 95% methanol was added and the mixture was shaken vigorously and after 30 min the absorption was measured at 517 nm. Ascorbic acid was used as the standard. The radical scavenging capacities of the test samples were expressed as percentage inhibition and calculated according to the equation: % inhibition of DPPH activity = (Absorbance control - Absorbance)/(Absorbance control) × 100.

Plotting was done of percentage inhibition *vs* concentration, and the concentration of sample required for 50% inhibition is regarded as IC_{50} value for each of the test samples.

Total antioxidant activity (FRAP assay): Total antioxidant activity was determined by the FRAP assay as described by Pracheta *et al*[56] and Shahwar *et al*[57]. It is a direct test of antioxidant capacity. The assay of reducing activity is based on the reduction of ferric to ferrous form in the presence of antioxidants in the tested samples (extracts of *Eucalyptus* species). The stock solutions included 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol HCl and 20 mmol $FeCl_3$, and 300 mmol acetate buffer (pH 3.6). The working solutions were freshly prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL of $FeCl_3$. The temperature of the solution

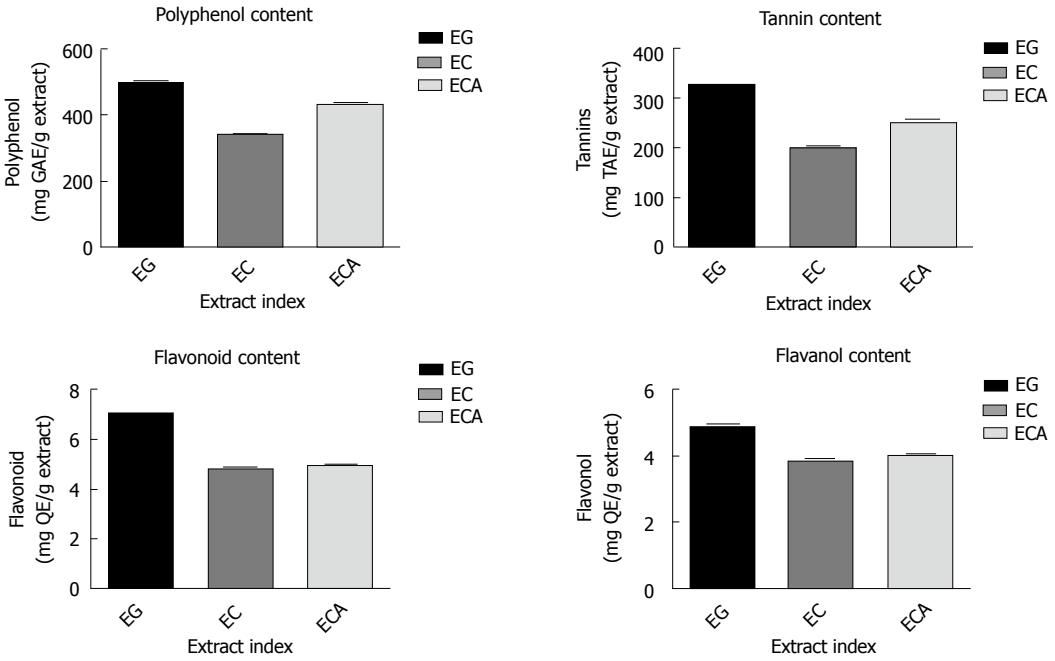

Figure 1 Graphical presentations of the presence of phytochemicals in Eucalyptus extracts. Data are presented as the mean ± SD of each triplicate test. EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis*.

Table 2 Total polyphenol, flavonoid, flavonol and tannin contents of *E. globulus*, *E. citriodora* and *E. camaldulensis*

Extract[1]	Polyphenol (mg/g extract[2])	Tannins (mg/g extract[2])	Flavonoid (mg/g extract[2])	Flavonol (mg/g extract[2])
EG	496.85 ± 3.98	329.06 ± 6.25	7.15 ± 0.02	4.98 ± 0.01
EC	341.75 ± 3.63	199.75 ± 5.49	4.89 ± 0.01	3.87 ± 0.05
ECA	429.91 ± 4.03	253.15 ± 4.96	5.01 ± 0.02	4.09 ± 0.01

[1]Content expressed per gram of relevant extracts (EG, EC and ECA); [2]Values are expressed as mean ± SD from triplicate determination. EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis*.

Table 3 Color test results for the presence of 1,8-cineole in *E. globulus*, *E. citriodora* and *E. camaldulensis* extracts

Extracts	Marquis test	Gallic acid test	Concentrated H_2SO_4	Phloroglucinol
EG	Orange	Yellow	Dark yellow	No color
EC	Orange	Dark Yellow	Dark yellow	No color
ECA	Orange	Yellow	Bright orange-yellow	Pink

EG: *E. globulus*; EC: *E. citriodora*; ECA: *E camaldulensis*.

was raised to 37 ℃ prior to use. *Eucalyptus* extracts (200 μL) were allowed to react with FRAP solution (2900-3000 μL) for 30 min in the dark. Absorbance of the colored product formed (ferrous tripyridyl triazine complex) was recorded at 595 nm. Results were expressed in μM equivalent to $FeSO_4$ by extrapolation from the calibration curve.

Statistical analysis

The experimental results were expressed as mean ± SD of three replicates. The data were subjected to one way analysis of variance (ANOVA) using commercially available software (Prism version 5.0; Graph Pad Software, San Diego, CA, United States). Results were analyzed by Student's *t* test (paired or unpaired, as appropriate) or Tukey's multiple comparison test. Statistical analysis was performed by using GraphPad Prism where $P < 0.05$ was considered statistically significant.

RESULTS

The yield of the *Eucalyptus* leaf extracts (extractions carried out in triplicates) were 49% ± 3.3% for EG, 46.5% ± 4.2% for EC, and 45.8 ± 3.9% for ECA. The details of phytochemicals amongst *Eucalyptus* Spp. and the enzymes inhibited by them are presented in Tables 1 and 2 and Figure 1. The color test results for the presence of 1,8-cineole in the extracts of EG, EC and ECA are presented in Table 3. GC analysis of the oils extracted from three species (EG, EC and ECA) showed the highest 1,8-cineole content in EG (about 50%). ECA also showed the presence of 1,8-cineole in addition to several other peaks indicating the presence of other compounds. In EC citronellal was found to be the major component.

All three extracts (EG, EC and ECA) showed promising inhibitory potentials for enzymes, including α-amylase, α-glucosidase, AR, ACE and DPP4. The antioxidative potential of the extracts were determined by DPPH radical scavenging and the total antioxidative capacity by the FRAP assay. The results of all such inhibitory assays are presented in Figure 2 and the summary of the IC_{50} values of tested samples in Table 4.

