ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 9, 2007

DOI: 10.1089/ars.2007.1692

Forum News & Views

Ceramide: A Novel Player in Reactive Oxygen Species-Induced Signaling?

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ABSTRACT

Generation of ceramide in the plasma membrane, with the subsequent formation of large ceramide-enriched membrane platforms, serves signal transduction via receptors, but also nonreceptor-mediated activation of cells. Recent studies demonstrate that enzymes mediating release of reactive oxygen species (ROS) localize to membrane rafts, and the integrity of these rafts is required for cellular ROS release. The authors and others noted that in a feed-forward mechanism, ROS are able to stimulate ceramide-releasing enzymes, for instance, acid sphingomyelinase, resulting in the formation of ceramide-enriched membrane platforms that may mediate cellular activation initiated by oxidative stress. *Antioxid. Redox Signal*. 9, 1535–1540.

MEMBRANE DYNAMICS AND CERAMIDE

THE PLASMA MEMBRANE OF MAMMALIAN CELLS consists primarily of sphingolipids, cholesterol, and other (glycero)phospholipids. Singer and Nicholson (24) proposed a weak interaction of lipids, resulting in a statistical distribution of lipid molecules and a free flotation of proteins in the cell membrane. However, studies in the last 10 years modified this model and suggested that sphingolipids and cholesterol interact via hydrogen bonds and hydrophobic van der Waal interactions (23). In addition, the head groups of sphingolipids bind to each other via hydrophilic interactions. The result of these interactions is a lateral association of sphingolipids and cholesterol, and the formation of very small, distinct membrane domains in the cell membrane named rafts (23). Cholesterol seems to stabilize these microdomains by filling the voids between the large bulky glycerosphingolipids (23), since interference with the cholesterol metabolism destroys rafts. However, it needs to be pointed out that existence of rafts is still controversial (14). In contrast, caveolae are well-defined membrane domains that constitute small invaginations within the cell membrane (5). Caveolae consist of the protein caveolin, as well as sphingolipids and cholesterol.

Studies in recent years demonstrated a novel membrane domain type, *i.e.,* ceramide-enriched membrane domains. Ceramide is generated in membranes by hydrolysis of sphingomyelin catalyzed by acid, neutral, or alkaline sphingomyelinases, which exhibit their peak activity at the respective pH values (6). Further, ceramide is generated *de novo* via the ceramide synthase pathway (6). The biophysical properties of ceramide molecules predict a tight interaction of ceramide molecules with each other, resulting in the formation of stable and tightly packed ceramide-enriched membrane microdomains that spontaneously fuse to form large ceramide-enriched membrane macrodomains, also named platforms (15).

Ceramide and ceramide-enriched membrane domains, respectively, are involved in many signaling pathways, for instance via different receptors, or signaling induced by stress-stimuli and pathogens (Fig. 1). The biological function of these membrane platforms was recently discussed in detail (8). Here, we will focus on the interaction between membrane domains and reactive oxygen species (ROS), as well as on ROS regulation of the ceramide metabolism.

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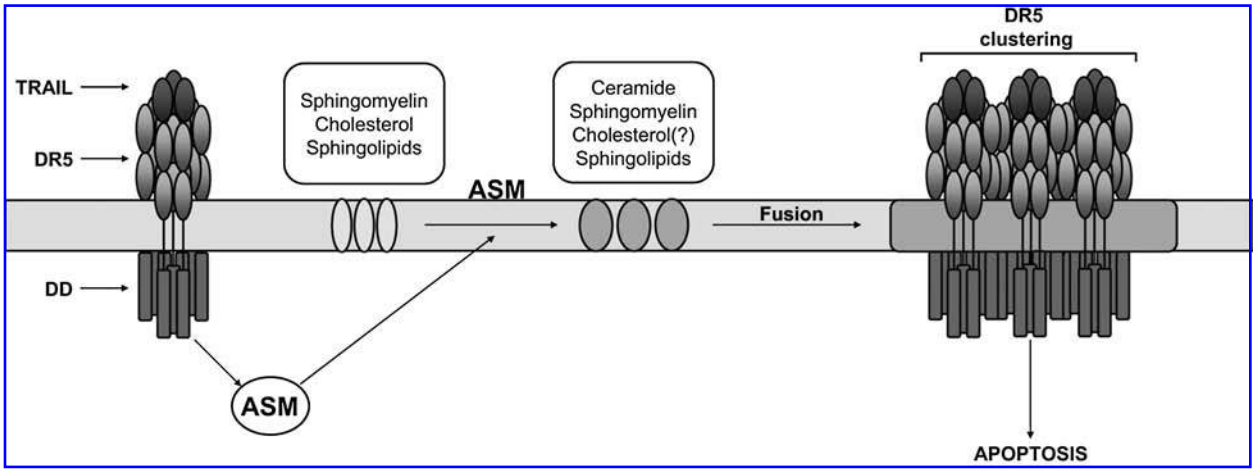

