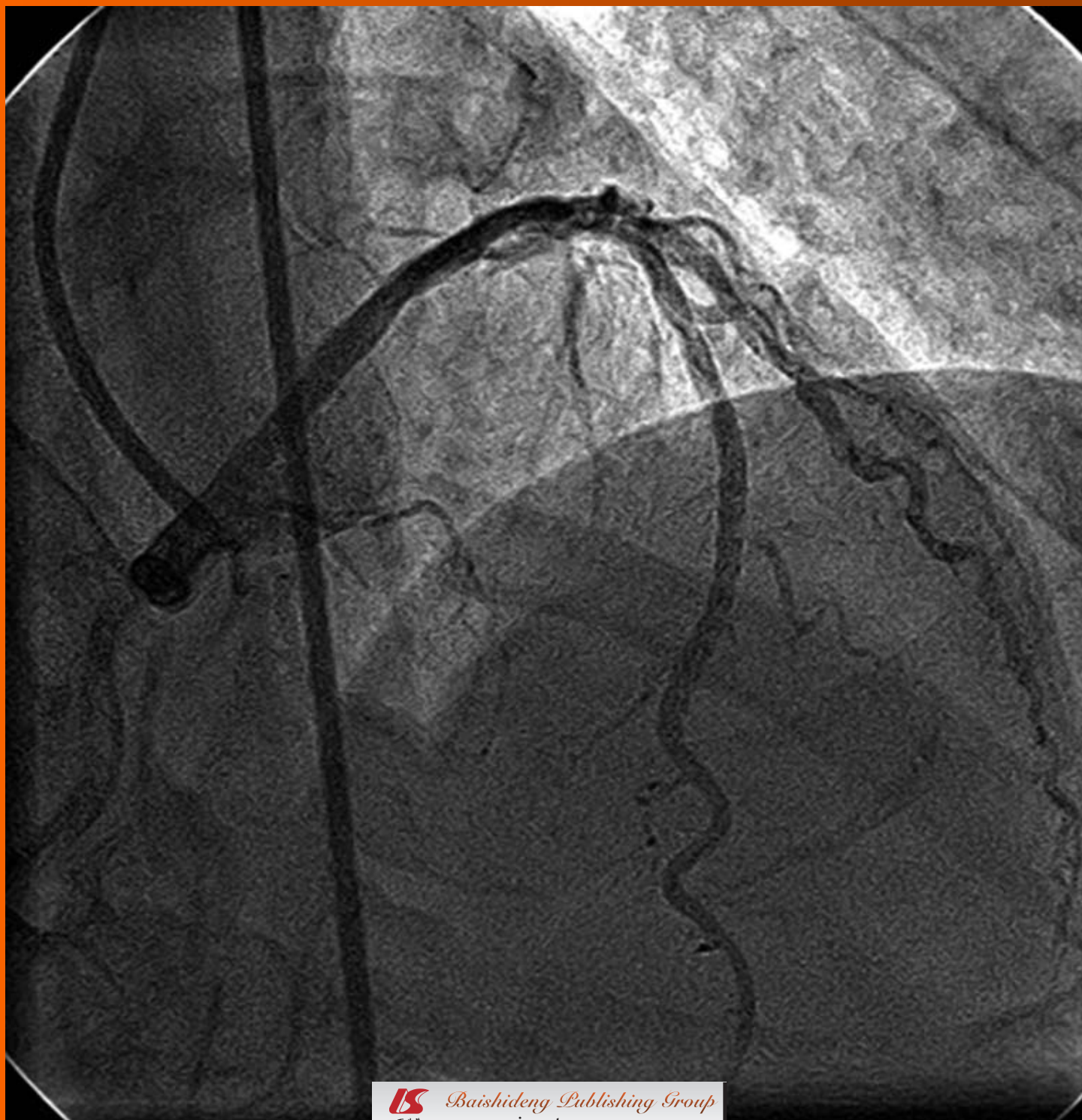


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Parallel effects of β -adrenoceptor blockade on cardiac function and fatty acid oxidation in the diabetic heart: Confronting the maze

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Abstract

Diabetic cardiomyopathy is a disease process in which diabetes produces a direct and continuous myocardial insult even in the absence of ischemic, hypertensive or valvular disease. The β -blocking agents bisoprolol, carvedilol and metoprolol have been shown in large-scale randomized controlled trials to reduce heart failure mortality. In this review, we summarize the results of our studies investigating the effects of β -blocking agents on cardiac function and metabolism in diabetic heart failure, and the complex inter-related mechanisms involved. Metoprolol inhibits fatty acid oxidation at the mitochondrial level but does not prevent lipotoxicity; its beneficial effects are more likely to be due to pro-survival effects of chronic treatment. These studies have expanded our understanding of the range of effects produced by β -adrenergic blockade and show

how interconnected the signaling pathways of function and metabolism are in the heart. Although our initial hypothesis that inhibition of fatty acid oxidation would be a key mechanism of action was disproved, unexpected results led us to some intriguing regulatory mechanisms of cardiac metabolism. The first was upstream stimulatory factor-2-mediated repression of transcriptional master regulator PGC-1 α , most likely occurring as a consequence of the improved function; it is unclear whether this effect is unique to β -blockers, although repression of carnitine palmitoyltransferase (CPT)-1 has not been reported with other drugs which improve function. The second was the identification of a range of covalent modifications which can regulate CPT-1 directly, mediated by a signalome at the level of the mitochondria. We also identified an important interaction between β -adrenergic signaling and caveolins, which may be a key mechanism of action of β -adrenergic blockade. Our experience with this labyrinthine signaling web illustrates that initial hypotheses and anticipated directions do not have to be right in order to open up meaningful directions or reveal new information.

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Key words: Diabetes; Heart failure; β -blockers; Cardiac metabolism; Fatty acid oxidation

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INTRODUCTION

Diabetic cardiomyopathy is a disease process in which diabetes produces a direct and continuous myocardial insult even in the absence of ischemic, hypertensive or valvular disease. It can act synergistically with hypertension or ischemia to damage heart muscle, but can also cause heart failure in its own right. The clinical course is long, and can be divided into three stages^[1]. In the early stage, the cardiomyopathy presents with mild asymptomatic diastolic dysfunction which is associated with ultrastructural changes in tissue architecture, impaired calcium handling, oxidative stress and changes in cardiac metabolism. As the disease progresses, evidence of left ventricular hypertrophy appears which is associated with more severe diastolic dysfunction and mild systolic dysfunction. Cardiomyocyte apoptosis and necrosis, myocardial fibrosis, mild autonomic neuropathy and activation of the renin-angiotensin system appear at this stage. Finally, combined systolic and diastolic dysfunction occur which are associated with cardiac microvascular disease, severe autonomic neuropathy and systemic sympathetic nervous system activation. This late stage is frequently associated with hypertension and the onset of ischemia^[1]. The mechanisms underlying the process are poorly understood, but an overall picture is emerging. The sustained diabetic cardiac insult appears to be produced by two major factors: hyperglycemia, a major mediator of many diabetic complications, and a shift in energy substrate selection by the heart^[2]. This disease process impairs both passive and active mechanical properties of the myocardium; the compliance of the heart wall decreases (due to increased cross-linking of collagen, cardiac hypertrophy and fibrosis^[2,3]), and contractility also decreases.

Heart failure is associated with activation of the sympathetic nervous system. The sympathetic drive to a failing resting heart is equivalent to the maximum drive a normal heart is subjected to during severe exercise; spillover of catecholamines increases as much as 50-fold, producing marked elevation of cardiac and systemic catecholamine levels^[4-7]. This large response is initiated in an effort to maintain systemic perfusion, but sympathetic activation is harmful to the failing heart, regardless of the cause of the failure, and correlates inversely with survival^[8]. Conversely, the β -blocking agents bisoprolol, carvedilol and metoprolol have been shown in large-scale randomized controlled trials to reduce heart failure mortality by a third or more^[9]. β -blocking drugs produce negative chronotropic and inotropic responses when administered acutely. For this reason, they were contraindicated in heart failure for many years. However, in the 1970s, β -blockers were pioneered as heart failure treat-

ments^[10], and they are now among the agents of choice for the treatment of heart failure^[9].

Putative mechanisms for the chronic effect of β -blockers include antiarrhythmic effects, amelioration of cardiomyocyte hypertrophy, necrosis and apoptosis, reversal of the fetal gene program (thereby improving calcium handling and force of contraction), increases in cardiac receptor density (for some β -blockers including metoprolol; the effect is likely to be mediated both by increased receptor expression and decreased receptor internalization), antiinflammatory effects (β -blockers lower serum C-reactive protein levels) and partial restoration of cardiac glucose oxidation^[11,12]. Metoprolol^[13], carvedilol^[14] and bucindolol^[15] have all been shown to induce a switch from fatty acid to glucose oxidation in non-diabetic patients with heart failure. Metabolic effects of nebivolol, another commonly used β -blocker, have not been investigated. Metoprolol was shown to increase lactate uptake in heart failure patients, an effect which is consistent with an increase in carbohydrate oxidation^[16]. A study in dogs with microembolism-induced heart failure revealed a potential mechanism for this effect: carnitine palmitoyltransferase (CPT)-1 was inhibited by chronic treatment with metoprolol^[17].

There had been no clinical or experimental studies examining whether β -blocking agents are beneficial in diabetic cardiomyopathy when we initiated our studies. A number of clinical studies had examined the effects of these agents in the context of ischemia and shown benefit^[18-25]. Our initial aim was to investigate whether a benefit could be seen in diabetic cardiomyopathy. Recently, however, the use of β -blockers as antihypertensive agents has been associated with an increased risk of new-onset diabetes, leading to concern about their use in this context^[26]. Hepatic glucose output is controlled by the β_2 adrenoceptor, and blockade of this receptor, which does occur with the β_1 selective agents, decreases hepatic glucose output and delays recovery from hypoglycemia^[27,28]. The risks and benefits of using β -blockers to treat diabetic cardiomyopathy would need to be very carefully weighed, and we do not believe that sufficient evidence exists at present on which to base this decision. Our studies in rats revealed that the effects of β -blocker treatment on metabolism and function are wide-ranging, and provided some new insights into the regulation of fatty acid oxidation. In this review, we summarize the effects of β -blocker treatment in diabetic cardiomyopathy and what they reveal about the interconnected signaling web which controls metabolism and function. The main pathways involved in cardiac metabolism are summarized in Figure 1^[29].

β -ADRENOCEPTOR SIGNALING

In 1948, Ahlquist^[30] first demonstrated the existence of two broad subtypes of adrenoceptors: α -adrenoceptors and β -adrenoceptors. Two subtypes of β -adrenoceptors, β_1 and β_2 , were identified and characterized in the late

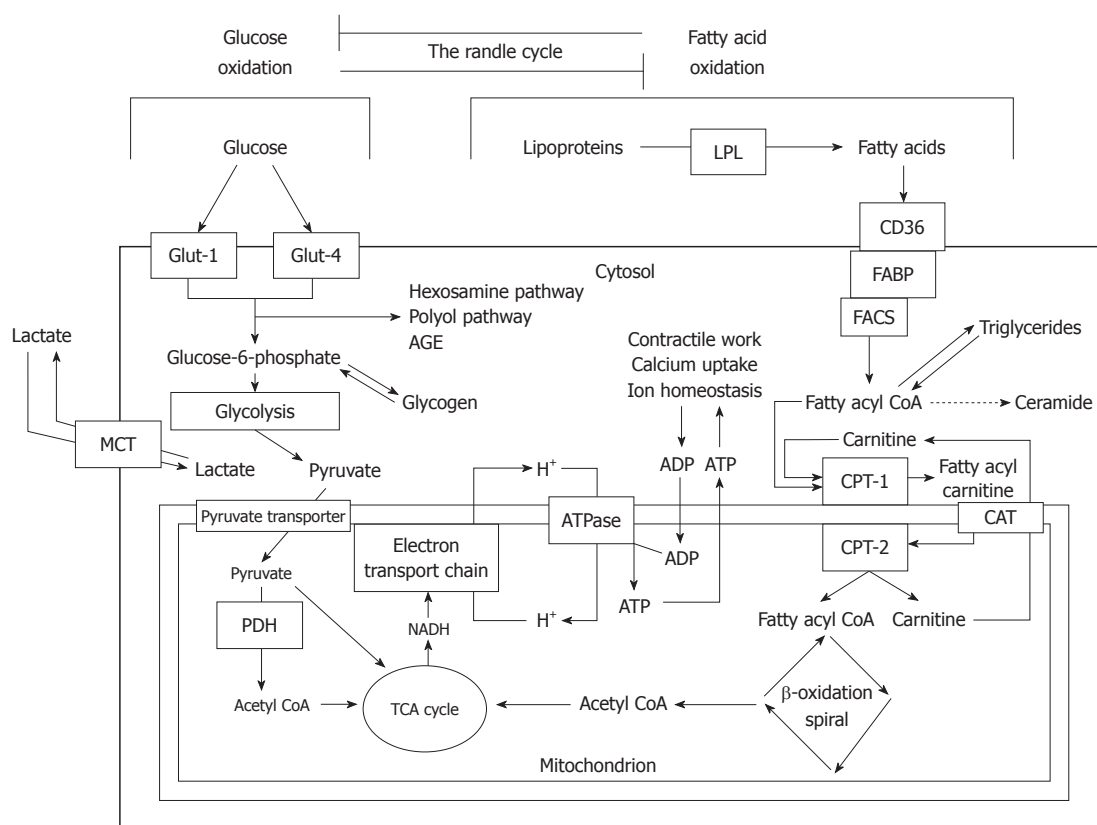


Figure 1 Summary of fatty acid and glucose metabolism. Glucose is taken up by Glut-1 and Glut-4 transporters and is converted by glycolysis to pyruvate which enters the mitochondria to be oxidized, producing acetyl coenzyme A (CoA). Fatty acids are liberated from lipoproteins by lipoprotein lipase (LPL) and taken up by fatty acid translocase (CD36) and fatty acid binding protein (FABP). Long-chain fatty acyl-CoA synthetase (LCAS) converts the fatty acid to a CoA ester which is then taken up by the carnitine shuttle system to the mitochondria. The fatty acyl CoA undergoes β -oxidation, removing two carbons per turn of the cycle and generating acetyl CoA. Acetyl CoA, generated by either pathway, enters the tricarboxylic acid (TCA) cycle to generate reducing equivalents [reduced nicotinamide adenine dinucleotide (NADH)]. These pass electrons to the electron transport chain which creates an electrochemical proton gradient to drive adenosine triphosphate (ATP) synthesis. ATP synthesis is coupled to the systems which create the ATP demand. FACS: Fatty acyl-CoA synthetase; CPT: Carnitine palmitoyltransferase; CAT: Carnitine acyl transferase; AGE: Advanced glycosylation end-product; PDH: Pyruvate dehydrogenase; MCT: Monocarboxylate transporter; ADP: Adenosine monophosphate. Modified from: Sharma *et al*^[29].

1960s^[31], while a third, β_3 , was isolated and cloned in 1989^[32]. All three subtypes are expressed in the heart, but the major subtypes are β_1 and β_2 , the ratio of β_1 : β_2 being approximately 60%-70%:40%-30%, with very low β_3 expression^[33]. The effects of the putative β_4 adrenoceptor are now believed to be mediated by a low-affinity state of the β_1 adrenoceptor^[34,35]. The receptor reserve is low because the absolute expression levels are in the femtomolar range (50-70 fmol/mg protein for the β_1 adrenoceptor)^[11]. The affinities of these receptors for their ligands differ: β_1 (adrenaline = 4 μ mol/L, noradrenaline = 4 μ mol/L, isoproterenol = 0.2 μ mol/L), β_2 (adrenaline = 0.7 μ mol/L, noradrenaline = 26 μ mol/L, isoproterenol = 0.5 μ mol/L), β_3 (adrenaline = 130 μ mol/L, noradrenaline = 4 μ mol/L, isoproterenol = 2 μ mol/L)^[36].

β -adrenoceptors are G-protein coupled receptors. In the classical β -adrenoceptor pathway, β_1 and β_2 adrenoceptors, acting *via* Gs, produce an acute positive inotropic response mediated by increased cAMP levels and stimulation of protein kinase A (PKA). PKA then phosphorylates several key proteins involved in calcium handling and calcium sensitivity of myofilaments. Phosphorylation

and activation of L-type calcium channels and ryanodine receptors increases calcium uptake and release, while phosphorylation of phospholamban relieves inhibition of SERCA, thereby increasing sarcoplasmic reticulum calcium uptake^[37-39]. Finally, PKA modulates the calcium sensitivity of myofilaments through phosphorylation of troponin I and myosin binding protein B^[40,41]. PKA also activates protein phosphatase inhibitor-1, sustaining its effects by preventing dephosphorylation of its targets^[42].

