Review

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Ceramide in Redox Signaling and Cardiovascular Diseases

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Abstract
Lipid rafts are distinct cell membrane microdomains that consist of cholesterol, sphingolipids, and some associated proteins. Accumulating evidence suggests that activation of sphingomyelinase and generation of ceramide mediates clustering of lipid rafts to form large ceramide-enriched platforms, in which transmembrane signals are transmitted or amplified. Ceramide and reactive oxygen species (ROS) are involved in the modulation of the cell membrane and intracellular ion channels, cell proliferation and apoptotic cell death, neutrophil adhesion to the vessel wall, and vascular tone and in the development of cardiovascular diseases to name some important examples. Ceramide triggers the generation of ROS and increases oxidative stress in many mammalian cells and animal models. Moreover, inhibition of ROS generating enzymes or treatment of antioxidants impairs sphingomyelinase activation and ceramide production. Thus, a new concept has been proposed that ceramide-enriched raft platforms are important redox signaling platforms that amplify activation of ROS generating enzymes (e.g. NADPH oxidase family enzymes) and sphingomyelinases. The general function of ceramide to form redox signaling platforms amplifying oxdative stress might be critically involved in the dysfunction of vascular cells induced by death receptor ligands and stress stimuli contributing to the development of cardiovascular diseases.

Ceramide generation and ceramide-enriched membrane domains

The plasma membrane of mammalian cells consists primarily of sphingolipids, cholesterol, and other (glycero)phospholipids. Accumulating evidence indicates that sphingolipids and their metabolites, in particular ceramide, sphingosine, and sphingosine-1-phosphate, serve as signaling molecules that regulate a great variety of cell functions [1, 2].

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Ceramide is generated by several enzymatic pathways in mammalian cells. It can be generated in several ways: from sphingomyelin by the activities of the neutral, acid, and alkaline sphingomyelinases [1, 3]; via the *de novo* synthesis pathway, which begins with serine and palmitoyl-CoA and synthesizes ceramide by ceramide synthases (Fig. 1) [4]; by the hydrolysis of complex glycosylated sphingolipids; by the reverse action of ceramidases on sphingosine; and by hydrolysis of ceramide-1-phosphate [5-7]. Sphingomyelinases belong to the family of sphingomyelin-specific phospholipase C (PLC), which hydrolyzes sphingomyelin to ceramide and phosphorylcholine. Sphingomyelin can be regenerated from ceramide by sphingomyelin synthase. Ceramide can also be converted into other sphingolipids, such as ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate.

The generation of ceramide within the cell membrane dramatically alters membrane properties. The classic fluid mosaic model, introduced by Singer and Nicolson in 1972, predicts free movement of proteins in the lipid bilayer; this model was based on biophysical experiments that determined the melting temperatures of lipids [8]. However, in the past 10 to 15 years, this concept has been revised; it currently indicates the existence of distinct membrane domains that are enriched by sphingolipids and cholesterol [9]. Sphingolipids consist of a hydrophobic ceramide moiety and a hydrophilic headgroup. The ceramide moiety is composed of D-erythro-sphingosine and a fatty acid whose acyl chain contains 2 to 28 carbon atoms and is connected via an amide ester bond. Sphingolipids interact with each other and with cholesterol molecules via hydrophilic interactions between the headgroups of the sphingolipids and the hydroxy group of the cholesterol molecule. In addition, hydrophobic van der Waal interactions bind the ceramide moieties to the sterol ring system.

The tight interactions between sphingolipids and cholesterol promote the transition of membrane lipids into a liquid ordered status and the formation of very small distinct domains in the cell membrane, called rafts. Recent microscopy studies demonstrated that the size of these rafts is approximately 20 nm. Hydrolysis of sphingomyelin results in the generation of ceramide within cell membranes, which alters these rafts and results in the formation of large ceramide-enriched membrane domains. Ceramide molecules self-associate to small ceramide-enriched membrane microdomains, which tend to fuse spontaneously to large ceramide-enriched membrane domains [10-12]. These ceramide-enriched membrane platforms serve to organize receptor and signaling molecules in the cell and, thus, serve the spatial and temporal organization of the cell's signaling machinery. In the present review we will focus on the regulation of reactive oxygen species (ROS) by ceramide and ceramide-enriched membrane domains.

