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REVIEW

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REVIEW

Mutual interaction between oxidative stress and endoplasmic reticulum stress in the pathogenesis of diseases specifically focusing on non-alcoholic fatty liver disease

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Abstract

Reactive oxygen species (ROS) are produced during normal physiologic processes with the consumption of oxygen. While ROS play signaling roles, when they are produced in excess beyond normal antioxidative capacity this can cause pathogenic damage to cells. The majority of such oxidation occurs in polyunsaturated fatty acids and sulfhydryl group in proteins, resulting in lipid peroxidation and protein misfolding, respectively. The accumulation of misfolded proteins in the endoplasmic reticulum (ER) is enhanced under conditions of oxidative stress and results in ER stress, which, together, leads to the malfunction of cellular homeostasis. Multiple types of defensive machinery are activated in unfolded protein response under ER stress to resolve this unfavorable situation. ER stress triggers the malfunction of protein secretion and is associated with a variety of pathogenic conditions including defective insulin secretion from pancreatic β-cells and accelerated lipid droplet formation in hepatocytes. Herein we use nonalcoholic fatty liver disease (NAFLD) as an illustration of such pathological liver conditions that result from ER stress in association with oxidative stress. Protecting the ER by eliminating excessive ROS *via*

the administration of antioxidants or by enhancing lipid-metabolizing capacity *via* the activation of peroxisome proliferator-activated receptors represent promising therapeutics for NAFLD.

Key words: Oxidative stress; Reactive oxygen species; Endoplasmic reticulum stress; Nonalcoholic fatty liver disease; Peroxisome proliferator-activated receptor

Core tip: Accumulated experimental data indicate that oxidative stress causes endoplasmic reticulum stress, which together leads to pathogenic damage to cells. The lipid metabolism in the liver is a sensitive target of these types of stress, which appears to be associated with non-alcoholic fatty liver disease.

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INTRODUCTION

Most eukaryotic cells, excluding enucleated cells such as mammalian red blood cells, synthesize proteins based on genetic information from nuclear DNA during their lifespan. Translated secretory and membrane proteins become functional when they undergo appropriate oxidative folding, posttranslational modification, and cellular localization. While the redox status in the cytosol is in a reduced state, the extracellular space is largely in an oxidized state. Premature secretion and exposure to an extracellular environment can cause aberrant conformational changes in proteins due to inappropriate disulfide bond formation. Hence, the appropriate oxidative folding of polypeptides by means of the oxidation of sulfhydryl groups to produce disulfide bridges in proteins within cells is a prerequisite process for producing secretory proteins and the membrane proteins prior to their secretion and translocation to the plasma membrane, respectively (Figure 1). The signaling process for these types of oxidative protein folding reactions was not understood well until the end of the last century[1,2].

The accumulation of misfolded proteins in the endoplasmic reticulum (ER) causes ER stress, which then leads to the malfunction of the ER[3]. Without proper resolution of ER stress, affected cells become dysfunctional and, if not resolved properly, they die. To avoid this unfavorable scenario, multiple defensive machineries, referred to as an unfolded protein response (UPR), are activated under such conditions and play roles in preventing this and permit the cells to recover from this fatal situation[4,5].

Multiple factors, either internally or externally, can cause the accumulation of misfolded proteins in the ER[6,7]. Reactive oxygen species (ROS) are produced during conventional physiological processes accompanied by oxygen consumption and the levels are enhanced under a variety of pathological conditions such as inflammation, high temperature, and a deficit in the antioxidative system, and result in the development of oxidative stress[8]. Both low molecular weight antioxidant compounds and antioxidative enzymes function to control the levels of ROS and reduce their levels to acceptable ranges. However, the antioxidant levels in the ER are relatively low compared to the cytoplasm or other organelles, despite the robust production of hydrogen peroxide *via* active reduction-oxidation (redox) reactions[9]. Oxidative stress perturbs the usual oxidative protein folding, which results in the ER stress and organ failure. Thus, among a variety of stressful conditions, oxidative stress can occur in any cell and is also responsible for ER stress, and they together lead to the development of a pathogenic state.

Herein we overview recent progress in our understanding of the relationships between oxidative stress and ER stress and attempt to clarify the pathogenic pathways that are involved, by focusing on fatty liver diseases.

OXIDATIVE FOLDING OF SECRETORY AND MEMBRANE PROTEINS IN THE ER

Protein conformation is supported by several types of chemical bonds, among which the disulfide bonds formed between cysteine sulfhydryl groups are the primary determinants of overall protein structure for secreted proteins and membrane proteins that face the extracellular space. Endoplasmic reticulum oxidoreductin 1 (ERO1), which contains flavin adenine dinucleotide (FAD) as a redox cofactor, is an evolutionarily conserved protein[10] and, in conjunction with molecular oxygen consumption, catalyzes disulfide formation in nascent proteins *via* protein disulfide isomerase (PDI) in the ER[11,12]. PDI is a member of the family of chaperone molecules that are specifically responsible for disulfide bond formation in proteins in the ER lumen[13,14]. In addition to PDI, several chaperone molecules are also present in the ER lumen and participate in maintaining ER homeostasis[15]. Mammals have two ERO1 genes, ERO1α and ERO1β, that are transcriptionally regulated by the CCAAT-enhancer-binding protein homologous protein (CHOP)[16]. ERO1 first introduces a disulfide bond in PDI and its family members using molecular oxygen as the oxidant[13,14]. Hydrogen peroxide is produced as a byproduct in this oxidative protein folding process. Because not only secretory proteins but also many membrane proteins that face the extracellular space must undergo oxidative protein folding in the ER, hydrogen peroxide is inevitably produced in the ERO1-mediated sulfoxidation reaction. Thus, the ER is exposed to an oxidative insult to a greater or lesser extent as the result of this type of oxidative protein folding[17].

ERO1 is prerequisite for oxidative protein folding

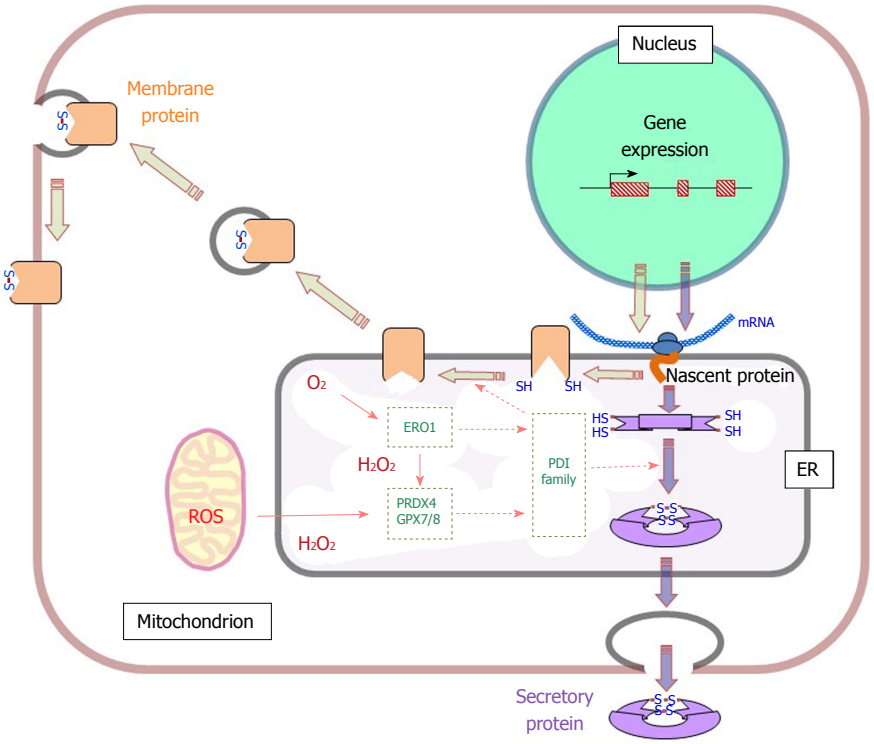

Figure 1 Process for the synthesis and oxidative folding of secretory proteins and membrane proteins within endoplasmic reticulum. During synthesis in the endoplasmic reticulum, both secretory proteins and membrane proteins need to be oxidatively folded before being translocated to final destination *via* the Golgi body. While endoplasmic reticulum oxidoreductin 1 utilizes molecular oxygen to oxidize reactive sulfhydryl groups on protein disulfide isomerase family proteins, peroxiredoxin 4 and glutathione peroxidase 7/8 oxidize them by means of hydrogen peroxide. ROS: Reactive oxygen species; ER: Endoplasmic reticulum; ERO1: Endoplasmic reticulum oxidoreductin 1; PDI: Protein disulfide isomerase; PRDX4: Peroxiredoxin 4; GPX: Glutathione peroxidase.

in yeast and the genetic ablation of the gene results in yeast that are hypersensitive to reducing agents such as dithiothreitol[18,19]. However the genetic ablation of genes encoding ERO1α and ERO1β cause only moderate effects in mice, *e.g.*, a decrease in ERO1 activity blunts the cardiomyocyte inotropic response to adrenergic stimulation and sensitized mice to adrenergic blockade[20]. This rather mild phenotype compared to the case of yeast can be explained by the presence of other redundant sulfoxidase enzymes in mammalian cells. Quiescin sulfhydryl oxidase (QSOX), a flavoprotein, catalyzes disulfide formation in PDI family members using an oxygen molecule and produces hydrogen peroxide[21]. The enzymatic properties are somewhat similar to ERO1. However, since QSOX is localized primarily in the Golgi apparatus and secreted fluids it appears to be unique as a multidomain enzyme[22]. Mammalian cells also possess other sulfoxidases that introduce disulfide bonds to PDI by means of hydrogen peroxide or other oxidizing power, such as peroxiredoxin (PRDX)4 and glutathione peroxidase (GPX)7/GPX8[23], as described below.

CAUSES OF AND RESPONSES TO ER STRESS

The accumulation of inappropriately folded proteins is caused by unfavorable conditions, including the excessive synthesis of proteins, the inhibition of protein glycosylation, and Ca^{2+} depletion[24]. ER homeostasis is maintained by a variety of factors that include ionic and metabolic conditions. For example, ER is the organelle that stores millimolar ranges of Ca^{2+} which is maintained by Ca^{2+}-ATPase localized at the ER membrane. Cardiac and skeletal muscle possess specialized forms for this Ca^{2+} storage, designated as the sarcoplasmic reticulum (SR), that regulates excitation-contraction coupling in these muscular tissues[25]. Ca^{2+}-ATPase in the SR membrane contributes to Ca^{2+} uptake, so that the inhibition of Ca^{2+} uptake by thapsigargin decreases the levels of Ca^{2+} stored in the SR lumen. Similarly thapsigargin inhibits the non-muscular form of Ca^{2+}-ATPase and leads to pathological conditions *via* ER stress[26]. Some antibiotics, *e.g.*, tunicamycin, inhibit the formation of N-linked glycosylation, leading to the suppression of the translocation of nascent proteins to the Golgi body[27].

Heat stress impairs the proper folding of proteins by affecting the free energy needed for correct protein folding and thereby produces misfolded proteins with a high frequency[28]. As a compensatory mechanism, heat shock proteins (HSPs), a large protein family, are induced at high temperatures and ameliorate protein folding *via* their ability to function as a chaperone. Repetitive or sustained stimulation of hormonal secretion, such as the case of insulin secretion under hyperglycemic conditions, is a suspected cause for defected insulin production and β-cell dysfunction, which typically leads to type Ⅱ diabetes[24].

Because the accumulation of misfolded proteins causes dysfunction of the ER and, if not properly resolved,

ultimately results in cell death, several systems in the UPR are activated to resolve the stress. Genes involved in ameliorating ER stress are largely induced by several mechanisms *via* sensor proteins that are localized on the ER membrane. Because this process has been overviewed extensively by experts in this research field[5,29], we briefly mention major machineries and related matters here.

Control of protein synthesis on the rough ER

Both secretory proteins and membrane proteins are synthesized on the rough ER where ribosome-mRNA complexes are attached. Eukaryotic initiation factor 2α (eIF2α) plays a key role in the regulation of the translational process from mRNA on the cytoplasmic surface of the rough ER. Upon ER stress, double-stranded RNA-activated eIF2α kinase-like ER kinase (PERK) is activated upon autophosphorylation and phosphorylates eIF2α, which results in the selective translation of activating transcription factor 4 (ATF4)[30]. ATF4 then transcribes activating transcription factor 3 (ATF3), CHOP, and growth arrest and DNA damage-inducible 34 (GADD34). In the meantime phosphorylated eIF2α causes the synthesis of many other protein to be suppressed and restricts further protein influx to the ER lumen *via* attenuating the guanine nucleotide exchange factor eIF2B[31].