Recently, a major paradigm shift has occurred in adrenoceptor biology. The β -adrenoceptors are now known to form complex "signalomes" which are temporally and spatially organized. A signalome can be defined as all genes, proteins and ligands which are involved in the transduction and response to a biological signal. With regard to temporal organization, it is well established that β -adrenoceptors, and most particularly the β_2 -adrenoceptor, desensitize by uncoupling from their G-proteins. This dissociation occurs following receptor phosphorylation and is mediated by β -arrestins acting together with G protein-coupled receptor kinases or PKA itself^[43-45]. In addition to receptor desensitization, proteins

and ligands are involved in the transduction and response to a biological signal. With regard to temporal organization, it is well-established that β -adrenoceptors, and most particularly the β_2 -adrenoceptor, are desensitized by uncoupling from their G-proteins. This dissociation is stimulated by β -adrenoceptors changing their coupling to downstream signaling pathways. Prolonged activation of β_1 adrenoceptors causes a switch from PKA- to calcium/calmodulin-dependent protein kinase-II (CAMK II)-dependent signaling, leading to CAMK-II-mediated apoptosis and pathological hypertrophy^[46]. In contrast, prolonged activation of β_2 -adrenoceptors switches G-protein coupling from Gs to Gi, which is cardioprotective^[47].

Whereas β_1 adrenoceptor signaling is widely disseminated throughout the cell, β_2 adrenoceptor signaling is compartmentalized, and the positive inotropic effect elicited by β_2 /Gs signaling is therefore smaller^[48,49]. β_2 adrenoceptor compartmentalization is partly achieved by the selective enrichment of β_2 adrenoceptors in caveolae^[50,51]. It has been suggested that translocation of β_2 adrenoceptors out of caveolae following sustained stimulation causes the switch from Gs to Gi association^[52]. β_2 adrenoceptor-Gi signaling activates the phosphoinositol-3 kinase (PI3K)-protein kinase B (Akt) pathway and phosphodiesterase 4^[48]. Phosphodiesterase 4 increases the breakdown of cAMP generated by β_1 -adrenoceptor-Gs stimulation, enabling the β_2 -adrenoceptor-Gi pathway to functionally antagonize the β_1 -adrenoceptor-Gs pathway. The PI3K-Akt pathway protects the cardiomyocyte against apoptosis^[48]. Recently, a role for the extracellular-signal-regulated kinase 1/2 in mediating β_2 -adrenoceptor-Gi cardioprotection has been suggested^[53]. Taken together, these data indicate that the coupling of β -adrenoceptors to downstream signaling pathways is compartmentalized and time-dependent. Sustained activation of β_1 adrenoceptors is harmful, whereas sustained activation of β_2 adrenoceptors could be cardioprotective.

Another consequence of PI3K/Akt activation is stimulation of nitric oxide (NO) production. NO is synthesized from the terminal guanidine nitrogen atom of the amino acid L-arginine and molecular oxygen by nitric oxide synthase (NOS). This process requires tetrahydrobiopterin (BH₄) as a cofactor; without BH₄, eNOS becomes “uncoupled”, and produces reactive oxygen species, including peroxynitrite, instead of NO. Endothelial NOS (eNOS) is constitutively expressed in adult cardiomyocytes, producing physiological NO signaling in the nanomolar range. Inducible NOS (iNOS) is expressed in response to inflammatory stimuli^[54,55] and produces higher levels of NO, mediating pathophysiological effects^[56,57]. NO and related reactive nitrogen species (RNS, e.g., peroxynitrite) covalently modify target proteins in one of three ways: nitrosylation, oxidation or nitration. Binding of NO to a protein, termed nitrosylation, is a reversible reaction and the modification produced is labile. Oxidation (e.g., glutathiolation of cysteine residues) or nitration of a protein (on tyrosine residues) produces more stable co-

valent modifications^[58]. Tyrosine nitration, nitrosylation and oxidation can be stimulatory or inhibitory depending on the target protein and residue affected. Nitrosylation of the heme moiety of soluble guanylyl cyclase by NO activates the enzyme, stimulating the production of cyclic 3',5'-guanosine monophosphate (cGMP) from guanosine triphosphate^[59]. Just as cAMP activates PKA, cGMP activates protein kinase G isoforms. The NO/cGMP signaling pathway induces a negative inotropic effect in the heart^[54]. β_3 -adrenoceptors always couple to Gi, activating the PI3K/Akt pathway. β_3 -adrenoceptors produce a negative inotropic effect which is mediated by NO. Therefore, β_2 adrenoceptor-Gi signaling and β_3 adrenoceptor-Gi signaling both stimulate NO production^[60,61].

The effects of diabetes on cardiac β -adrenergic responsiveness have been studied for many years, but the results obtained have been conflicting. Vadlamudi *et al.*^[62] showed a decrease in the cardiac relaxant effects without an effect on heart rate or contractility. Zola *et al.*^[63] showed a decrease in the chronotropic response in rabbit heart *in vivo*. Foy *et al.*^[64] demonstrated an increased chronotropic response and a decreased inotropic response in atria. Most recent studies report decreased sensitivity to β -adrenergic stimulation in cardiac tissues^[65,66]. The effects of diabetes on β -receptor expression and downstream signaling are also controversial^[66-70]. Fourteen weeks but not 8 wk of diabetes blunted the chronotropic response to noradrenaline, but the response to fenoterol, a selective β_2 agonist, was preserved^[71]. This suggests that β_1 -mediated responses are selectively blunted in the diabetic heart. The expression of β_1 is markedly decreased and that of β_2 adrenoceptors modestly decreased in the diabetic heart, whereas the expression of β_3 adrenoceptors is increased two-fold^[66]. A similar increase in β_3 adrenoceptor expression has been reported in failing human hearts^[72]. The significance of this shift in receptor subtypes towards β_3 adrenoceptors remains to be determined; it is possible that this shift contributes to cardiac dysfunction by promoting a negative inotropic effect; on the other hand, a cardioprotective effect may also result if β_3 adrenoceptor-mediated activation of the PI3K/Akt pathway also prevents apoptosis (Figure 2).

EFFECTS OF METOPROLOL ON CARDIAC FUNCTION AND METABOLISM

We demonstrated that metoprolol ameliorates the cardiac dysfunction produced by diabetic cardiomyopathy in rats^[73]. This improvement was evident both from Starling curves generated by direct left ventricular pressure measurements and from measurements of cardiac output and hydraulic power taken over the course of an hour-long perfusion at constant preload and afterload. However, when we repeated the measurements *in vivo* using echocardiography, a robust improvement in function was not seen; although metoprolol improved stroke volume and cardiac output, it also increased end-diastolic volume, so

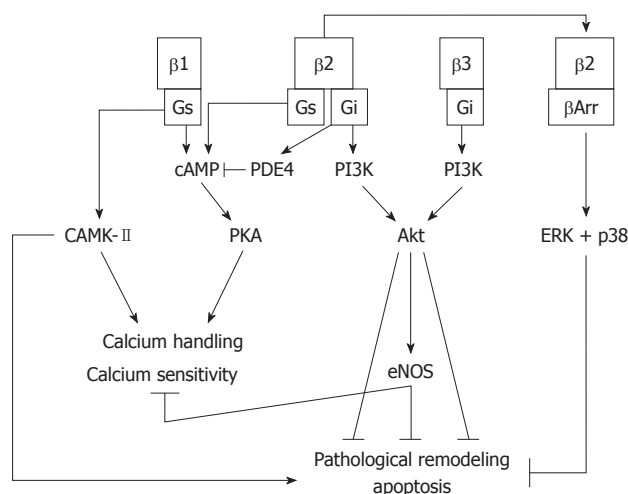


Figure 2 β -adrenergic signaling pathways. β 1-adrenergic receptors activate protein kinase A (PKA), which regulates calcium sensitivity and calcium handling. Prolonged activation of this receptor activates a harmful calmodulin-dependent protein kinase (CAMK)-II pathway which is pro-apoptotic and induces pathological remodeling. β 2-adrenergic receptors also activate PKA, but prolonged activation causes a switch to Gi signaling which activates PDE4, inhibiting cAMP formation, and activates the cardioprotective phosphoinositol-3 kinase (PI3K)/protein kinase B (Akt) pathway. Desensitization of β 2-adrenergic receptors by β -arrestin can recruit p38 and extracellular-signal-regulated kinase (ERK), which protect the cell from apoptosis. β 3-adrenergic receptors produce a negative inotropic effect which is mediated by nitric oxide produced via the PI3K/Akt pathway.

it was unclear whether the underlying cardiac dysfunction was being attenuated or worsened^[74].

We began our investigation of the metabolic effects of metoprolol by investigating its effects on known cardiac fuels. As expected, metoprolol had no effects on plasma glucose levels. To account for the putative ability of β -blockers to suppress lipolysis, we measured circulating plasma lipids. Metoprolol had no effect on plasma free fatty acids, triglycerides or cholesterol. Surprisingly, however, metoprolol attenuated the observed increase in plasma ketone bodies in the diabetic rats^[73]. It is not clear why metoprolol would produce such an effect.

The rate of ketogenesis is determined by the rate of fatty acid delivery to the liver, the rate of fatty acid oxidation in the liver, and the activity of mitochondrial β -hydroxy- β -methylglutaryl-CoA synthase (HMG CoA synthase), which catalyses the first step in ketogenesis. Stimulation of HMG-CoA synthase is the underlying mechanism by which low insulin levels, starvation and low-carbohydrate/low protein/high fat diets increase ketogenesis^[75]. β -adrenergic stimulation is known to increase lipolysis in adipocytes. The effect is mediated partly by hormone-sensitive lipase and partly by a poorly understood alternative pathway^[76]. β -blockers, acting on the same pathway, may decrease lipolysis, thereby decreasing the delivery of fatty acids to the heart. The fact that metoprolol treatment had no effect on circulating plasma lipids argues against a major effect on lipolysis. However, there are two components to lipolysis: a pulsatile release under the control of the sympathetic nervous system

and a basal release which is independent of the sympathetic nervous system^[77]; attenuation of the pulsatile release could be sufficient to reduce ketogenesis without affecting cholesterol synthesis by the liver. Alternatively, the effect could be mediated by inhibition of CPT-1 in the liver. It is also possible that metoprolol increases the peripheral utilization of ketones. However, present evidence suggests that ketone utilization by peripheral tissues is largely unregulated, being determined solely by ketone body supply^[78]. It is therefore difficult to postulate a mechanism by which metoprolol could directly increase the peripheral utilization of ketones. Further studies are needed to investigate the mechanism of this intriguing effect of metoprolol on plasma ketones.

We next measured *ex vivo* fatty acid and glucose metabolism in the heart to explore the acute and chronic effects of metoprolol^[73]. By studying the effects of chronic metoprolol treatment, we hoped to gain insights into the sustained changes in cardiac metabolism that accompany the improvement in cardiac function. By studying the rapid effects of acute metoprolol perfusion, we hoped to gain insights into positive events that could occur *in vivo* immediately following the commencement of treatment, preceding the improvements in function. The pattern of changes we observed in our studies was complex and depended on the disease state and the duration of metoprolol exposure.

The heavy reliance of the diabetic heart on fatty acid oxidation observed in our studies was expected and agrees with previous experimental findings in isolated perfused hearts; palmitate oxidation was markedly increased, glycolysis was decreased by 50% and glucose oxidation was negligible^[79-85]. Surprisingly, however, myocardial energetics, as indicated by tissue adenine nucleotide levels, were not altered. Chronic diabetes is known to be associated with a fall in cardiac ATP production^[86]. It is possible that 6 wk of diabetes is too soon to observe a fall in ATP levels as this is the timepoint at which cardiac dysfunction first appears. Furthermore, we did not observe any activation of AMPK in the diabetic heart. This is consistent with previous reports^[87,88], and a recent study suggested that AMPK activation is prevented by high circulating and tissue lipids^[88]. However, circulating fatty acids were only mildly elevated in our studies.

Contrary to expectations, we observed that chronic metoprolol treatment increased palmitate oxidation and decreased glucose oxidation in control hearts^[73]. However, in diabetic hearts, chronic metoprolol treatment had the expected effect of lowering fatty acid oxidation and increasing glucose oxidation. Before attempting to resolve this apparent paradox, it was important to establish whether the main target of metoprolol was in the fatty acid or in the glucose oxidation pathway. In a preliminary study, we found that chronic metoprolol treatment had no effect on glycolysis, but increased coupling between glucose oxidation and glycolysis in the diabetic heart by increasing glucose oxidation. To determine whether the observed changes in glucose oxidation were direct, or

mediated through the Randle Cycle by direct changes in fatty acid oxidation, we repeated the perfusions in the absence of insulin to reduce glucose uptake and utilization to low levels. When this was done, the effect of metoprolol on glucose oxidation was abolished while the effect on palmitate oxidation was preserved^[73]. This strongly suggests that fatty acid oxidation is the direct target of metoprolol. It also indicates that the effect of metoprolol is independent of insulin.

Short term perfusion with metoprolol inhibited fatty acid oxidation and produced marked stimulation of glucose oxidation in both control and diabetic hearts, and was associated with a decrease in lactate production, reflecting a marked improvement in glycolytic/glucose oxidation coupling, and an increase in tissue ATP levels^[73]. When the perfusions were repeated in the absence of insulin, the effect of metoprolol on glucose oxidation was attenuated in control hearts and abolished in diabetic hearts. However, the effect on palmitate oxidation was preserved^[73]. Once again, this suggests that fatty acid oxidation is the direct target of metoprolol, and inhibition of fatty acid oxidation occurs immediately following exposure to the drug. Further evidence for this was provided by the fact that metoprolol concomitantly inhibited PDC catalytic activity. Any stimulatory effect would therefore have to be mediated by the Randle cycle.

Intriguingly, we found that acute metoprolol perfusion and chronic metoprolol treatment lowered tissue triglyceride levels regardless of whether fatty acid oxidation was increased or decreased. This effect cannot be explained on the basis of fatty acid oxidation changes alone. Indeed, inhibition of CPT-1 by metoprolol in dogs produced an increase in tissue triglycerides^[17]; treatment of rats with CPT-1 inhibitors also increased tissue triglyceride levels^[89]. However, our tissue triglyceride measurements were carried out in hearts that had been perfused *ex vivo*. The heart is known to utilize its endogenous triglyceride pool over the course of an *ex vivo* perfusion, which may partly account for the difference. Nevertheless, inhibition of CPT-1 would be expected to decrease the utilization of fatty acids from all sources, so the decrease in tissue triglyceride levels following metoprolol treatment is unlikely to be attributable to increased triglyceride utilization. It is possible that metoprolol decreases the uptake of fatty acids into the cytoplasm. Uptake of long chain fatty acids into the cytoplasm is known to be stimulated by contraction, but the effect is likely to be mediated by AMPK (which was unaffected in our studies) and PKC isoforms^[90]. Triglyceride levels can be decreased by the secretion of lipoproteins by the heart itself. Indeed, overexpression of apolipoprotein B (apo B) prevents triglyceride accumulation in the diabetic heart^[91,92]. However, there is presently no evidence to suggest that β -adrenoceptors regulate this process.

The β -blocker propranolol was reported to induce an increase in CPT-1 activity in normal Sprague-Dawley rats^[93]. Metoprolol, by contrast, was reported to decrease CPT-1 activity in conscious dogs with micro-embolism-

induced heart failure^[17]. In dogs with pacing-induced heart failure, glucose uptake was improved by carvedilol but not by metoprolol^[94]. However, in clinical studies, metoprolol, carvedilol and bucindolol^[13-15] have all been shown to inhibit fatty acid oxidation. The wide variation in responses reported in the literature reflects the complexity observed in our own studies in which the effect of metoprolol on fatty acid oxidation varied according to the length of exposure to the drug and the disease state.

An increase in diastolic filling increases cardiac work and oxygen consumption in direct proportion *via* the Frank-Starling mechanism. However, in the normal heart, ATP supply is maintained at a steady level regardless of cardiac work or oxygen consumption. This means that cardiac metabolism is driven by cardiac function^[95]. What is less clear, however, is how cardiac function influences cardiac energy substrate selection. It is possible that some of the effects of metoprolol on cardiac metabolism may be attributable to, rather than responsible for, its effects on cardiac function. When palmitate and glucose oxidation rates were normalized to cardiac function, the pattern of changes observed was preserved, and, in the case of palmitate oxidation, even accentuated. However, to fully account for effects of function on metabolism, future studies are needed to investigate whether the effect of metoprolol is preserved in isolated cardiomyocytes, in which the effects of cardiac function and the Frank-Starling mechanism do not apply.