The correlation coefficient (R^2) between polyphenol and flavonoid content and IC_{50} inhibitory values of the enzymes ranged between 0.81-0.99 and 0.57-0.99 respec-

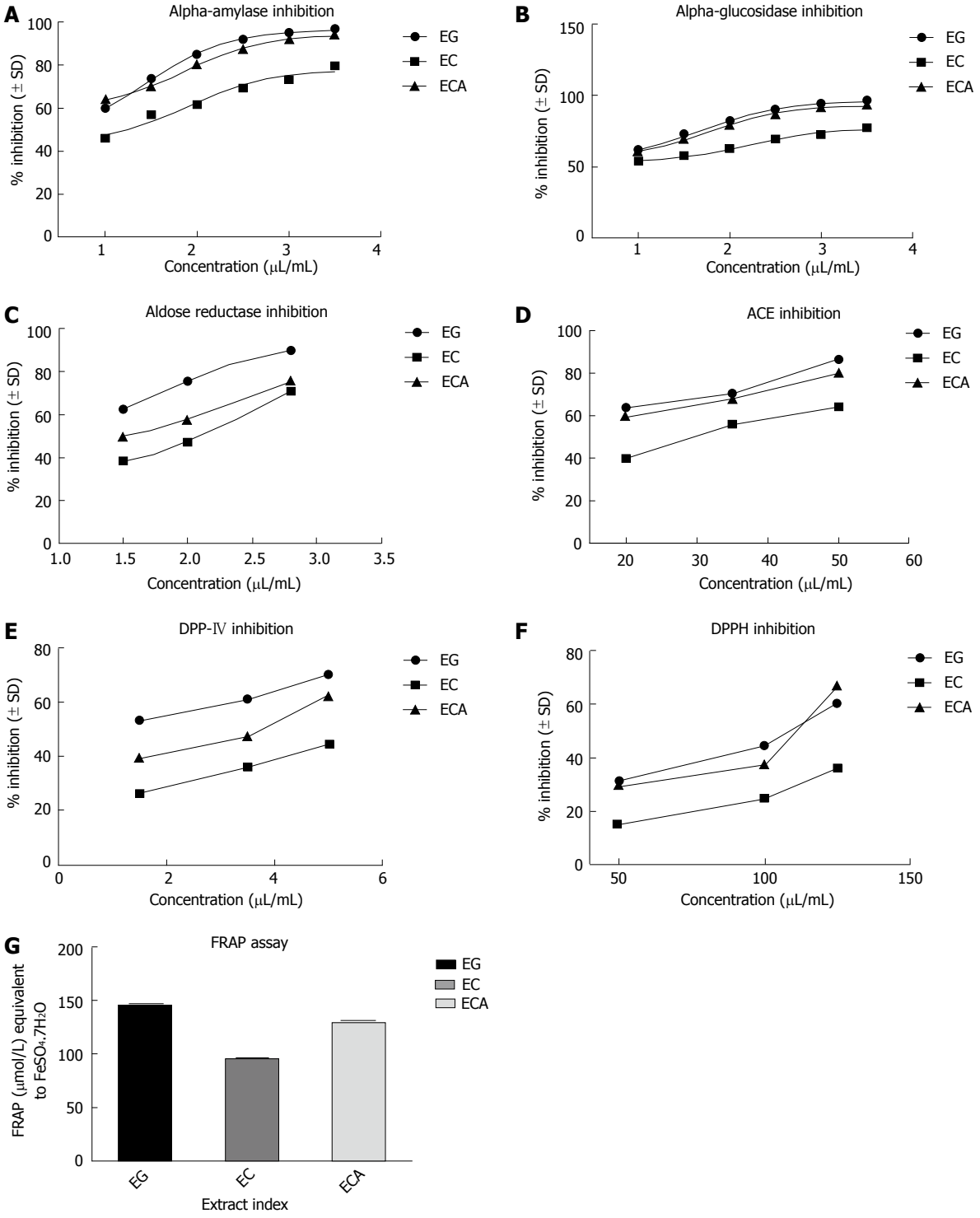

Figure 2 Eucalyptus extracts. A: Alpha-amylase; B: Alpha-glucosidase; C: Aldose-reductase; D: Angiotensin converting enzyme; E: Dipeptidyl peptidase 4; F: 1,1-Diphenyl-2-picrylhydrazyl; G: FRAP assay. Data are presented as the mean ± SD of each triplicate test. EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis;* FRAP: Ferric reducing antioxidant power.

tively.

The polyphenol content of three *Eucalyptus* Spp. (EG, EC and ECA) was compared with the IC_{50} values of different inhibitory assays using Tukey's multiple comparison test (one-way ANOVA), considering $P < 0.05$ as significant. All P values were found to be < 0.05. The results suggested that the inhibitory potentials of the extracts are largely dependent upon the polyphenol content in *Eucalyptus* Spp.

DISCUSSION

Qualitative and quantitative phytochemical investigations of the *Eucalyptus* leaf extracts EG, ECA and EC showed appreciable levels of bioactive components like polyphenols and flavonoids. From the IC_{50} values of *Eucalyptus*

Table 4 IC_{50} inhibitory values of Eucalyptus extracts *E. globulus*, *E. citriodora* and *E. camaldulensis* in different assays

Assays	EG	EC	ECA
α-amylase	3.01 ± 0.01	4.13 ± 0.09	3.65 ± 1.04
α-glucosidase	2.08 ± 0.01	2.68 ± 0.11	2.11 ± 0.19
Aldose reductase	2.06 ± 0.03	6.72 ± 0.65	2.56 ± 0.84
Angiotensin converting enzyme	4.31 ± 0.09	30.83 ± 0.45	6.85 ± 0.98
Dipeptidyl peptidase	3.098 ± 0.09	6.138 ± 0.68	3.99 ± 0.91
1,1-diphenyl-2-picrylhydrazyl (DPPH)	12.32 ± 0.91	68.42 ± 0.05	14.44 ± 1.91

EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis*.

extracts in different assays (Table 4), it appears that all three extracts showed significant inhibitory potentials against the six enzymes assayed, in the order EG > ECA > EC. Based on the results of FRAP assay, the reducing ability of EG was highest and that of EC lowest (Figure 2). 1,8-cineole is the major constituent of the volatile fractions in EG and ECA, whereas in EC the major constituent is citronellal with citronellol and spathulenol. According to the literature, compounds with highest reducing ability have delocalized chemical bonds[56-60]. Prior research suggested a strong positive correlation (R^2 = 0.99) between phenolic content and antioxidative potential[12,18,58,59]. Polyphenols received wide attention because of their antioxidant properties which refers to their ability to prevent damage from ROS through radical scavenging or prevent the generation of these species by iron chelation[61]. Polyphenols also bind and inhibit the enzymes α-amylase and α-glucosidase[61]. Polyphenols have also been shown to facilitate insulin response and attenuate secretion of glucose dependent insulinotropic polypeptide and glucagon like GLP-1. Other suggested mechanisms for the hypoglycemic actions of polyphenols were down regulation of the expression of liver glucokinase, upregulation of phosphoenolpyruvate carboxykinase (PEPCK), induction of the AMP-activated protein kinase (AMPK) pathway, enhancing peripheral glucose utilization by stimulating glucose transporter subtype 4 (GLUT-4), *etc.*[62]. In this context, it is to be mentioned that green tea extract (GTE) contains polyphenols like catechin, epicatechin, *etc.* Epigallocatechin gallate (EGCG), an abundant form of catechin, is the major attributable factor for the beneficial effects of green tea. EGCG inhibits adipocyte proliferation, increases fat oxidation and enhances the expression of GLUT-4, as shown in animal studies[63,64].