Sensing and signaling to nucleus

The inositol requiring enzyme 1 (IRE1) was originally reported as a yeast membrane protein that was predicted to be a kinase involved in inositol metabolism[32]. The potential role of IRE1 in the transmission of the unfolded protein signal across the ER or the inner nuclear membrane was then rediscovered later[33]. IRE1 undergoes activation *via* oligomerization in response to unfolded proteins and autophoshorylation. Active IRE1 then completes the splicing of the X-box-binding-protein 1 (XBP1) precursor mRNA by its RNase activity[34]. The XBP1 protein translated from the mature XBP1 mRNA moves to the nucleus and then transcriptionally activates genes for a protein chaperone, such as the glucose-regulated protein of 78 kDa (GRP78 also referred to as Bip)[35] and PDIs[36].

Activating transcription factor 6 (ATF6) was also originally found in yeast[37], but its localization in the ER membrane was recognized at later period. The localization of ATF6 to the ER membrane in its inactive form and its subsequent transcriptional activation in response to UPR have been reported[38]. ER stress stimulates the translocation of the precursor form of ATF6 to the Golgi membrane where Site-1 (S1P) and the Site-2 proteases (S2P) are located[39,40]. These proteases release the transcriptional activator domain from the precursors, and the resulting active ATF6 moves to the nucleus where it transcriptionally activates the corresponding genes[41]. Astrocytes specifically express a unique ER stress transducer, the old astrocyte specifically induced substance (OASIS), that regulates the signaling of UPR and contributes to the resistance to ER stress[42].

ER-associated degradation of misfolded proteins

Misfolded proteins that are formed within the ER lumen undergo either refolding to the correct conformation in a chaperone-mediated manner or undergo proteolytic degradation, designated as ER-associated degradation (ERAD)[43]. PDI is the first enzyme that was reported to catalyze protein folding[44]. Nearly 20 PDI family proteins are present in the ER lumen and are involved in correcting disulfide bonds and also the oxidative folding of nascent proteins in the ER in association with ERO1 and other sulfoxidases[45].

Some unfolded proteins are exported out from the ER lumen or the ER membrane, polyubiquitinated and then degraded by proteasomes[46]. ERAD pathways, which are further classified into ERAD-L, -M, and -C, are responsible for misfolded proteins in the ER lumen, within the membrane, or on the cytosolic side of the ER membrane, respectively[43]. RING-finger ligase Hrd1, in association with other components appear to play a major role in ERAD-L and ERAD-M[47]. A retro-translocation channel is formed by Hrd1 in complex with the partner molecule Hrd3 and are involved in the polyubiquitination of target proteins[48]. Thus, among the several candidate channel molecules, the Hrd1-Hrd3 complex appears to play a *bona fide* role in polyubiquitination/protein translocation in the ERAD. Derin 1 (Der1), which reportedly initiates the export of aberrant proteins from the ER[49], appears to aid luminal substrates to be moved to the Hrd1 channel. The potential involvement of other ubiquitin ligases that span the ER membrane in this manner has also been proposed.

The machinery responsible for ERAD is also involved in cholesterol homeostasis by controlling the protein levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase in the ER membrane[50]. HMG CoA reductase, which is embedded in the ER membrane, limits cholesterol synthesis. The presence of a sufficient amount of cholesterol results in an accelerated degradation of HMG CoA reductase *via* the activation of polyubiquitination followed by proteasome-catalyzed degradation[51], a process that is independent from ER stress. Hence, the ERAD system appears to be involved in, not only the proteolytic removal of misfolded proteins, but also regulation of the cholesterol metabolic process by means of the same ER-associated machinery. Moreover, because some ER-resident proteins such as Bip[35], PDI[36], and PRDX4[52,53] are reportedly present in the cytosol, the ERAD machinery may be partly responsible for the retro-translocation of these proteins from the ER to the cytosol.

Induction of cell death

Cell death is induced when these protective mechanisms against the ER stress functions insufficiently and do not rescue ER function[54]. CHOP, also referred to as GADD153, is a developmentally regulated nuclear protein that has a strong sequence similarity to transcription factors C/EBP. CHOP functions as a dominant-negative inhibitor of the activity of C/EBP-like proteins[55]. CHOP induces the production of ERO1α, which then activates the inositol 1,4,5-triphosphate (IP_3) receptor and stimulates the release of Ca^{2+} from the ER *via* the IP_3 receptor channel. The increased levels of cytosolic Ca^{2+} then trigger apoptosis in cells that are under ER stress[56], and induction

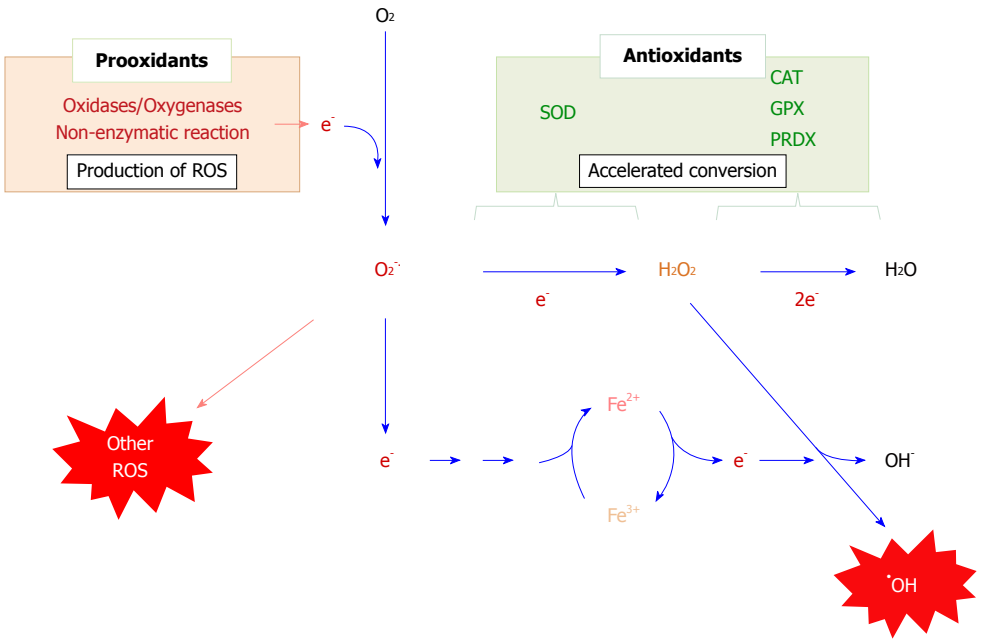

Figure 2 Production, conversion, and suppression of reactive oxygen species. The production of reactive oxygen species is largely initiated by a one-electron donation to an oxygen molecule, resulting in the production of superoxide ($O_2^{\cdot-}$). Superoxide undergoes spontaneous dismutation to hydrogen peroxide, a process that is markedly accelerated by superoxide dismutase. The resulting hydrogen peroxide can be rapidly eliminated by peroxidases, such as catalase, glutathione peroxidase and peroxiredoxin In the meantime, a one-electron reduction of a transition metal, notably ferric to ferrous ion, results in the conversion of hydrogen peroxide to hydroxyl radicals ($^{\cdot}OH$). ROS: Reactive oxygen species; SOD: Superoxide dismutase; CAT: Catalase; GPX: Glutathione peroxidase; PRDX: Peroxiredoxin.

of cell death is a well-established function of CHOP[57]. ERO1α was found to be enriched in the mitochondrial associated membrane (MAM), the interface area of the ER with mitochondria[58], and may also regulate mitochondrial Ca^{2+} flux *via* controlling redox homeostasis in the ER[59]. The pro-apoptotic roles of CHOP are supported by the finding that the disruption of the gene that encodes CHOP causes delays in the development of ER stress-mediated diabetes and decreases the sensitivity of pancreatic islet cells to nitric oxide toxicity[60,61]. PERK and the IRE1 pathway activate CHOP, which, after translocation to the nucleus, inhibits the expression of anti-apoptotic genes, such a BCL-2, and activates the pro-apoptotic genes, Bim and DR5[62,63]. Nevertheless, CHOP appears to have an additional function, which needs clarification[64].

OXIDATIVE STRESS AND ER STRESS

Superoxide radicals are produced primarily *via* a one-electron donation to molecular oxygen, which is mediated either enzymatic reactions such as oxygenases or non-enzymatic reactions such as glycoxidation[65,66] (Figure 2). While ROS, notably hydrogen peroxide, produced by stimuli function as signal regulators[67], excessively produced ROS cause oxidative stress that can exert a variety of effects over cellular functions. Transferring an unpaired electron together with oxygen to an unsaturated lipid causes lipid peroxidation, which then triggers a radical chain reaction[68]. Superoxide is not reactive as a radical, but it may trigger the production of more reactive free radicals by a reaction referred to as Fenton chemistry, a process that is initiated by the donation of an electron to a transition metal ion, such as Fe^{3+}[69]. The resultant Fe^{2+} reacts with hydrogen peroxide, leading to the production of hydroxyl radicals, one of the most harmful molecules, which oxidizes lipids that contain unsaturated fatty acids, bases in nucleic acids, and proteins.

Sources for oxidative stress in the ER

Based on the amount of consumed oxygen molecules, mitochondria are generally regarded as the main source of ROS production. MAM appears to be the place where these organelles communicate and provide ROS to the ER from mitochondria[58]. NADPH-oxidases (NOX) are another source of ROS and frequently cause oxidative stress, especially under inflammatory conditions[70]. After mitochondria, the ER is the organelle that consumes the most molecular oxygen because there are several oxygenases that are associated with this organelle. While most NOX members are located in the plasma membrane and produce superoxide as primary ROS, NOX4, a ER-resident enzyme, faces the ER lumen and releases hydrogen peroxide by the two-electron reduction of an oxygen molecule[71]. Cytochrome P450 is largely associated with the ER membrane and releases ROS to the cytoplasmic side[72,73]. However, these genes are selectively expressed in tissues, such as the liver and steroidogenic organs.

In the meantime, oxygen molecule is also consumed inside the ER by ERO1[74,75], which is involved in oxidative protein folding in most cells, as mentioned above. In fact, the ER is the organelle that produces the highest levels of hydrogen peroxide[76]. The levels of hydrogen peroxide increase under conditions where the secretion of proteins is enhanced due to ERO1-mediated oxidative protein

folding[77]. Insulin production is maintained at high levels under prolonged hyperglycemic conditions and hydrogen peroxide levels are also kept high, which may cause oxidative stress together with ER stress in pancreatic β-cells and ultimately aberrant insulin secretion[78]. Hydrogen peroxide may also come from other cellular compartments or reactions other than ERO1-catalysed PDI oxidation[79].

Machineries maintaining ER homeostasis against oxidative stress

To avoid oxidative stress, ROS are either eliminated *via* interactions with low molecular weight antioxidants, such as glutathione (GSH) and vitamin C, or converted to less-reactive compunds by antioxidative enzymes. Superoxide is exclusively converted to hydrogen peroxide by superoxide dismutase (SOD). In the case of the elimination of hydrogen peroxide, GSH peroxidase (GPX) and peroxiredoxin (PRDX), which are encoded by respective gene families[80,81], largely contribute to the reductive detoxification of hydrogen peroxide. While conventional GPX utilizes GSH as the electron donor, PRDX largely utilizes thioredoxin for this purpose. Extracellular superoxide dismutase (SOD3) and the plasma form of GPX (GPX3) are produced in the ER in the same manner as other secretory proteins. However, while some of them may be retained in the ER lumen, most are excreted. Thus, only few antioxidative systems are present in the ER lumen compared to cytoplasm and mitochondria.

Conversion of superoxide to hydrogen peroxide

Superoxide is converted to hydrogen peroxide by dismutation, either by a spontaneous reaction between two superoxide molecules or by accelerated production *via* the SOD-catalyzed reaction[82]. The latter reaction is about 3000 times faster than the spontaneous dismutation and causes superoxide levels to be decreased to less than 0.5% of that of the SOD1-free erythrocytes[83]. However, the physiological relevance of SOD is sometimes a topic of debate because hydrogen peroxide, which is not a radical but is still a reactive compound, is the product of the catalytic conversion of superoxide. While hydrogen peroxide does not itself cause a radical chain reaction, on accepting one electron, hydrogen peroxide is converted to a highly toxic molecule, hydroxyl radical, *via* the Fenton reaction[69]. The Fenton reaction involves a one-electron reduction of a transition metal ion in the preceding step and can be prevented by either the removal of the free transition metal ion or the elimination of the radical species that serves as the electron donor. Thus, the physiological significance of SOD can be rationally explained by the rapid elimination of superoxide before donating its unpaired electron. The resulting hydrogen peroxide can be reduced immediately to water by peroxidases that abundant in the body.