ROS metabolism: enzymes involved in ROS production

Redox signaling is a fundamental signaling mechanism in cell biology, particularly in cardiovascular cell biology. Various ROS, including $O_2^{-\bullet}$, H_2O_2, OH, and $ONOO^{-\bullet}$, participate in cell signaling under certain physiological or pathological conditions. The most important of these ROS is $O_2^{-\bullet}$, which is unstable and short-lived because it has an unpaired electron, and it is highly reactive with a variety of cellular molecules, including proteins and DNA. $O_2^{-\bullet}$ is reduced to H_2O_2 by superoxide dismutase (SOD), and both $O_2^{-\bullet}$ and H_2O_2 can diffuse from their sites of generation to other cellular locations. H_2O_2 is further reduced to generate the highly reactive ·OH through the Haber-Weiss or Fenton reaction under pathological conditions. In contrast to $O_2^{-\bullet}$ and H_2O_2, ·OH is highly reactive and, therefore, causes primarily local damage. In addition, $O_2^{-\bullet}$ can also interact with NO to form another reactive oxygen free radical, $ONOO^{-\bullet}$. Under physiological conditions, $O_2^{-\bullet}$ preferably produces H_2O_2 via the dismutation reaction. However, when excess $O_2^{-\bullet}$ is produced, a substantial amount of $O_2^{-\bullet}$ reacts with NO to produce $ONOO^{-\bullet}$. Taken together, these ROS constitute a redox regulatory network that controls cellular activity and function. Unbalanced or enhanced production of ROS, impaired function of the antioxidant system, or both result in oxidative stress.

$O_2^{-\bullet}$ has been considered the progenitor of other ROS. In mammalian cells, many pathways are involved in the production of $O_2^{-\bullet}$, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, mitochondrial respiration chain, and NO synthase-uncoupling. NADPH oxidase was first discovered in phagocytic cells, in which the host defense, phagocytic NADPH oxidase, is massively activated to promote the generation of large amounts of bacteria-killing ROS. To date, NADPH oxidase has been detected in nearly every tissue, and in many cells, such as those in the vasculature, it is the primary source of ROS. Recent studies suggest that NADPH oxidase localizes to specific subcellular compartments, including lamellipodial focal complexes and