Literature surveys have shown that flavonoids and its subfamilies significantly inhibit the ACE enzyme by generating chelate complexes within the active center of ACE[65]. Flavonoids were found to attenuate hepatic gluconeogenesis by decreasing the activity of glucose-6-phosphate and PEPCK, subsequently improving glycemic control[65]. Our research data are in accordance with this phenomenon. A strong correlation was found between polyphenol (R^2 = 0.81-0.99) and flavonoid contents (R^2 = 0.57-0.99) with the antioxidative and enzyme inhibitory potentials of the extracts.

NEIs can serve as an important therapeutic tool against type 2 DM. The current research aims to provide the state-of-the-art search of NEIs amongst *Eucalyptus* Spp. by *in vitro* assays which can be further utilized for bioactivity-guided isolations of such enzyme inhibitors. Our research results show the hypoglycemic potential of the *Eucalyptus* Spp. (extracts) for future exploitations in phytotherapy of type 2 DM. However, further extensive pharmacology and toxicological studies in animal and human models are warranted.

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COMMENTS

Background

The current research aims to explore the presence of biomolecules by *in vitro* assays amongst three eucalyptus species acting as natural enzyme inhibitors for enzymes with significant pathogenic roles in type 2 diabetes.

Research frontiers

Enzymes like α-amylase, α-glucosidase, aldose reductase, angiotensin converting enzyme and dipeptidyl peptidase 4 play important pathogenic roles in type 2 diabetes. Phytomolecules acting as inhibitors of such enzymes can act as effective therapeutic targets in type 2 diabetes. Volatile and non-volatile fractions of *Eucalyptus* Spp. include biomolecules like terpenes, triterpenoids, flavonoids, polyphenols, *etc.* The exploration of enzyme inhibitors amongst *Eucalyptus* Spp. by *in vitro* assays will help in bioactivity guided isolation of such inhibitors to be targeted as natural hypoglycemics.

Innovations and breakthroughs

Enzymes play a vital role in mediating essential biochemical life processes. However, hyper or hypo activity of such enzymes leads to malfunctions of the respective biochemical processes, which in many cases are the underlying causes of diseases like diabetes. The current research aims to provide the state-of-the-art search of natural enzyme inhibitors amongst *Eucalyptus* Spp. by *in vitro* assays which can be further utilized for bioactivity-guided isolations of such enzyme inhibitors. Those research findings have shown that the *Eucalyptus* Spp. under study have immense hypoglycemic potentials with high IC_{50} values against the targeted enzymes. Moreover, the inhibitory potentials of the species are also well correlated with the polyphenol-flavonoid contents of the species.

Applications

The *Eucalyptus* Spp. (extracts) under study showed significant hypoglycemic potentialities for future exploitations in phytotherapy of type 2 DM.

Terminology

Natural Enzyme Inhibitors: Malfunctions of certain enzymes are the root causes of many diseases. Effective enzyme inhibitors have great clinical significance and a substantial role in the drug delivery process. Such enzyme inhibitors of natural origin are more acceptable due to safety and lower incidences of side effects on short and long term treatment modalities.

Peer review

Dey *et al* investigated the potential hypoglycemic actions of Eucalyptus extracts *in vitro*. The extracts were found to significantly inhibit a number of enzymes related to T2DM, such as amylase, glucosidase, dipeptidyl peptidase 4, *etc.* The rationale of this study and methodology were adequately described. The selection of enzymes and antioxidant activity is based on the hypothesis that these activities are involved in the pathogenesis of type 2 diabetes. The three extracts show broad enzyme inhibitory activity and antioxidant activity, which differs in

magnitude between the three extracts. The authors conclude that the extracts might serve as starting material for new therapeutic modalities for type 2 diabetes and that their data fit with the idea that leaves from trees could provide a base material for drug discovery and development programs.

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CLINICAL TRIALS STUDY

Toll-like receptor expression and signaling in human diabetic wounds

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Abstract

AIM: To examine the contribution of toll-like receptors (TLRs) expression and activation to the prolonged inflammation often seen in human diabetic wounds.

METHODS: Debridement wound tissue was collected from diabetic patients with informed consent. Total RNA and protein were isolated and subjected to real-time polymerase chain reaction and Western blot analyses.

RESULTS: TLR1, 2, 4, and 6 mRNA expressions were increased significantly in wounds of diabetic patients compared with non-diabetic wounds ($P < 0.05$). MyD88 protein expression was significantly increased in diabetic wounds compared to non-diabetic wounds. Interleukin-1beta, tumor necrosis factor-alpha concentration nuclear factor-kappa B activation, and thiobarbituric acid reactive substances were increased in diabetic wounds compared to non-diabetic wounds ($P < 0.01$).

CONCLUSION: Collectively, our novel findings show that increased TLR expression, signaling, and activation may contribute to the hyper inflammation in the human diabetic wounds.

Key words: Interleukin-1β; Inflammation; Toll-like receptors 2; Toll-like receptors 4; Tumor necrosis factor-α; Type 2-diabetes mellitus; Wound healing

Core tip: Increased TLR2/4-MyD88-nuclear factor-kappa B expression and signaling with attendant oxidative stress may contribute to the hyperinflammation frequently seen in human diabetic wounds.

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INTRODUCTION

Diabetes mellitus (DM) is a constellation of metabolic aberrations that collectively manifest as debilitating pathological complications affecting the quality of life in DM patients. Around 348 million people worldwide and 36 million people in United States have DM and 40%-60% of these patients develop foot wounds accounting to more than 20% of all hospitalizations equating to one amputation every 30 s[1-3]. Emerging experimental data and human studies suggest that systemic inflammation orchestrated by innate immune receptors plays a role in the pathogenesis of DM complications[4]. Toll-like receptors (TLRs) are pivotal innate immune receptors that induce inflammatory responses[5] and their expression and activation is increased in a plethora of inflammatory disorders including DM and its complications[6-9]. Recent data from our group and others have provided evidence that TLR expression, activation, and signaling are significantly increased in monocytes of DM patients, non-obese diabetic (NOD) mice, and db/db mice (see review, 4). In addition, we showed that genetic ablation of TLR2/4 in diabetic mice attenuates inflammation as indicated by decreased circulating cytokine/chemokine

levels and improved wound healing[8-10]. However, it is not known if TLR expression and activation contributes to the uncontrolled inflammation seen in wounds of DM patients. Thus, in the present study, we examined TLR expression, signaling, and inflammation in human DM wounds.

