Biological Roles of Acid and Neutral Sphingomyelinases and Their Regulation by Nitric Oxide

Generation of the pleiotropic sphingolipid mediator ceramide by acid and neutral sphingomyelinases is a key event in many cellular pathophysiological processes including survival, death, proliferation, and differentiation, in which also the short-lived gaseous messenger nitric oxide plays a crucial role. This review describes how the outcome of these key cellular processes is finely tuned by surprising and complex interplays among nitric oxide, ceramide, and their effectors.

All living cells, prokaryotic and eukaryotic, have a plasma membrane (PM) that encloses their contents and serves as a semi-porous barrier to the outside environment. The membrane acts as a boundary, holding the cell constituents together and keeping other substances from entering. For many years, proteins of the PM have been considered to act both as signaling and structural molecules, whereas lipids have been viewed to act mostly as inert structural components of the cellular membranes.

This notion about the role of lipids resisted until the late 1970s: the discovery of the phosphoinositide cycle (5) represented a major breakthrough, pointing for the first time to lipids as key players also in signal transduction events. From that point on, other lipid molecules found acceptance as biological mediators and modulators; among these, a class of lipids has emerged as a group of important signaling molecules: the sphingolipids.

Lips of the sphingolipid class contain a long-chain sphingoid base backbone (such as sphingosine), an amide-linked, long-chain fatty acid, and one of various polar head groups. The structure of these head groups defines the various classes of sphingolipid subtypes, with a hydroxyl group found in ceramide, phosphorylcoline in sphingomyelin (SM), and carbohydrates in glycosphingolipids. Their synthesis begins in the endoplasmic reticulum and continues in the Golgi apparatus, and they are thereafter found in all intracellular membrane structures (for a review, see Ref. 33).

Richard Kolesnick’s and Yussuf Hannun’s groups were the first to point attention to SM-based signaling pathway: the former showed in 1987 the rapid activation of sphingomyelinases (SMases) in response to 1,2 dyacylglycerol treatment (54) and proposed the existence of a SMase-generated signaling pathway (55); the latter showed that this pathway could be activated by receptor-mediated mechanisms and provided first evidence for ceramide as a cellular mediator (73). From then on, several papers have been published on the biological roles of sphingolipids and of sphingolipid-metabolizing enzymes; besides their recognized structural roles in mechanical stabilization and chemical resistance, they were found to contribute to membrane trafficking and vesicle formation, such as in exosome budding, phagocytosis, autophagy, and endo-/exo-cytosis (43, 91). Finally, sphingolipids were proven to act as signaling molecules in complex intracellular pathways presiding over cell functions as diverse as growth, migration, differentiation, and death, with relevant consequences for human diseases (for an extensive review, see Ref. 40).

Among sphingolipids, ceramide (66) functions both as a key player in cell signaling and as the precursor of other sphingolipids. In contrast to many sphingolipids, which contain a hydrophilic region, such as phosphate in the case of sphingosine-1-phosphate (S1P) and ceramide-1-phosphate, phosphorylcholine in SM, and sugar residues in glycosphingolipids, ceramide has a minimal hydrophilic region comprising two OH groups. Ceramide signaling has been intimately involved in the regulation of cell growth, differentiation (72), senescence (92), necrosis (42), proliferation (1), and apoptosis (71).

Despite the possible de novo formation of ceramide, in the vast majority of cells, SM appears to be the primary sphingolipid source for bioactive ceramide, thereby emphasizing the critical role for SMases in initiating ceramide-activated apoptotic signaling.

Two of these enzymes, the lysosomal phosphodiesterase acid SMase (A-SMase) and the membrane-bound neutral SMase (N-SMase), have been...
shown to contribute substantially to apoptosis triggered by death receptors, although their contribution to apoptosis appears to vary depending on the tissues and cells investigated (11, 60, 63, 76, 88). The mechanisms of activation of SMases have been extensively studied. The enzymes are stimulated early after death receptor activation, downstream to recruitment to these receptors of proteins involved in initiating their apoptotic signaling, i.e., FAN (for N-SMase) and FADD (for A-SMase) (63, 85), with possible involvement of other early signals (20, 51, 88). Expression of dominant negative FAN and FADD gives rise to long-lasting inhibition of apoptosis, restored only by exogenous ceramide (60, 88).