With these studies, we had established that metoprolol acts directly on fatty acid oxidation. We also found that neither diabetes nor chronic metoprolol treatment had any effect on the activities of acyl-CoA dehydrogenase or citrate synthase^[96]. Based on these findings, and previous reports of the effects of β -blockers on CPT-1, we investigated whether the observed effects of metoprolol on fatty acid oxidation are mediated by CPT-1.

CPT-1 ACTIVITY AND REGULATION BY MALONYL COA

Mechanisms linking β -adrenergic signaling with cardiac metabolism had not been investigated in great detail. We therefore employed a combination of “bottom-up” (known rate-limiting enzymes) and “top-down” (known β -adrenoceptor pathways) approaches to unravel the pathways involved. As discussed above, a previous study in microembolism-induced heart failure in rats demonstrated that chronic metoprolol treatment decreased the activity of CPT-1^[17]. In the heart, the major mechanism by which CPT-1 is regulated is through modulation of malonyl CoA levels. Isoproterenol had previously been shown to lower malonyl CoA levels by increasing PKA-mediated phosphorylation of acetyl CoA carboxylase (ACC)^[97]. We therefore hypothesized that β -adrenergic blockade could have the opposite effect, preventing ACC phosphorylation and increasing malonyl CoA levels. A study in isolated cardiomyocytes using activators and in-

hibitors of cAMP revealed that stimulation of fatty acid oxidation by contraction was PKA-dependent^[98].

However, we found that malonyl CoA levels were decreased by metoprolol in control hearts and were unchanged in diabetic hearts. The mechanism of this effect is unclear, because ACC and malonyl CoA decarboxylase (MCD) expression were unchanged, and we found no evidence of changes in AMP-activated protein kinase (AMPK) or PKA-mediated phosphorylation of ACC. Dobutamine, a non-selective β -agonist, was previously found to decrease malonyl CoA levels without an effect on AMPK, ACC or MCD^[99,100]. In addition to the activities of ACC and MCD, malonyl CoA levels are also dependent on the cytosolic supply of acetyl CoA^[100]. Most of the acetyl CoA in the cardiomyocyte is present in the mitochondria^[101], and cytosolic acetyl CoA is derived from peroxisomal β -oxidation, citrate and acetylcarnitine^[102]. Intriguingly, acute inhibition of CPT-1 has been shown to produce a fall in malonyl CoA levels independent of ACC and MCD^[102]. The fall in malonyl CoA levels observed in control hearts could, therefore, have been secondary to the inhibition of CPT-1. It is unclear why such a mechanism would only lower malonyl CoA levels in control hearts. One possibility is that fatty acid oxidation rates, and therefore the acetyl CoA/CoA ratio, are higher in the diabetic heart, and the fall in cytosolic acetyl CoA levels produced by CPT-1 inhibition in this context may not be sufficient to decrease malonyl CoA levels. Metoprolol tended to decrease tissue acetyl CoA levels in our studies, but measurements of the cytosolic and mitochondrial acetyl CoA pools would be required to confirm these speculations. Overall, however, malonyl CoA levels did not correlate with the observed changes in fatty acid oxidation. The action of metoprolol, therefore, could not be explained solely on the basis of malonyl CoA regulation^[73].

We next investigated the effects of metoprolol on CPT-1 itself^[73]. Metoprolol had no effect on CPT-1 catalytic activity or malonyl CoA sensitivity when incubated with CPT-1 *in vitro*. Effects were only seen following perfusion of the isolated working heart with metoprolol or chronic treatment. Metoprolol decreased the maximum capacity of CPT-1 activity as measured *in vitro*. This effect was observed following both short-term perfusion with metoprolol and chronic metoprolol treatment, and was seen in both control and diabetic hearts. Since allosteric effects are lost during sample preparation, these effects could only be explained by a decrease in CPT-1 expression or a covalent modification. Surprisingly, metoprolol also decreased the sensitivity of CPT-1 to malonyl CoA. To our knowledge, this was the first demonstration that regulation of CPT-1 sensitivity occurs in the heart. Long-term changes in CPT-1 catalytic activity and malonyl CoA sensitivity were previously believed to occur only in the liver^[103,104].

Taken together, the time and disease-dependent changes in fatty acid oxidation can be described as fol-

lows. In control hearts, acute metoprolol perfusion causes malonyl CoA levels to fall. The sensitivity of CPT-1 to malonyl CoA decreases, and the activity of CPT-1 is markedly decreased. With chronic treatment, malonyl CoA levels remain low but the sensitivity of CPT-1 to malonyl CoA is restored and the inhibition of CPT-1 activity is less marked. Fatty acid oxidation is therefore inhibited following acute exposure to the drug, but this effect is lost with time. In diabetic hearts, acute metoprolol perfusion markedly reduces CPT-1 activity. With chronic treatment, this reduction is sustained and produces inhibition of fatty acid oxidation despite a concomitant decrease in malonyl CoA sensitivity. The major determinants of the fatty acid oxidation rate are CPT-1 activity and malonyl CoA levels. Using metabolic control analysis, it has been shown that CPT-1 only becomes rate-limiting when its activity is inhibited by approximately 50%^[105]. Consistent with this observation, in our studies, fatty acid oxidation was always inhibited if CPT-1 activity was inhibited by approximately 50%. The observed changes in CPT-1 sensitivity would be expected to increase flux through CPT-1; however, they may represent a fine tuning mechanism of the system since at no point do they hold sway over the overall fatty acid oxidation rate.

Both CPT-1A and CPT-1B are present in the heart^[106,107]. The net IC₅₀ of malonyl CoA in the heart is intermediate between the high sensitivity of CPT-1B and the low sensitivity of CPT-1A; in our studies, the IC₅₀ of control hearts was approximately 30 μ mol/L malonyl CoA^[73,108]. Catalytic activity and malonyl CoA sensitivity could change for several reasons. Firstly, total CPT-1 expression could be altered. Secondly, isoform switching between CPT-1A and CPT-1B could alter sensitivity; the fetal heart expresses CPT-1A, and CPT-1B expression is asserted during development, eventually becoming the major isoform^[107]. However, CPT-1 isoform switches in the adult heart have not been reported. Finally, two splicing variants of CPT-1 have been identified in the heart and are predicted to be malonyl CoA-insensitive^[109,110].

The N- and C-termini of CPT-1 both face the cytosol, separated by a loop region inserted into the outer mitochondrial membrane which contains two membrane spanning domains. The C-terminus is the catalytic region, and residues which regulate malonyl CoA sensitivity have been found within the C-terminus, the N-terminus and the loop region^[111-114]. In the liver, regulation of CPT-1A sensitivity is more important than regulation of malonyl CoA levels, and has been attributed to regulation by cytoskeletal elements^[115], changes in the membrane environment^[116] and direct phosphorylation of CPT-1^[117]. Peroxynitrite-mediated nitration of CPT-1B has been shown to decrease CPT-1B catalytic activity following endotoxemia in the heart^[118]. However, no other covalent modifications of CPT-1B have been identified. We therefore pursued two lines of enquiry. Firstly, we investigated the effects of chronic metoprolol treatment on CPT-1A and CPT-1B expression. Secondly,

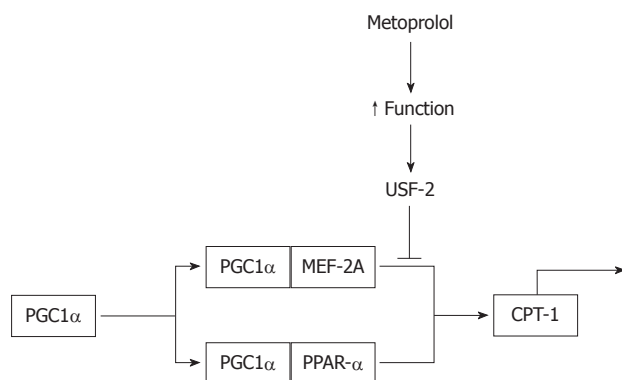


Figure 3 Repression of carnitine palmitoyltransferase-1 by metoprolol. Improved contractile function in rat heart leads to stimulation of upstream stimulatory factor (USF)-2, which represses PGC1- α transcriptional complex and reduces the expression of carnitine palmitoyltransferase (CPT)-1. PPAR: Peroxisome proliferator-activated receptor.

we investigated the effects of short-term metoprolol perfusion and chronic metoprolol treatment on CPT-1B covalent modifications.

REGULATION OF CPT-1 EXPRESSION

Chronic metoprolol treatment decreased total CPT-1 expression in rat heart, and this was attributable to a decrease in CPT-1B expression. The decrease was only seen in diabetic hearts and was associated with a modest increase in CPT-1A expression which provides a partial explanation for the decrease in CPT-1 malonyl CoA sensitivity. The expression of CPT-1 is controlled by peroxisome proliferator-activated receptor (PPAR)- α , but the PPAR- α /RXR complex produces only modest induction of CPT-1 when acting alone^[119-121]. PGC1 α greatly enhances CPT-1 induction by PPAR- α , but can also induce CPT-1 independently by binding to MEF-2A^[122]. PGC1 α -mediated expression of CPT-1 has been shown to be repressed in isolated cardiomyocytes by upstream stimulatory factor (USF)-2. USFs are transcription factors of the basic helix-loop-helix leucine zipper family which bind to the E-box consensus sequence CANNTG^[123]. In the heart, USFs are involved in excitation-transcription coupling, responding to sustained increases in electrical stimulation by increasing the expression of sarcomeric genes such as sarcomeric mitochondrial creatine kinase and MHC^[124,125].

PGC1 α occupancy of the CPT-1 promoter was increased in diabetes and decreased by metoprolol treatment; this was associated with increased binding of USF-2 to PGC1 α ^[96]. The data obtained to date only establish associative effects, but we proposed the following model. In control hearts, USF-2 maintains a constant level of tonic repression of CPT-1 expression, and CPT-1 expression is modulated through the activation of PGC1 α and PPAR- α . This produces modest changes in CPT-1 expression. Even though USF-1 and 2 expression are increased by metoprolol in control hearts, MHC

expression is unaffected, indicating that USF activity is unchanged. In the diabetic heart, USF expression, and USF activity as indicated by MHC expression, are both decreased in the diabetic heart, and metoprolol increases USF expression and activity. The result is that tonic repression of PGC1 α by USF-2 is lost in the diabetic heart, and restoration of USF-2 repression produces marked changes in CPT-1 expression (Figure 3). The role of USF-2 could be tested in transgenic mice with USF-2 knockout targeted to the heart, or alternatively in a conditional knockout model. Alternatively, USF-2 could be silenced using an interfering RNA approach. If USF-2 mediates repression of CPT-1 by metoprolol, the effect would be attenuated or lost following USF-2 knockout and mimicked by USF-2 overexpression. Furthermore, we would expect USF-2 knockout to be associated with an increase in CPT-1 expression. We were able to demonstrate that both USF-2 and MEF-2A co-immunoprecipitate with PPAR- α , suggesting that PGC1 α , PPAR- α , MEF-2A and USF-2 could form a single transcriptional complex.

Moore *et al.*^[122] demonstrated that PGC1 α /myocyte enhancing factor (MEF) 2A-dependent induction of CPT-1 was repressed by USF-2 in isolated rat cardiomyocytes. We have demonstrated that binding of USF-2 to PGC1 α occurs in the heart with the native proteins, and that this is associated with functionally significant repression of CPT-1 expression. Our results suggest that binding of USF-2 can be induced by the transcriptional activation of USF-2 itself, since USF-2 binding to PGC1 α always changed in the same direction as USF activity. USF is activated by increases in electrical stimulation^[125]. It is therefore likely that activation of USF by metoprolol is mediated by the increase in electrical stimulation that accompanies the improvement in function; this explains why the effect is only seen in the diabetic heart. Function did not change in control hearts. However, the more global regulation of the PGC1 α transcriptional complex observed in our studies is not explicable solely on the basis of USF binding. The decrease in PGC1 α association with PPAR- α and MEF2A could be an indirect effect of the acute changes in fatty acid metabolism. However, it is more likely that active regulation of the complex is occurring. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) increases both PGC1 α /PPAR- α coactivation and downstream signaling to PGC1 α and PPAR- α targets^[126-129]. It has been suggested that phosphorylation by p38 MAPK may serve to integrate and coordinate contractile and metabolic gene expression^[122].

We found that the association of MEF-2A with the CPT-1 promoter was obliterated by both metoprolol and diabetes. In diabetes, this may reflect generalized repression of MEF-2A targets, which is known to occur. With metoprolol treatment, it may represent sequestration of MEF-2A to higher priority gene targets. Alternatively, the decreased association could be due to covalent modifications of MEF-2A or PGC1 α , perhaps mediated by p38,

or competition between PPAR- α and MEF-2A for their consensus sites, which are known to overlap. Activation of β 2-adrenoceptors in the heart has been shown to increase signaling through the p38 MAPK pathway^[130]. It is therefore possible that metoprolol decreases p38 phosphorylation by blocking β 2-adrenoceptors, leading to a decrease in the association of PGC1 α with its coactivators. Further studies are required to investigate the role of stress-kinase signaling in the regulation of the PGC1 α transcriptional complex.

We investigated whether metoprolol influenced covalent modifications of PGC1 α ^[96]. Metoprolol, by promoting glucose oxidation, reduced pyruvate levels which in turn led to a decrease in the binding of the pyruvate-activated deacetylase SIRT-1 to PGC1 α . The binding of the acetylase p300 was increased by metoprolol. Both of these changes would have been expected to result in an increase in the acetylation state of PGC1 α . The opposite effect was observed; PGC1 α acetylation was decreased! We speculate that USF-2 interferes with the acetylation reaction, because the acetylation site is very close to the USF-2 binding region. Decreased acetylation may be another mechanism of the repressive effect of USF-2. We also investigated the phosphorylation of PGC1 α by PKA, and found, surprisingly, that this was increased by metoprolol in the diabetic heart. We have no explanation for this effect, except to speculate that PGC1 α is a low priority target of PKA and is only available to it when is not interacting with its primary targets.

α -MHC expression is decreased as part of the fetal gene program induction. In the diabetic heart, a fall in both α -MHC and SERCA expression was observed, both of which were improved by metoprolol^[73]. This improvement in fetal gene program expression is consistent with what is known about the mechanism of action of β -blockers. α -MHC is regulated by USFs, while SERCA has been shown to be induced by MEF-2A^[131] and is proposed to be induced by PPAR- α ^[132]. It is therefore possible that the PGC1 α /PPAR α /MEF2A/USF complex may be able to prevent or reverse the induction of at least some components of the fetal gene program. In other words, improvement of gene expression and modulation of cardiac metabolism could occur in parallel as a result of modulation by the same transcriptional complex.

NO/RNS - INDUCED COVALENT MODIFICATIONS OF CPT-1

In recent years, there has been increasing interest in the ability of NO and its associated RNS to directly regulate protein function in a similar manner to phosphorylation. Residues that are targeted by NO and RNS are cysteine, methionine and tyrosine^[133,134]. The unique redox chemistry of protein thiol groups confers specificity and reversibility to thiol covalent modifications. The attachment of NO to thiol groups on critical cysteine residues within a protein, termed S-nitrosylation, is a major mechanism by which NO acts as a signaling molecule. Intriguingly, there

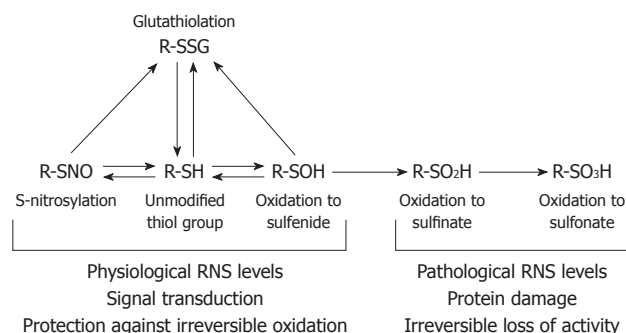


Figure 4 Nitric oxide and reactive nitrogen species-mediated modifications of thiol residues. Thiol (SH) residues undergo a series of reversible modifications in response to changes in the redox potential or exposure to physiological levels of reactive nitrogen species or nitric oxide. Oxidation of the thiol to the corresponding sulfenic acid or the formation of a disulfide bond between the thiol and glutathione (glutathiolation) are reversible either by changes in the equilibrium, or enzymatic restoration of the thiol group by thiol transferases. Further oxidation of a glutathiolated residue is not possible, so glutathiolation confers protection against oxidative damage for as long as it persists. However, exposure of the thiol group or the sulfenic acid to pathological levels of reactive nitrogen or oxygen species results in the formation of sulfinate and then sulfonate; these are irreversible modifications which result in protein damage and loss of activity. RNS: Reactive nitrogen species. Modified from: Figure 2, Klatt *et al.*^[133].

is a consensus sequence, analogous to kinase consensus sequences, which confers site specificity on NO-mediated thiol modifications^[135]. Furthermore, S-nitrosylation is a reversible reaction, and a number of enzymatic and non-enzymatic reactions have been identified which can remove NO from cysteine thiols^[136-138]. S-nitrosylation activates guanylate cyclase, the classical NO target. The list of targets proposed to be regulated by S-nitrosylation is growing, and, in the heart, includes GAPDH and SERCA^[139].