FIG. 1. Schematic representation of ASM and ceramide-mediated receptor clustering in membrane platforms. Stimulation via TRAIL/DR5, CD95, or CD40 activates ASM and triggers a translocation of the enzyme onto the cell surface. The release of ceramide in small rafts correlates with the fusion of these small membrane domains to large platforms and the trapping of receptor molecules within ceramide-enriched membrane platforms. Receptor clustering then functions as a prerequisite for specific signal transduction via the receptor molecule.

OXIDATIVE STRESS AND LIPID RAFTS

Oxidative stress refers to the imbalance of enhanced production of ROS and/or impaired function of the antioxidant system. ROS include superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals, and hydrogen peroxide (H_2O_2). The generation of ROS usually starts with production of $O_2^{\cdot -}$, which rapidly dismutates into H_2O_2 at low pH or via catalysis by superoxide dismutase (SOD). H_2O_2 can be further converted into highly reactive hydroxyl radicals via iron-catalyzed Fenton reactions under pathological conditions. $O_2^{\cdot -}$ also rapidly reacts with nitric oxide (NO) to form a more stable free radical, peroxynitrite ($OONO^{\cdot -}$), which is a potent cytotoxic oxidant. Therefore, in this respect, $O_2^{\cdot -}$ has been considered as the progenitor of other ROS. In mammalian cells, many pathways are involved in the production of $O_2^{\cdot -}$ including NADPH oxidase, xanthine oxidase, mitochondrial respiration chain, NO synthase (NOS) uncoupling, or other systems. NADPH oxidase was first discovered in phagocytic cells, where in host defense, phagocytic NADPH oxidase is massively activated to generate large amount of ROS to kill bacteria. To date, NADPH oxidase has been found in almost every tissue and in many cases it is the major sources of ROS, for instance in vasculature. Recent studies suggest that NADPH oxidase localizes in specific subcellular compartments including lamellipodial focal complexes and focal adhesions, membrane ruffles, caveolae and lipid rafts, endosomes, sarcoplasmic reticulum, and the nucleus (26, 29, 30). Given that ROS are short-lived and diffusible, localization of ROS signals in specific subcellular compartments suggests the existence of temporal and spatially organized redox signaling pathways in mammalian cells which regulate various cellular functions.

Oxidative stress and ROS can elicit and modulate various physiological and pathological processes including cell death. However, molecular mechanisms mediating ROS-induced cellular effects still require definition. Accumulating evidence demonstrates that lipid rafts and lipid raft-derived membrane platforms are involved in oxidative cellular stress. ROS are capable to react with and damage various molecular targets including DNA, proteins, and lipids and, thus, ROS may directly or indirectly interact with raft components and regulate signaling functions of lipid rafts. On the other side, membrane platforms may regulate ROS-generating enzymes including NADPH oxidase and thereby amplify ROS production. The significance regarding the role of lipid rafts and membrane platforms in ROS production will be discussed in a parallel review by Li *et al.* in this forum series.