Among the three SOD isozymes present in mammalian cells, SOD1, which is localized in the cytoplasm and the intermembrane space in mitochondria as well, is expressed ubiquitously in the body and the best characterized from the biochemical and pathological viewpoints[82]. A missense mutation in SOD1 is reportedly a cause of familial amyotrophic lateral sclerosis (ALS)[84]. More than 150 mutations have been identified in the *SOD1* gene in familial ALS, and several model mice have been established by the transgenic overexpression of the mutant gene and have been employed in attempts to elucidate the etiology of ALS and to develop possible drugs[85]. On the contrary, the genetic ablation of SOD1 does not induce ALS in mice[86], indicating that ALS develops as a consequence of the gain-of-function of mutant SOD1. Among several proposed mechanisms, an attractive hypothesis has been proposed from viewpoint of ER stress-mediated motor neuron death as follows. ALS-linked mutations of SOD1 cause chronic ER stress through interactions with Der1, a component proposed for ERAD[87], as described above. In addition it is noteworthy that, under zinc-depleted conditions, the wild-type SOD1 interacts with Der1[88,89], suggesting that SOD1 plays a role in the interaction with Der1. Therefore SOD1 may be post-translationally converted into a pathogenic molecule under zinc insufficient conditions and could also cause sporadic ALS. In the meantime, mutant SOD1 interacts with subunits of the coatomer coat protein Ⅱ (COPⅡ) complex, which is essential for ER-Golgi transport[90]. Thus, mutant SOD1 may aggravate ER stress *via* the suppression of membrane transport from the ER to the Golgi body.

Reductive detoxification of hydrogen peroxide and oxidative folding by PRDX4

Because ERO1 and other oxidoreductases that are involved in oxidative protein folding produce hydrogen peroxide, which would be expected to lead to ER stress *via* oxidative modification, especially sulfhydryls in nascent proteins. While peroxiredoxins exert peroxidase activity using the reducing power derived from reducing agents, such as thioredoxin, PRDX4 possesses a hydrophobic signal peptide[91] and is the only form present in the ER lumen[92,93]. PRDX4 eliminates peroxides in a thioredoxin-dependent manner *in vitro* analogous to other family members[94], but the thioredoxin-redox system is much less active in the ER compared to the cytosol. Hence, it is not likely that thioredoxin supports the elimination of hydrogen peroxide by PRDX4 in the ER.

PRDX4-catalyzed disulfide formation in PDI proteins by means of hydrogen peroxide was first demonstrated *in vitro*[95,96]. PRDX4 utilizes hydrogen peroxide to oxidize reactive sulfhydryls in the PDI, rescues an ERO1 deficiency in yeast[95], and catalyzes hydrogen peroxide-mediated oxidative protein folding in the ER[97]. Among the PDI family members, two proteins, P5 and ERp46, are preferential targets of PRDX4[98]. While ERO1 utilizes molecular oxygen as an electron acceptor and produces hydrogen peroxide[95], PRDX4 utilizes electrons from sulfhydryl groups in PDI family proteins and reduces hydrogen peroxide. Thus PRDX4 is beneficial from the standpoint of antioxidation as well[99]. PRDX4 regulates disulfide formation in glycerophosphodiester phosphodiesterase

(GDE)2, a membrane protein, and determines its cell surface expression and consequently neurogenesis in mouse embryos as well[100].

The genetic ablation of PRDX4 causes a defect in the development of testis but otherwise shows a moderate phenotypical abnormality in mice[101]. This can be attributed to a functional redundancy of other molecules in the ER. The induction of PRDX4 is observed during the terminal differentiation of B cells to plasma cells after a lipopolysaccharide treatment[102]. In PRDX4-deficient plasma cells, large aggregates of IgM are actually formed in the ER. The ablation of both ERO1 and PRDX4 causes the consumption of ascorbic acid, which donates electrons for the hydroxylation of prolines in collagen, and results in atypical scurvy, which is characterized by an insufficient production of collagen[103]. The pancreas express PRDX4 abundantly[104] probably because protein secretion both as exocrine and endocrine mechanisms is critical in this organ. In fact mice that overexpress PRDX4 show protection against high-dose streptozotocin-induced diabetes in transgenic mice[105,106]. The biosynthesis and secretion of insulin are improved by the overexpression of PRDX4 in INS-1E cells[107]. Moreover, atherosclerosis in apolipoprotein E-knockout mice is attenuated by the excessive expression of PRDX4[108].

Issues regarding the physiological functions of PRDX4 other than ER sulfoxidase remain ambiguous and should be resolved. A portion of the PRDX4 is present in blood plasma[104] and is elevated under some pathological conditions[109]. However, its release from cultivated cells is suppressed by oxidative stress[110], so that the organ source and the mechanism responsible for the release of the PRDX4 are unknown. Moreover, sexually matured testes express a variant form of PRDX4 that is transcribed from the alternative exon 1[111]. The translated variant protein does not possess a secretory signal peptide, which results in this protein being localized in the cytosol. The variant form of PRDX4 exhibits an antioxidative function[112], but its physiological significance in testes is unknown.

Other proteins involved in disulfide formation in the ER

Several other proteins that are present in the ER lumen also appear to participate in disulfide formation[113]. GPX7 and GPX8 exhibit sulfoxidase activity in the ER[81,114], and GPX8 is reportedly more selective for the reduction of hydrogen peroxide produced by ERO1. GPX8 appears to form a complex with Ero1α and exerts peroxidase activity that prevents the diffusion of Ero1α-derived hydrogen peroxide into and out of the rough ER[115]. Similar to PRDX4, GPX7 and GPX8 are not expressed in β cells but improves insulin secretion when overexpressed in INS-1E β-cells[116]. Vitamin K epoxide reductase (VKOR) catalyzes the reduction of vitamin K 2,3-epoxide and vitamin K to vitamin K hydroquinone, which is required for the γ-glutamyl carboxylation of coagulation factors[117]. VKOR is an ER protein consisting of four transmembrane helices, employs an electron transfer pathway similar to the bacterial homologue and preferably couples with membrane-bound thioredoxin-like redox partners[118]. The combined depletion of ERO1, PRDX4, and VKOR actually causes cell death, and VKOR alone is capable of supporting cell viability and protein secretion in the absence of ERO1 and PRDX4 activities[119]. Thus, VKOR appears to function sufficiently in oxidative protein folding and compensates for the lack of other sulfoxidases.

Low molecular redox compounds

Some small molecules other than proteins are also reportedly involved in redox homeostasis in the ER. GSH is the most abundant non-protein thiol in cells and plays pleiotropic roles, such as in antioxidation and in the detoxification of toxicants[120]. The antioxidative functions of GSH are effectively expressed by a donation of electrons to peroxides *via* GPX[81]. Hence, a GSH insufficiency triggers a redox imbalance and makes cells more vulnerable to an oxidative insult, leading to cell death in severe cases. ER contains 15 mM GSH[121] which reportedly supports the secretion of proteins by its redox capacity. However, GSH depletion, which is caused by expressing an exogenous GSH-metabolizing enzyme in the ER lumen, shows no measurable effect on the induction of UPR, suggesting the existence of an alternative molecule capable of supporting redox reactions in the ER[122].

Ascorbic acid is also present abundantly in the ER and appears to support oxidative protein folding. As mentioned above, a double deficiency of ERO1 and PRDX4 causes atypical scurvy in mice due to ascorbic acid consumption and defects in collagen synthesis[103]. However, the genetic ablation of gulonolactone oxidase (GULO), which is the enzyme that catalyzes the last step of ascorbic synthesis but which is lacking in primates, shows normal collagen status in mouse skin[123]. In mice that lack aldehyde reductase encoded by AKR1A, ascorbic acid synthesis is decreased to approximately 10% of wild-type mice[124] but normal collagen synthesis in the fibrotic kidney after unilateral ureteral obstruction was observed[125]. Thus, these reports suggest the presence of other electron donors, other than ascorbic acid, to support collagen synthesis.

NON-ALCOHOLIC FATTY LIVER DISEASE AS A REPRESENTATIVE INJURY MEDIATED BY OXIDATIVE /ER STRESS

Non-alcoholic fatty liver disease (NAFLD) is a condition where the fat content in the liver exceeds 5%-10% by weight without a history of an excessive ingestion of alcohol[126]. A variety of metabolic dysfunctions including diabetes, dyslipidemia, and the metabolic syndrome are associated with NAFLD, which then degenerates to non-alcoholic steatohepatitis (NASH), a condition associated with inflammation and fibrotic liver damage[127].

Roles of oxidative/ER stress in fatty liver disease

Oxidative stress is commonly regarded as "the second hit" for NASH development[128]. Signaling pathways for the ER stress-induced inflammatory process to NAFLD de-

velopment has been extensively overviewed[129]. Oxidative stress triggers ER stress by stimulating the formation of misfolded proteins, and they together coordinately trigger NAFLD[130,131]. Below we briefly summarize the processes for lipid droplet accumulation triggered by these types of stress.

Sterol regulatory element-binding proteins (SREBPs) are the main transcriptional regulatory factors that control the synthesis of fatty acids and cholesterol under the control of the sterol status in the ER membrane[51]. Two SREBP genes, SREBP1 and SREBP2, are present in mammals. SREBP1 is alternatively transcribed into two forms SREBP1a and SREBP1c and is mainly involved in fatty acid synthesis. SREBP2 regulates a series of genes that are responsible for cholesterol synthesis. SREBPs are membrane proteins predominantly exposed to the cytoplasmic side and bind to another ER protein, the SREBP cleavage-activating protein (SCAP), which is located in the ER membrane[132]. Intermembrane domains on the SCAP protein bind cholesterol, which enable the formation of the SCAP/SREBP complex. Insig-1, another ER membrane protein, block the lateral movement of SCAP/SREBP into COPⅡ-coated vesicles on ER membranes and prevent them from reaching the Golgi body[51,133]. Upon the dissociation of cholesterol due to an insufficient presence within the ER membrane, the SCAP/SREBP complex is released from Insig-1, translocated to the Golgi apparatus and then cleaved by S1P and S2P there[134]. This proteolytic activation recruits the cytoplasmic domain of the proteins to the nucleus, which then results in the induction of the expression of a series of lipogenic genes[135]. As described above, S1P and S2P are processing enzymes for the ER membrane-bound transcriptional regulatory protein ATF6 and activate it under conditions of ER stress[41]. Because the proteolytic machinery is shared, ER stress actually upregulates SREBP-1c, leading to the accumulation of lipid in hepatic cells[136]. Glycogen synthase kinase (GSK)-3 appears to be involved in signaling downstream of ER stress[137]. In the meantime HMG CoA reductase limits cholesterol synthesis, irrespective of ER stress. The protein levels of HMG CoA reductase are indirectly regulated by cholesterol *via* an Insig-mediated reaction; *i.e.*, the presence of sufficient amounts of cholesterol consequently drives the polyubiquitination of HMG CoA reductase and proteolytic degradation by proteasomes[50].

The fact that ER stress induces the activation of SREBPs, in turn, suggests that SREBP-mediated lipogenesis also activated under conditions of oxidative stress. In fact, oxidative stress induces SREBP1c activation and lipid accumulation[138]. Thus, oxidative stress and ER stress interdependently stimulates the *de novo* synthesis and accumulation of triglycerides and cholesterol but, on another front, inhibits the secretion of lipoproteins[139]. Because cell cultures are typically performed under atmospheric oxygen, the spontaneous activation of SREBP1 and the associated expression of genes fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase (SCD)1 are observed in primary hepatocytes and this is greatly enhanced in SOD1-knockout hepatocytes[140]. For the excretion of triglyceride-rich lipoproteins, appropriate oxidative folding of the apoB protein is essential[141,142]. In fact, oxidative stress appears to suppress lipoprotein secretion, which would be most likely caused *via* the misfolding of apoB and an impaired microsomal transfer protein function[143]. Stimulation of lipogenesis and the inhibition of lipoprotein secretion would cooperatively elevate lipid droplet accumulation, which would consequently result in the development of liver steatosis (Figure 3). This interdependent work of dealing with oxidative stress and ER stress in liver steatosis is further supported by recent observations showing the double knockout of SOD1 and PRDX4 result in aggravated liver damage compared to singly knockout mice[144].

An examination of fatty acid metabolism in NAFLD patients indicates that the inability of the liver to regulate changes in lipogenesis during the transition from the fasted to fed state is the underlying mechanism responsible for this[145]. Fasting induces the formation of lipid droplets, not only in the liver but also other tissues that are dominantly involved in active β-oxidation. While feeding a high fat diet leads to lipid droplet accumulation in the liver[146] and intestinal epithelia[143] more intensely in SOD1-knockout mice than the wild-type mice, fasting induces severe and irreversible damage not only to the liver but to other aerobic organs as well[147,148]. Thus fasting may aggravate oxidative damage in these organs and become a serious pathogenic factor for NAFLD.