Reversible oxidation or nitrosylation of thiol groups is mediated by physiological levels of NO and RNS, and typically produces the following reversible modifications: S-nitrosylation (addition of NO), glutathiolation (formation of mixed disulfides between the thiol group and glutathione) or oxidation from thiol to sulfenate. Any of these modifications can regulate protein function, but glutathiolation and S-nitrosylation have been most frequently implicated in the regulation of enzyme activity^[133]. Higher levels of RNS induce further oxidation of the sulfenate (one oxygen) to sulfinate (two oxygens) and sulfonate (three oxygens). This is toxic, causing irreversible loss of function. Glutathiolation, by committing the thiol to an alternate reaction pathway, protects critical thiol residues against irreversible oxidation^[133] (Figure 4).

Tyrosine nitration is classically regarded as an inhibitory modification. However, some proteins are activated by tyrosine nitration including cytochrome C, fibrinogen and PKC^[140-143]. As with thiol modification, tyrosine nitration also exhibits site-specificity^[144]. Tyrosine nitration is frequently used as a biomarker of peroxynitrite^[140], and we also used it as such. Previous studies have demonstrated that incubation of CPT-1 with continuous peroxynitrite, NO or hydrogen peroxide producing systems produces a decrease in CPT-1 activity which is associated

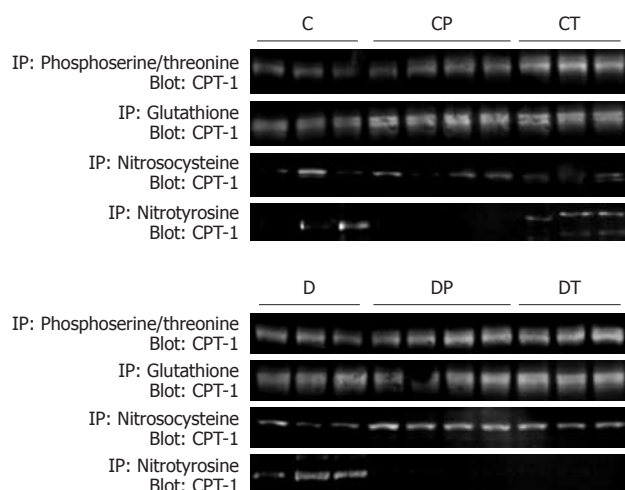


Figure 5 Covalent modifications of carnitine palmitoyltransferase-1 measured by immunoprecipitation. Rat heart samples underwent immunoprecipitation with combined pan-specific anti-phosphoserine and anti-phosphothreonine antibodies, anti-glutathione antibodies or anti-nitrosocysteine antibodies, followed by immunoblotting with pan-specific carnitine palmitoyltransferase (CPT)-1 antibodies. Densitometric analyses of these data are presented in Figure 6. C: Control, $n = 5$; CP: Control perfused with metoprolol, $n = 5$; CT: Control treated with metoprolol, $n = 5$; D: Diabetic, $n = 5$; DP: Diabetic perfused with metoprolol, $n = 5$; DT: Diabetic treated with metoprolol, $n = 5$.

with tyrosine nitration^[145]. Furthermore, endotoxemia produced inhibition and nitration of CPT-1 in suckling rats^[118].

Tyrosine nitration of CPT-1 by peroxynitrite has been shown to inhibit CPT-1 activity following endotoxemia^[118]. Furthermore, superoxide, NO and peroxynitrite were all shown to inhibit CPT-1 activity *in vitro* when CPT-1 was co-incubated with systems which continuously generated these reactive oxygen and nitrogen species^[145]. This suggests that CPT-1 can be regulated by covalent modifications mediated by nitrogen species. It is likely that, in addition to tyrosine nitration, cysteine nitrosylation and glutathiolation also occur. Indeed, cysteine-scanning mutagenesis of the muscle isoform of CPT-1 revealed that cysteine 305 was important for catalysis; nitrosylation or glutathiolation of this residue could conceivably increase or decrease the catalytic activity of the enzyme.

Cysteine-scanning mutagenesis of CPT-1 revealed that cysteine 305 is critical for catalytic activity of the enzyme^[146]. We therefore tested whether nitrosylation or glutathiolation of cysteine residues, or nitration of tyrosine residues, inhibits CPT-1 activity. To test the effects of the modifications *per se* on CPT-1 activity, we incubated isolated mitochondria from rat heart with increasing concentrations of peroxynitrite ranging from 100 nmol/L to 1 mmol/L. At neutral pH, peroxynitrite is rapidly degraded, but even brief exposure to peroxynitrite is sufficient for it to induce the full range of its target modifications. We also incubated mitochondria with a peroxynitrite-generating system. Finally, we used dithiothreitol to remove the covalent modifications induced by peroxynitrite. We found that CPT-1 was stimulated by peroxynitrite at low

physiological levels but inhibited at high levels, and that peroxynitrite could induce tyrosine nitration, cysteine nitrosylation and cysteine glutathiolation. By a process of elimination, we found that activation of CPT-1 was most consistently associated with glutathiolation of CPT-1B. We hypothesized that the key residue involved was cysteine 305, but our efforts to confirm this by mass spectroscopy (MS) were unsuccessful^[108].

We also successfully detected cysteine-nitrosylation, glutathiolation and nitration of CPT-1 in whole rat heart homogenates. Acute metoprolol perfusion increased nitrosylation and glutathiolation in diabetic hearts, but decreased tyrosine nitration in both control and diabetic hearts (Figures 5-7). In control hearts, nitrosylation was low and glutathiolation increased only following chronic treatment. An increase in CPT-1 glutathiolation would be expected to increase CPT-1 activity based on our *in vitro* studies. The change in CPT-1 activity cannot therefore be explained on the basis of CPT-1 glutathiolation. Furthermore, the mechanism of the changes in CPT-1 nitrosylation, glutathiolation and nitration is not explicable on the basis of cell-wide changes in NO and RNS production, because the observed patterns in systemic NO/RNS and CPT-1 covalent modifications do not match. There is a mitochondrial isoform of NOS (mtNOS), but NO and peroxynitrite produced by mtNOS affect targets within the mitochondrial matrix and the inner mitochondrial membrane^[147]. CPT-1 predominantly faces the cytosol, so it is likely that regulation of CPT-1 by NO/RNS is mediated by eNOS and possibly iNOS. eNOS has been proposed to translocate to the mitochondria^[148,149]; mitochondrial eNOS translocation could therefore be a major determinant of NO/RNS mediated effects on CPT-1.

PHOSPHORYLATION OF CPT-1

Phosphorylation of CPT-1A has been demonstrated *in vitro*^[117], and the stimulation of CPT-1 by okadaic acid in rat hepatocytes was prevented by a specific inhibitor of CAMK II, indicating that CAMK II is involved in stimulation of CPT-1A activity^[150]. Activation of the sympathetic nervous system centrally by cerulenin was found to stimulate CPT-1B activity in soleus muscle within 3 h^[151]. This effect must have been mediated by an as yet unidentified covalent modification of CPT-1B; the modification in question could conceivably be phosphorylation. It is possible that phosphorylation of CPT-1 requires the presence of other proteins present on or recruited to the outer mitochondrial membrane. Compartmentalization of PKA signaling in the cardiomyocyte is achieved in part by the action of A-kinase anchoring proteins (AKAPs), a group of proteins which bind to PKA targets in order to regulate PKA-dependent phosphorylation of those targets^[152]. Three mitochondrial AKAPs have been identified - AKAP121, D-AKAP-1 and AKAP149 - but functional studies of their role in the heart are awaited^[152]. It is possible that mitochondrial AKAPs mediate, and are

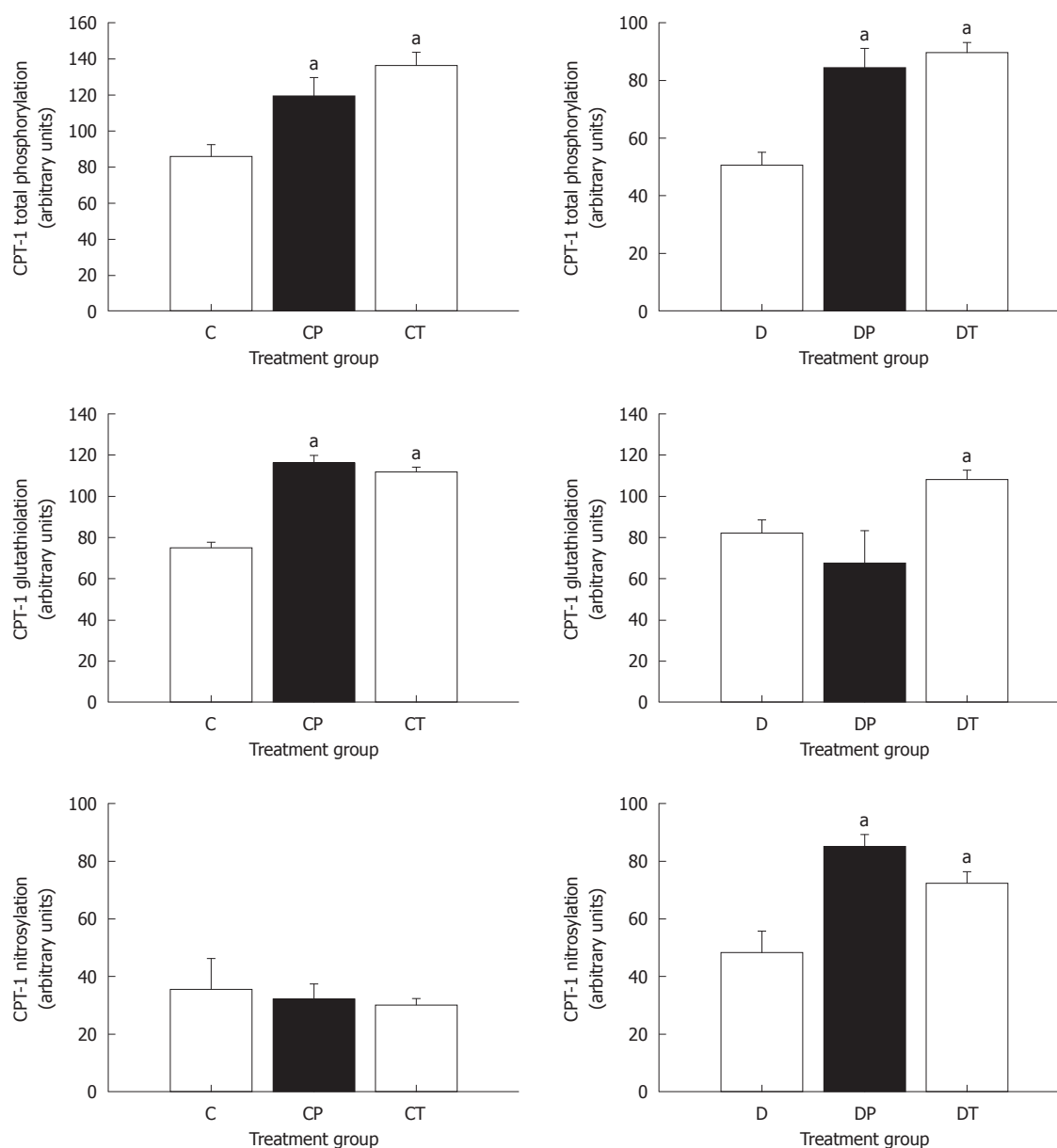


Figure 6 Densitometric analysis of carnitine palmitoyltransferase-1 covalent modifications. Data represent mean \pm SE. Data were analyzed using one-factor analysis of variance with the Neumann-Keuls *post hoc* test. *Significantly different from C or D ($P < 0.05$). CPT: Carnitine palmitoyltransferase; C: Control, $n = 5$; CP: Control perfused with metoprolol, $n = 5$; CT: Control treated with metoprolol, $n = 5$; D: Diabetic, $n = 5$; DP: Diabetic perfused with metoprolol, $n = 5$; DT: Diabetic treated with metoprolol, $n = 5$.

even essential for, CPT-1 phosphorylation. This possibility has never been investigated.

We speculated that the reason phosphorylation of CPT-1B had never been reported is because the kinases involved require other mediators to be present in order to bind their targets. This was our rationale for using isolated mitochondria rather than purified enzyme preparations which are usually used for investigating enzyme phosphorylation. When PKA was incubated with isolated mitochondria, it bound and phosphorylated CPT-1A, and increased the binding of AKAP-149; the functional effect was a decrease in CPT-1 sensitivity without any effect on catalytic activity.

When CAMK-II was incubated with isolated mitochondria, it bound and phosphorylated CPT-1B; however,

the functional effect in this case was an increase in CPT-1 sensitivity without any effect on catalytic activity^[108]. Our mitochondrial isolation produced a variation in the initial sensitivity of CPT-1, most likely produced by membrane effects. This unintended effect revealed that the magnitude of the increase in malonyl CoA sensitivity depended on the initial sensitivity. In other words, CAMK-II tended to restore CPT-1 sensitivity to a “set-point” represented approximately by an IC_{25} of 20 $\mu\text{mol/L}$, an IC_{50} of 100 $\mu\text{mol/L}$ and an IC_{75} of 150 $\mu\text{mol/L}$ malonyl CoA. In this case, the scaffolding protein turned out to be α -actinin, whose binding was decreased by CAMK-II phosphorylation of CPT-1. It is unlikely that α -actinin is anchored in the mitochondrial membrane. CPT-1B probably forms a point of attachment of the cytoskel-

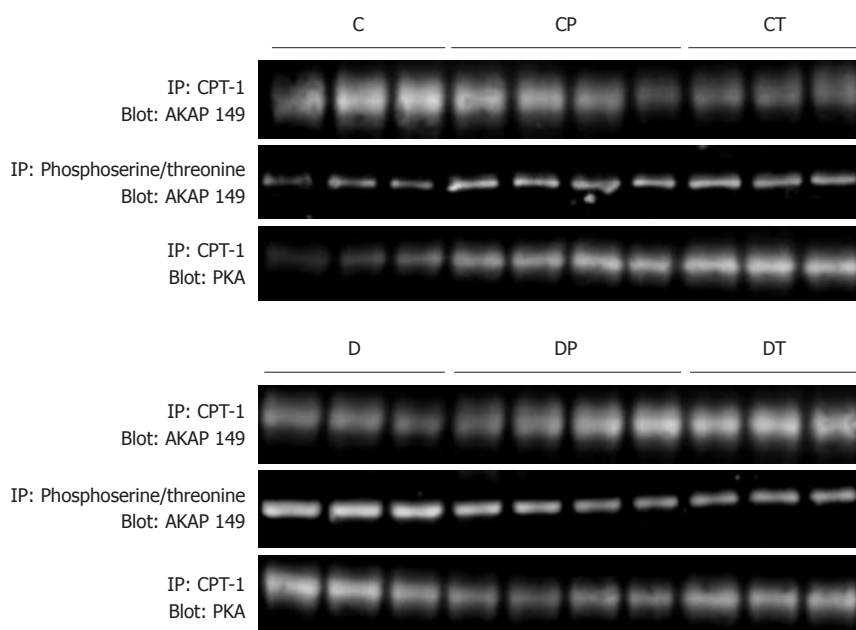


Figure 7 Co-immunoprecipitation of protein kinase A and A-kinase anchoring protein-149 with carnitine palmitoyltransferase-1, and phosphorylation state of A-kinase anchoring protein-149. Densitometric analyses of these data are presented in Figure 8. AKAP: A-kinase anchoring protein; PKA: Protein kinase A; CPT: Carnitine palmitoyltransferase; C: Control, $n = 5$; CP: Control perfused with metoprolol, $n = 5$; CT: Control treated with metoprolol, $n = 5$; D: Diabetic, $n = 5$; DP: Diabetic perfused with metoprolol, $n = 5$; DT: Diabetic treated with metoprolol, $n = 5$.

eton to the mitochondria. We speculate that AKAP-149 and α -actinin restrict access to the malonyl CoA binding site, and changes in the strength of their association with CPT-1 isoforms produce changes in their malonyl CoA sensitivity.