CERAMIDE AND LIPID RAFTS MEDIATE ROS-INDUCED CELL DEATH

Previous studies demonstrated a role of ROS in the induction of apoptosis by CD95 (19). Recently, we determined the release of ROS when BJAB or splenocytes were stimulated with TRAIL (3). TRAIL induced a very rapid release of ROS, which was prevented by preincubation with the antioxidants Tiron or *N*-acetylcysteine, respectively. Importantly, Tiron or *N*-acetylcysteine also inhibited the formation of ceramide-enriched membrane platforms and apoptosis triggered by TRAIL (3). These results indicate that rapid ROS production is an upstream event of the formation of ceramide-enriched membrane platforms, which is essential for the induction of apoptosis. Shen and colleagues recently reported that receptor-interacting protein (RIP) and tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2), two key effector molecules of TNF signaling, are essential for ROS-induced cell death (22). In mouse embryonic fibroblasts, they observed that exogenous H_2O_2 induced a complex formation by RIP and TRAF2 and a transient colocalization of RIP within GM1-enriched raft clusters (22). These data further indicate that clustered raft microdomains serve as important signaling platforms to mediate apoptotic response induced by ROS.

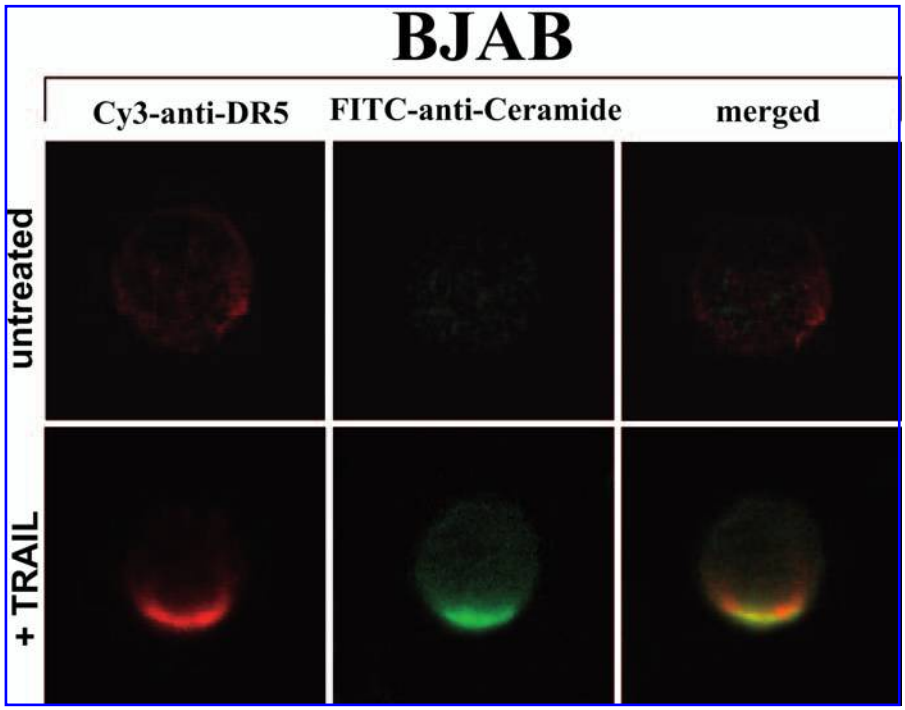

FIG. 2. TRAIL induces DR5 clustering in ceramide-enriched membrane platforms. Stimulation of BJAB lymphocytes with TRAIL results in clustering of the DR5 receptors within ceramide platforms. Receptor clustering and the presence of ceramide-enriched membrane platforms were shown by confocal microscopy and fluorescence microscopy. (Printed with permission from *Oncogene*).