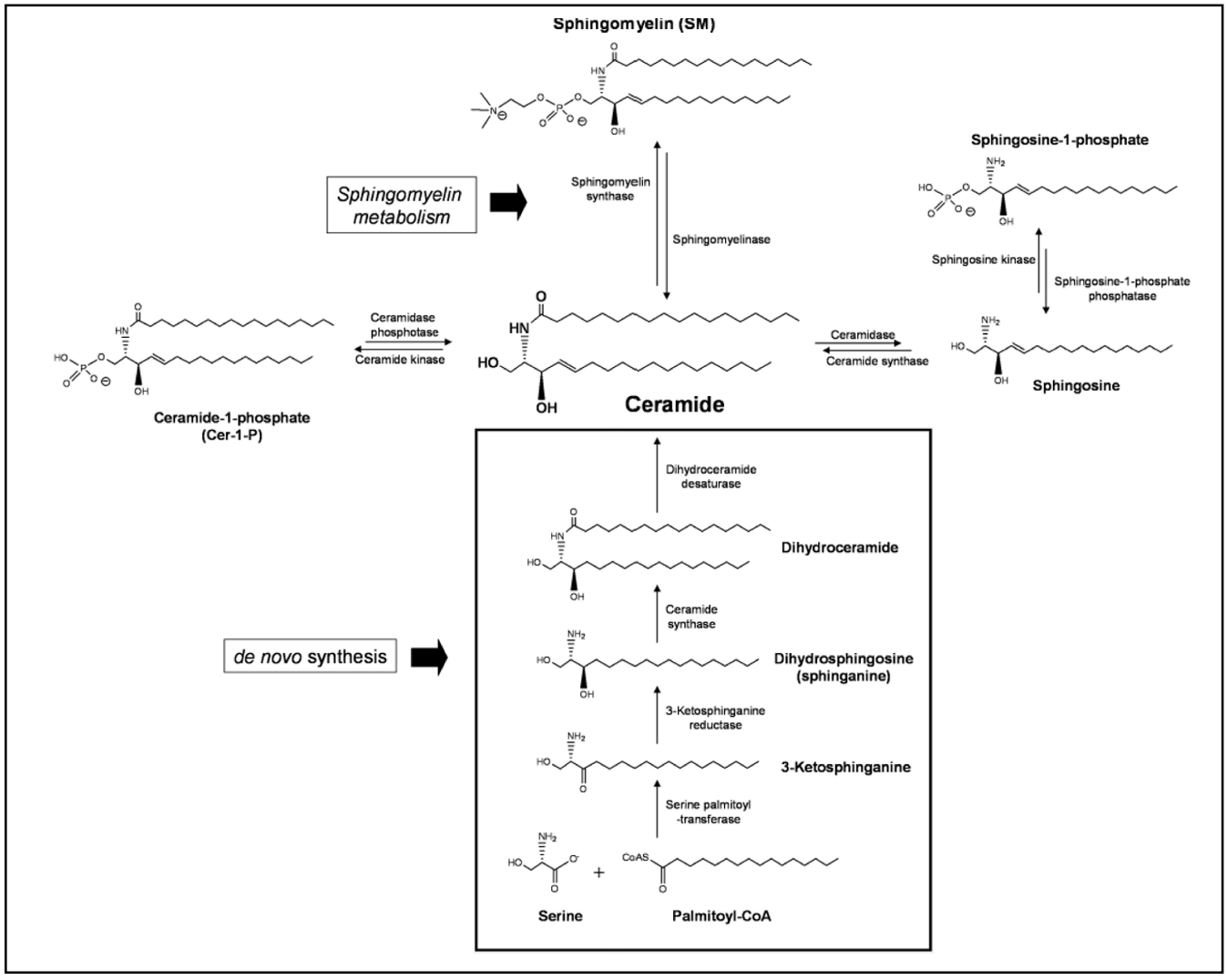

Fig. 1. Ceramide synthesis and metabolism. Ceramide is generated from sphingomyelin or via the *de novo* synthesis pathway. Ceramide is further converted into other sphingolipids such as ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate.

focal adhesions, membrane ruffles, caveolae and lipid rafts, endosomes, sarcoplasmic reticulum, and the nucleus [13-17]. NO synthases normally localize in caveolae and function as homodimers to synthesize NO [18, 19]. When exposed to oxidative or nitrosative stress, NOS becomes structurally unstable ("uncoupling state") and exhibits NADPH oxidase activity resulting in $O_2^{-\bullet}$ formation. Given that ROS are short-lived and diffusible, the localization of ROS signals in specific subcellular compartments suggests that mammalian cells contain temporally and spatially organized redox signaling pathways that regulate various cellular functions.

Role of ROS in sphingomyelinase activation

Recently, the results of several studies have indicated that the generation of ROS may be involved in the activation of the enzyme in response to various stimuli [20-23]. Scheel-Toellner and colleagues demonstrated that acid sphingomyelinase (ASM) activation, ceramide generation, and CD95 clustering play a crucial role in the spontaneous apoptosis of neutrophils; apoptosis was substantially delayed in Asm-deficient mice [23]. On the basis of the observation that the intracellular redox balance

changes in aging neutrophils, the authors investigated the possibility that ROS may be involved in ASM activation. Their study demonstrated that pretreating neutrophils with the antioxidants N-acetylcysteine (NAC) and desferrioxamine substantially inhibited the events downstream of ASM, such as ceramide generation and CD95 clustering, thus indicating that ROS release is required for ASM activation [23]. Similarly, pretreatment with the antioxidant pyrrolidine dithiocarbamate (PDTC) abolished ASM activation by ultraviolet (UV)-C light in U937 cells [20].

Neuronal stimulation with soluble oligomers of amyloid-beta peptide results in the release of ROS and the subsequent activation of the sphingomyelinases [22]. The authors demonstrated that both the acid and the neutral sphingomyelinases play a crucial role in neuronal apoptosis induced by amyloid-beta oligomers, as shown by enzymatic activity assays, neutral and acid sphingomyelinase inhibitors, and antisense oligonucleotides for gene knockdown. Because treatment with antioxidant molecules and a $cPLA_2$-specific inhibitor or antisense oligonucleotide led to inhibition of sphingomyelinase activation and subsequent apoptosis, the results of their study suggest that amyloid-beta oligomers induce neuronal death by activating neutral and acid sphingomyelinases in a redox-sensitive $cPLA_2$-arachidonic acid pathway [22].

Dumitru and colleagues have recently demonstrated the involvement of ROS in TRAIL-induced activation of ASM and apoptosis [21]. Stimulation with TRAIL/DR5 led to the activation of ASM and the subsequent formation of ceramide-enriched membrane platforms, DR5 clustering, and, finally, apoptosis. Pretreatment with the antioxidants NAC and Tiron substantially inhibited TRAIL-induced ASM activation, ceramide/DR5 clustering, and apoptosis, thus demonstrating the important role of ROS in this signaling pathway [21]. Finally, studies investigating the cellular effects of Cu^{2+} showed that Cu^{2+} also promotes the ROS-dependent activation of ASM and leads to the death of hepatocytes [24]. The results of these studies demonstrate that the accumulation of Cu^{2+}, as in Wilson disease, activates ASM in hepatocytes and triggers the release of ceramide in these cells. This process results in Cu^{2+}-induced hepatocyte death, which can be prevented by a deficiency in ASM [24].