The action of ceramide in these intracellular pathways often occurs through coordinate interplays with other messenger molecules and their generating enzymes (12, 41, 44). One such molecule is the short-lived gaseous messenger nitric oxide (NO).

NO is generated in cells by specific enzymes, the NO synthases (NOS) (2). Because of its chemical reactivity and high diffusibility, NO production by NOS is under complex, tight control designed to dictate specificity to its signaling and to limit toxicity toward other cellular components. Studies in recent years have uncovered an increasingly important role of physical association of the NOS isoforms with a variety of regulatory and structural proteins (56). Of importance, these protein-protein interactions, as well as regulating NOSs activity, often target them to cellular membranes. The NH2 terminus of neuronal NOS I contains a PDZ (postsynaptic density protein-95, discs-large, ZO-1) domain that allows interactions of the enzyme with other PDZ-containing proteins at the PM including α1-syntrophin, PSD-95, and PSD-93 (10). Endothe
tial NOS III is localized at both the PM and the Golgi complex through its ability to be myristoylated and palmitoylated (31). In addition, both NOS II (i.e., the inducible isoform) and III may interact with caveolin 1 and/or 3, proteins responsible not only for the localization of these enzymes at the PM but also for the regulation of their activity (NOS III) and expression (NOS II) in an inhibitory fashion (27, 32). The localization at cellular membranes is a key aspect of NOS and constitutes the structural basis of the functional interaction among NO, sphingolipids, and their generating enzymes. In particular, regulation of A-SMase and N-SMase by NO is an event of high biological relevance.

The aim of this review is to highlight the biological roles of A-SMase and N-SMase and their interplay with NO.

**ACID SMase**

A-SMase (OMIM 607608) was originally identified as a cation-independent hydrolase (EC 3.1.4.12) contributing to the catabolism of SM in lysosomes and in Niemann-Pick types A and B diseases, where a deficiency of the enzyme leads to lysosomal accumulation of SM (46). The enzyme is a 629-amino acid sequence; at the NH2 terminus of the protein, there is a signal peptide that directs the translation product into the endoplasmic reticulum (48). Studies carried out in COS-1 cells transfected with A-SMase cDNA have identified two major forms of apparent molecular mass of 75 and 72 kDa (peptide chain of 64 and 61 kDa, respectively) and a minor one with molecular mass of 57 kDa (peptide chain of 47 kDa); this smaller form is generated by cleavage of the 75-kDa precursor, which is a pre-polypeptide with little if any enzymatic activity in the endoplasmic reticulum (29). Also the 57-kDa form has little enzymatic activity (29, 47). The 72-kDa precursor is generated into the endoplasmic reticulum/Golgi apparatus via a proteolytic cleavage of the 75-kDa pre-polypeptide and then transported to the endo/lysosomal compartments (48), where it is processed to its mature form of 70 kDa. The mature enzyme is degraded to an inactive form of 52 kDa. The mature form and its precursors have six potential N-glycosylation sites, and at least five of them are important for proper folding, protection against proteolysis, and enzymatic activity (28, 84). Additionally, A-SMase has a mannose 6-phosphate residue that is required for lysosomal targeting of the enzyme via the mannose 6-phosphate receptor (48).

Recent studies have indicated that A-SMase localization is in fact not limited to the interior of lysosomes. Indeed, from the gene of A-SMase, another product may be generated, i.e., the secretory SMase, which is very similar to A-SMase, differing only in the intracellular trafficking and in the need of zinc for activation.