The MAPK p38 bound and phosphorylated CPT-1B *via* the scaffolding protein JIP-2, stimulating CPT-1 catalytic activity without affecting malonyl CoA sensitivity^[108]. By contrast, Akt did not bind or phosphorylate CPT-1, and had no effect on the activity or sensitivity of the enzyme.

We were able to confirm the associations of the kinases and their scaffolding proteins in rats *in vivo* using rigorous colocalization studies^[108]. We then examined the effects of metoprolol on the associations. Both acute metoprolol perfusion and chronic metoprolol treatment increased the total phosphorylation state of CPT-1 (Figures 1 and 2). However, when PKA and CAMK-II interactions with CPT-1 were examined in greater detail, an intriguing pattern emerged (Figures 8 and 9). Firstly, we found, for the first time, that PKA and CAMK-II physically associate with CPT-1. Furthermore, we also found that AKAP-149 also physically associates with CPT-1 and appears to mediate PKA binding. AKAPs bind the regulatory subunit of PKA, and activation of PKA occurs following release of the catalytic subunit. The PKA antibodies we used in our studies recognize the catalytic subunit of PKA. Phosphorylation of AKAP-149 was always associated with a decrease in AKAP-149/CPT-1 association and an increase in PKA/CPT-1 binding. Conversely, loss of AKAP-149 phosphorylation was always associated with an increase AKAP-149/CPT-1 binding and a decrease in PKA/

CPT-1 binding. Based on these findings, we propose the following scheme. PKA is targeted to the mitochondria by AKAP-149. Phosphorylation of AKAP-149 by PKA causes AKAP-149 to disassociate from CPT-1, enabling PKA to bind and phosphorylate CPT-1. The pattern of changes differed between control and diabetic hearts; in control hearts, the association between AKAP-149 and CPT-1 was decreased by chronic metoprolol treatment, whereas in diabetic hearts, it was increased by both acute metoprolol perfusion and chronic metoprolol treatment. It is clear that the compartmentalization and response of PKA and its target proteins is markedly different in the diabetic state. CAMK-II binding followed a different pattern, consistently decreasing in response to any duration of metoprolol exposure. There are other mitochondrial AKAPs which could mediate similar effects as AKAP-149: for example, AKAP-121 has also been shown to target PKA to mitochondria^[153].

The effects of metoprolol, and the basal state of the system, differed between control and diabetic hearts. In control hearts, acute metoprolol perfusion and chronic metoprolol treatment increased PKA binding to CPT-1. Chronic metoprolol treatment also modestly decreased CAMK-II binding to CPT-1. In diabetic hearts, PKA-binding to CPT-1 was decreased by metoprolol, but CAMK-II binding was also decreased, and, following chronic treatment, the decrease in CAMK-II binding was marked. Taken together with our findings in isolated mitochondria, these data explain the sensitivity changes produced by metoprolol; metoprolol increases PKA-mediated desensitization in control hearts, and decreases CAMK-II-mediated sensitization in diabetic hearts. Acute metoprolol perfusion, but not chronic treatment,

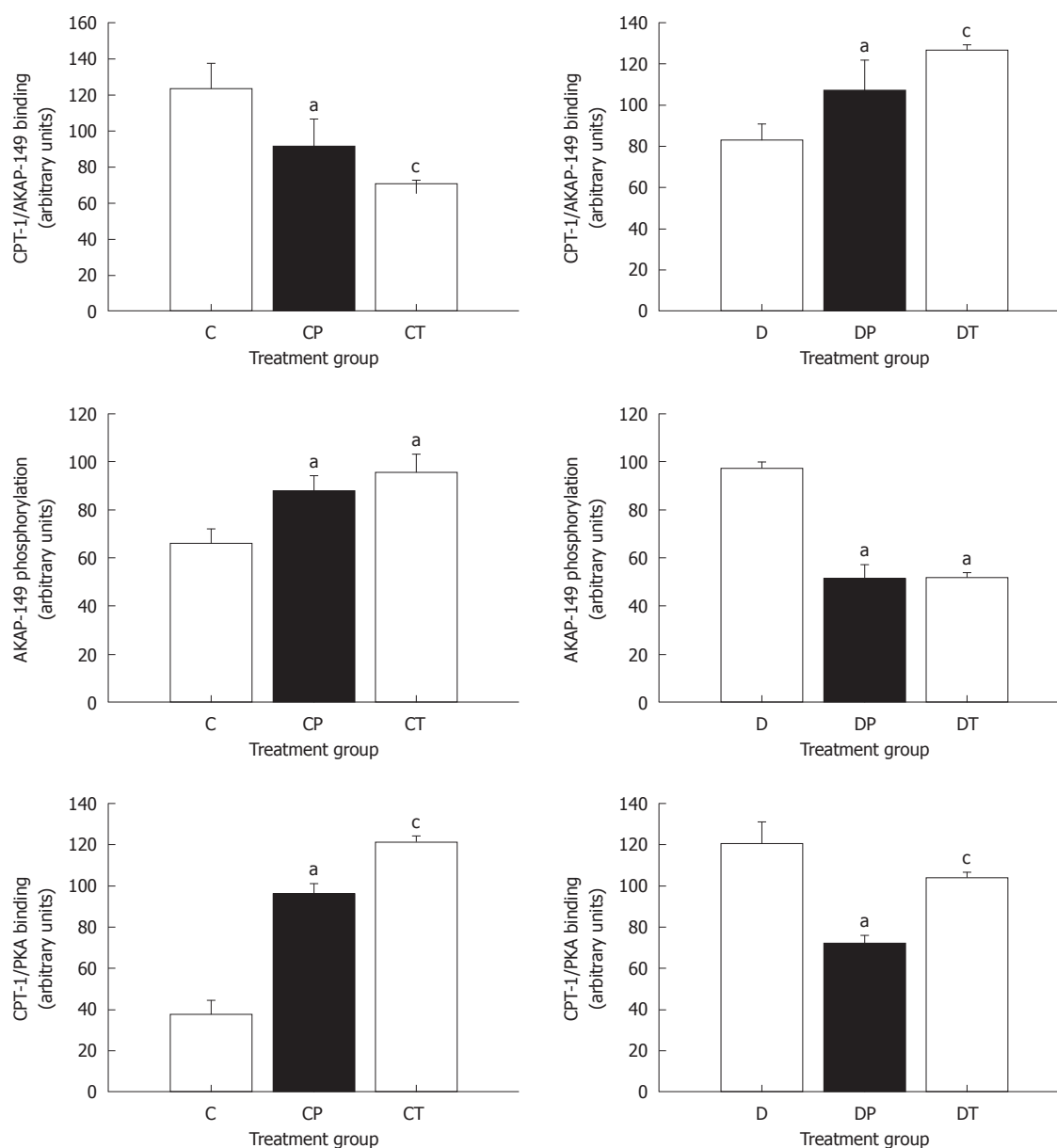


Figure 8 Densitometric analysis of the binding of protein kinase A and A-kinase anchoring protein-149 to carnitine palmitoyltransferase-1, and phosphorylation state of A-kinase anchoring protein-149. Data represent mean \pm SE. Data were analyzed using one-factor analysis of variance with the Neumann-Keuls *post hoc* test. AKAP: A-kinase anchoring protein; PKA: Protein kinase A; CPT: Carnitine palmitoyltransferase; C: Control; CP: Control perfused with metoprolol; CT: Control treated with metoprolol; D: Diabetic; DP: Diabetic perfused with metoprolol; DT: Diabetic treated with metoprolol. *Significantly different from C or D; ^aSignificantly different from C and CP or D and DP ($P < 0.05$).

abolished the association of p38 with CPT-1; this may explain why acute metoprolol perfusion inhibits CPT-1 activity in control hearts. However, the association of p38 with CPT-1 was abolished in the diabetic heart. It is noteworthy that the binding of PKA, CAMK-II and p38 to CPT-1 bear no relation to the overall activities of these kinases measured in the whole heart. It is the translocation of these kinases to the mitochondria which is crucial. The mechanisms by which β -adrenoceptors might regulate such a translocation process are unknown and require further investigation. Clearly the compartmentalization and regulation is altered in the setting of diabetes.

Having confirmed that functionally significant phosphorylation of CPT-1 occurs, we attempted to identify

specific phosphorylation sites on CPT-1. Consensus sites were identified for PKA, CAMK I, CAMK II and p38 MAPK, so we proceeded to use liquid chromatography-tandem MS (LC-MS/MS) to examine phosphorylation of these sites. Identifying phosphorylation events is challenging due to the labile nature of the modification and the overwhelming number of peptides generated by tryptic digestion. In order to maximize our chances of finding the phosphorylation sites, we used many different methods to purify the isolate and digest the sample. Although coverage of CPT-1 was sufficient to identify the presence of the protein, it was not sufficient to examine the phosphorylation sites of interest; all the sites except one were missed. Several factors may account for this.

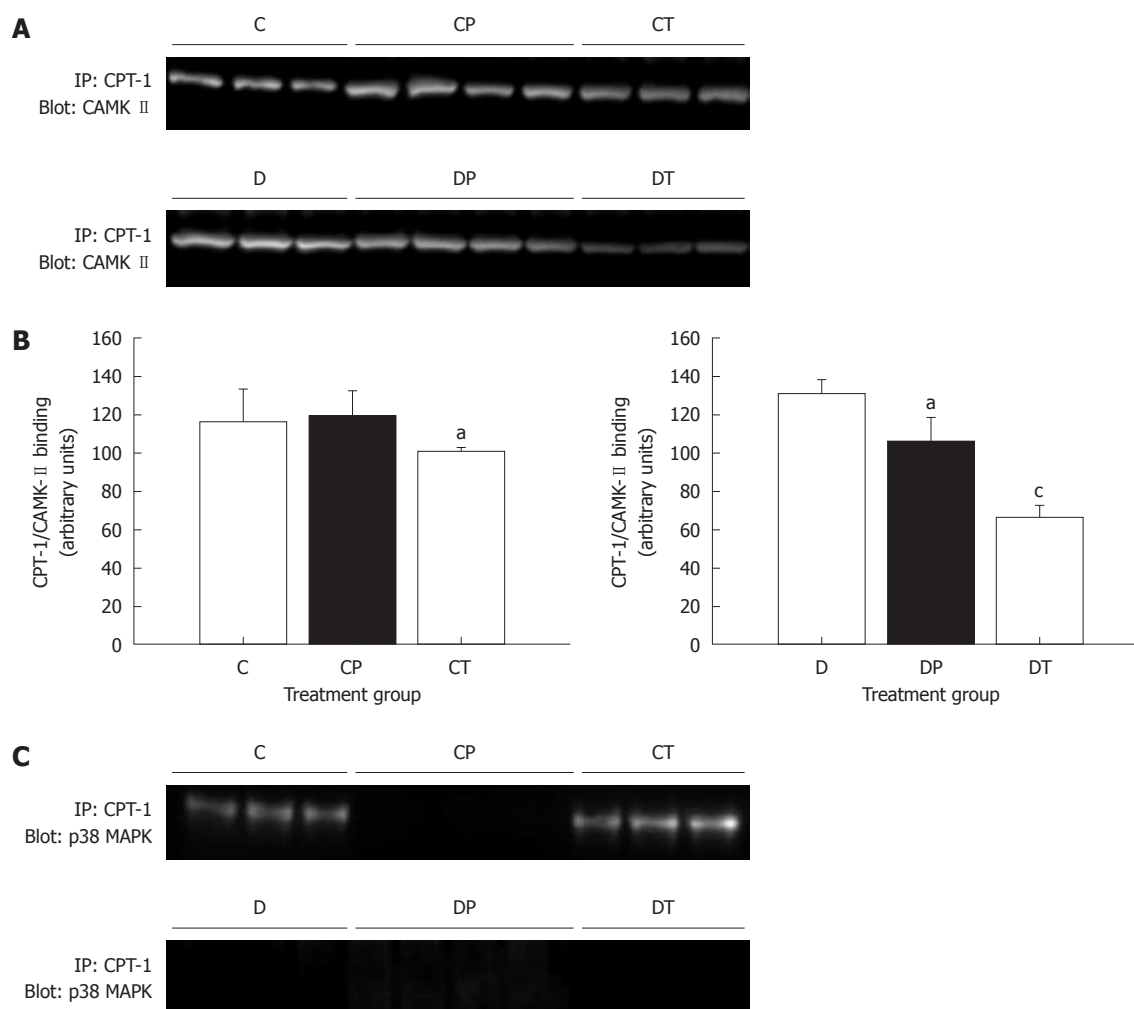


Figure 9 Binding of calmodulin-dependent protein kinase-II and p38 MPK carnitine palmitoyltransferase-1. Data represent mean \pm SE. Data were analyzed using one-factor analysis of variance with the Neumann-Keuls *post hoc* test. CAMK: Calmodulin-dependent protein kinase; CPT: Carnitine palmitoyltransferase; MAPK: Mitogen-activated protein kinase; C: Control; CP: Control perfused with metoprolol; CT: Control treated with metoprolol; D: Diabetic; DP: Diabetic perfused with metoprolol; DT: Diabetic treated with metoprolol. *Significantly different from C or D; ^aSignificantly different from C and CP or D and DP ($P < 0.05$).

CPT-1 abundance in the whole-cell homogenate may have been too low. Also, there were relatively few trypsin cutting sites on CPT-1. Many of the CPT-1 peptides could have been too large to be eluted from the column during the LC-MS/MS procedure (peptides over 2500 Da in mass are retained). We attempted to use an alternative cutting agent, but this rendered CPT-1 undetectable. In rat livers, 99% coverage of CPT-1 has been achieved, but the amount of starting material required could never be feasibly obtained from rat hearts. Forced overexpression of CPT-1 would be another approach, but CPT-1 only has catalytic activity if it is correctly folded and inserted into the mitochondrial membrane. The interactions of CPT-1 with kinases and scaffolds would probably not be preserved in forced overexpression. These difficulties have hampered efforts to study CPT-1 for many years.

Overall, changes in CPT-1 sensitivity produced by metoprolol could be explained by decreased CAMK-II phosphorylation of CPT-1 and/or increased phosphorylation of CPT-1 by PKA and are mediated by changes in the as-

sociation of CPT-1 with scaffolding proteins (Figure 10). The acute decrease in CPT-1 catalytic activity in control hearts could be due to obliteration of the CPT-1/p38 interaction. However, there is no explanation for the acute decrease in CPT-1 catalytic activity seen in the diabetic heart. The association with p38 MAPK was obliterated, and glutathiolation of CPT-1 was increased. The effects of the covalent modifications identified *in vitro* may not reflect their effects *in vivo*. Also, it is possible that the covalent modifications we identified produce more complex and varied effects on CPT-1 when acting together as opposed to in isolation. It is also possible that there are other mechanisms for regulating CPT-1 catalytic activity which have not yet been identified.