The above-mentioned studies demonstrate that ROS are involved in the activation of ASM; however, direct oxidation of the enzyme has been described only in a biochemical study by Qiu and co-workers [25]. The authors demonstrated that C-terminal cysteine (Cys^{629}) plays a crucial role in the enzymatic activity of recombinant human ASM (rhASM). In particular, it appears that any change that causes a loss of the free sulfhydryl group on this amino acid also results in activation of the enzyme: i.e., copper-promoted dimerization of rhASM by C-terminal cysteine, thiol-specific chemical modification of this cysteine to form a mixed disulfide bond or a sulphur-carbon linkage, deletion of this cysteine by carboxypeptidase or recombinant DNA technology, and site-specific mutation to change the cysteine to a serine residue.

Because zinc is required for ASM activity, the authors proposed a model that explains the effect of C-terminal cysteine modification on the activation of ASM. In its low-activity form, free C-terminal cysteine is involved in active-site zinc coordination, either by competing with a water molecule for coordination with zinc or by forming a non-optimal 5-ligand coordination structure. Thus, C-terminal cysteine decreases zinc's ability to ionize water for the nucleophilic attack and decreases enzymatic activity. Because thiol is a better zinc ligand than water, the non-optimal structure may be energetically favorable as long as the cysteine is freely available. In the high-activity form of rhASM, however, the free cysteine is lost either by either chemical modification or by deletion and is no longer available for coordination. As a result, zinc coordinates with a water molecule to produce an optimal structure for catalysis. This model is essentially identical to the "cysteine switch" activation mechanism described previously for the matrix metalloproteinase family [26]. Although the "terminal cysteine" model could explain the effect of ROS on the mechanism of ASM activation, the requirement of dimerization, further molecular events, or both for enzyme stimulation *in vivo* should be addressed in future studies.

A redox mechanism of activation has been also described for the other main type of sphingomyelinase, neutral sphingomyelinase (NSM). Several studies have demonstrated the activation of this enzyme and subsequent ceramide generation, events that were inhibited by pretreatment with antioxidants [27-29]. Furthermore, the natural antioxidant glutathione (GSH) prevented the activation of NSM; this finding indicates that ROS are involved in this pathway [30-33]. Recently, Martin and colleagues clarified the GSH-dependent mechanism of NSM redox regulation in an *ex vivo* system [34]. The authors demonstrated that reducing total GSH without substantially altering the ratio between GSH and oxidized glutathione (GSSG) did not affect NSM activity. However a transient decrease in the GSH/GSSG ratio resulted in

temporary activation of NSM, whereas a permanent decrease in total GSH and in the GSH/GSSG redox ratio produced sustained activation of NSM. Taken together, these findings indicate that altering the GSH/GSSG ratio by increasing GSSG levels or decreasing GSH levels modulates NSM activity [34].

Role of ceramide in ROS production

Several other studies have demonstrated that ASM activation and ceramide production are upstream signals of ROS production. Inhibiting ASM blocks the release of ROS, a finding suggesting that ROS functions downstream of ASM in hepatocytes [35]. Similarly, inhibiting ASM attenuates the ceramide and ROS production induced by histone deacetylase/perifosin, fenretinide, or sodium nitroprusside [36-38]. However, this finding does not necessarily contradict the ASM-oxidation model presented above. It has been shown that in CD95, which releases and requires ROS for the induction of apoptosis [35, 39, 40], ligation of the receptor primarily induces a very weak recruitment of FADD and the stimulation of caspase 8; however, this stimulation reaches only approximately 1% of the levels that are observed for maximal activation of caspase 8 [41]. This weak activation of caspase 8 is observed in ASM-deficient cells, but it is insufficient to trigger apoptosis in these cells. However, the low activity of caspase 8 is sufficient to trigger the translocation and activation of ASM within seconds, with the subsequent formation of ceramide-enriched membrane platforms that cluster CD95 [41]. Receptor clustering leads to the formation of death-inducing signaling complex (DISC) and full activation of caspase 8. A similar model may also apply to the regulation of ROS release by ASM, with a primary weak activation of ASM and a feed-forward loop by ROS.

Ceramide induces the activation of ROS-generating enzymes, including NADPH oxidase, xanthine oxidase, NO synthase, and the mitochondrial respiratory chain [42-45]. In particular, ceramide has been shown to activate NADPH oxidase and to increase the production of $O_2^{-\bullet}$ in a variety of mammalian cells, including human aortic smooth muscle cells, endothelial cells, and macrophages [46-48]. Because many stimuli activate NADPH oxidase by aggregating its subunits, it has been proposed that ceramide mediates the fusion of small raft domains to ceramide-enriched membrane platforms and the aggregation of subunits of NADPH oxidase, thereby stimulating the production of $O_2^{-\bullet}$ [17]. In addition, ceramide has also been shown to interact with the mitochondrial electron transport chain, thereby leading to the generation of ROS [42, 43].