Stress is believed to activate A-SMase to generate ceramide, which serves as a cellular mediator in initiating apoptotic response. The first evidence for this paradigm was provided in 1996 by Santana et al., who showed that lymphoblasts from Niemann-Pick patients fail to respond to ionizing radiation with ceramide generation and apoptosis (81). Later, Grassme et al. demonstrated in human epithelial cells and primary fibroblasts the activation of phospholipase C and A-SMase with the release of diacylglycerol and ceramide in the hepatocellular apoptosis induced by Neisseria gonorrhoeae (36). Genetic and/or pharmacological inhibition of A-SMase caused inhibition of cellular invasion by the pathogen (36).
Recently, A-SMase has emerged as a biochemical mediator of stimuli as diverse as ionizing radiation, chemotherapeutic drugs, UVA light, heat, CD95 activation, reperfusion injury, as well as infection with some pathogenic bacteria and viruses (18, 25, 26, 34, 38, 67, 75, 80). A-SMase activity is also crucial for developmental programmed cell death of oocytes (39). A comprehensive model has been proposed, enticing a role of A-SMase to explain the function of ceramide in CD95-induced apoptosis. Posttranslational palmitoylation of CD95 allows the targeting of the CD95 receptor to lipid raft (precisely, “chole-raft”), where, on stimulation, CD95 ligand binds to CD95, inducing the translocation of A-SMase to the raft and the generation of ceramide by hydrolysis of SM. Ceramide transforms cholest-raft to “cer-raft” and leads to the coalescence of cer-rafts into larger rafts, where the connection between CD95 receptor and actin cytoskeleton does occur. This interaction is mediated via the association of CD95 with ezrin, ultimately responsible for the initiation of cer-raft-CD95-CD95L internalization (D), a fundamental step for the formation of the Death Inducing Signaling Complex (DISC), which leads to an efficient caspase activation and cell death (for review, see Ref. 67).

In a recent paper, Bianco et al. have discovered a new role for A-SMase: the release of PM-derived microparticles (MPs) (7). Vesicles shedding is emerging as a relevant mechanism of intercellular communication. Cells constitutively release microvesicles transporting biologically active molecules, among which are cell surface receptors, cytokines (IL1-β), microRNA, and adhesion molecules that contribute to inflammation, coagulation, and tumor invasiveness and metastasis (for a review, see Ref. 17).

Bianco et al. found that A-SMase, either in microglia or in cortical astrocytes, on stimulation of the ATP receptor P2X7R, is recruited to the PM and activated, leading to the budding of the membrane and the ensuing shedding of MPs, indicating a fundamental role for A-SMase in the event of intracellular communication involving the generation of MPs (8, 24, 70).

Neutral SMase

Neutral magnesium-dependent SMase (N-SMase) activity was described initially in 1967 (83). The indication of the existence of an enzyme with a different pH optimum from A-SMase generated by a different gene came from studies indicating the total preservation of N-SMase activity in A-SMase knockout mice (46, 74) and in cells from patients affected by Niemann-Pick type A disease (59). From then on, many groups have published articles about the purification of this enzyme (4, 19, 61), and Kim and co-workers identified multiple N-SMases in bovine brain, suggesting the existence, at least in brain, of several isoforms of this enzyme (52).

In 1998, Tomiuk et al. cloned and characterized in mouse and human tissues an enzyme with Mg$^{2+}$-dependent N-SMase activity, now known as N-SMase1 (90). The murine protein consists of 419 residues with a molecular mass of 47.5 kDa, and the human isoform is a protein of 423 residues corresponding to 47.6 kDa; both are membrane proteins with two putative transmembrane domains at the COOH terminus. The physiological function of this enzyme as a SMase was denied by subsequent studies in which it was observed that N-SMase1 overexpressed in cells did not modify the SM metabolism despite its in vitro activity (82) but instead acted as a lyso-platelet activating factor (lyso-PAF) phospholipase C.

In 2000, another mammalian brain-specific N-SMase has been cloned and named N-SMase2 (45). The gene encodes a protein of 655 amino acids with a molecular mass of 71 kDa, a transmembrane region at the COOH terminus, and the catalytic domain of the COOH terminus. This enzyme has SMase activity, dependent on Mg$^{2+}$, both in vitro and in vivo (64), and is involved in cell growth regulation. The enzyme cellular localization is still controversial since the Stoffel’s group showed a Golgi localization, whereas the Hannun’s group showed a localization at the level of the PM (45, 65).