LIPID RAFTS MEDIATE ROS-INDUCED PRO-SURVIVAL SIGNALING

In addition to pro-apoptotic signaling, ROS can also induce prosurvival signaling pathways. In cultured bovine aortic endothelial cells, Yang and co-workers reported that H_2O_2 activates prosurvival signaling pathways including activation of PI3 kinase/Akt and ERK1/2 (28). Pretreatment of endothelial cells with methyl-β-cyclodextrin or filipin, agents that disrupt lipid rafts, attenuated these signaling events (28). Reconstitution of raft domains restored H_2O_2-induced Akt and ERK1/2 phosphorylation. Other studies showed that ROS can activate many signaling molecules and receptors involved in cell survival signaling such as Src-kinases, Ras, and epidermal growth factor receptor, which are enriched or partially localized in lipid raft microdomains. Taken together, these findings suggest that lipid rafts also serve as signaling platforms for propagation of compensatory survival pathways in response to damaging levels of H_2O_2. Thus, lipid rafts seem to have a dual function, being involved in cell death and survival after ROS, probably depending on the set of receptors and signaling molecules that are sorted into lipid rafts after stimulation.

ROS INTERACT WITH CAVEOLIN-1

Biochemical and morphological experiments have shown that at least two subtypes of lipid domains are present in mammalian cells: caveolar and noncaveolar lipid domains. The size of noncaveolar lipid rafts is ~50–100 nm and each lipid raft may contain 10–30 protein molecules. Caveolae, cave-like plasma-membrane subdomains, are considered as another subtype of lipid domains. Caveolin-1 is the major protein component of caveolae. Polymerization of caveolin-1 forms a rigid scaffold that maintains the characteristic cave-like morphology. In addition to its structural function, caveolin-1 has several important regulatory activities through direct interaction with other functional proteins and signaling molecules. Caveolin-1 is subject to two types of post-translational modification that might be critical for regulating its intracellular activity and localization, namely phosphorylation and palmitoylation. Recent studies indicate that both phosphorylation and palmitoylation of caveolin-1 can be regulated by ROS and ultimately affect caveolar functions. In vascular endothelial cells, H_2O_2 causes increased tyrosine phosphorylation of caveolins (27). In addition, Parat and colleagues showed that exogenous H_2O_2 did not alter the intracellular localization of caveolin-1 in endothelial cells but, instead, H_2O_2 inhibited the trafficking of newly synthesized caveolin-1 to membrane raft domains (16). They further demonstrated that H_2O_2 did not alter the rate of caveolin-1 depalmitoylation, but rather decreased the 'on-rate' of palmitoylation (16). Functional studies substantiated that caveolin-1 is a sensitive target of oxidative stress. Smart and coworkers reported that the oxidation of caveolar membrane cholesterol causes the translocation of caveolin-1 from the plasma membrane to the Golgi apparatus (25). In a separate study, treatment of endothelial cells with ROS caused a release of caveolin-1 from membranes and also a decrease in the number of caveolae detected by electron microscopy (17). In summary, these results suggest that oxidative stress modulates caveolin-1 function and cellular levels, which may ultimately affect caveolar function and plasma membrane composition, *i.e.*, alteration in ratio of caveolar *vs*. noncaveolar lipid domains.

ROS INTERACT WITH CHOLESTEROL

The formation of liquid ordered microdomains, lipid rafts, is driven by tight packing between cholesterol and sphingomyelin and other sphingolipids. Oxysterols are derivatives of cholesterol that contain a second oxygen atom as a carbonyl, hydroxyl, or epoxide group (13). Cytotoxic oxysterols formed by nonspecific oxidative mechanisms can affect many cellular processes that contribute to the pathogenesis of disease. According to their biophysical properties, which can be distinct from those of cholesterol, oxysterols can promote or inhibit the formation of membrane microdomains or lipid rafts (1, 20). For instance, the activities of receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor and the insulin receptor, which are found in lipid raft/caveolae, can be modulated by changes in cellular cholesterol content. EGF stimulation-induced phosphatidylinositol 4,5-bisphosphate turnover is inhibited by depletion of cholesterol, but the effects of repletion with different oxysterols varied according to their structure. Turnover was not restored by 25-hydroxycholesterol, while 7-ketocholesterol and 5α,6α-epoxycholesterol restored turnover (13). Likewise, desmosterol, another oxysterol, impaired raft-dependent signaling via the insulin receptor, while nonraft-dependent protein secretion was not affected (13). Therefore, these studies suggest that ROS may oxidize cholesterol to various oxysterols, which affect the stability of lipid raft microdomains and formation of lipid raft-derived signaling platforms.