Ceramide in cardiovascular diseases

Recently, the role of ceramide in the regulation of cardiovascular function and in the development of cardiovascular diseases has been extensively studied. Ceramide has been shown to regulate cardiovascular functions, including cardiac contractility, vasomotor responses, and endothelial function [45, 49, 50]. In general, ceramide has been shown to mediate the detrimental actions of many cardiovascular pathogenic factors [51-53], although in only a very few conditions was ceramide found to exert a protective effect [54]. The effects of ceramides are associated with ceramide-induced modulation of the cell membrane, intracellular ion channels, cell proliferation, apoptotic cell death, neutrophil adhesion, and alterations in a number of signaling pathways [49].

Ceramide has been implicated in the regulation of vascular tone or vasomotor responses in a variety of vascular beds. However, whether ceramide induces vasodilator or vasoconstrictor effects is still controversial. There is evidence that treatment with cell membrane-permeable short-chain ceramides, sphingomyelinase, or both produce contractions in canine cerebral arteries, bovine coronary resistance arteries, and capacitance vessels [45, 55-57]. In contrast, some studies have demonstrated that, in phenylephrine-contracted rat thoracic aorta, ceramide induced a relaxation that was associated with decreased intracellular calcium mobilization [58, 59]. This ceramide-induced vasodilation was also seen in rat mesenteric microvessels, although it was only transient. To the best of our knowledge, the discrepancies in these findings may reflect the heterogeneity of vascular ceramide actions between vascular beds or between species. In addition, the differences in vessel preparations may also contribute to the inconsistencies between these results.

In small coronary arteries, ceramide signaling contributes to the impairment of endothelium-dependent vasodilation by reducing NO bioavailability in coronary arteries [44, 45]. The results indicate that ceramide-mediated signaling is a novel mechanism underlying the endothelial dysfunction associated with the overproduction of cytokines during ischemic heart disease. Therefore, ceramide signaling could become the target of a

therapeutic strategy aimed at reducing or preventing endothelial dysfunction during myocardial ischemia and reperfusion. Ceramide consistently reduces the release of bioactive NO (as measured by the stimulation of soluble guanylyl cyclase) in human umbilical vein endothelial cells (HUVECs), and a ceramide-dependent activation of endothelial NO synthase (eNOS) occurs when endothelial cells are exposed to tumor necrosis factor (TNF)-α or high-density lipoprotein [60]. Thus, ceramide decreases endothelial NO levels and thereby inhibits NO-mediated endothelium-dependent relaxation in response to various endothelium-dependent vasodilator agonists. In this respect, ceramide may be a pathogenic factor resulting in endothelial dysfunction in the coronary circulation.

The reduction of NO bioavailability is associated with ceramide-induced oxidative stress. Endothelial cells have been found to generate all forms of ROS, including $O_2^{-\bullet}$, H_2O_2, OH, and $ONOO^{-\bullet}$, as well as other radicals [61-63]. Recent studies have suggested that the generation of ROS, in particular $O_2^{-\bullet}$, by endothelial cells is both physiologically and pathophysiologically relevant to the function of these cells [61-63]. ROS levels are tightly regulated by antioxidants such as SOD, catalase, thioredoxin, glutathione, antioxidant vitamins, and other small molecules [61-63]. Under normal conditions, the rate of ROS production is balanced with the rate of its elimination. However, a mismatch between the production and the elimination of ROS will result in the increased bioavailability of ROS and a state of oxidative stress. One of the pathogenic outcomes of oxidative stress is oxidative damage resulting in endothelial dysfunction.

Ceramide-mediated oxidative stress is primarily due to the activation of endothelial NADPH oxidase, which requires assembly of its subunits to form an integrated enzyme complex [17]. Ceramide-enriched membrane domains serve as redox signaling platforms that facilitate the aggregation and activation of NADPH oxidase [17, 64]. It is plausible that the formation of this redox signaling platform makes important contributions to the endothelial dysfunction associated with various death receptor agonists, such as TNF-α or CD95 ligand, which are importantly involved in the pathogenesis of various vascular diseases, such as atherosclerosis, hypertension, and ischemia/reperfusion injury. The formation of this ceramide-enriched redox signaling platform on the membrane of endothelial cells may be implicated in the development of these diseases.

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