These observations raise the next query, *i.e.*, why is lipogenesis elevated as a consequence of UPR in cases of ER stress if lipid accumulation is unfavorable for the liver? We have a clue to this, in that feeding a lard-containing high-calorie diet increases the accumulation of lipid droplets but improves the longevity of the SOD1-deficient mice compared to mice fed a normal diet[149]. This is unexpected because lipid accumulation is generally recognized to be an exacerbating factor for liver function. Based on these observations we hypothesized that lipid droplets that accumulate in response to oxidative stress may have a protective role against the hepatotoxic effects of ROS. Experimental data actually suggest that lipids that transiently accumulate in the liver have a protective function against oxidative injury caused by a liver toxicant thioacetamide in mice and by hydrogen peroxide in cultivated cells[150]. Thus, the accumulation of lipids under ER stress conditions may also be regarded as an adoptive response of hepatocytes to oxidative stress conditions[151], although liver steatosis at a more advanced stage is hazardous and should be treated appropriately.

Therapeutics to combat against NAFLD

Multiple processes appear to underlie the pathogenesis of NAFLD, so that a variety of agents and treatment may be applicable for therapeutic purposes. Antioxidants directly eliminate ROS and result in the suppression of oxidative stress and consequently ER stress. Hydrophobic antioxidants, such as vitamin E[68] and coenzyme Q_{10}[152], and their derivatives and hydrophilic antioxidants, such as vitamin C (ascorbic acid) and *N*-acetylcysteine, a precursor

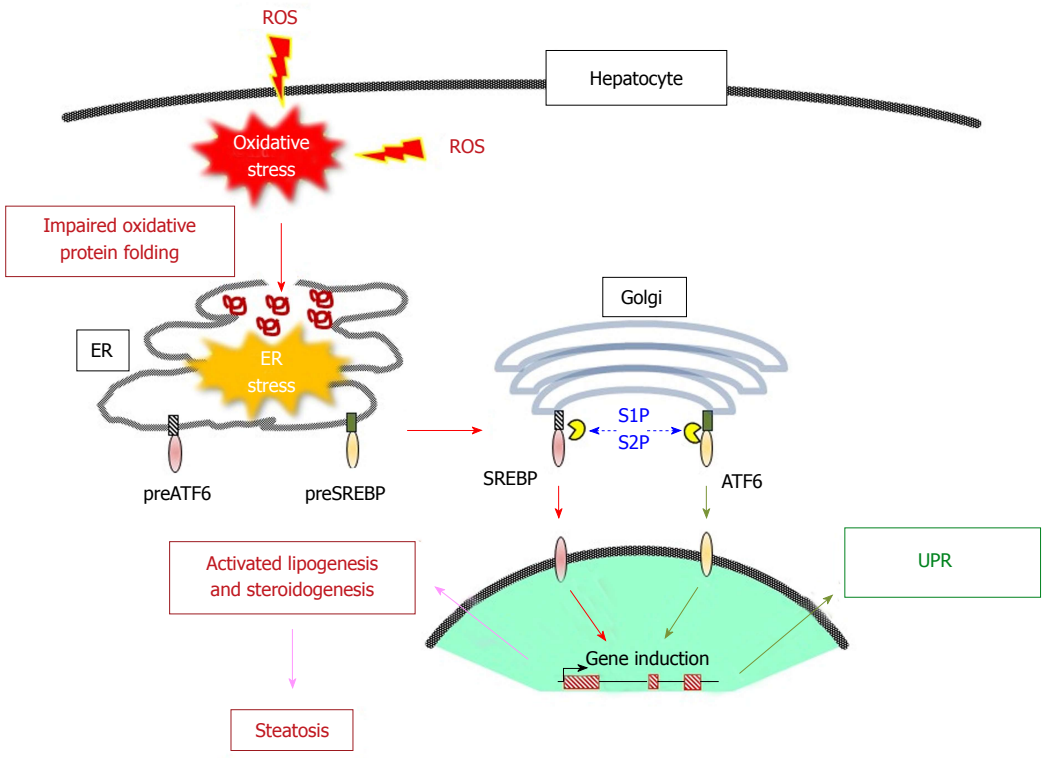

Figure 3 Coordinate action of oxidative stress and endoplasmic reticulum stress in liver steatosis. Excessively produced reactive oxygen species (ROS) cause the misfolding of proteins in the endoplasmic reticulum (ER), which leads to ER stress. The precursor forms of activating transcription factor 6 (ATF6) and sterol regulatory element-binding proteins (SREBPs) in the ER membrane are translocated to the Golgi body by an independent mechanism but are proteolytically activated there by site-1 protease (S1P) and S2P. The transcriptionally active ATF6 and SREBP then move to the nucleus. While ATF6 exerts a protective function by activating the genes involved in the unfolded protein response, SREBPs activates genes that are involved in lipogenesis and steroidogenesis, which may lead to the development of nonalcoholic fatty liver disease. ROS: Reactive oxygen species; ER: Endoplasmic reticulum; ATF6: Activating transcription factor 6; SREBP: Sterol regulatory element-binding protein; S1P: Site-1 protease; UPR: Unfolded protein response.

for cysteine and GSH[153], may be promising agents for use in ameliorating the effects of NAFLD. In fact, vitamin E and polyphenol have been reported to be useful in the treatment of NAFLD patients, while the issue of whether vitamin C is beneficial is ambiguous at this moment[154,155].

While antioxidants decrease the levels of pathogenic ROS directly, it would be helpful if cellular capacity to resist malfunctions in lipid metabolism could be additionally enhanced. In this sense, peroxisome proliferator-activated receptors (PPARs) and their binding partner retinoid X-receptors (RXR), appear to have roles in maintaining lipid homeostasis and, hence, represent promising targets for ameliorating the pathogenesis of NAFLD[126]. PPAR family proteins of which PPAR-α, PPAR-β/δ, PPAR-γ are members, can be either activated or inhibited by lipid metabolites and other lipophilic agents *in vivo*. Regarding the distribution of PPARs in tissues, the highest expression of PPAR-α is observed in brown adipose tissue, liver, kidney, and heart[156]. The expression of PPAR-γ is higher in adipose tissues than other tissues. Compared to these isoforms, the expression of the PPAR-β/δ isoform is rather ubiquitous. Mice in which PPAR-α is knocked out are viable and fertile and show no detectable gross phenotypic defects[157]. Contrary to PPAR-α knockout mice, a PPAR-γ deficiency showed impaired placental vascularization, leading to embryonic death by embryonic day E10.0[158]. Similarly, the genetic ablation of PPAR-β/δ also impairs the placenta and leads to embryonic death at E9.5 - E10.5[159].

The roles of PPAR-β/δ are less established compared to those of PPAR-α and PPAR-γ. In 2003 it was reported that PPAR-δ contributes to fat metabolism[160,161], which then attracted more interest in PPAR-δ than before. For example, PPAR-δ activation enhances fatty acid oxidation and rescues ER stress in pancreatic β-cells[162]. The PPAR-α/δ agonist GFT505 prevents high fat diet-induced liver steatosis and protects the liver from inflammatory reactions in mice[163]. The activation of PPARβ/δ by GW501516 also prevents the inflammation associated with ER stress in skeletal muscle cells and ameliorates insulin resistance in mice through an adenosine monophosphate-activated protein kinase (AMPK)-dependent mechanism[164]. The PPAR-β/δ agonist GW0742 also reportedly attenuates ER stress by improving hepatic energy metabolism in the livers of high fat diet-administered mice[165]. The effectiveness of agonists for PPAR-α/δ has been confirmed in NASH patients as well[166]. Thus, the application of PPAR agonists, notably those for PPAR-β/δ isoforms, appears to be promising therapeutics for the treatment of NAFLD[167]. While the molecular mechanisms for how PPAR-β/δ controls insulin signaling are largely

unknown, a stable interaction of PPAR-β/δ with nuclear T-cell protein tyrosine phosphatase 45 (TCPTP45) isoform has been demonstrated as the most upstream component that resolves the downregulation of insulin signaling[168]. Forthcoming experiments directed at elucidating the pathway in more detail would provide a clearer vision on this issue.

As described above, the activation of PPARβ/δ is a largely promising area of therapeutics for fatty live diseases in animal experiments[169]. However, it should be remembered that the most potent and specific activator for PPAR-β/δ, GW501516, also accelerates intestinal adenocarcinoma in Apc^{min} mice that are predisposed to developing intestinal polyposis[170]. The activation of PPAR-β/δ by GW501516 is also associated with the induction of cancer development, while the PPAR-γ agonist GW7845 causes a moderate delay in tumor formation[171]. At the present stage, the activation of PPAR-β/δ does not always lead to consistent results; *i.e.*, it is either pro-carcinogenic or anti-carcinogenic, depending on carcinogenic model and the animals being used[172,173]. Therefore the development of activating agents that exert a beneficial action on PPAR-β/δ, but do not have other side effects, is awaited for using synthetic activators for therapeutic purposes in the treatment of fatty liver diseases.

PERSPECTIVES

Oxidative stress and ER stress are frequently linked and become pathogenic in some diseases such as NAFLD[174]. Antioxidants suppress oxidative stress directly and also subsequent ER stress and, hence, can mitigate the causal factors for NAFLD. In the meantime, the activation of PPARs renders the liver more resistant against stress caused by lipotoxicity. Coordinated treatment with these agents may exert therapeutic effects more efficiently in NAFLD as well as in other diseases that are caused by aberrant lipid metabolism.

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MINIREVIEWS

Role of STIM1 in neurodegeneration

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Abstract

STIM1 is an endoplasmic reticulum (ER) protein with a key role in Ca^{2+} mobilization. Due to its ability to act as an ER-intraluminal Ca^{2+} sensor, it regulates store-operated Ca^{2+} entry (SOCE), which is a Ca^{2+} influx pathway involved in a wide variety of signalling pathways in eukaryotic cells. Despite its important role in Ca^{2+} transport, current knowledge about the role of STIM1 in neurons is much more limited. Growing evidence supports a role for STIM1 and SOCE in the preservation of dendritic spines required for long-term potentiation and the formation of memory. In this regard, recent studies have demonstrated that the loss of STIM1, which impairs Ca^{2+} mobilization in neurons, risks cell viability and could be the cause of neurodegenerative diseases. The role of STIM1 in neurodegeneration and the molecular basis of cell death triggered by low levels of STIM1 are discussed in this review.

Key words: Calcium; Neurodegeneration; Parkinson's disease; Alzheimer's disease; STIM1; Voltage-operated Ca^{2+} channels

Core tip: STIM1 is an endoplasmic reticulum protein that regulates store-operated Ca^{2+} entry, which is a Ca^{2+} influx pathway involved in a wide variety of signalling pathways. Growing evidence supports a role for this protein, STIM1, in long-term potentiation and the formation of memory. In this regard, the loss of STIM1 observed in brain tissue from Alzheimer's disease patients risks cell viability and could be the cause of neurodegenerative diseases. This is the reason for discussing the role of STIM1 in neurodegeneration in this review.

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STIM1 AND CALCIUM MOBILIZATION

STIM1 (stromal interaction molecule 1) is a type I transmembrane protein located mainly in the endoplasmic reticulum (ER), with a significant pool of approximately 20% at the plasma membrane. Due to its Ca^{2+}-sensitive EF-hand domain close to the N-terminus, STIM1 acts as an ER-intraluminal Ca^{2+} sensor[1,2]. This EF-hand domain shows an apparent dissociation constant for Ca^{2+} of 250 μmol/L[3]. The decrease of the ER-intraluminal Ca^{2+} concentration, with the subsequent dissociation of Ca^{2+} from the EF-hand domain, triggers the oligomerization and the conformational change of STIM1. These two events are critical for STIM1 activation.

The rapid decrease of the ER-intraluminal Ca^{2+} concentration is a common event in cells under diverse stimuli, such as the activation of growth factor receptors or the activation of G protein-coupled receptors. In both cases, phosphoinositide-specific phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. The generation of IP_3 activates its receptor at the ER, with the subsequent release of Ca^{2+} through this channel/receptor and the rise of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$). As mentioned above, the emptying of intracellular Ca^{2+} stores (mainly the ER) activates STIM1, which is then able to bind and activate STIM1-dependent Ca^{2+} channels[4], such as ORAI1[5]. The activation of ORAI1 leads to the transient increase of Ca^{2+} influx and to the rise of $[Ca^{2+}]_c$, which is required for the refilling of the ER and for the sustainability of this system in successive stimulations. Thus, STIM1 protein and STIM1-dependent Ca^{2+} channels ensure Ca^{2+} mobilization and the stimulation of Ca^{2+}-dependent signaling pathways by activating the "store-operated Ca^{2+} entry" (SOCE), *i.e.*, the Ca^{2+} influx pathway activated by the decrease of the ER-intraluminal Ca^{2+} level.