ROS AND ACID SPHINGOMYELINASE ACTIVATION

Although activation of acid sphingomyelinase (ASM), with the subsequent release of ceramide, has been reported for a great number of stimuli, the exact mechanism by which this enzyme is activated has not been completely elucidated. Initial studies described a diacylglycerol (DAG)-induced ASM activation (7). DAG was shown to activate ASM in a protein kinase C-independent manner, since these effects of DAG occurred in cells downmodulated for protein kinase C (7). At least in the case of TNF stimulation, ASM has been shown to be activated via some adaptor proteins (21). The authors developed a model in which the activation of the ASM is signalled via the death-domain of the TNF-receptor-1 through recruitment of the TNF-receptor-associated proteins TRADD and FADD, but not TRAF2 nor RIP (21).

In the last years, a new concept for the mechanism of ASM activation emerged. Several recent studies indicated that generation of ROS may be involved in activation of the enzyme in response to various stimuli (2, 3, 9, 20). Scheel–Toellner and colleagues demonstrated a crucial role of ASM activation, ceramide generation and CD95 clustering for spontaneous apoptosis of neutrophils, since apoptosis was significantly delayed in ASM-deficient mice (20). Based on the observation that the intracellular redox balance changes in aging neutrophils, the authors investigated the possibility of ROS involvement in ASM activation. Their study demonstrated that pretreatment of neutrophils with the antioxidants *N*-acetylcysteine (NAC) and desferrioxamine significantly inhibited the events downstream of ASM, such as ceramide generation and CD95 clustering, thus indicating a requirement of ROS release for ASM activation (20). Similarly, pretreatment with the antioxidant PDTC abolished UV-C light-induced ASM activation in U937 cells (2). Our group has recently demonstrated the involvement of ROS in the TRAIL-induced apoptotic pathway (3). Stimulation via TRAIL/DR5 led to activation of the ASM with the subsequent formation of ceramide-enriched membrane platforms, DR5 clustering, and finally apoptosis (Fig. 2). Pretreatment with antioxidants NAC and Tiron significantly inhibited TRAIL-induced ASM activation, ceramide/DR5 clustering, and apoptosis, thus demonstrating the important role of ROS for this signaling pathway (3). Finally, studies investigating cellular effects of Cu^{2+} also revealed a ROS-dependent activation of the ASM by Cu^{2+} eventually leading to death of hepatocytes (9).

The above-mentioned studies show an involvement of ROS in ASM activation mechanism; however, a direct oxidation of the enzyme has only been described in a biochemical study by Qiu and co-workers (18). The authors demonstrated a critical role of the C-terminal cysteine (Cys^{629}) in the enzymatic activity of recombinant human ASM. Particularly, 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, that is, copper-promoted dimerization of rhASM via the C-terminal cysteine, thiol-specific chemical modification of this cysteine to form a mixed disulfide bond or a sulfur–carbon linkage, deletion of this cysteine by carboxypeptidase or recombinant DNA technology, and site-specific mutation to change the cysteine to a serine residue. Based on the fact that zinc is required for the activity of the ASM, the authors proposed a model which explains the effect of C-terminal cysteine modification regarding the activation of the ASM. In the low activity form, the free C-terminal cysteine is involved in the active site zinc coordination, either by competing with a water molecule for coordination with zinc or by forming a nonoptimal five-ligand coordination structure. This decreases the ability of zinc to ionize water for the nucleophilic attack and decreases enzymatic activity. As thiol is a better zinc ligand than water, the nonoptimal 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 by either chemical modification or deletion and is no longer available for coordination. As a result, zinc coordinates with a water molecule, resulting in an optimal structure for catalysis.