MATERIALS AND METHODS

Patients

The study population consisted of type 2 DM patients presenting for care of a diabetic ulcer located anywhere on the foot and non-DM patients (controls) with a leg ulcer, aged between 45-65 years. We collected wound tissues from diabetic (n = 8) and non-diabetic subjects (n = 4) during initial debridement as part of standard of care, with informed patient consent at the Sacramento VA clinics. Patient evaluations consisted of a medical history, physical examination, and wound site measurements (including location, size, presence of periulcerative tissue, and clinical infection) were recorded. Serum glucose and HbA1c levels were extracted from patient charts that were done within the last 60 d. All the human study protocols were approved by the Institutional Review Board at University of California at Davis and VA of Northern California, MatherField CA.

Collection of debridement wound tissue

Study inclusion criteria were as follows: age 18 or older; ulcer size > 2 cm^2 and < 25 cm^2; ulcer duration of ⩾ 4 wk; no clinical signs of infection; glycosylated haemoglobin (HbA1c) < 12%; and adequate circulation to the affected extremity Patients were excluded if any of the following preexisting conditions: presence of charcot foot, index ulcer probing to bone; currently receiving radiation or chemotherapy; known or suspected malignancy of current ulcer; diagnosis of autoimmune connective tissue disease; received a biomedical or topical growth factor for their wound within the previous 30 d; taking medications considered to be immune system modulators, antibiotics, with C-reactive protein levels (> 10 mg/dL), and CBC (white blood cells < 4 to > 11 K/mm^3) indicative of infection. Debridement tissue was collected using sharp debridement technique[11] and immediately snap frozen in liquid nitrogen for mRNA and protein analyses.

Real time-polymerase chain reaction

Total RNA was isolated from all the snap frozen wound tissues and mRNA expression was determined by REal time-polymerase chain reaction (RT-PCR) using commercial sequence-specific primers and probes purchased from SA Biosciences, Gaithersburg, MD, United States). The first strand of cDNA was synthesized using total RNA (1 μg per reaction). cDNA (50 ng) was amplified using primer probe sets for TLR1, TLR2, TLR4, TLR6, Myeloid differentiation factor-88 (MyD88), Interleukin receptor activated kinase-1 (IRAK-1), myeloid differentiation protein-2 (MD2), nuclear factor-kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α) and 18s (SA Biosciences) following the manufacturer's cycling parameters. Data were calculated using the $2^{-\Delta\Delta Ct}$ method and are presented as ratio of transcripts for *TLR* gene normalized to 18s as described previously[8,9].

Western blot and ELISA

For Western blot assays, wound tissues were homogenized in tissue lysis buffer and total protein was determined using bicinchoninic acid protein quantitation method[8-10]. Equal amounts of protein (25 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and were probed with MyD88 (Imgenix, United States) and β-actin (Santa Cruz, United States) antibodies as reported earlier[8,9]. Densitometric ratios of the bands were calculated as reported earlier[8,9] and expressed as MyD88/β-actin ratio. Interleukin-1beta (IL-1β) and TNF-α levels were measured in the wound tissue lysates using ELISA (R and D systems) assay as reported earlier[8-10]. Intra- and interassay coefficient of variation (CV) of ELISA assays were determined to be < 10%[8-10]. Nuclear extracts were used to perform NF-κB transcription factors activation assays (Active Motif, Carlsbad, CA, United States) to verify activation of NF-κB in the diabetic wounds, indicative of increased inflammation. Assays were performed in accordance to the manufacturer's protocols. Intra- and inter-assay CV for transcription factor assays was < 8%[8-10].

Thiobarbituric acid reactive substances

We measured oxidative stress through lipid peroxidations [Thiobarbituric acid reactive substances (TBARs)] in wound tissues to reflect the pathogenic mechanisms in impaired wound healing in DM wounds compared with control wounds. TBARs are a surrogate marker of oxidative stress and malondialdehyde equivalents were determined by reading the absorbance at 532 nm using 1,1,3,3-tetramethoxypropane as an external standard[8,12]. Results were expressed as malondialdehyde equivalents (nmol/mg protein) as reported previously[8,12].

Statistical analyses

Data are presented as mean ± SD. We used two-tailed t tests with appropriate *post hoc* analyses. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism software[8-10].

RESULTS

All the patients had DM for > 5 years (mean glucose of 132 ± 10 mg/dL and HbA1c of 7.5% ± 0.8%) and are on routine standard care for a chronic diabetic foot ulcer of at least 4-wk duration and showed no signs of clinical infection. We first examined mRNA levels of TLRs and associated inflammatory signaling mediators in DM and control wound tissue to test the hypothesis that increased TLR expression and activation accentuate inflammation in diabetic wounds, using RT-PCR. TLR1, TLR2, TLR4, TLR6, MyD88, IRAK-1, NF-κB, IL-1β, and TNF-α mRNA expression were significantly increased compared

Table 1 Toll-like receptor pathway genes expressed in debridement wound tissue

Gene	Non-diabetic wounds mRNA/18s ratio	Diabetic wounds mRNA/18s ratio[a]
TLR1	0.6 ± 0.1	1.9 ± 0.4
TLR2	1.2 ± 0.3	3.6 ± 0.5
TLR4	1.3 ± 0.2	3.8 ± 0.2
TLR6	0.2 ± 0.1	2 ± 0.5
MyD88	1.4 ± 0.2	3.1 ± 0.4
IRAK-1	1.1 ± 0.1	2.8 ± 0.6
MD2	0.2 ± 0.04	1.6 ± 0.3
NF-κB	0.8 ± 0.05	2.3 ± 0.2
TNF-α	1 ± 0.4	2.6 ± 0.6

Human diabetic wounds (n = 8) show significantly higher mRNA/18s ratio compared to non-diabetic wounds (n = 4) ([a]P < 0.05 *vs* non-diabetic wounds). TLR: Toll-like receptor.

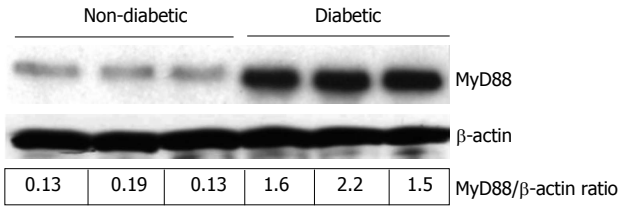

Figure 1 Representative Western blot showing the MyD88 protein expression in non-diabetic and diabetic wound tissues. Wound tissues were collected, lysed and 25 μg protein was blotted for MyD88 and β-actin. Densitometric ratios (MyD88/β-actin) are indicated below. Each lane presents protein from an individual patient wound debridement tissue (n = 3/group).