The activation of plasma membrane Ca^{2+} channels by STIM1 is carried out in ER-plasma membrane contact sites (ER-PM junctions)[6], where STIM1 relocalizes in response to Ca^{2+} store depletion. When the ER-intraluminal Ca^{2+} concentration is high, STIM1 remains bound to the growing tip of microtubules and moves freely on the ER surface[7]. However, activated STIM1 becomes phosphorylated at three ERK1/2-target sites (Ser575, Ser608, and Ser621) and this phosphorylation is critical for enhancing the dissociation from microtubules[8,9]. Oligomers of active STIM1 are less mobile and phospho-STIM1 is found at the cell periphery[10], close to the plasma membrane, where it binds ORAI1. Because STIM1 and ORAI1 are ubiquitous, they are involved in a wide range of signaling pathways that regulate many cellular functions[11]. However, the number of studies about the role of STIM1 in neuronal tissue is much more limited.

STIM1 EXPRESSION AND FUNCTION IN NEURONAL CELLS

STIM1 is widely expressed in the brain according to databases such as Expression Atlas (from the European Bioinformatics Institute, http://www.ebi.ac.uk/gxa) or UniGene (from the National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/unigene). Indeed, it is well known that STIM1 becomes activated upon depletion of intracellular Ca^{2+} stores in the brain in a similar fashion to that found in any other cell or tissue[12,13]. The role of STIM1 in neuronal function was initially suggested in *Drosophila melanogaster* neurons. Shortly after the description of STIM1 as the main regulator of SOCE, it was proved that STIM1 was required for normal flight and associated patterns of rhythmic firing of the flight motoneurons[14], and that SOCE regulates spatial and temporal Ca^{2+} mobilization in vertebrate photoreceptor cones, suggesting a role in the generation of excitatory signals across the retinal synapse[15].

A key finding was reported in 2010 by Ricardo Dolmetsch's and Donald L. Gill's labs. They found that STIM1 directly suppresses depolarization-induced opening of the voltage-operated Ca^{2+} channel (VOCC) $Ca_V1.2$[16,17]. What was striking was the fact that STIM1 binds to $Ca_V1.2$ through the same domain that activates ORAI1, the Ca^{2+} release-activated Ca^{2+} activation domain, and also triggers the internalization of the channel from the membrane. These findings provided the molecular explanation for the shared control of Ca^{2+} entry through ORAI1 and $Ca_V1.2$, making it possible for them to operate independently. In HEK293 cells, it was later reported that Homer proteins are required for the binding between STIM1 and $Ca_V1.2$ channels upon Ca^{2+} store-depletion conditions triggered by thapsigargin[18], an inhibitor of the ER-Ca^{2+} pump.

T-type VOCCs, such as $Ca_V3.1$, are also modulated by STIM1. This was first observed not in neurons but in cardiomyocytes, where it was reported that STIM1 co-precipitated with $Ca_V1.3$ channels, and that the knocking-down of STIM1 expression

increased $Ca_V1.3$ surface expression and the current density of T-type VOCCs[19].

Given the abundance of STIM1 and STIM2 in neuronal tissues and their role in Ca^{2+} mobilization, it is not surprising to learn that they have a direct impact on cognitive functions. In mice with conditional deletion of *Stim1* or *Stim2* genes in the forebrain (conditional knock-outs or cKO), the analysis of spatial reference memory revealed a mild learning delay in Stim1 cKO mice, no effect in Stim2 cKO mice, and a deep impairment in spatial learning in the double cKO[20]. This striking effect was explained by the regulation of the phosphorylation of the AMPA receptor subunit GluA1, the transcriptional regulator CREB and the $Ca_V1.2$ on protein kinase A-target sites, leading to the proposal that the upregulation of cAMP/PKA signaling impairs the development of spatial memory[20]. Kuznicki's lab reported that STIM1 protein in neurons can control AMPA-induced Ca^{2+} entry, based on the inhibition of Ca^{2+} entry observed with AMPA receptors (AMPAR) inhibitors and the finding that STIM1 physically binds GluA1/GluA2 AMPAR[21].

On the other hand, in transgenic mice overexpressing STIM1 in neurons it was reported a reduction of long-term depression in hippocampal slices, as well as a decrease in anxiety-like behavior and an increase in contextual learning improvement[22]. All of this further confirms the role of STIM1 in the modulation of synaptic strength and memory formation.

Closely related to the above statement, the control of L-type VOCCs by STIM1 has functional consequences that were reported for dendritic spine structural plasticity. In hippocampal neurons, depolarization by the neurotransmitter glutamate activates postsynaptic N-methyl-D-aspartate receptors and L-type VOCC-dependent Ca^{2+} influx, as well as the release of Ca^{2+} from the ER. The consequent activation of STIM1 inhibits VOCCs, an event that leads to the enlargement of ER content in spines, which is believed to help in the stabilization of mushroom spines that have become enlarged during long-term potentiation[23].

STIM1 IN NEURONAL CELL DEATH

There are some examples of the involvement of STIM1 and SOCE in neuronal injury. For instance, cell death due to diffuse axonal injury is preceded by an increase of STIM1 expression in neurons of the rat cerebral cortex after lateral head rotational injury[24]. In this regard, STIM1 expression was significantly increased in a traumatic brain injury model, and STIM1 knock-down inhibited apoptotic cell death after traumatic injury by decreasing the upregulation of mGluR1-dependent Ca^{2+} signaling[25]. However, Berna-Erro *et al*[26] demonstrated that STIM2, but not STIM1, was essential for ischemia-induced cytosolic Ca^{2+} accumulation in neurons using hypoxic conditions for culturing neurons from wild-type and $Stim2^{-/-}$ mice, hippocampal slice preparations, as well as in $Stim2^{-/-}$ mice subjected to focal cerebral ischemia.

Oxytosis, a type of cell death characterized by an increase of reactive oxygen species (ROS) and augmented Ca^{2+} influx, can be triggered in neurons in culture by depleting reduced glutathione content. Henke *et al*[27], reported that the Ca^{2+}-influx pathway in this cell death could be mediated by ORAI1, the CRAC channel activated by STIM1. Similarly, in PC12 cells exposed to 6-hydroxydopamine (6-OHDA), an experimental model to trigger ROS-dependent cell death, the knockdown of STIM1 was able to attenuate apoptotic cell death by limiting the mitochondrial Ca^{2+} uptake induced by 6-OHDA. This resulted in the protection of PC12 cells against the oxidative stress generated by ER stress and mitochondrial dysfunction[28]. On the other hand, the inhibition of SOCE or the knock-down of STIM1 limited ROS production and the activation of apoptosis in PC12 cells exposed to 1-methyl-4-phenylpyridinium or MPP^+[29], the toxic metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a well-known inducer of Parkinsonism. These studies revealed that oxidative stress induces an increase of $[Ca^{2+}]_i$ mediated by the activation of SOCE. Indeed, STIM1 is a redox-sensitive protein, and it is known that Cys56 becomes S-glutathionylated during oxidative stress[30], a residue located near its luminal EF-hand domain. Hawkins *et al*[30], demonstrated that S-glutathionylation lowered the affinity of STIM1 for Ca^{2+}, thereby activating STIM1 in a store-independent fashion. Similarly, ORAI1 is a redox sensor through the Cys195 located in the second extracellular loop. Although it was initially shown that the oxidation of this Cys residue inhibited Ca^{2+} current through this channel[31], other researchers found that exposure to H_2O_2 increased influx through ORAI1[32], suggesting that ROS has multiple redox-sensitive targets in the SOCE machinery.

Mitochondrial dysfunction is an early event in neurotoxicity triggered by massive Ca^{2+} influx, as observed during glutamate neurotoxicity[33]. Following acute increase in

$[Ca^{2+}]_i$, Ca^{2+} uptake by mitochondria contributes to the protection against cell death. However, Ca^{2+} overload in mitochondria triggers the opening of the mitochondrial permeability transition pore (mPTP), an event that led to cell death in different neuronal cell types[34,35]. It is accepted that the overproduction of ROS modulates the opening of the mPTP, but it has also been shown this opening at physiological levels of ROS. Recently, Agarwal *et al*[36], reported that astrocytes show transient cytosolic Ca^{2+} spikes generated by the Ca^{2+} release from mitochondria when the mPTP opens by a mechanism that involves ROS generated during the electron transfer in the respiratory systems. Electron transport rates are strongly dependent on the availability of NADH, and therefore dependent on the Krebs cycle status, which is tightly controlled by the mitochondrial $[Ca^{2+}]$. Therefore, there is a strong correlation between dysregulation of Ca^{2+} entry through ORAI1, mitochondrial Ca^{2+} overload, ROS generation, mPTP opening and cell death.

STIM1 IN NEURODEGENERATIVE DISEASES

Alzheimer's disease

Taking into consideration the information summarized above, it should not be surprising that the dysregulation of STIM1 could underlie the pathogenesis of some of the most frequent neurodegenerative diseases. In 1907 Alzheimer[37] described a disease in a 51-year-old woman with presenile dementia who displayed diffuse cortical atrophy, nerve cell loss, plaques, and tangles. Nowadays, Alzheimer's disease (AD) patients are classified within 3 groups: Early-onset AD (up to 5% of all patients with AD), late-onset or sporadic AD (the most common form of the disease), and familial AD (FAD, less than 1% of AD patients). FAD is linked to known genes, such as the amyloid beta precursor protein gene (*APP*), the apolipoprotein E gene (*APOE*), presenilin1/2 genes (*PSEN1, PSEN2*), or the alpha-2-macroglobulin gene (*A2M*), and most early-onset AD patients are FAD patients. There is no significant pathological difference between sporadic AD and FAD, but symptoms progress more rapidly in FAD[38].

The major risk for sporadic AD is aging, which increases the difficulty of finding a suitable model animal that recapitulates all the hallmarks of the human disease in the absence of mutated genes as in FAD. However, there is a growing consensus regarding the hypothesis that Ca^{2+} dysregulation is in the pathogenesis of AD[39-42]. This hypothesis is supported by evidence that revealed how diverse Ca^{2+} mobilization systems are impaired in AD, including VOCCs, IP_3 receptors, store-operated Ca^{2+} channels (SOCs), and mitochondrial Ca^{2+} transporters[43].

Regarding STIM1 and SOCE, it is known that SOCE is reduced and that STIM1 and ORAI1 expression are downregulated in long-term cultures of hippocampal neurons, an experimental approach intended to mimic *in vivo* neuronal aging[44]. Also, reduced expression of STIM2 was observed in hippocampal neurons from the presenilin-1 M146V knock-in mouse model of FAD. As it is assumed that STIM2 and the activation of the calmodulin-dependent protein kinase II (CaMKII) mediates the stabilization of mushroom spines, this decrease in STIM2 levels could explain the loss of dendritic spines and the defects in the development of long-term potentiation LTP and memory development in AD patients[45]. In this regard, it is known that the gamma-secretase protein complex interacts with STIM1 in SH-SY5Y neuroblastoma cells, skin fibroblasts from FAD patients, and in mouse primary cortical neurons[46]. Tong *et al*[46], also reported that cultured hippocampal neurons expressing the mutant PSEN1 M146L, showed reduced dendritic spines, together with diminished SOCE. Because the wild-type phenotype was rescued by overexpressing STIM1, or by inhibiting gamma-secretase activity, they hypothesized that STIM1 could be a substrate for the gamma-secretase complex. Finally, they proved that the transmembrane domain of STIM1 shows a target domain for the proteolytic activity of the gamma-secretase complex and that the reduced SOCE in PSEN1-mutant neurons was due to the higher rates of STIM1 proteolysis. Although this proteolysis needs to be studied further to confirm cleavage sites on STIM1, this data fits well with the recent observation that there is a sharp decline of STIM1 protein levels in brain tissue from non-familial (sporadic) AD patients[47]. This is supporting evidence for a common hallmark in sporadic AD and FAD, *i.e.*, reduced STIM1 could be severely affecting Ca^{2+} mobilization in neurons in both groups of patients. Thus, it is necessary to study the consequences of the reduced STIM1 expression in neurons in order to understand how neuronal cell physiology develops in the absence of STIM1 and to find possible targets for clinical interventions. An approach to studying the patho-physiological consequences of a limited level of STIM1 in neurons has been recently reported by our group[47]. In this report, we modified *STIM1* gene locus using CRISPR/Cas9-mediated

editing techniques, and we found that the differentiation of SH-SY5Y cells to neuronal-like cells was not impaired by the absence of STIM1. However, the loss of STIM1 triggered significant cell death due to the impairment of mitochondrial respiratory chain complex I, and to reduced mitochondrial Ca^{2+} concentration. These two events led to high levels of senescence. STIM1-KO cells showed potentiation of Ca^{2+} entry through L-type VOCCs[47], further confirming earlier observations that demonstrated the inhibitory role of STIM1 on $Ca_V1.2$ channels[16,17]. Consequently, the knocking-down of *CACNA1C* gene transcripts (for $Ca_V1.2$ channel) rescued the wild-type phenotype, confirming that the upregulation of Ca^{2+} entry through $Ca_V1.2$ channels was deleterious in STIM1-deficient cells[47] (Figure 1). In this regard, higher Ca^{2+} entry through VOCCs had been recorded in CA1 pyramidal neurons from the hippocampus in aged rats[48], an effect that resulted in the down-regulation of short-term neuronal plasticity.