β -ADRENOCEPTOR SIGNALING PATHWAYS: NO AND SURVIVAL SIGNALING

Consistent with previous reports, diabetes produced a decrease in β 1-adrenoceptor expression and a marked in-

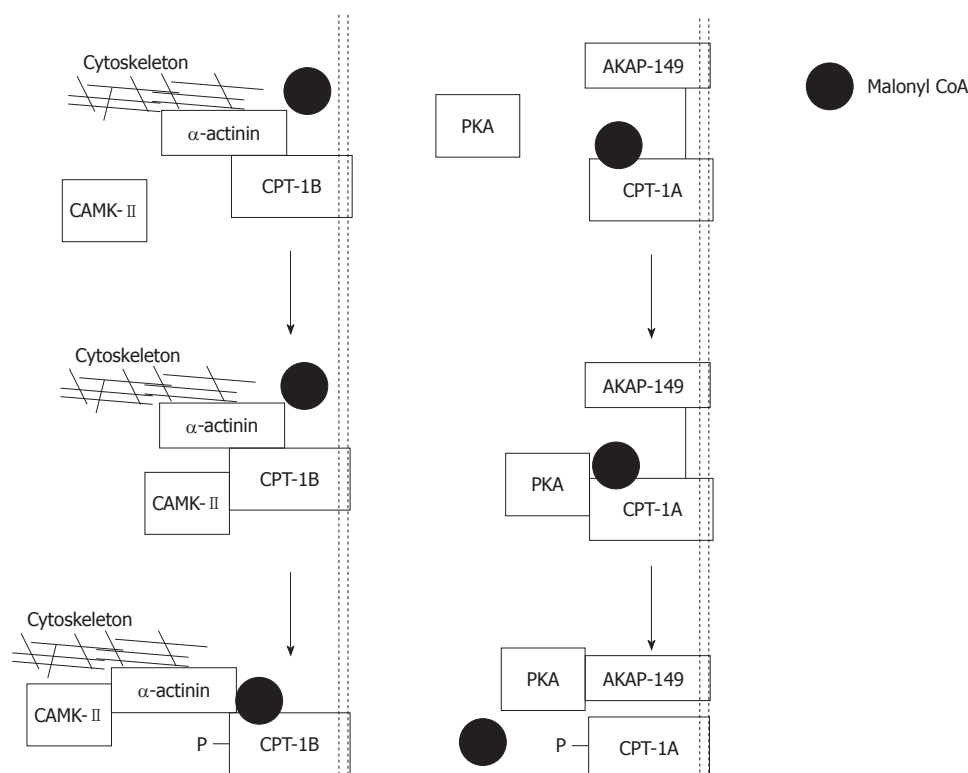


Figure 10 Proposed model of the actions of protein kinase A and calmodulin-dependent protein kinase-II. Left panel: Exogenously applied protein kinase A (PKA) phosphorylates carnitine palmitoyltransferase (CPT)-1A and is then captured by its scaffolding protein. Phosphorylation produces a conformational change tightening the interaction between A-kinase anchoring protein (AKAP)-149 and CPT-1A. As a result, malonyl CoA is denied access to its binding site, and the sensitivity of CPT-1 to malonyl CoA is reduced. Note that CPT-1 and AKAP-149 are both anchored in the mitochondrial membrane; Right panel: Exogenously applied calmodulin-dependent protein kinase (CAMK)-II phosphorylates CPT-1B and is then captured by its scaffolding protein α -actinin. Phosphorylation produces a conformational change, loosening the interaction between α -actinin and CPT-1B. As a result, malonyl CoA has improved access to its binding site and the sensitivity of CPT-1 to malonyl CoA is increased. Note that CPT-1B is anchored to the mitochondrial membrane whereas α -actinin is anchored to the cytoskeleton. Modified from: Figure 4, supplementary information, Sharma *et al.*^[108]

crease in β 3-adrenoceptor expression in rat heart. Metoprolol increased the expression of all three adrenoceptor subtypes. PKA activity was decreased by both acute metoprolol perfusion and chronic metoprolol treatment, whereas PI3K activity, as indicated by Akt phosphorylation, was increased by metoprolol only following chronic treatment. CAMK activity was not significantly affected by metoprolol. There was no clear shift in β 2-adrenoceptor association with Gs or Gi; association with both G-proteins was detected. These results indicate that, in the whole heart, the major acute effect of metoprolol is to decrease classical cAMP/PKA signaling. Chronic treatment with metoprolol, in addition, increases PI3K/Akt signaling, and we speculate that this is primarily due to the marked increase in β 3-adrenoceptor expression. Activation of Akt was associated with pro-survival effects. The pro-apoptotic factors FOXO-3 and Bad were inhibited, and the anti-apoptotic factor BCL-2 was stimulated^[74,154]. Another intriguing pro-survival effect was that metoprolol increased the sequestration of activated caspase-3 by caveolins^[74].

eNOS is regulated by two main mechanisms; phosphorylation of Ser 1177, mediated by the PI3K/Akt pathway, was shown to increase eNOS activity in transfected COS cells^[155,156]; phosphorylation of Thr 495, me-

diated by PKA, partially blocks the phosphorylation of Ser 1177 in bovine aortic endothelial cells^[157]. Calcium-dependent translocation of eNOS from caveolae in the plasma membrane to calmodulin in the cytosol is also associated with an increase in eNOS activity^[158,159]. Activation of eNOS by β 3-adrenoceptors has been shown to be due both to an increase in Ser 1177 phosphorylation and to translocation from caveolae, but the importance of these mechanisms is region-specific; in atria, translocation is the predominant mechanism whereas, in the left ventricle, phosphorylation is the predominant mechanism^[160]. Intriguingly, β 3-adrenoceptor stimulation has also been shown to uncouple eNOS and increase oxygen free radical formation^[160].

Our efforts to measure NOS activity in the heart were unsuccessful. We therefore measured nitrate/nitrite levels and total protein glutathiolation as biomarkers of NO and physiological RNS production respectively, and correlated these with changes in the phosphorylation and expression of NOS isoforms. The pattern of changes produced for both markers was the same, with the exception of the diabetes treatment group, and can be interpreted as follows. In control hearts, acute metoprolol perfusion increased NO/RNS production by decreasing the inhibitory phosphorylation of Thr 486, a PKA site.

Stimulatory phosphorylation of Ser 1177 by Akt was also decreased, but we speculate that the decrease in PKA-mediated phosphorylation exerted a greater effect on activity. Following chronic treatment with metoprolol in control hearts, NO production remained high despite a surprising decrease in eNOS expression and a loss of any effect on eNOS phosphorylation. We speculate that this increase in activity could be due to increased eNOS translocation from caveolae to the cytosol.

In the diabetic heart, NO production is reduced and is dependent on iNOS rather than eNOS. iNOS is not regulated by β -adrenoceptors acutely, so acute perfusion with metoprolol has no effect on NO production. Chronic metoprolol treatment prevented the induction of iNOS without restoring eNOS expression. The net result was that chronic treatment with metoprolol had no effect on NO production. However, as indicated by the fall in glutathiolation, prevention of iNOS induction by chronic metoprolol treatment did decrease RNS production. The changes produced by metoprolol on the phosphorylation of eNOS by Akt did not correlate with the changes in Akt phosphorylation produced by the same treatment; this suggests that the elevated Akt signal was compartmentalized, and that eNOS was not its primary target. It is important to note that several cell types would have been present in the whole heart homogenate, including cardiomyocytes and endothelial cells; we did not differentiate between endothelial and cardiomyocyte NO signaling in our studies.

When tyrosine nitration, a marker of peroxynitrite, was measured, levels of total protein tyrosine nitration remained constant as long as either eNOS or iNOS were present. When eNOS expression was low and iNOS was absent, total protein tyrosine nitration fell. These data indicate that nitrosative stress was not significantly increased in diabetic hearts, although eNOS expression was low and NO levels had fallen. The fact that prevention of iNOS induction had a marked effect on RNS production but no effect on NO production suggests that iNOS was producing predominantly RNS. As discussed above, NO has been reported to inhibit glucose utilization predominantly through inhibition of glycolysis^[161]. However, chronic metoprolol treatment had no effect on glycolysis in control hearts despite the fact that it increased NO production.

The effects of metoprolol on pro-survival signaling provide the most likely explanation for its pro-survival effects, even though apoptosis itself was not detectable (we observed artefactual TUNEL staining, a cautionary example of this assay's non-specificity). Although metoprolol inhibits CPT-1, it has no effect on CD-36 translocation and increases the cytoplasmic accumulation of long-chain acyl CoAs. Metoprolol also did not prevent oxidative stress or the resulting DNA damage. It is therefore clear that the stimulus for cell damage remains unaltered, and metoprolol acts by preventing the sequelae of the stimulus (Figure 11).

SIGNIFICANCE OF THE PRESENT STUDIES

We chose to investigate metoprolol because it had previously been found to inhibit fatty acid oxidation, it is a clinically useful drug and its known range of actions is narrower than that of carvedilol. Several other β -blockers have been reported to have effects on metabolism. However, several key questions remain to be answered. Is this effect a class effect or is it mediated only by certain β -blockers? Are the effects mediated by β -adrenoceptors, and if so, what is the contribution of each receptor? Comparative studies of a wider range of β -blockers, as well as studies in which β -adrenoceptor expression is silenced, must be carried out in order to answer these questions.

We have shown that metoprolol improves cardiac function in diabetic cardiomyopathy in rats, raising the question as to whether the drug should be used earlier in diabetic patients. However, its *in vivo* effects on function were equivocal. There are also a number of concerns with the administration of β -blockers to diabetic patients which need to be weighed against the benefits of introducing the drug so early. First and foremost are concerns about the effects of β -blockers on glycemic control. Co-administration of a β -blocker with a thiazide has been reported to worsen glycemic control since the 1980s when the effects of propranolol and hydrochlorothiazide were reported^[162]. Recently, however, the use of β -blockers as antihypertensive agents has been associated with an increased risk of new-onset diabetes, leading to concern about their use in this context^[26]. Hepatic glucose output is controlled by the β_2 adrenoceptor, and blockade of this receptor, which does occur with β_1 selective agents, decreases hepatic glucose output and delays recovery from hypoglycemia^[27,28]. Attenuation of the symptoms of hypoglycemia by β -blockade is no longer considered to be a problem, because the symptoms of sweating and paresthesias are preserved, and patients can be educated to recognize these signs^[28,163].

Another concern with chronic β -blockade is the presence of sustained unopposed α_1 -adrenoceptor stimulation. This is problematic in two situations. Firstly, activation of the sympathetic nervous system by hypoglycemia increases unopposed α_1 -adrenoceptor stimulation to the point where a hypertensive crisis can be precipitated^[28]. Secondly, unopposed α_1 -adrenoceptor stimulation produces peripheral vasoconstriction which could worsen peripheral vascular disease and, by decreasing muscle flow, increase insulin resistance^[12,164]. Indeed, use of β -blockers in diabetic patients increases glucose levels and triglycerides and lowers high density lipoprotein cholesterol levels by decreasing insulin sensitivity^[12]. None of these concerns are considered great enough to deny β -blockers to patients with systolic heart failure because these drugs are lifesaving in this context. However, the risks and benefits of earlier β -blocker use will need to be weighed carefully

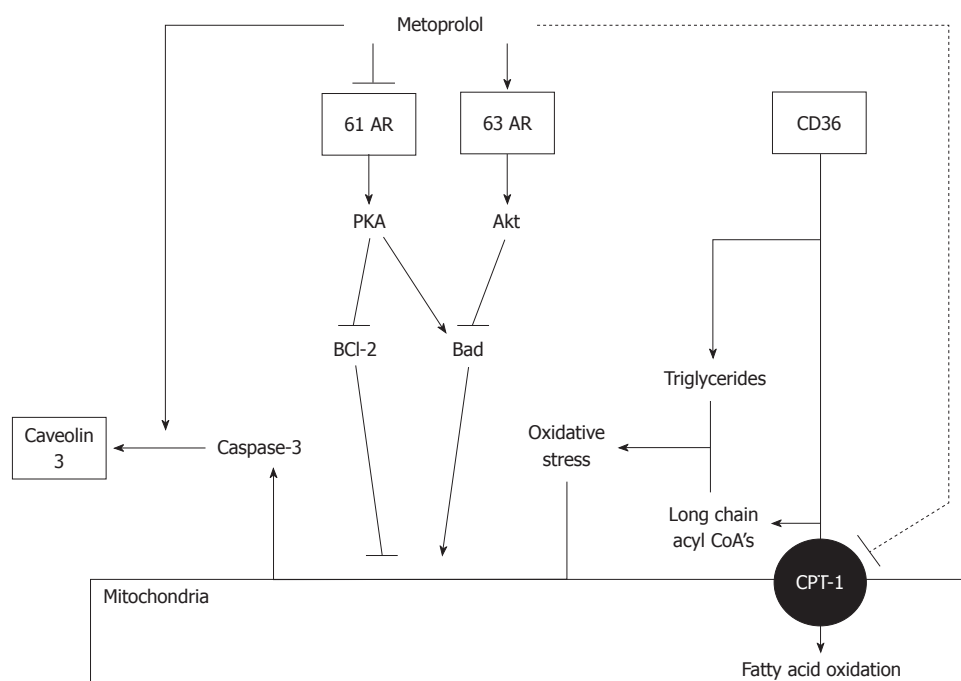


Figure 11 Mechanisms of action of metoprolol. Metoprolol inhibits fatty acid oxidation by inhibiting carnitine palmitoyltransferase (CPT)-1, but has no effect on CD36. Triglyceride and long chain acyl CoA accumulation, and stimulation of oxidative stress, are therefore unaltered. Metoprolol also promotes β_3 adrenoceptor signaling, leading to inhibition of Bad and stimulation of BCL-2, and inhibition of caspase-3 activation. Finally, metoprolol stimulates sequestration of caspase-3 by caveolins. The net effect is a prevention of caspase activation. Modified from: Figure 7, Sharma *et al.*^[108]

and no evidence currently exists on which to base these considerations. β -blockers with antioxidant activity, such as carvedilol, may be superior agents to metoprolol as they could also prevent the injury caused by oxidative stress, rather than just preventing its sequelae.

These studies have expanded our understanding of the range of effects produced by β -adrenergic blockade and show how interconnected the signaling pathways of function and metabolism are in the heart. Although our initial hypothesis that inhibition of fatty acid oxidation would be a key mechanism of action was disproved, unexpected results led us to some intriguing regulatory mechanisms of cardiac metabolism, some of which were hitherto unsuspected. The first was USF-2-mediated repression of PGC-1 α , most likely occurring as a consequence of the improved function. The second was the identification of a range of covalent modifications which can regulate CPT-1 directly, mediated by a signalome at the level of the mitochondria. We also identified an important interaction between β -adrenergic signaling and caveolins, which may be a key mechanism of action of β -adrenergic blockade. Our experience with this labyrinthine signaling web illustrates that initial hypotheses and anticipated directions do not have to be right in order to open up meaningful directions or reveal new information.

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Coronary computed tomography angiography in coronary artery disease

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Abstract

AIM: To investigate the research directions of coronary computed tomography (CT) angiography in the diagnosis of coronary artery disease (CAD) based on a systematic review of the literature.

METHODS: A search of articles on coronary CT angiography in the diagnosis of CAD was performed during a 6-year-period between 2005 and 2010 from five main radiology journals namely, Radiology, American Journal of Roentgenology, European Radiology, European Journal of Radiology and British Journal of Radiology. Analysis of the references was focused on the research directions of coronary CT angiography with regard to the type of studies in terms of diagnostic value, application of dose-reduction strategies and resultant effective radiation doses with use of these techniques.

RESULTS: One hundred and forty two studies were identified which met the selection criteria and were included in the analysis. 64-slice CT (single source and

dual-source CT) dominated 78% of the coronary CT angiography studies. Prior to 2007, research was focused on the diagnostic value of coronary CT angiography, but since 2008 more attention has been paid to radiation dose reduction. Radiation dose was reported in 64 studies, representing 45% of total studies published in the five radiology journals. Various dose-saving strategies have been implemented and prospective electrocardiography-triggering and high pitch techniques were found to be the most effective approaches for radiation dose reduction, with the corresponding mean effective dose being 3.5 ± 1.9 mSv and 1.7 ± 0.6 mSv, respectively.

CONCLUSION: This review shows that the current research in coronary CT angiography has shifted from the previous focus on diagnostic accuracy in CAD to more emphasis on radiation dose reduction.

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Key words: Coronary artery disease; Diagnostic value; Multislice computed tomography; Radiation dose; Risk

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INTRODUCTION

Coronary artery disease (CAD) is the leading cause of morbidity and mortality in many advanced countries and its prevalence is increasing in developing countries^[1,2].

According to recent World Health Organization statistics for 2007, cardiovascular deaths account for 33.7% of all deaths worldwide, whereas cancer represents 29.5%, other chronic diseases 26.5%, injury 7%, and communicable diseases 4.6%^[3]. CAD is the leading cause of cardiovascular death throughout the world. In light of the current global focus on healthcare utilization, costs, and quality, it is essential to monitor and understand the magnitude of healthcare delivery and costs, as well as the quality of healthcare delivery in relation to the CAD.

Invasive coronary angiography (ICA) is widely used as a reliable technique to diagnose CAD because of its superior spatial and temporal resolution. However, it is an invasive and expensive procedure with an associated small percentage of morbidity and mortality^[4]. As an alternative to ICA, coronary computed tomography (CT) angiography has been increasingly used for the investigation of suspected CAD, and rapid technological developments of multislice CT (MSCT) have led to both improved spatial and temporal resolution^[5-7]. Studies have shown that coronary CT angiography has a high diagnostic accuracy for the detection of significant CAD ($\geq 50\%$ lumen stenosis) when compared to ICA^[8-11], and in selected patients, coronary CT angiography is recommended as a reliable alternative to ICA in the diagnosis of CAD.