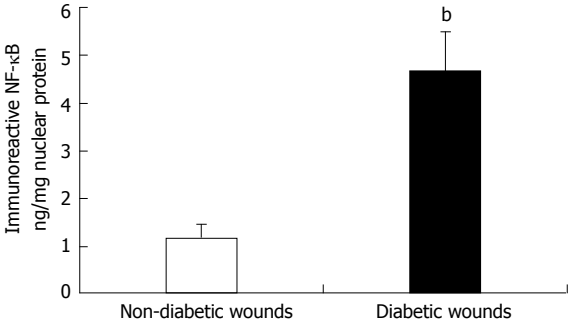

Figure 2 The DNA-binding activity of nuclear nuclear factor-kappa B p65 in wound tissues was determined using ELISA technique. Values are normalized to mg nuclear protein and expressed as mean ± SD. [b]P < 0.001 *vs* non-diabetic.

to non-diabetic wounds (P < 0.05) (Table 1) implicating a role for TLR-MyD88-NF-κB signaling on hyperinflammatory phenotype often seen in DM wounds[7-9]. The mRNA data was validated using Western blot and enzyme-linked immunosorbent assay (ELISA) assays[7-9]. MyD88 is an immediate and common downstream adaptor molecule recruited by activated TLRs through their TIR domain. MyD88, in turn, recruits IRAK-1, leading to the activation of NF-κB transcription factor, and attendant inflammatory cytokine gene expression[5]. Thus, we chose MyD88 for further validation. As shown in Figure 1, MyD88 protein expression was significantly higher in DM wounds compared to the non-diabetic wounds (P < 0.05 *vs* non-diabetic wounds). Figure 2 depicts significantly increased NF-κB activation in the nuclear extracts of diabetic wounds compared to non-diabetic wounds (P < 0.001). Next, local IL-1β and TNF-α levels known to be expressed as a result of TLR-MyD88-NF-κB activation, were determined using ELISA assay. Figure 3 shows significantly increased IL-1β and TNF-α levels in DM wounds compared to non-diabetic wounds (P < 0.05) supporting our hypothesis that TLR signaling and activation contribute to the prolonged inflammation seen in DM wounds[7-9]. Because oxidative stress and inflammation are linked by TLRs[13] as a surrogate index of oxidative stress, we measured TBARS formation during an acid-heating reaction in wound tissues as described earlier[8,12]. Figure 4 depicts significantly higher TBAR levels in diabetic wounds compared to non-diabetic wounds (P < 0.01). Thus our data for the first time attests to the concept that persistent activation of TLR-MyD88-NF-κB signaling pathway and increased oxidative stress contribute to the hyperinflammation frequently seen in human DM wounds.

DISCUSSION

The interactions among increased glucose levels elevated free fatty acids and resultant proinflammatory cytokines in DM have clear implications for the immune system[14,15]. A diabetic foot ulcer is primarily comprised of keratinocytes, dermal cells, and leukocytes with a coexisting paucity for angiogenesis[16]. All the evidence point towards uncontrolled inflammation and frequent bacterial colonization at the site of injury as the main causes for foot ulcers not healing in a timely manner or not heal at all[7,16]. In addition, chronic diabetic ulcers may also persist due to disrupted formation of granulation tissues and deep tissue necrosis[7,16,17]. Along with cell specific abnormalities, inflammatory cytokine expression such as IL-1β and TNF-α are elevated and sustained by hyperglycemia implying the role of innate immunity[14,18]. TLRs in the wound bed environment play an important role in mediating innate immune functions and inflammation whereby potential healing may be impaired[6,8,9].

Studies in animal models as well as humans have suggested that inflammation is a major contributing factor to DM pathology primarily orchestrated by the innate immune receptors[8-10]. Mohammad *et al*[19] reported increased TLR2 and TLR4 expression in bone marrow derived macrophages of non-obese diabetic (NOD) mice, correlating with increased NF-κB activation and increased pro-inflammatory cytokines. Kim *et al*[20] using TLR2$^{-/-}$, TLR4$^{-/-}$ knockouts, and NOD mice have demonstrated that TLR2 senses beta cell death and contributes to the instigation of autoimmune diabetes. Recently, we showed increased TLR2 and TLR4 expression, intracellular signaling, and TLR2/4 mediated inflammation in monocytes with significant correlation to HbA1c levels in DM patients[21,22]. Creely *et al*[23] showed increased TLR2 expression in the adipose tissue of type 2 diabetes (T2DM) patients with strong correlates to plasma endotoxin levels. Also, Song *et al*[24] reported increased TLR4 mRNA expression in differentiating adipose tissue of

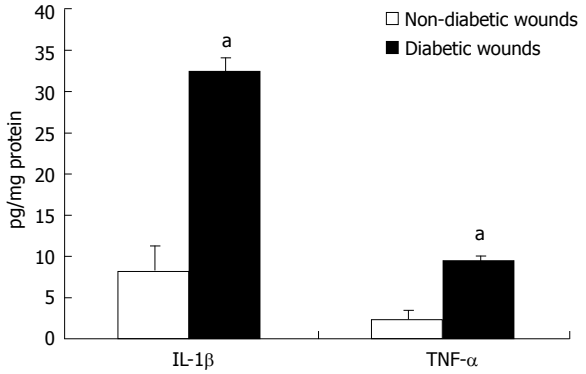

Figure 3 Interleukin-1β and tumor necrosis factor-α concentration in wound tissues were determined by ELISA assay. Values are normalized to mg protein and expressed as mean ± SD. $^{a}P < 0.05$ *vs* non-diabetic. IL-1β: Interleukin-1beta; TNF-α: Tumor necrosis factor-alpha.

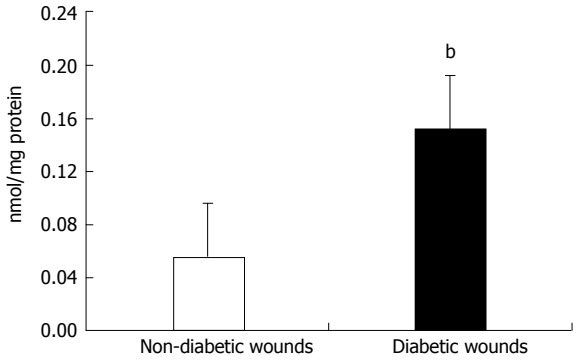

Figure 4 Lipid peroxidation in wound tissue lysates were determined using thiobarbituric acid reactive substances assay as described in Materials and methods. Values are normalized to mg protein and expressed as mean ± SD. $^{b}P < 0.01$ *vs* non-diabetic.

db/db mice. Furthermore, Davis *et al*[25] have shown that the TLR4-deficient 10ScN mouse strain fed with diet rich in saturated fat is protected from systemic inflammation. Taken together, these observations suggest a potential role for TLR2 and TLR4 in the pathology of DM. Furthermore, recent findings have shown increased TLR2/4 expression, signaling, ligands, and functional activation in DM subjects with and without complications[20,26]. All the above studies suggest that TLR activation and signaling contribute to the prolonged inflammatory condition seen in DM and may lead to complications in line with our current data.