Accumulation of beta amyloid peptides (Aβ) begins earlier than most of the clinical symptoms associated with FAD. However, clinical interventions to prevent this accumulation have been inconclusive so far. Accumulation of Aβ directly affects Ca^{2+} mobilization, and the possibility that an increase of PKA-dependent phosphorylation of $Ca_V1.2$ channels could underlie the upregulation of Ca^{2+} influx through these channels has been discussed[49,50]. Therefore, an alternative clinical intervention is the blocking of excessive Ca^{2+} entry in neurons. Transgenic mice have been designed to accumulate Aβ and hyperphosphorylation of tau protein in CA1 pyramidal neurons, as an experimental approach to mimic some clinical features of FAD patients. Using these mice (known as 3xTgAD mice) it has been shown that Ca^{2+} current through L-type VOCCs became higher in these hippocampal neurons, supporting the possible role of VOCCs in neuronal degeneration in FAD patients[51]. In addition, the long-term treatment of subjects receiving active treatment with L-type VOCCs blockers (nitrendipine) reduced sporadic dementia by 55% during aging[52], suggesting that the enhanced Ca^{2+} entry through VOCCs could be in the pathogenesis of sporadic AD. Protection against the loss of working memory has also been monitored in rats treated with the VOCC blocker nimodipine, a treatment that reduced Ca^{2+} current through $Ca_V1.3$ in CA1 neurons[53]. Finally, isradipine, another dihydropyridine, attenuated Aβ accumulation toxicity by reducing $Ca_V1.2$ expression and Ca^{2+} influx in MC65 neuroblastoma cells[54]. Interestingly, isradipine also showed a neuroprotective effect in models of Parkinson's disease (PD) and stroke[50,55].

Whereas a decline in STIM1 level is deleterious, in part due to the upregulation of VOCCs, high levels of STIM1 and SOCE might be protective, as suggested by the reduced Aβ secretion observed in cells expressing a constitutively activated STIM1 mutant (D76A)[56]. On the other hand, Aβ seems to affect STIM1-dependent Ca^{2+} entry because knocking-down APP transcripts delayed the binding of STIM1 to ORAI1 in response to store depletion[57], and SOCE was largely reduced in cultured astrocytes from APP-KO mice[58], confirming the crosstalk between SOCE and APP.

PD

A recent report showed that neurotoxins that trigger PD symptoms targeted TRPC1 expression and increased Ca^{2+} influx through $Ca_V1.3$ channels (L-type VOCC) which led to degeneration of dopaminergic (DA) neurons[59]. Because of the key role of $Ca_V1.3$ in the regulation of basal single-spike firing in DA neurons[60], the reported inhibition of $Ca_V1.3$ by the STIM1-TRPC1 complex[59] could explain the disruption of neuronal Ca^{2+} homeostasis in PD patients. Indeed, in mice treated with MPTP, the expression of $Ca_V1.2$ and $Ca_V1.3$ in the substantia nigra increased after 2 wk of treatment, and isradipine (L-type VOCC blocker) prevented this upregulation and the loss of DA neurons[61]. Similarly, nimodipine prevented cell death triggered by MPP^+ in SH-SY5Y cell in culture and Parkinsonism in MPTP-treated mice[62]. Because dihydropyridines are not highly selective in discriminating between $Ca_V1.2$ and $Ca_V1.3$, Wang *et al*[61], reported a high-throughput screening that led to 1-(3-chlorophenethyl)-3-cyclopentylpyrimidine-2,4,6-(1H,3H,5H)-trione as the first potent and highly selective $Ca_V1.3$ antagonist with potential utility in clinical approaches. However, Ortner *et al*[63], reported later that this specific compound showed inhibitory activity of $Ca_V1.3$ only in a minority of cells.

On the other hand, antagonists of SOCE and depletion of STIM1 by siRNA increased cell viability, reduced intracellular ROS production as well as lipid peroxidation and prevented mitochondrial dysfunction in MPP^+-treated PC12 cells[29], supporting the hypothesis that augmented Ca^{2+} entry through STIM1-activated channels mediates toxicity of MPP^+. This result, however, is in conflict with the observation that treatment with this neurotoxin decreased TRPC1 expression, TRPC1

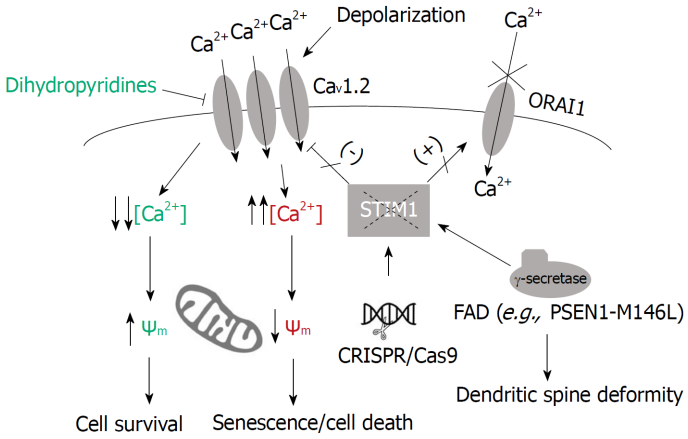

Figure 1 Deficiency of STIM1 and neurodegeneration. Neurons expressing the mutant PSEN1 M146L showed higher rates of STIM1 proteolysis, reduced levels of STIM1, reduced store-operated Ca^{2+} entry and diminished dendritic spines[46]. Deficiency of STIM1 has been observed in non-familial (sporadic) Alzheimer's disease (AD) patients, and can be mimicked by genome edition of STIM1 locus in SH-SY5Y cells[47]. Because STIM1 is a negative regulator of $Ca_V1.2$ channels, this deficiency triggered the upregulation of Ca^{2+} entry through $Ca_V1.2$ channels which was responsible for the loss of inner mitochondrial membrane polarization, senescence, and cell death[47]. This higher rate of Ca^{2+} influx through $Ca_V1.2$ channels has also been monitored in 3xTgAD mice[51]. The long-term treatment with dihydropyridines, known blockers of $Ca_V1.2$, reduced sporadic dementia by 55% during aging[52], pointing out the decrease of STIM1 as a possible mechanism to explain neurodegeneration in sporadic and familial AD.

interaction with STIM1, and Ca^{2+} entry in SH-SY5Y cells[64], making further study necessary to discover the role of STIM1, SOCE, and VOCCs in the pathogenesis of PD.

CONCLUSION

Neurodegenerative diseases are devastating for the elderly population and no fully efficient therapies are available to treat some of them, particularly AD. However, a growing body of evidence supports a role for excessive Ca^{2+} entry through VOCCs in neurodegeneration. Recent reports proposed that the specific loss of STIM1 in neuronal tissue fully explains the observed Ca^{2+} homeostasis disruption in neurons during sporadic AD and FAD. In this regard, STIM1 deficiency triggered upregulation of Ca^{2+} entry through $Ca_V1.2$ in differentiated SH-SY5Y cells, which can be explained by the role of STIM1 in the inhibitory control of $Ca_V1.2$. This augmented Ca^{2+} influx led to the inhibition of the mitochondrial respiratory chain complexI activity, mitochondrial inner membrane depolarization, reduced mitochondrial free Ca^{2+} concentration, and to higher levels of senescence and cell death. All these effects were prevented by silencing $Ca_V1.2$ expression, emphasizing the upregulation of these channels as a major cause of neuronal cell death (Figure 1).

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MINIREVIEWS

Arrestin-mediated signaling: Is there a controversy?

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Abstract

The activation of the mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase (ERK)1/2 was traditionally used as a readout of signaling of G protein-coupled receptors (GPCRs) *via* arrestins, as opposed to conventional GPCR signaling *via* G proteins. Several recent studies using HEK293 cells where all G proteins were genetically ablated or inactivated, or both non-visual arrestins were knocked out, demonstrated that ERK1/2 phosphorylation requires G protein activity, but does not necessarily require the presence of non-visual arrestins. This appears to contradict the prevailing paradigm. Here we discuss these results along with the recent data on gene edited cells and arrestin-mediated signaling. We suggest that there is no real controversy. G proteins might be involved in the activation of the upstream-most MAP3Ks, although *in vivo* most MAP3K activation is independent of heterotrimeric G proteins, being initiated by receptor tyrosine kinases and/or integrins. As far as MAP kinases are concerned, the best-established role of arrestins is scaffolding of the three-tiered cascades (MAP3K-MAP2K-MAPK). Thus, it seems likely that arrestins, GPCR-bound and free, facilitate the propagation of signals in these cascades, whereas signal initiation *via* MAP3K activation may be independent of arrestins. Different MAP3Ks are activated by various inputs, some of which are mediated by G proteins, particularly in cell culture, where we artificially prevent signaling by receptor tyrosine kinases and integrins, thereby favoring GPCR-induced signaling. Thus, there is no reason to change the paradigm: Arrestins and G proteins play distinct non-overlapping roles in cell signaling.

Key words: G protein-coupled receptors; Arrestin; G protein; Signaling; Extracellular signal-regulated kinase 1/2; c-Jun N-terminal kinase 3

Core tip: Both arrestins and G proteins play important roles in G protein-coupled receptor (GPCR) signaling, including GPCR-initiated activation of mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase 3 (JNK3). Their roles do not overlap. G proteins participate in signal initiation, by activating MAP3Ks. Arrestins, free and GPCR-bound, function as scaffolds of the three-tiered MAP kinase cascades, facilitating signal transduction. Cells express other scaffolds, so that no MAPK cascade relies solely on arrestins. Different experimental paradigms highlight the role of G proteins or arrestins in this process, and

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neither can be discounted based on available evidence.

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INTRODUCTION

G-protein-coupled receptors (GPCRs) respond to hormones, neurotransmitters, light, odorants, taste molecules, extracellular calcium, extracellular protease activity, cell adhesion, and a variety of other stimuli[1]. All members of the GPCR super-family (which includes hundreds of receptors encoded by different genes in animals) share a common transmembrane domain consisting of seven α-helices, which are connected by intra- and extra-cellular loops of variable lengths[2]. Their extracellular N-termini and intracellular C-termini also differ widely in size and structure[2]. Upon activation by an appropriate input most GPCRs serve as guanyl nucleotide exchange factors of heterotrimeric G proteins, facilitating the release of guanosine diphosphate (GDP) bound to their inactive α-subunits and its exchange for guanosine triphosphate (GTP), which is a lot more abundant in cells. Activated G proteins then dissociate from the receptors, their α- and βγ-subunits separate and activate or inhibit various effectors. Active GPCRs can sequentially activate several molecules of G proteins, providing signal amplification at this level. Active GPCRs are also specifically phosphorylated by G protein-coupled receptor kinases (GRKs)[3], of which most mammals have seven. Nocturnal rodents only have six, as they are missing GRK7, specialized GRK expressed in cone photoreceptors, which function in relatively bright light.

ARRESTIN-MEDIATED GPCR DESENSITIZATION

The first arrestin family member (current systematic name arrestin-1) was discovered in the visual system as the protein that specifically binds active phosphorylated rhodopsin and suppresses its signaling[4]. Thus, desensitization, *i.e.*, the suppression of G protein-dependent signal transduction, was the first arrestin function discovered. Subsequently the first[5] and then the second non-visual arrestin[6-8] were cloned. The demonstration that the first non-visual arrestin preferentially desensitized phosphorylated β2-adrenergic receptors (β2AR) (which gave it the original name, β-arrestin; systematic name arrestin-2), whereas visual arrestin-1 preferentially desensitized phosphorylated rhodopsin[9], suggested the idea that all arrestins desensitize cognate GPCRs *via* specific binding to their active phosphorylated state[10]. Thus, the field came to believe that the model of two-step desensitization, phosphorylation of active GPCRs by specific GRKs, reviewed in[3], followed by arrestin binding to the active phosphorylated receptor, applies to all GPCRs[10-12]. In this paradigm, the role of arrestins is to stop GPCR signaling *via* G proteins. This remains the best characterized biological function of all arrestin proteins[11]. Subsequent findings that receptor-associated non-visual arrestins directly bind clathrin[13] and clathrin adaptor, adaptor protein 2 (AP2)[14], the key components of the coated pit, and that the binding to both is enhanced by arrestin-receptor interactions[15], suggested that arrestins participate in the next step of desensitization, *i.e.* receptor removal from the plasma membrane *via* internalization.