Despite promising results having been achieved with coronary CT angiography, it has the disadvantage of high radiation dose, which leads to the concern of radiation-associated risks^[12,13]. It is generally agreed that CT is an imaging modality with high radiation exposure, as it contributes up to 70% of the radiation dose of all radiological examinations, although it comprises only 15% of all radiological examinations. Radiation-induced malignancy is a problem that has raised serious concern in the medical field and this has been addressed by the National Research Council of the United States^[14]. It is reported that radiation dose from a CT scan has been significantly underestimated by both radiologists and physicians^[12,15]. Despite the increased awareness of radiation risk, many clinicians and researchers have not realized the amount of radiation exposure associated with coronary CT angiography, or the possibility of tailoring the scanning protocols to reduce radiation exposure to patients. Thus, the purpose of this article was to investigate the current research directions of coronary CT angiography in CAD, based on a systematic review of the literature published in five main radiology journals over the past 6 years.

MATERIALS AND METHODS

References searching

A search of articles on coronary CT angiography in cardiac imaging was performed during a 6-year-period between 2005 and 2010 in five main radiology journals, *Radiology*, *American Journal of Roentgenology* (AJR), *European Radiology* (ER), *European Journal of Radiology* (EJR) and *British Journal of Radiology* (BJR). The reason we started

our search in 2005 is because 64-slice CT was introduced in 2004. The keywords used in searching the references in each journal website included: MSCT in CAD, diagnostic value of MSCT angiography in CAD, coronary CT angiography and CT coronary angiography, prospective electrocardiography (ECG)-gating/triggering in cardiac imaging, and cardiac CT with radiation dose reduction. In addition, a search for relevant articles was conducted by electronically checking the monthly issue of each journal's publications, and eligible full-text articles were obtained for data extraction and analysis. Prospective and retrospective studies were included if they met the following criteria: (1) **studies conducted on human subjects** or phantom with use of MSCT or coronary CT angiography in CAD; (2) **either coronary calcium scoring, diagnosis of CAD, prognostic value of CAD, or myocardial perfusion imaging** must be addressed in each study; (3) coronary CT angiography in terms of prospective ECG-triggering or retrospective gating must be clearly stated in each study; and (4) **any strategies to reduce radiation dose** must be provided. Since it is possible that many studies would not meet the third and fourth criteria, studies were still eligible for inclusion in the analysis as long as they met the first two criteria. Exclusion criteria were: studies on coronary stenting or coronary artery-bypass surgery; review articles or case reports; a letter or comment to the editor.

Data extraction and analysis

All articles were reviewed and extracted by two independent authors with regard to study design and procedure techniques. Each author independently assessed the retrieved articles according to the selection criteria and inter-observer agreement was reached by consensus. The authors looked for the following characteristics in each study: year of publication; type of scanning unit used for coronary CT angiography; type of studies in terms of prospective or retrospective studies; researcher background, e.g. radiologists, cardiologists, physicians or other health care professionals (for example, medical imaging specialists); number of research centres where studies were performed. In addition, more focus was placed on the research directions of coronary CT angiography with the aim of seeking information on the application of dose-saving strategies. This included the common approaches for dose reduction: adjustments of kVp and mAs (tube current modulation, either anatomy-based or ECG-controlled tube current modulation); high pitch value; prospective ECG-triggering *vs* retrospective ECG-gating.

Statistical analysis

All variables are expressed as mean \pm SD. Statistical tests were performed using SPSS V 18.0 (SPSS, Inc., Chicago, IL, United States). Comparisons were performed using one sample *T* test. A *P* value less than 0.05 was defined as statistically significant difference.

RESULTS

General information

One hundred and forty-two studies were identified which met the selection criteria and were included in the analysis. One hundred and thirty seven studies were conducted at a single centre, while three studies were performed at 3 centres and the remaining two studies at 9 and 20 study sites, respectively. The majority of these studies were published in *ER* with the highest number being 62 articles, and the least number of studies was found in the *BJR* with only 11 studies being identified. For the remaining three journals, *Radiology*, *AJR* and *EJR*, the corresponding studies were 23, 24 and 22, respectively.

Analysis of author background indicated that most of the studies were performed by radiologists, followed by cardiologists, and other health care professionals such as physicians and medical imaging specialists. Radiologists accounted for 84% of the authors who performed the studies, while cardiologists were in collaboration with radiologists as a research team in all of the studies, except in two studies published in *ER*, where cardiologists alone conducted the research.

Of 142 studies, 126 were performed on patients (89%), and the remaining 16 studies were conducted on anthropomorphic phantoms with the aim of investigating the effect of heart rate on diagnostic image quality, or radiation dose or high pitch value on image quality. 71 studies (50%) were performed to study the diagnostic value (67 studies) and prognostic value (4 studies) of coronary CT angiography in CAD, while the remaining studies were mainly focused on radiation dose reduction.

Type of MSCT scanners

Variable MSCT scanners were used in these studies, with 64- or more slice-scanners representing the majority (85%) of the studies. Figure 1 shows the distribution of different generations of MSCT scanners that were identified in the analysis. Despite rapid technological developments of MSCT scanners, such as the increased availability of 256- and 320-slice CT, single source 64-slice and dual-source CT (DSCT) still dominated 78% of the coronary CT angiography studies. Coronary CT angiography was compared with integrated single photon emission computed tomography/CT and positron emission tomography/CT in two studies to investigate the myocardial perfusion value of coronary CT angiography.

Research directions of coronary CT angiography

The issue of radiation dose has been addressed since 2007 in the publications, *Radiology*, *AJR* and *BJR*, while this has been addressed since 2008 in the *EJR* publications. In comparison, radiation dose was first reported in some studies published in 2006 in the journal of *ER*, although more attention has been paid to the issue of dose since 2007. Prior to 2006 and 2007, research was focused on the diagnostic value of coronary CT angiography in CAD, regardless of the studies performed with 8-slice,

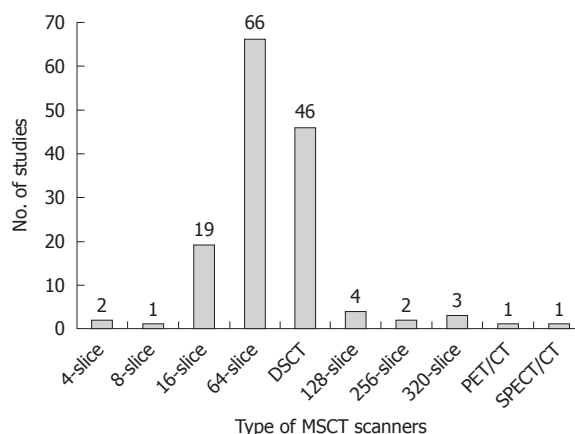


Figure 1 The number of studies performed with different generations of multislice computed tomography scanners. CT: Computed tomography; DSCT: Dual-source CT; MSCT: Multislice CT; PET: Positron emission tomography; SPECT: Single photon emission computed tomography.

16- or 64-slice CT scanners. From 2008, more and more studies started to address radiation dose reduction in these five radiology journals.

Dose-saving strategies

Various dose-saving strategies have been implemented in these studies, and have included adjustments of tube voltage (kVp), tube current (tube current modulation), pitch value, prospective ECG-triggering *vs* retrospective ECG-gating, addition of padding in patients with high heart rate variability, and application of adaptive statistical iterative reconstruction (ASIR) algorithms for image noise reduction. Radiation dose was reported in 64 studies, representing 45% of the total studies published in the five radiology journals. Of these 64 studies, nearly 50% were published in the journal of *ER*.

Prospective ECG-triggering was the most commonly applied technique for dose reduction, with 25 studies reported in these five radiology journals. Of these 25 studies, a direct comparison between prospective ECG-triggering and retrospective gating was performed in 16 studies. The mean effective dose was 3.5 ± 1.9 mSv for prospective ECG-triggering, which is significantly lower ($P < 0.0001$) than that acquired with retrospective ECG-gating, which is 16.3 ± 6.3 mSv.

ECG-controlled tube current modulation was the second most common approach utilized in 22 studies for dose reduction. However, the effective dose value was only reported in 7 studies, with the mean dose being 8.8 ± 2.6 mSv. Application of pitch (up to 3.4) in coronary CT angiography was reported in five studies with the mean effective dose being 1.7 ± 0.6 mSv. Lower kVp of 100 *vs* the standard 120 kVp was compared in four studies with the resultant mean effective dose of 5.4 ± 2.9 mSv for 100 kVp protocols, and 9.4 ± 5.1 mSv for 120 kVp protocols, leading to a dose reduction of 42%. Figure 2 shows the mean effective dose associated with the above-mentioned commonly used strategies for dose reduction. As shown in the figure, prospective ECG-trig-

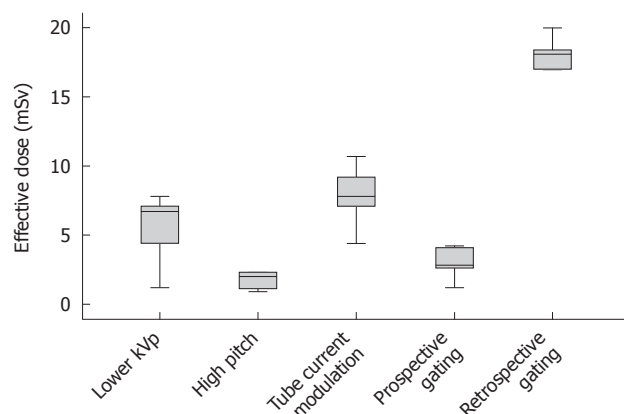


Figure 2 Box plot shows the mean effective dose associated with different dose-reduction techniques. Coronary computed tomography angiography with the use of high pitch and prospective electrocardiography-triggering leads to the lowest radiation dose. The boxes indicate the first to third quartiles, and each midline indicates the median (second quartile) and the whiskers represent the maximum and minimum values of effective dose.

gering and high pitch resulted in the lowest doses when compared to other dose-saving strategies.

ASIR was reported in two studies and the mean effective dose was less than 3.0 mSv. A comparison of radiation dose with use of the International Commission on Radiological Protection (ICRP) 103 and ICRP 60 documents was conducted in two studies with a significant difference observed, indicating that the radiation dose was underestimated if dose calculation is based on the weighting factors that were reported in the ICRP 60.

Radiation dose corresponding to different heart rates was reported in three studies and the analysis shows that DSCT is superior to single-source CT in the diagnosis of CAD in patients with higher heart rates, with resultant lower doses. Padding was applied in one study performed with prospective ECG-gating. The purpose of adding padding is to provide additional phase information to compensate for variations in heart rate by adding time before and after the centre phase of the acquisition. Padding is described in the range of 0-200 ms and is added to both sides of the centre of the acquisition with padding 0 corresponding to a window of 100 ms scanning time and padding 100 corresponding to a window of 200 ms scanning time. In that study, the effective dose was 2.3 mSv for 0 padding, and the dose increased to 3.8 mSv for 1-99 padding and to 5.5 mSv for 100-150 padding.

The remaining study involved the investigation of the effect of adjusting the scan length of coronary CT angiography using the calcium scoring images instead of the scout view with regard to radiation dose. The effective dose associated with the calcium scoring-derived length (9.0 ± 0.6 mSv) was significantly lower than that using the scout view-derived length (10.7 ± 1.2 mSv), corresponding to a radiation dose reduction of 16%.

DISCUSSION

This analysis presents three findings which are considered

important for the clinical application of coronary CT angiography in CAD. Firstly, there is a change in research direction with a shift of focus from the previous diagnostic value of coronary CT angiography in CAD to an emphasis on radiation dose reduction. This indicates the increased awareness of radiation dose to patients during coronary CT angiography examinations. Secondly, more dose-saving strategies have been implemented to reduce radiation dose associated with coronary CT angiography while still maintaining diagnostic image quality. Of these strategies, prospective ECG-triggering and high pitch protocols represent the most effective approaches with a significant reduction of radiation dose compared to conventional retrospective gating or other approaches. Thirdly, coronary CT angiography performed with 64-slice CT (single-source or DSCT) dominated the majority of studies that were reviewed, and this indicates that 64-slice CT still plays a key role in the clinical diagnosis of CAD, despite the emergence of 256- and 320-slice CT.

Over the last decade a great deal of interest has been focused on the imaging and diagnosis of CAD using coronary CT angiography, due to its less invasive nature and improved spatial and temporal resolution. Moderate to high diagnostic accuracy was achieved with 64- or more slice CT, owing to further technical improvements^[16-20]. These studies have indicated that coronary CT angiography has high accuracy in the diagnosis of CAD and could be used as an effective alternative to ICA in selected patients. However, coronary CT angiography has the disadvantage of high radiation dose which raises concerns for both clinicians and manufacturers. This is reflected in the changing research directions from the early research focus on the diagnostic value of coronary CT angiography in CAD to the increasingly reported studies on dose reduction, as demonstrated in this analysis.

Radiation dose is becoming a major issue for coronary CT angiography, since 64- or more-slice CT shows improved and promising results in the diagnosis of CAD^[21-25]. The radiation risks associated with coronary CT angiography have raised serious concerns and have become a hot topic of debate in the literature^[12,13,26]. The general view about radiation dose is that CT is associated with a risk of cancer development. Therefore, coronary CT angiography should be performed with dose-saving strategies whenever possible to reduce the radiation dose to patients.

One of the most effective approaches for dose reduction is adjustment of the tube current according to ECG signal, which is defined as ECG-controlled tube current modulation. This approach represents the most significant improvement in minimizing radiation exposure from coronary CT angiography. It has been reported that radiation dose can be reduced by 30%-50% through modulation of the tube current output to decrease the dose given during the systolic phase^[27,28]. The estimated radiation dose reduction is similar to or less than that of an ICA examination with use of this dose saving strategy^[27,29]. The mean effective dose of ECG-controlled tube current modulation in

this analysis was 8.8 mSv, and this was comparable to that of ICA, which was between 3-9 mSv^[30].

Prospective ECG-triggering has been confirmed to be another efficient technique for radiation dose reduction in coronary CT angiography^[31-33]. Use of prospective ECG-triggering with 64-slice or DSCT has been reported to reduce the effective radiation dose by up to 90% when compared to retrospective ECG-gating, with diagnostic image quality being achieved in more than 90% of cases^[34-39]. This was confirmed in this analysis, as the mean effective dose of prospective triggering was 3.5 mSv, which is equivalent to or even lower than that of ICA.

Another effective method currently undertaken in coronary CT angiography to reduce radiation dose is to lower the tube voltage, since radiation dose varies with the square of the kV. Modern CT scanners include tube voltages of 120 or 140 kV, reflecting the settings which most often result in adequate image quality. However, acquisition of cardiac CT images with 100 kV, or even lower, is possible and has been suggested as an effective means to reduce radiation dose in coronary CT angiography^[40,41]. Up to 70% dose reduction has been reported in the literature when the X-ray tube voltage is decreased from 120 kVp to 100 kVp or even 80 kVp^[42,43]. This analysis showed that a 42% dose reduction was observed in coronary CT angiography with the use of 100 kVp when compared to the routine 120 kVp protocol, and this is consistent with the results reported in the literature. Therefore, tube voltage can be adjusted in cardiac CT angiography without affecting diagnostic image quality, and this should be applied whenever possible in clinical practice^[44,45].

For coronary CT angiography, very low pitch values (0.2-0.4) are typically required for coronary data acquisition to ensure continuous z-axis coverage between image stacks reconstructed from consecutive cardiac cycles. The main disadvantage of this approach is higher radiation exposure since the table is advanced by less than one detector width during each gantry rotation, thus, the same anatomic area is exposed to X-ray radiation during consecutive rotations of the gantry. Increasing the pitch to a higher value was made possible by the development of the second generation of DSCT scanners, Siemens Definition Flash, which enabled acquisition of 128 slices simultaneously (flying focal spot)^[46-50]. This DSCT mode allows coronary CT angiography to be performed at high pitch values of up to 3.4 with a significant reduction in radiation dose. A high pitch was applied in five studies in this analysis with a mean effective dose of 1.72 mSv, and a radiation dose less than 1.0 mSv was also reported in one study, indicating the effectiveness of this dose-reduction approach. The new scan mode, with a superior temporal resolution of 75 ms, is regarded as an attractive alternative to ICA due to the very low dose and high image quality, although more studies based on a large cohort are required.

Although nearly 50% of the studies in this analysis were conducted with the use of single-source CT, DSCT was used in about one-third of the studies, indicating the increased use of this improved technique. The main

limitation of single-source CT coronary angiography is that β -blockers are frequently used to lower the heart rate to less than 65 beats per minute. The introduction of DSCT marked another technological improvement of MSCT in cardiac imaging, as the temporal resolution was further increased from 165 ms to 83 ms, thus eliminating the need to control the heart rate during the scan^[43,44,51]. Studies comparing DSCT with single-source CT demonstrated that DSCT maintains high diagnostic accuracy in the diagnostic examination of a wide range of patient subsets, e.g., patients with higher and even irregular heart rates (including atrial fibrillation)^[52-54]. Despite slightly lower per-segment evaluability in patients with higher heart rates, DSCT did not show decreased diagnostic accuracy for the detection of coronary stenoses^[54,55]. DSCT improves temporal resolution which is vital in patients who cannot have β blockade.