Functional activation of TLRs includes dimerization and this results in cytokine production. TLR2 requires heterodimerization with TLR1 or TLR6 for activity[27]. We have previously shown that hyperglycemia induces TLR2/TLR6 heterodimerization resulting in cytokine secretion in human monocytes[14] consistent with the increased mRNA expression as seen in this study. However, it is to be noted that characterization of dimerization events *in vivo* is technically challenging. Besides, we also observed changes in TLR1 mRNA expression and it is not known if either TLR1 or TLR6 by themselves are inflammatory and if TLR2/1 heterodimerization play a role in the peristent inflammation. TLR2 primarily activates MyD88-dependent signaling pathway[28]. The activation of MyD88-dependent signaling pathway leads to the induction of inflammatory cytokines[28]. There are studies showing delayed dermal wound healing in nondiabetic MyD88-deficient mice[29], suggesting that alternate TLR pathways may be active in diabetic milieu (for example, TLR4/MD2). Here, we provide the first evidence, that in human DM wounds, there is increased TLR2 and TLR4 expression, with corresponding increased NF-κB activity, increased expression of downstream adapter proteins such as MyD88 and IRAK-1, resulting in increased local pro-inflammatory cytokines. Similar findings were found when cells were treated *in vitro* under hyperglycemic, dyslipidemia, and increased oxidative stress conditions[4,6,27]. Thus, we suggest that abrogating inflammation in human DM wounds using TLR2/4 as a target appears to be a reasonable approach to alleviate inflammation accelerating DM wound-healing process.

Collectively, these findings are best valued when recognizing that TLR activation, signaling, and inflammation may be undesirable for proper healing of wounds in DM patients. The limitations of the current study include the lack of correlative evidence between hyperglycemia, duration of diabetes, wound size, and TLR expression due to small sample size. Future and ongoing studies are focussed on collecting sequential wound debridement specimens, infected wound tissues to record the relationship between TLR activation and wound healing as this will aid in establishing the timing of the receptor expression and activation and the relationship between innate immunity and infection in manifesting the impaired wound healing phenotype. At the same time, TLR expression and activation may be used as a cue for healing. Prolonged and exacerbated cytokine production leads to sustained inflammatory responses and impaired healing, causing extensive tissue damage (amputations in case of diabetic wounds). Therefore, it is important to understand local inflammatory mechanisms that might be useful in developing therapeutic strategies for the management of difficult wounds burdened by excessive inflammation. Our findings suggest a role for TLRs in the human DM wound pathology and emphasize the importance of understanding the various pathogenic mechanisms involved in a complicated wound-healing process.

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COMMENTS

Background

Toll-like receptors (TLRs) are sentinel pathogen recognition receptors with a pivotal role in inflammation, tissue injury, diabetes and its complications.

Innovations and breakthroughs

Increased TLR expression, signaling, and activation may contribute to the hyper inflammation in the human diabetic wounds.

Peer review

This manuscript is well written and shows results of potential interest.

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CASE REPORT

Gas-forming liver abscess associated with rapid hemolysis in a diabetic patient

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Abstract

We experienced a case of liver abscess due to *Clostridium perfringens* (*CP*) complicated with massive hemolysis and rapid death in an adequately controlled type 2 diabetic patient. The patient died 6 h after his first visit to the hospital. *CP* was later detected in a blood culture. We searched for case reports of *CP* septicemia and found 124 cases. Fifty patients survived, and 74 died. Of the 30 patients with liver abscess, only 3 cases survived following treatment with emergency surgical drainage. For the early detection of *CP* infection, detection of Gram-positive rods in the blood or drainage fluid is important. Spherocytes and ghost cells indicate intravascular hemolysis. The prognosis is very poor once massive hemolysis occurs. The major causative organisms of gas-forming liver abscess in diabetic patients are *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*). Although *CP* is relatively rare, the survival rate is very poor compared with those of *K. pneumoniae* and *E. coli*. Therefore, for every case that presents with a gas-forming liver abscess, the possibility of *CP* should be considered, and immediate aspiration of the abscess and Gram staining are important.

Key words: Liver abscess; Gas-forming; *Clostridium perfringens*; Hemolysis; Diabetes

Core tip: Gas-forming liver abscess caused by *Clostridium perfringens* can result in massive hemolysis and death within several hours. For survival, urgent surgical intervention and antibiotic administration are necessary.

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INTRODUCTION

Gas-forming infections are an example of a severe type of infection in diabetic patients. Although life threatening, there still remains time for treatment[1,2]. However, in rare cases of *Clostridium perfringens* (*CP*) infection, the time remaining for the patient is very limited[3-7]. *CP* is an anaerobic Gram-positive rod that is found in the soil and the human gastrointestinal and urogenital tracts. *CP* causes septicemia in cases of food intoxication, wound-associated soft tissue infections, liver abscess, and lung abscess. *CP* may cause septicemia without any apparent wound through bacterial translocation[5-8]. Patients typically have an underlying condition such as diabetes, malignancy, liver cirrhosis, or an immunosuppressive state[4-23].

In some reports, *CP* septicemia occurred after an invasive procedure in the hepatobiliary tract[24-26] or gastrointestinal tract or following gynecological treatment[27,28] or line insertion[29]. Early diagnosis is difficult because only nonspecific inflammation and gas formation in the focus are present. However, once α-toxin triggers hemolysis, it progresses very rapidly and is followed by acidosis and renal failure[30,31]. According to the literature, the mortality rate ranges from 70% to 100%[3]. For survival, surgical removal of the focus, appropriate antibiotics, control of hemolysis, and supportive care including hemodialysis are necessary. These treatments should be started before the blood culture result is returned. For early diagnosis, the detection of spherocytes and Gram-positive rods in the blood is important[5,32,33]. We experienced a case of liver abscess in an adequately controlled diabetic patient without any triggering event. The patient died within hours following massive hemolysis and cardiac arrest. Although the majority of gas-forming infections in diabetics are caused by *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*)[34], the possibility of *CP* infection should be considered.

CASE REPORT

The patient was a 65-year-old Brazilian of Japanese origin. He had a 3-day history of fever, appetite loss, nausea, and upper abdominal pain. The patient had type 2 diabetes treated with an oral hypoglycemic agent. He also had hypertension and dyslipidemia. He had a history of coronary stenting but no history of liver cirrhosis or malignancy. On physical examination, consciousness was clear, his blood pressure was 157/90 mmHg, and hyperventilation and coldness of the limbs were noted. Slight scleral jaundice and slight tenderness of the abdomen were noted. Laboratory examinations indicated mild liver dysfunction and elevation of serum bilirubin, C-reactive protein, and the white blood cell count (Table 1). At this time, the serum did not show any sign of intravascular hemolysis (Figure 1A). CT of the abdomen revealed a liver abscess 4 cm in diameter with gas formation in the right lobe (Figure 1C). A blood culture sample was taken, and ceftriaxone injection was started immediately. The patient briefly returned to his dormitory to prepare for admission and was found unconscious by a fellow worker. He was transferred to the hospital, and CPR was performed in vain. The serum color at this time point revealed strong hemolysis (Figure 1B). He died 6 h after his first visit to the hospital. The remarkably high levels of serum potassium (11.8 mEq/L) and lactate dehydrogenase (LDH) (6203 IU/L) during CPR suggested massive intravascular hemolysis. *CP* was later detected in the blood culture. Autopsy was refused, and we were unable to determine whether he had an occult malignancy.