GPCR-DEPENDENT ARRESTIN SIGNALING

The arrestin-mediated cellular signaling was first discovered upon GPCR stimulation, and therefore was assumed to be strictly receptor-dependent. The binding of non-visual arrestins to their cognate receptors was shown to facilitate the activation of protein kinases proto-oncogene tyrosine-protein kinase Src (c-Src)[16], c-Jun N-terminal kinase 3 (JNK3)[17], then extracellular signal-regulated kinase (ERK)1/2[18]. As JNKs and ERKs are mitogen-activated protein kinases (MAPKs) activated *via* the three-tiered kinase cascade (in general terms, MAP3K, MAP2K, and MAPK[19,20]), the latter two cases suggested that receptor-bound arrestins scaffold the three-kinase modules,

thereby facilitating signal transduction in them. Initial studies detected direct arrestin binding to both MAP3Ks, proto-oncogene serine/threonine-protein kinase (cRaf) (a.k.a. Raf1) and apoptosis signal-regulating kinase 1 (ASK1), and corresponding MAPKs, ERK1/2 and JNK3, but not to the MAP2Ks of these cascades, MEK1 or MKK4/7[17,18]. However, subsequently arrestin interactions with MEK1[21], as well as with MKK4 and MKK7[22,23] were documented. Thus, the idea of scaffolding of MAP kinase cascades by arrestin bound to a GPCR received further experimental support. The binding of arrestins to ERK1/2 is barely detectable in the absence of activated GPCR[24], and both arrestin binding to ERK1/2 and arrestin-dependent ERK1/2 activation are greatly facilitated by GPCR stimulation[18]. Therefore, arrestin-dependent ERK1/2 activation following GPCR stimulation in the experimental conditions excluding other inputs (see below) became a readout of choice for arrestin-mediated signaling. It has been shown that GPCRs that form stable complexes with arrestins tend to increase ERK1/2 activity in the cytosol, presumably *via* retaining ERK1/2 activated by the GPCR-bound arrestin scaffold in that compartment, whereas GPCRs that form transient complexes with arrestins induce mitogenic response due to the translocation of active ERK1/2 to the nucleus, where it acts on its nuclear substrates[25]. Moreover, using siRNA knockdown ERK1/2 activation by angiotensin II type 1A receptor *via* G proteins (likely $G_{q/11}$) was found to be transient, peaking at 2 min and then rapidly declining, whereas arrestin-mediated activation of ERK1/2 was shown to peak later and last much longer[26]. Even though ERK1/2 can be activated *via* a variety of pathways in the cell[27], it became widely accepted that the late phase (10-30 min after the stimulus) of ERK activation reflects GPCR signaling *via* arrestins[28,29]. However, it has been shown that G protein-mediated ERK1/2 activation can also have a late phase (see as the first report of this phenomenon[30], reviewed in[31]). As the late phase of ERK1/2 activation was subsequently shown to be mediated by G proteins in several other studies involving different GPCRs, the time course of ERK1/2 activation cannot be regarded as an indication of it being G protein- or arrestin-dependent.

The molecular mechanism of arrestin-mediated connection between GPCRs and proteins containing Src homology 3 (SH3) domains was recently extensively investigated using biophysical methods[32]. The data suggest that arrestin "unwinds" the auto-inhibited conformation of c-Src, thereby directly activating this kinase. Free arrestin-2 was found to have detectable effect, but an increase in Src activity upon binding to the arrestin-2 engaged by an appropriately phosphorylated GPCR, or, to a lesser extent, receptor-derived phosphopeptide, was much greater[32]. The authors detected notable differences between conformational changes in arrestin-2 induced by differentially phosphorylated peptides[32], which is consistent with the barcode hypothesis positing that GPCRs phosphorylated at different sites by different GRKs have differential effects on arrestin conformation, which is translated into the activation of distinct branches of arrestin-mediated signaling[33,34].

GPCR-INDEPENDENT ARRESTIN SIGNALING

Interestingly, whereas both non-visual arrestin-2 and -3 (a.k.a. β-arrestin1 and 2) appeared to facilitate the activation of c-Src and ERK1/2, only one subtype, arrestin-3, facilitated the activation of JNK3[24,35,36], as well as at least some isoforms of ubiquitously expressed JNK1 and JNK2[37]. Early studies revealed that arrestin-3 can facilitate JNK3 activation even in receptor-independent manner, when the upstream-most kinase, MAP3K ASK1, is overexpressed[24,35]. This finding was confirmed by documenting that arrestin-3 mutant incapable of GPCR binding (which has a deletion in the inter-domain hinge region, precluding domain movement[38,39] necessary for the binding to GPCRs[40-42]) promotes JNK3 activation as effectively as wild type (WT) arrestin-3[43]. It was also shown that replacement of certain residues in the arrestin-3 with their homologues from closely related arrestin-2[6,7] impedes its ability to activate JNK3, rendering it arrestin-2-like[36]. Systematic comparison of the effects of β2AR ligands acting *via* endogenous receptor on the activation of ERK1/2 and JNK3 in the same cells expressing various forms of arrestin-3 proved beyond reasonable doubt receptor-independence of this arrestin-3 function: While the levels of active phosphorylated ERK1/2 reflected the functional state of the receptor, the levels of active phospho-JNK3 did not depend on it, reflecting only the nature of arrestin-3 mutant expressed[43]. Arrestin-3-mediated scaffolding of the two modules of the JNK3-activating cascade, MKK4-JNK3 and MKK7-JNK3, was demonstrated using purified proteins *in vitro* in the absence of any GPCRs, confirming yet again that receptors are not necessary for this arrestin-3 function[22,23]. Recent structure of the arrestin-3 trimer crystallized in the presence of a fairly abundant intracellular small molecule, inositol-

hexakisphosphate (IP6)[44] revealed that all three protomers in the trimer are in the "active" (receptor-bound-like) conformation. It was similar to the conformation of arrestin-1 in complex with rhodopsin[41,42], as well as the conformations of constitutively active arrestin-1 splice variant p44[45] and C-terminally truncated arrestin-2 in complex with the phosphopeptide derived from the angiotensin receptor C-terminus[40], thereby suggesting a molecular mechanism of receptor-independent activation of arrestin-3. These data suggest that at least one of non-visual arrestins, arrestin-3, can assume "active" (GPCR bound-like) conformation without the help of GPCRs[44]. Curiously, the molecular mechanism of arrestin-3 activation in this case appears to resemble the mechanism of activation of all arrestins by GPCRs: The phosphates of IP6 engage the same positively charged side chains in arrestin as receptor-attached phosphates[46]. It is also noteworthy that GPCRs might activate arrestins catalytically, *i.e.*, that arrestins can maintain active conformation after dissociation from GPCRs[47]. Thus, multiple mechanisms can generate "active" arrestins in the cytoplasm that are not bound to GPCRs.

The propensity of arrestin-3 mutants to form trimers in the presence of IP6 appeared to correlate with their ability to facilitate JNK3 activation in cells[44]. However, no IP6 was used in the experiments where MKK4-JNK3 and MKK7-JNK3 modules were reconstituted *in vitro* from purified proteins with arrestin-3[22,23], suggesting that this subtype can assume active (at least in terms of the ability to facilitate signaling in the JNK3 activation cascade) conformation spontaneously, without the help of IP6. Indeed, structural data[48] and molecular dynamics simulations[49] indicate that arrestin-3 is more flexible than other arrestin subtypes. A short arrestin-3-derived peptide comprising the first 25 residues was found to facilitate JNK3 activation both *in vitro* and in cells[50]. This peptide is unlikely to trimerize, as it does not contain most of the inter-protomer interfaces observed in the crystal trimer[44]. It was expressed as a fusion with well-folded proteins (MBP in *E. coli* and YFP in mammalian cells), which suggests that it simply needs to have loose conformation to function as a scaffold[50]. Interestingly, the arrestin-3 N-terminus, containing this peptide, does not appear to be particularly loose in the crystal trimer[44], so that the detailed molecular mechanism of arrestin-3-mediated scaffolding of the ASK1-MKK4/7-JNK3 cascade still remains to be elucidated.

The facilitation of JNK3 phosphorylation by arrestin-3 is not the only receptor-independent function of arrestin proteins documented. It was recently shown that arrestin-2-(1-380) fragment generated by caspase cleavage in the absence of receptor stimulation translocates to the mitochondria, where it assists caspase-cleaved tBid in releasing cytochrome c, thereby promoting apoptotic cell death[51]. Both non-visual arrestins and their receptor binding-deficient mutants affect cell spreading and motility *via* disassembly of focal adhesions and regulation of small GTPases[52,53]. Thus, several signaling functions of arrestins do not appear to be dependent on GPCRs, and, by extension, on G proteins.

ROLE OF G PROTEINS

As discussed above, some signaling functions could be performed by free arrestins independently of their interaction with GPCRs and, consequently, of G proteins activated by these receptors. However, arrestin-dependent signaling has long been considered to require arrestin binding to GPCR but at the same time to be G protein-independent serving as an alternative pathway of the GPCR signaling (*e.g.*, see[54]; recently reviewed in[55,56]). Indeed, free arrestins have minimal effect on certain signaling pathways, such as ERK1/2 activation, whereas arrestins bound to agonist-activated phosphorylated GPCRs are able to facilitate signaling in these pathways. Recently the notion of G protein independence of the GPCR-initiated arrestin signaling function has been called into question.

The use of CRISPR-Cas9 gene editing enabled the creation of cells lacking individual G proteins or several G proteins at the same time[57]. The only class of G proteins that could not be eliminated by CRISPR-Cas9 gene editing was $G_{i/o}$ subtypes, but these G proteins can be inactivated by pertussis toxin[57,58]. Thus, the combination of inactivation of $G_{i/o}$ proteins by pertussis toxin in cells where all other G protein subtypes were knocked out by CRISPR-Cas9 made possible the construction of cells lacking all G protein-mediated signaling (termed "zero functional G" cells). A comprehensive study was performed in these cells, with numerous GPCRs, including β2AR and angiotensin1 receptor often used to demonstrate arrestin-dependent ERK1/2 activation[58]. The results showed that neither arrestin recruitment to GPCRs nor receptor internalization requires G protein signaling. However, the authors did not detect any arrestin-mediated ERK1/2 activation in "zero functional G" cells using

a variety of methods, including label-free dynamic mass redistribution and ERK1/2 phosphorylation in response to receptor stimulation[58]. Interestingly, the authors documented the role of arrestins in ERK1/2 activation by comparing "zero arrestin" cells with parental line, but only when at least some G protein-mediated signaling remained (illustrated by the Supplementary Figure 4 in Grundmann *et al*[58]). The main take-home message of that study was that while GPCR-induced arrestin-mediated signaling exists, it requires G protein action. The results suggested that without G proteins arrestins do not regulate ERK1/2 activation. Thus, the field has to decide whether these data call for yet another paradigm change.

MAPKS ARE ACTIVATED BY VARIOUS INPUTS

It is important to note that *in vivo* the main activators of MAPK cascades are not GPCRs. In most cases upstream MAP3Ks are activated by growth factor receptors[19,59], death receptors[60], integrins[61], or various stressors[62]. We should keep in mind that experimental paradigms used to study arrestin-mediated signaling actually exclude non-GPCR inputs. Cultured cells are usually plated on supports that do not activate integrins. In addition, cells where GPCR-induced MAPK activation is studied are routinely serum-starved, *i.e.*, maintained in growth factor-deficient conditions, which prevents MAPK activation *via* growth factor and/or death receptors, likely the prevalent mechanisms *in vivo*. Mammals have 20 different MAP3Ks that integrate signaling inputs[20]. The mechanisms of MAP3Ks activation are usually complex. For example, one of the MAP3Ks of the ERK1/2 cascade, cRaf (a.k.a. Raf1) is activated by active (GTP-liganded) small G proteins of Ras family, which recruit it to the membrane and promote its dimerization. cRaf dimerizes with other members of RAF family and kinase suppressor of Ras (KSR). An element adjacent to the Ras-binding domain, cysteine-rich domain stabilized by zinc, binds phosphatidylserine, facilitating membrane anchoring[63]. Several additional events contribute to cRaf activation[63]: Ras binding facilitates dephosphorylation of the site upstream of the kinase domain that in the inactive state of Raf1 binds 14-3-3 protein. Dimerized cRaf molecules apparently phosphorylate the activation segment, which stabilizes the active form of the kinase. For full activity, the negatively charged N-terminal region and the C-terminal 14-3-3 binding site also need to be phosphorylated. Another example of complex activation mechanism is ASK1, one of the MAP3Ks of JNK1/2/3 cascades. It is activated by oxidative stress, endoplasmic reticulum (ER) stress, calcium influx, or mechanical stress, and inhibited by the interactions with reduced thioredoxin and 14-3-3 protein[62]. Its phosphorylation on three different serines in the N- and C-terminal elements is inhibitory, whereas the phosphorylation of the three threonines in the kinase domain is stimulatory[62]. Thus, in addition to being phosphorylated on threonines, for full activation the three serines in ASK1 must be dephosphorylated, and both thioredoxin and 14-3-3 protein must dissociate[62]. To the best of our knowledge, none of these events is regulated by heterotrimeric G proteins. It is entirely possible that when MAP3Ks are activated *via* GPCR-independent mechanisms by integrins, death or growth factor receptors, or stressors, G proteins are not involved, whereas arrestins might still act as scaffolds bringing the three kinases of MAPK cascades together.