Expansion of MSCT systems from a 64-slice to 256- and 320-slice system has allowed for the accurate assessment of stenosis severity and atherosclerotic plaque composition, or even the acquisition of whole-heart coverage in one gantry rotation, thus, eliminating the restrictions and limitations associated with 64-slice CT scanners^[22,24]. Studies performed with 256- and 320-slice CT demonstrated the high diagnostic value of coronary CT angiography for the quantification of coronary lumen stenosis and assessment of plaque morphology and distribution in the coronary arteries^[22,56,57]. In patients with atrial fibrillation, 320-slice CT allows for excellent visualization of all coronary segments with sufficient images to enable a diagnosis, and acquisition of images with consistent CT attenuation along the different coronary arteries^[23,57]. Despite these advantages, 256- and 320-slice CT has the limitations of reduced gantry speed (270-350 ms), high radiation dose and cone beam effects which need to be addressed in future technical developments.

Some limitations exist in this review. Firstly, our search for references only focused on the five main radiology journals. However, research articles on cardiac CT imaging are also published in cardiac journals such as *European Heart Journal*, *Journal of American College of Cardiology*, *Circulation*, *American Journal of Cardiology* and *International Journal of Cardiovascular Imaging*. Therefore, it is possible that some relevant references were not included in this analysis. Secondly, although tube current modulation is one of the most effective methods for radiation dose reduction, a number of studies did not report the actual effective dose, despite the application of this technique in their studies. This emphasises the importance of awareness of radiation exposure to patients by physicians; thus, radiation dose values (effective dose or dose length product) should be recorded in each study to enable the comparison of dose-saving techniques between different studies. Thirdly, we tried to include as many references as possible that were available over the last few years; however, some articles which were accepted for publication were excluded from the analysis. Finally, although this analysis shows that 64-slice CT coronary

angiography dominated the majority of the studies, there is no doubt that more and more studies are being performed with the latest models such as 256- and 320-slice CT scanners. Further research to include these studies is necessary.

In conclusion, this systematic review of the literature from five main radiology journals shows that the current research in coronary CT angiography has shifted from the previous focus on diagnostic accuracy in CAD to more emphasis on radiation dose reduction. This review also indicates the increased awareness of radiation dose associated with coronary CT angiography in the literature. Various dose-saving strategies have been undertaken in the past few years to lower radiation exposure to patients undergoing coronary CT angiography. Effective dose reduction has been achieved by employing techniques with a radiation dose of around 10 mSv to as low as 1 mSv in some studies. It is important to note that the current effective doses from coronary CT angiography are at the same level or even lower than those acquired from ICA. Therefore, according to this analysis, the achievements in radiation dose reduction in coronary CT angiography have been tremendous.

COMMENTS

Background

Coronary computed tomography (CT) angiography is increasingly used to diagnose coronary artery disease (CAD) due to its reduced invasiveness and improved diagnostic accuracy. Despite the high diagnostic value of coronary CT angiography, it has the disadvantage of high radiation dose which contributes to radiation-induced malignancy. This study was conducted to investigate the current research directions of coronary CT angiography in the diagnosis of CAD based on a systematic review of the radiology literature.

Research frontiers

Current research in coronary CT angiography has shifted from the previous focus on diagnostic accuracy in CAD to more emphasis on radiation dose reduction. This reflects the increased awareness of radiation dose associated with coronary CT angiography in the literature.

Innovations and breakthroughs

Various dose-saving strategies have been proposed in the literature with regard to radiation dose reduction associated with coronary CT angiography. This study shows that these dose-saving strategies have been very effective in dose reduction, and it is possible to lower the level of radiation dose to that of invasive coronary angiography without compromising diagnostic image quality.

Applications

Coronary CT angiography is mainly performed with the use of 64-slice CT, however, with technological developments in CT scanning techniques, the latest models such as 256- and 320-slice CT are increasingly used to accurately diagnose patients with suspected CAD due to whole-heart coverage in one gantry rotation.

Terminology

Effective dose is a single parameter used to reflect the relative risk of exposure to ionizing radiation. Calculation of the effective dose of coronary CT angiography takes into account the biological effect of the radiation on the heart, because each organ is given a tissue weighting depending on its individual susceptibility to the effects of ionizing radiation.

Peer review

This paper reviews recent developments in technology of CT-angiography of the coronary arteries and elute on future directions in development of this technology. The paper covering this relatively new non-invasive technology is in general interest for the readers of the journal.

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Percutaneous revascularization in a patient with anomalous origin of left main coronary artery

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Abstract

Anomalous origin of the coronary artery from opposite coronary sinus is infrequently observed during coronary angiography. Percutaneous coronary intervention (PCI) of anomalous coronary artery is technically difficult and challenging. It requires appropriate selection of guide catheters for adequate stability, coaxial alignment and backup support during the intervention. We hereby report a rare case of anomalous origin of left main coronary artery (LM) from the right coronary sinus, having a retro-aortic course to the left side before its bifurcation into left anterior descending (LAD) and circumflex artery. The 59-year-old man had successful PCI of atherosclerotic LAD lesions. A 64-slice Multi-Detector Computed Tomography (MDCT) performed at 4 years of follow-up demonstrated patency of coronary stents and also delineated the origin and course of the anomalous LM. The case illustrates the rarity of anomalous LM, and describes technical issues during PCI and the role of MDCT in coronary anomaly imaging.

INTRODUCTION

Anomalous origin of the coronary artery from opposite coronary sinus is infrequently observed during coronary angiography. Percutaneous coronary intervention (PCI) of anomalous coronaries is technically difficult and challenging. It requires a proper selection of hardware and adequate/appropriate expertise of the operator to perform the intervention. We hereby report a rare case of anomalous origin of left main coronary artery (LM) from the right coronary sinus, in which successful PCI of the left anterior descending (LAD) artery was performed. A long term 5-year follow-up of favorable clinical outcome and coronary arterial imaging is also demonstrated.

CASE REPORT

In August 2006, a 59-year-old male presented to the out-

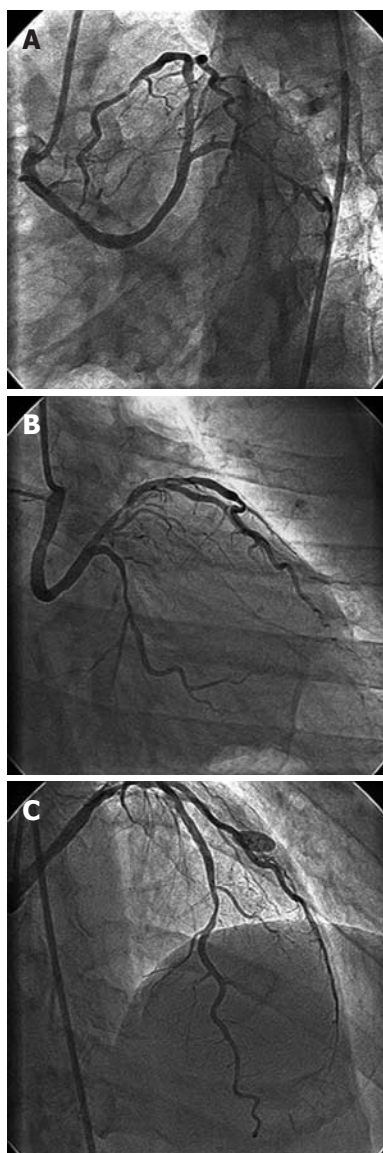


Figure 1 Left coronary angiogram after injection from right coronary sinus. A: Separate ostial origin of anomalous left main coronary artery from right coronary sinus, having retro-aortic course; B: Proximal left anterior descending (LAD) artery having type B 70% tubular stenosis, proximal left circumflex artery having 50% diffuse stenosis; C: Distal LAD artery having type B 70% tubular stenosis.

patient department with a history of chest pain, which first occurred 4 d previously during morning hours. He was a non-smoker, non-diabetic and had normal blood pressure. An ECG done by a local physician showed left bundle branch block (LBBB). A repeat ECG at our institute was within normal limits. His general physical and systemic examinations were unremarkable. His routine biochemistry was normal. Fasting lipid profile revealed total cholesterol 198 mg%, HDL 42 mg%, LDL 110 mg%, and triglycerides 236 mg%. Two-dimensional echocardiogram showed a left ventricular ejection fraction of 0.60, no regional wall motion abnormality and no mitral regurgitation. He underwent coronary angiography by trans-femoral route. The right coronary artery (RCA), which was cannulated with a Judkins Right 4, 6F catheter, was a

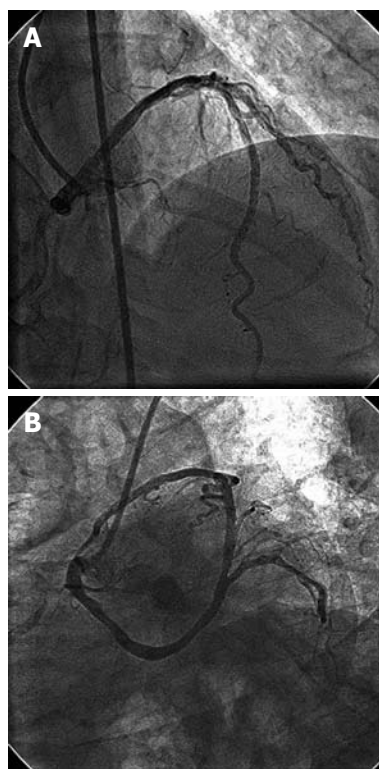


Figure 2 Left coronary angiogram following coronary stenting of left anterior descending artery. A: Distal left anterior descending (LAD) lesion stented with 2.5 mm × 23 mm sirolimus-eluting stent; B: Proximal LAD lesion stented with 3 mm × 23 mm sirolimus-eluting stent.

normal and dominant vessel. The LM could not be visualized in the left coronary sinus with the Judkins Left 4, 6F catheter. Subsequently, it was found to be arising from the right coronary sinus. Both RCA and LM were arising from separate ostia. The LM had a retro-aortic course from the right coronary sinus before its bifurcation into LAD and left circumflex (LCx) arteries (Figure 1A and B). The LAD showed two type B lesions in proximal (Figure 1B) and distal segments (Figure 1C). The LCx, which was a non-dominant vessel, showed insignificant stenosis in the distal segment. A left ventriculogram showed an ejection fraction of 0.60 and no regional wall motion abnormality.

Following a written informed consent for percutaneous revascularization, the patient was subjected to PCI of the LAD artery, in a second stage after 48 h. The anomalous LM was cannulated with an Eric Cohen Right (ECR) 4, 6F coronary guide catheter (an extra-backup support catheter for RCA from Medtronic, Inc., Minneapolis, Minnesota) and LAD lesions were crossed with an All Track Wire (ATW) coronary guide wire (Cordis Co., Miami, Florida). Both proximal and distal LAD lesions were pre-dilated with a 2.5 mm × 15 mm Sprinter balloon (Medtronic). The distal LAD lesion was stented with a 2.5 mm × 23 mm sirolimus-eluting Cypher stent (Cordis) (Figure 2A) and the proximal LAD lesion was stented with a 3 mm × 23 mm sirolimus-eluting Cypher stent (Cordis) (Figure 2B). He had an uneventful recovery and was discharged on day 2 of intervention with dual anti-platelet and statin therapy. He remained asymptomatic

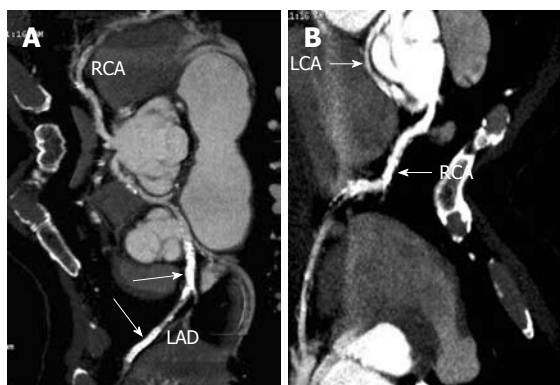


Figure 3 64-slice multi-detector computed tomography image of coronary arteries. A: Computed tomography (CT) image showing separate ostia of both right and left main coronary artery. The left anterior descending stents are marked with white arrow; B: CT image showing retro-aortic course of left main coronary artery. RCA: Right coronary artery; LCA: Left coronary artery; LAD: Left anterior descending.

on follow-up. A check angiogram at 9 mo of follow-up showed patent LAD stents with no endoluminal loss. The check angiogram was performed for demonstration of patency of stents for academic reasons only. At 4 years of follow-up, a 64-slice MDCT coronary scan revealed good antegrade flow across the stented LAD segments and also demonstrated the retro-aortic course of the LM (Figure 3A and B). After the PCI, he remained asymptomatic throughout his 5 years of follow-up.

DISCUSSION

Coronary artery anomalies are present since birth, but relatively few are symptomatic during childhood or later in life. Most of the anomalies are incidentally detected during selective coronary angiography. The incidence of coronary anomalies among individuals subjected for catheterization varies from 0.6% to 1.3% in various series^[1]. Anomalous LM arising from the right coronary sinus is a rare anomaly, having the incidence of 0.017% in an angiographic series^[1]. The course of anomalous LM can be inter-arterial, retro-aortic, septal or through the anterior free wall of the right ventricle^[1]. It is more common to have an inter-arterial or septal course than the retro-aortic course^[1-4]. The retro-aortic course, as present in the index case, is the less common anomaly of LM and is usually benign in nature in comparison to inter-arterial or septal course^[1,4]; however, a few authors have reported adverse outcomes even with retro-aortic course^[5-7]. This index case had angina secondary to atherosclerotic disease of the LAD and not because of anomalous retro-aortic course of LM, as the patient remained asymptomatic following intervention at long term follow-up. Anomalous coronary artery arising from the opposite coronary sinus is associated with angina, myocardial infarction and sudden cardiac death, even in the absence of significant atherosclerotic stenosis^[8]. The pathogenesis of these clinical manifestations can be multi-factorial including anomalous coronary compression with inter-arterial course, slit-like

coronary orifice with dynamic obstruction, acute angle take off, and presence and length of intramural segments of anomalous coronary vessel^[8-10].

PCI of anomalous coronaries is difficult, technically challenging, and requires significant experience for successful intervention^[11,12]. However, a failed PCI and subsequent bypass surgery in an acute myocardial infarction situation with anomalous left main coronary artery has even been described^[13]. During the intervention, there is a need for coaxial alignment and adequate backup support of the guide catheter for successful outcome. Various authors have described different types of guide catheters, such as Amplatz Right, Amplatz Left, Multipurpose catheter, *etc.*, for intervention in such situations^[11,14,15]. In the described case, we used an ECR guide catheter, which provided a good backup support and also coaxial alignment during intervention. Even though there are reports about PCI of anomalous LM with inter-arterial or septal course^[10,11,14,15], only a single case report of PCI of LM with retro-aortic course has been published^[5]. To our knowledge, the index case is the second one in the published literature about PCI with regard to anomalous LM having a retro-aortic course.

The role of multi-detector computed tomography (MDCT) for detection of coronary stent patency^[16] and delineation of anomalous coronary origin, course and relation^[17] has been described in the literature. In the index case, MDCT has demonstrated the patency of the LAD drug-eluting stents and delineated the origin and course of anomalous LM.

In conclusion, we hereby report a rare case of anomalous LM originating from the right coronary sinus and having a retro-aortic course, in which a successful coronary stenting of the LAD artery was performed. Follow-up MDCT imaging demonstrated the long term patency of drug-eluting stents, and also delineated the origin and course of anomalous LM.

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February 25-27

CardioRhythm 2011
Hong Kong, China

March 19-26

Cardiology Update: Caribbean
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San Diego, CA, United States

March 25

Cardiology for General Practice

London, United Kingdom

April 1-2

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Brussels, Belgium

April 14-16

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Genova, Switzerland

April 30-May 4

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May 11-14

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Córdoba, Argentina

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May 20-22

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May 21-24

Heart Failure Congress 2011
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June 2-5

CODHy 2011 - The 1st Asia Pacific
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Hypertension
Shanghai, China

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Format

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- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID: 2516377 DOI: 10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI: 10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI: 10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI: 10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiecezorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 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>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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