Recently, van Bunderen *et al*[3] reported 40 cases of *CP* septicemia and hemolysis between 1990 and 2010. In total, 80% of the patients had died; among the 11 cases with liver abscess, 10 (90.9%) had died. These 10 cases included two cases of microabscess. In one case, the focus of infection was removed, and the patient survived. On the other hand, Fujita *et al*[35] studied patients with systemic inflammatory response syndrome (SIRS) with *CP*-positive blood cultures and reported that 5 of 18 cases had died (27.8%). Yang *et al*[36] reported the prognosis of *CP* septicemia in a tertiary care hospital. They found 93 cases over 10 years, and the 30-d mortality rate was 26.9%. Therefore, the mortality rate of *CP* septicemia differs considerably. We hypothesized that the complication of liver abscess decreases the survival rate. We searched PubMed for papers published since 2010 and the database of the Japan Medical Abstract Society since 1994 with the keywords "Clostridium perfringens" and "septicemia". We found 20 cases from PubMed and 104 cases from Japan, including our case[4-33,35,37-39]. Fifty patients survived, and 74 (59.7%) died.

Several possible triggers of septicemia were found, including transarterial embolization of the hepatoma[24,25], laparoscopic cholecystectomy[26], amniocentesis[27], abortion[28], and intravenous line insertion[29]. Among the 30 cases with liver abscess, 27 (90%) died. Six cases underwent drainage or laparotomy, and three cases survived[8,30,38]. Among the cases with liver abscess, 23 were male and 7 were female; the average patient age was 67.2 years old, and 11 patients had diabetes. The median time from the first visit to death was only 6 h. Of the 74 deceased patients, 45 were male, 21 were female, and 8 were not described; the average age was 64.4 years old. Malignancy was the frequent underlying disease. Twenty-one cases had a history of cancer in the liver, stomach, colon, rectum, gall bladder, biliary duct, lung, pancreas, breast, prostate gland, or uterus. Ten cases had a history of leukemia, lymphoma, or multiple myeloma. One patient had

Table 1 Serial laboratory results for a patient with liver abscess and massive hemolysis caused by *Clostridium perfringens*

Parameter	Admission	On CPR	Reference range
White blood count (× 10^9/L)	24.8	26.0	3.5 to 9.7
Red blood count (× 10^9/L)	4980	1280	4380 to 5770
Hemoglobin (g/L)	135	81	136 to 183
Hematocrit (%)	40.7	10.8	40.4 to 51.9
Platelet (× 10^9/L)	243	118.8	140 to 379
Total bilirubin (mg/dL)	6.4	6.96	0.2 to 1.0
Aspartate aminotransferase (IU/L)	140	261	8 to 38
Alanine aminotransferase (IU/L)	102	297	4 to 44
Alkaline phosphatase (IU/L)	178	469	104 to 338
γ-glutamyl transpeptidase (IU/L)	25	6	18 to 66
Lactate dehydrogenase (IU/L)	373	6203	106 to 211
Creatine phosphokinase (IU/L)	220	438	104 to 338
Urea (mg/dL)	24.2	30.5	8 to 20
Creatinine (mg/dL)	1.33	1.12	0.63 to 1.03
Sodium (mEq/L)	134	128	137 to 147
Potassium (mEq/L)	4.6	11.8	3.5 to 5.0
Chloride (mEq/L)	95	84	98 to 108
C-reactive protein (mg/dL)	23.2	16.0	< 0.30
International normalized ratio	1.05	19.4	0.9 to 1.1
APTT (s)	38	122.9	25 to 40
Glucose (mg/dL)	226	129	

APTT: Activated partial thromboplastin time.

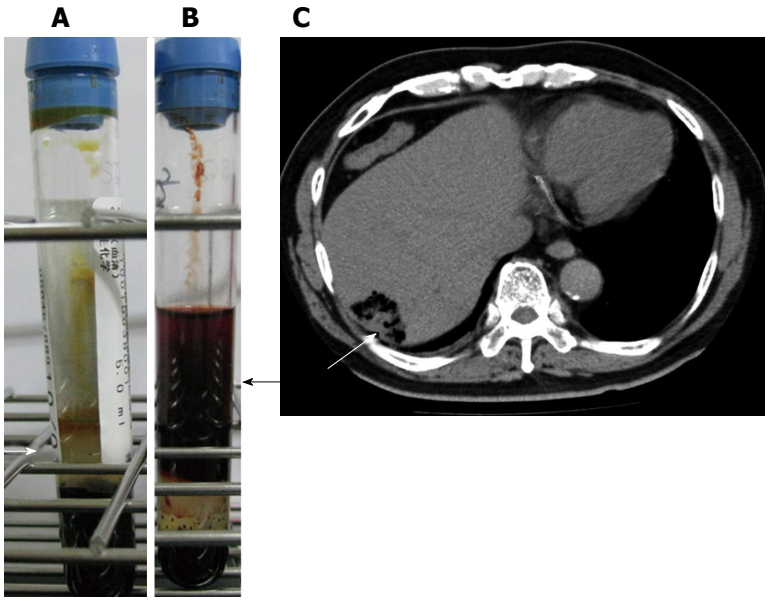

Figure 1 The serum color before and after massive hemolysis and computed tomography imaging results. A: Patient serum color on admission showed no sign of hemolysis (white arrow); B: The dark red color of serum taken during CPR indicated massive hemolysis (black arrow); C: Computed tomography of the abdomen revealed a 4 cm × 2 cm abscess with gas formation in the right lobe (white arrow).

a brain tumor. In total, 30 cases (45.5%) had a history of at least one malignancy. Eighteen cases had diabetes. Four cases had liver cirrhosis. The median time from the first visit to death was 6 h. Only 12 cases (16%) had undergone emergency surgery or drainage. Two patients received hemoperfusion using a polymyxin B-immobilized fiber column (PMX-F), which is used for endotoxin removal in Japan and Italy[40-43]. Of the 50 surviving patients, 16 were male, 19 were female, and 15 were not described. Females were significantly more prevalent among the survivors, according to a chi-squared test ($P < 0.05$). Three cases involved children younger than 2 years old. The average age, excluding these small children, was 58.1 years. The age difference between the deceased and surviving cases was not significant ($P = 0.06$), according to a two-sided t test. Six cases had leukemia, and 4 cases had cancer or sarcoma in the breast, uterus, or colon. Six cases had diabetes. Twenty (40%) cases underwent surgical removal or drainage of the focus. A significantly greater number of patients who underwent surgical debridement or drainage were among the surviving cases compared with the deceased cases, according to a chi-squared test ($P < 0.01$). PMX-F was used to treat 5 patients who survived. Among the surviving cases, steroid pulse therapy was performed in three cases and hyperbaric oxygen therapy was used in two.