MAPK ACTIVATION IN DIFFERENT SUBCELLULAR COMPARTMENTS

Another important aspect of MAP kinase signaling is related to cell compartmentalization. Most MAPKs phosphorylate transcription factors in the nucleus, although practically every MAP kinase has cytoplasmic or even plasma membrane-localized substrates[64]. Naturally, the biological impact of MAPK activity towards nuclear and non-nuclear proteins has very different biological meaning. Free arrestins are soluble cytoplasmic proteins, whereas GPCR-bound arrestins localize even more restrictively, to the plasma membrane and endosomes. Localization of scaffolds determines where active MAPKs are generated, thus directing their signaling towards substrates in a particular cellular compartment. The original studies suggested that ERK1/2 activated *via* arrestin scaffold remains in the cytoplasm[18], where it phosphorylates its non-nuclear substrates, whereas ERK1/2 activated *via* G protein- and growth factor receptor-mediated mechanisms translocates to the nucleus[18]. Indeed, in some cases arrestin-mediated activation of ERK1/2 was shown not to affect transcription[65]. However, a recent study showed that arrestin-2 in adrenocortical zona glomerulosa facilitates aldosterone production by ERK1/2

activation[66], apparently *via* transcription regulation. Similarly, ERK1/2 activation by angiotensin 1A receptor in vascular smooth muscle *via* both G_q and arrestin was shown to involve transactivation of EGF receptor[67]. Thus, direct biological consequences of ERK1/2 activation also cannot be used to distinguish between arrestin-dependent and -independent mechanisms of its activation.

BIASED GPCR SIGNALING

Recently GPCR ligands that bias the signaling towards G proteins or arrestins have attracted a lot of attention as tools that might help achieving desired therapeutic outcome while minimizing unwanted side effects[55,56]. Ligand-activated GPCRs[68], as well as light-activated prototypical GPCR rhodopsin[69], exist in an equilibrium of multiple conformational states (reviewed in[70]). Thus, distinct subsets of active GPCR conformations might preferentially bind particular signal transducers, such as different G proteins and/or arrestins. The data suggesting that G protein action is required for arrestin-mediated signaling appears to be inconsistent with the concept of arrestin-biased signaling. Indeed, if we envision a ligand with the 100% bias towards arrestin, then it might have to rely on alternative signaling inputs to provide an initial "push", at least, for some signaling pathways such as the ERK activation, before arrestins could step in. However, these findings do not contradict the idea that GPCR ligands that promote arrestin recruitment to a greater extent than G protein activation can yield signaling outcomes quite different from those generated by unbiased ligands promoting the activation of both G proteins and arrestins.

Indeed, it is likely that a relatively low level of G protein activity is sufficient to provide the initial activation of the MAPK pathways where arrestins play the role of scaffolds or signaling enhancers. Furthermore, practically all G proteins have measurable rate of spontaneous exchange of GDP for GTP[71], *i.e.*, activation. In addition, non-GPCR activators, such as AGS proteins[72], or other proteins containing G protein regulatory (GoLoco) motif[71], were shown to catalyze nucleotide exchange, leading to G protein activation. Thus, a fraction of the G protein pool in the cell is always active. In practical terms, considering that GPCRs and their endogenous ligands were designed by evolution to signal in both directions, it is highly unlikely that 100% effective bias can be achieved by manipulation of ligand structure. For example, it was recently shown that carvedilol, which was traditionally considered to be a "clean" arrestin-biased ligand of β-adrenergic receptors, actually promoted β1-adrenoreceptor coupling to Gi proteins, and this unconventional Gi activation by the receptor that was believed to be strictly Gs-specific is required for observed "arrestin-biased" signaling[73]. In the same vein, recent comprehensive analysis of 65 different ligands of β2AR identified many G protein-biased ones, but none specifically biased towards arrestin recruitment[74], reinforcing the notion that the evolution "designed" GPCRs primarily to activate G proteins. Thus, it appears likely that any synthetic arrestin-biased ligand will have sufficient ability to produce necessary G protein activation, so that the proposed model suggesting the involvement of G proteins in arrestin-mediated signaling does not negate the possibility of exploiting biased signaling for therapeutic purposes. For example, even weak partial agonism towards G proteins combined with a stronger agonism towards arrestins might generate sufficient "push" to activate MAP3Ks and enable arrestin-mediated scaffolding, but not enough G protein signaling to yield the biological effect of a full agonist. Conversely, a strong GPCR agonist biased towards G proteins might produce an effect without activating the arrestin brunch, thereby avoiding arrestin-mediated signaling.

Alternatively, when MAP3Ks are activated *via* G protein-independent mechanisms (which is the most likely scenario *in vivo*), GPCR-bound arrestins might function as signal-enhancing scaffolds, facilitating MAPK activation and other pathways independently of G proteins. Arrestin-mediated scaffolding would restrict the localization of generated active MAPKs to the vicinity of GPCRs, *i.e.*, to plasma membrane and endosomes, thereby directing them to substrates in these locales. Thus, arrestin-biased GPCR agonists would affect cell signaling in a different manner than unbiased ones. These ideas must be explored experimentally, preferably in cells that are meant to be targeted under the conditions where the cell receives all inputs, including stimulation *via* growth factor receptors and integrins.

COOPERATION OF ARRESTINS AND G PROTEINS

Conceivably, there might be situations *in vivo* where the bulk of MAP kinase

activation depends on GPCRs, similarly to the experimental conditions used to study arrestin-mediated signaling to MAPKs. In these cases, it might appear counter-intuitive that the signaling of arrestins, which suppress G protein coupling to GPCRs, might require G protein activity. However, it is very likely that any GPCR agonist, including those with arrestin bias, also activates G proteins to a certain extent, as arrestin binding to any GPCR is inevitably delayed by the need of receptor phosphorylation by GRKs to increase arrestin affinity[10,11]. There are known examples where particular biological outcomes, such as Rho A activation and stress fiber formation, require simultaneous input from active G proteins and arrestins[75]. Another known mechanism where G proteins and arrestins might cooperatively participate in signaling that requires both types of transducers involves complexes between certain GPCRs and growth factor receptors that create distinct signaling platforms (reviewed in[76]).

PUTTING PIECES TOGETHER

Here the focus of the discussion is GPCR-dependent activation of ERK1/2 in the experimental conditions used to study arrestin-mediated signaling, which exclude non-GPCR inputs. The data obtained with gene knockout appear to be less ambiguous than those obtained with siRNA knockdown often used earlier[28,29,54]: The knockdown is never complete and one can never be sure that only the targeted proteins were knocked down. A good example demonstrating problems with knockdown specificity are the two siRNA studies[77,78], where opposite conclusions regarding the role of arrestins and arrestin domain-containing proteins in β2AR trafficking were made based on the data. Thus, strictly speaking, without the demonstration of rescue by the expression of knockdown-resistant exogenous protein substituting for that targeted by siRNA, the results of knockdown cannot be unambiguously interpreted[79,80]. The same applies to knockout: Only rescue with knocked out protein proves that the phenotype observed emerged due to the elimination of an intended target. In addition, complete knockout of an important signaling protein has other caveats: The cells might be inadvertently selected for their ability to survive without eliminated protein due to changes in signaling pathways. For example, simultaneous knockout of both non-visual arrestins is embryonic lethal in mice[81], whereas mouse embryonic fibroblasts[81] and HEK293 cells[57,58,82,83] lacking arrestins are viable and can be transfected to generate cells exclusively expressing individual arrestins or particular mutants[52,53,84,85]. Thus, some cells can live without arrestins, whereas others cannot. It is possible (and very hard to check) that "zero functional G" cells also have unanticipated and uncontrolled changes in their signaling pathways. A recent study using three independently generated lines of "zero arrestin" HEK293 cells suggested that these lines are quite different, particularly in terms of signaling: The elimination of arrestins resulted in enhanced, reduced, or unchanged ERK1/2 phosphorylation in response to GPCR activation, as compared to parental cell lines[83]. These data clearly showed that non-visual arrestins do play a role in signaling, at least in GPCR-dependent ERK1/2 activation[83].

So, does arrestin-mediated signaling *via* GPCRs that is G protein-independent, as previously claimed[54], exist? While unambiguous answers require further experimentation, one plausible explanation for the apparent controversy between a large body of data describing arrestin-dependent signaling (reviewed in[55,56]) and recent findings in "zero functional G" and "zero arrestin" cells[57,58,82] can be proposed. An important point that was consistently overlooked in studies of GPCR-dependent arrestin-mediated signaling *via* MAP kinases is the issue of signal initiation. MAP kinase cascades are highly conserved in eukaryotes, from yeast to mammals, and always contain three protein kinases (MAP3K-MAP2K-MAPK) that sequentially activate each other by phosphorylation[86]. The signaling in these cascades is initiated by the activation of the upstream-most MAP3Ks[19]. Yet is was never taken into account that MAP3Ks of the ERK1/2 and JNK3 cascades (cRaf and ASK1, respectively), have to be activated to initiate signaling that eventually leads to the observed phosphorylation of ERK1/2 or JNK3, which usually depends on various protein scaffolds bringing the three kinases of each cascade together. Non-visual arrestins were found to serve as scaffolds but were never shown to facilitate MAP3K activation (reviewed in[12,87-89]). Thus, it is entirely possible that in "real life" the first "push" leading to the activation of MAP3Ks is provided by GPCRs *via* G proteins, or, more likely, by numerous non-GPCR signaling mechanisms, whereas signal propagation is facilitated by scaffolds, including receptor-bound or free arrestins. In case of MAP3K activation by growth factor receptors or integrins arrestin-dependent ERK1/2 activation might appear GPCR-dependent but G protein-independent. In

contrast, under experimental conditions so far used to study arrestin-mediated signaling G proteins activated in response to GPCR stimulation might be the only remaining source of MAP3K activation, which would translate into G protein dependence of arrestin signaling to the ERK pathway, as described recently[58]. The need, or lack thereof, of active G proteins for apparently receptor-independent JNK3 activation by arrestin-3 and arrestin-3-derived peptide has never been tested experimentally, although ASK1 activation *via* G protein-independent mechanisms is more likely in this case.

The existing evidence of the role of non-visual arrestins in cell signaling[83] does not actually contradict the idea that G protein activity might be necessary for the arrestin-mediated signaling under conditions where the inputs from growth factor receptors, integrins, and stressors are excluded. Arguably, the situation where GPCRs assume the leading role in the MAPK activation can be encountered only in rather artificial experimental conditions, although we cannot exclude that this situation sometimes exists *in vivo*. The data obtained in "zero functional G" cells do not contradict the notion that arrestin-mediated signaling exists and plays a role in cell biology. Regardless of the potential role of G proteins, signal propagation to MAPKs would still depend on scaffolds, possibly including non-visual arrestins. Experiments where the activity of MAP3Ks and MAP2Ks in each cascade, rather than only the phosphorylation state of downstream MAPKs, such as ERK1/2 and JNK3, is determined in cells expressing non-visual arrestins with or without functional G proteins are necessary to test this hypothesis. It would be instructive to test whether the activation of growth factor receptors, which are the main known activators of MAP3Ks[19], or the activation of integrins (*e.g.*, by plating cultured cells on fibronectin) bypasses the requirement for the G protein activity. If non-visual arrestin scaffolds contribute to MAPK activation under any of these conditions, their function is likely to be G protein-independent.

CONCLUSION

Available evidence strongly indicates that non-visual arrestins scaffold three-tiered MAP kinase cascades, facilitating signal propagation. Other signaling functions of arrestins are also well documented. However, arrestins were never implicated in the activation of upstream-most MAP3Ks. Cells have numerous MAP3Ks that are activated by various inputs, including, but not limited to, G protein-mediated GPCR signaling. Thus, arrestins and heterotrimeric G proteins have distinct non-overlapping functions in cell signaling. In MAPK cascades, under experimental conditions that exclude non-GPCR inputs, G proteins might play a role in MAP3K activation, whereas arrestins act as scaffolds facilitating signal transduction.

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