Although the 'terminal cysteine' model could explain the effect of ROS on the activation mechanism of the ASM, the requirement of dimerization and/or further molecular events for stimulation of the enzyme *in vivo*, needs to be addressed in future studies.

Several other studies demonstrated the involvement of ROS in the ASM/ceramide pathway; however, these studies propose that generation of ROS is also an event downstream of ASM activation. Recent experiments on hepatocytes demonstrated that an inhibitor of the ASM blocks the release of ROS, suggesting that ROS functions downstream of the ASM (19). However, this is not necessarily in contrast to the ASM-oxidation model presented above. It has been recently shown for CD95, which releases and requires ROS for induction of apoptosis (19), that ligation of the receptor primarily induces a very weak recruitment of FADD and stimulation of caspase 8, which

reaches ~1% of the levels that are observed for maximal activation of caspase 8 (4). This weak activation of caspase 8 is even observed in ASM-deficient cells, but it is insufficient to trigger apoptosis. However, the low activity of caspase 8 is sufficient to trigger the translocation and activation of the ASM within seconds, with the subsequent formation of ceramide-enriched membrane platforms that cluster CD95 (4). Receptor clustering leads to DISC formation and full caspase 8 activation. Thus, the ASM functions in a feed-forward loop to amplify signaling via death receptors, and a blockade of this forward loop by inhibition of the ASM would also prevent a further release of ROS. This could also explain the model of TNF-induced ASM activation described by Schwandner and co-workers, which proposed the adaptor proteins TRADD and FADD as upstream activators of the ASM (21).

A redox mechanism of activation has been also described for the other major type of sphingomyelinase, the neutral SMase (NSM), since activation of this enzyme and the subsequent ceramide generation were inhibited by pretreatment with antioxidants (11). Furthermore, the natural antioxidant glutathione (GSH) prevented activation of the NSM, indicating an involvement of ROS in this pathway (10). Recently, Martin and colleagues (2006) clarified the GSH-dependant mechanism of NSM redox regulation in an *ex vivo* system (12). The authors demonstrated that reduction of total GSH without significantly altering the GSH/oxidized glutathione (GSSG) ratio did not affect NSM activity. However, a transient decrease of the GSH/GSSG ratio resulted in a temporary activation of the NSM, while a permanent decrease of total glutathione and GSH/GSSG redox ratio produced a sustained activation of NSM activity. Taken together, these data indicate that altering the GSH/GSSG ratio by increasing GSSG or decreasing GSH levels, but not the total concentration of glutathione, modulates NSM activity (12).

CONCLUSIONS

Many recent studies have disclosed a critical function of membrane rafts, acid sphingomyelinase, and ceramide-enriched membrane domains in the induction of apoptosis by death receptors, stress stimuli, and during development; the infection of mammalian cells by bacteria, viruses, and parasites; and the regulation of endothelial functions. The concept of ceramide-enriched membrane domains and the ceramide-mediated transformation of very small "inactive" membrane rafts into large, "active" signaling platforms explain the function of ceramide for cellular activation by all these stimuli. Understanding the mechanisms that regulate activation of the acid sphingomyelinase and generation of ceramide platforms by ROS would be useful for developing therapeutic strategies to treat at least some forms of cancer, infectious diseases, or chronic degenerative disorders.

ACKNOWLEDGMENTS

The authors thank Siegfried Moyrer for excellent help with the manuscript. Studies described in the present review were supported by DFG grant Gu 335/10-3/4 and Deutsche Krebshilfe grant 10-2238-Gu2 to E.G.

ABBREVIATIONS

ASM, acid sphingomyelinase; DAG, diacylglycerol; DD, death domain; GM1, ganglioside G(M1); GSH, glutathione; GSSG, oxidized glutathione; NAC, *N*-acetylcysteine, NOS, NO synthase; PAF, platelet activating factor; ROS, reactive oxygen species; TNF, tumor necrosis factor; UV, ultraviolet.

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Date of first submission to ARS Central, April 6, 2007; date of final revised submission, April 6, 2007; date of acceptance, April 7, 2007.

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