DISCUSSION

Although our case did not show anemia at first presentation and the size of liver abscess was only 4 cm, he developed massive fatal hemolysis within hours, despite prompt treatment with the appropriate antibiotics. Therefore, *CP* septicemia should be considered in diabetic patients with fever and gas-forming lesions before any signs of hemolysis develop. van Bunderen *et al*[3] reported 40 cases of septicemia caused by *CP* during 1990-2010. Over half of the patients presented elevated bilirubin and LDH as well as anemia, suggesting hemolysis at the initial presentation. Thirty-two of the patients died, and the median time from admission to death was only 8 h. We searched new cases of *CP* septicemia. We found 124 cases, and the death rate was 59.7%. However, in cases with liver abscess, the death rate reached 90%, and the median time from visit to death was only 6 h. Rapid hemolysis caused by α-toxin is an important complication that makes rescue difficult. The α-toxin of *CP* has two domains plus one loop in between. The N-terminal domain has phospholipase activity, and the C-terminal domain is hydrophobic and inserts into the cell membrane[44]. The loop between the N- and C-terminal domains contains a GM1 ganglioside-binding motif and specifically binds GM1a. In addition to disrupting membrane phospholipids through phospholipase activity, α-toxin binding to GM1a triggers specific signaling events. The activation of a tyrosine kinase A (TrkA)[45] and the subsequent signaling cascade results in the release of tumor necrosis factor-α (TNF-α). The catastrophic events induced by α-toxin may in part be mediated by TNF-α signaling. The hemolysis of erythrocytes by α-toxin is reported to depend on Ca^{2+} uptake[46].

The key for patient rescue is how fast the appropriate treatments are started. At the moment of suspicion of *CP* septicemia, aggressive early management is warranted, including timely debridement or drainage of the focus, initiation of appropriate antibiotics without delay, and support of circulation with a multi-disciplinary team approach. For the early diagnosis of *CP* infection, Gram staining of the blood or drainage sample is important because *CP* is a Gram-positive rod, whereas *K. pneumoniae* and *E. coli* are Gram negative. The early signs of hemolysis are elevated LDH, total or indirect bilirubin, and potassium. Spherocytes or ghost cells may be found in the blood film. A red color of the serum or hemoglobinuria may be observed after substantial hemolysis.

Shah *et al*[47] reported 25 cases of *CP* septicemia in a tertiary-care hospital from 1995 to 2003 and classified antibiotics into two categories. The antibiotics classified as "appropriate" for *Clostridium* were penicillin G, clinda-

mycin, cefoxitin, metronidazole, ampicillin/sulbactam, piperacillin/tazobactam, and imipenem/cilastatin; other antibiotics were classified as "insufficient". Patients treated with "insufficient" antibiotics had a significantly higher 2-d mortality rate (75%) compared with patients treated with "appropriate" antibiotics (12.5%). Clindamycin, metronidazole, and rifampicin have been shown to be effective methods to reduce the release of α-toxin[48]. However, penicillin and cephalosporin do not have such activity. Oda *et al*[49] have reported that erythromycin pretreatment reduces the release of TNF-α from activated neutrophils and suppresses hemolysis.

Because α-toxin has enzymatic activity, methods to neutralize or eliminate this toxin are needed. Unfortunately, we were unable to find any established method of doing so. PMX-F is used in septic shock treatment. PMX-F binds endotoxin, monocytes, activated neutrophils, and anandamide, decreasing inflammatory cytokines and other mediators. A review by Cruz *et al*[40] analyzed 987 patients treated with PMX-F and 447 patients treated with conventional medical therapies. PMX-F increased the mean arterial pressure by 19 mmHg while reducing the dopamine/dobutamine dose by 1.8 μg/kg per min. PMX-F therapy was associated with a significantly lower mortality risk (RR = 0.53; 95%CI: 0.43-0.65). However, the number of reported cases is currently too small to discuss the effectiveness of PMX-F in the treatment of *CP* septicemia. Ochi *et al*[46] reported that flunarizine, a T-type Ca^{2+} channel blocker and tetrandrine, an L- and T-type Ca^{2+} channel blocker, inhibited hemolysis by α-toxin. Nagahama *et al*[50] reported that the C-terminal recombinant peptide of α-toxin was effective as a vaccine to protect against hemolysis in an animal experiment.

Empirical antibiotic therapy should be started before the culture results are returned. The major causative organisms of gas-forming liver abscesses are *K. pneumoniae* and *E. coli*[1,2,34]. These organisms can also cause fatal infections, and endophthalmitis or meningitis may occur[51], but the mortality rate is not as high as that of *CP*. A review of 46 cases reported death in *K. pneumoniae* liver abscess for 11 of 43 (25.6%) patients[51]. According to a report from China, 95% of the patients with liver abscess were eventually cured if treated radically[34]. Fortunately, *CP* septicemia is rare. Kasai *et al*[52] reported that among cases of severe infection in diabetic patients in Japan, 119 cases presented with a gas-forming abscess, and only 8 cases were positive for *Clostridium*. Kurai *et al*[53] reported that among 5011 blood samples that were positive for any bacteria, only 41 were positive for *Clostridium*. Of the 41 samples, 16 were confirmed as septicemia, and 9 of the 16 were positive for *CP*. According to a report from Canada, the incidence of *CP* septicemia in the community is 0.7 in 100000 per year[54]. Additionally, in hospital-based studies, *CP* septicemia is very rare. Zahar reported 45 cases of anaerobic bacteremia among 7989 positive blood cultures in a cancer center during 1993-1998[55]; seven of them were *CP* septicemia. Woo *et al*[53] reported 38 cases of *Clostridium* septicemia in a large hospital from 1998 to 2001; 79% of them were caused by *CP*, and the overall mortality was 29%. Younger age and gastrointestinal/hepatobiliary tract disease were associated with mortality. However, considering the very high mortality rate associated with liver abscess, excluding *CP* infection is important.

In summary, *CP* septicemia is a rare but well-known cause of massive intravascular hemolysis. Diabetic patients with fever and gas-forming lesions should always be suspected of having *CP* septicemia.

COMMENTS

Case characteristics

A 65-year-old male with treated diabetes presented with fever and upper abdominal pain.

Clinical diagnosis

Hypertension, hyperventilation, coldness of limbs, scleral jaundice, and tenderness of the abdomen were noted.

Differential diagnosis

Obstructive jaundice complicated with biliary infection and liver abscess.

Laboratory findings

White blood cell 24.8 × 10^9/L, hemoglobin 135 g/L, total bilirubin 6.4 mg/dL, aspartate aminotransferase 140 IU/L, alanine aminotransferase 178 IU/L, creatinine 1.33 mg/dL, C-reactive protein 23.2 mg/dL, and glucose 226 mg/dL.

Imaging diagnosis

Computed tomography imaging showed a gas-forming mass (4 cm × 2 cm) in the right lobe of the liver.

Pathological diagnosis

Autopsy was not allowed, and blood culture revealed infection by *Clostridium perfringens*.

Treatment

Injection of ceftriaxone was started immediately.

Related reports

The reported mortality rate of *Clostridium perfringens* septicemia varies widely from 26.9% to 80%; however, 90% of patients with liver abscess have been reported to die.

Term explanation

Polymyxin B-immobilized fiber column (PMX-F) is hemoperfusion with a polymyxin B-immobilized fiber column used to remove endotoxin in cases of septic shock.

Experiences and lessons

Although rare, fatal liver abscess patients should be under close observation, and the possibility of *Clostridium perfringens* infection should be considered upon the slightest sign of hemolysis.

Peer review

This is a well written manuscript in which the author gave detailed description of death report associated with *CP* infection.

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 200201 03498. 2002 Aug 1

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