**NO Controls Stress-Induced Apoptosis Through the Inhibition of A-SMase Activity**

Generation of NO by a constitutive NOS, such as NOS III, at low physiological concentration is one of the mechanism of inhibition of apoptosis when triggered by the activation of death receptors of the TNF-α (TNF-RI)/CD95 superfamily (62). By contrast, at high concentrations, NO can induce apoptosis per se. Various mechanisms have been proposed to account for these two apparently conflicting effects of NO (13, 62). Strong evidence indicates that a relevant mechanism resides in the ability of NO to regulate cellular levels of ceramide. Studies carried out in the U937 monocyte-derived cells and in clones of γ/δ T lymphocytes have shown that NO inhibits the apoptotic responses induced by CD95 and TNF-RI by reducing the ability of these receptors to generate ceramide (3, 14, 21, 86, 87). Although both A-SMase and N-SMase are targets of NO, protection from apoptosis is due to the inhibition of A-SMase (FIGURE 1). The mechanism of apoptosis protection outlined above appears to be widespread since it has been shown to be active also in human and murine dendritic
cells as well as in cancer cells. In particular, NO generation inhibited apoptosis of dendritic cells exposed to apoptogenic concentrations of lipopolysaccharide (LPS) in vitro and in a model of LPS-induced sepsis in vivo (26); in addition, it protected DCs from the toxic effect of the chemotherapeutic drug cisplatin, in vitro and in vivo, in a model of tumor chemotherapy (75). Also, in dendritic cells, the primary target of NO action appears to be A-SMase, activated by both LPS, acting via the Toll-like receptor 4 (26), and cisplatin, acting via CD95 activation (58, 75). Similar results were observed in studies on glioma cell in which A-SMase is activated by CD95 stimulation (Perrotta C, Clementi E, unpublished observations). Of importance, inhibition of activity of A-SMase and N-SMase and protection from apoptosis by NO appear to be mediated through activation of soluble guanylyl cyclase, cGMP generation, and activation of cGMP-dependent protein kinase G (3, 14, 26, 75).

The findings outlined above open an important question about the mechanism of SMases inhibition. A- and N-SMases are quite different in terms of their intracellular distribution, localization-activity relationship, and regulation by cellular messengers. Thus it is unlikely that NO operates to control them through the same mechanism. Although no studies have so far addressed this question specifically, we have preliminary evidence that regulation of activity of A-SMase is mediated through regulation of intracellular localization (Perrotta C, Clementi E, unpublished observations); no information is available about the mechanism of N-SMase inhibition by NO.

The exposure to high levels of NO, such as those generated by inducible NOS II in inflammation, have effects opposite to those of physiological NO concentrations. Indeed, it has been demonstrated in different cell lines that high concentrations of NO increase the generation of ceramide through the activation of both A- and N-SMase, leading to death via apoptosis (16, 49, 78, 89) (FIGURE 1). The mechanisms involved in SMases activation by NO have not been investigated; however, they are clearly distinct from those involved in SMase inhibition, since the former are independent of cGMP and require a caspase-3-dependent step (16, 49, 89), possibly involving generation of arachidonic acid-derived eicosanoids (77).

**N-SMase-Generated Ceramide Induces Activation of NOS III**

In the case of N-SMase, the interplay between NO and the SMase/ceramide pathway is bidirectional, since NOS III activation may be stimulated in signaling pathways generated following N-SMase activation. The functional coupling between NOS III, N-SMase, and SMase-activating receptors might be facilitated by their common subcellular localization at PM rafts (35, 37, 50, 53). How N-SMase activates NOS III has been studied in placental and microvascular endothelial cells (23) and in HeLa...
cells (3, 14) stimulated by TNF-α. Such activation occurs through a complex pathway that includes sequential stimulation of N-SMase 2 and sphingosine kinase 1 (SK1). The product of SK1, sphingosine-1-phosphate (Sph1P), activates Akt (protein kinase B) through its PM receptors, S1P1 and S1P3. Akt, in turn, triggers phosphorylation of NOS III at Ser1179 (3, 22, 23). Phosphorylation of Ser1179 is a mode of activation of the enzyme shared by several signaling pathways, including shear stress (32). Also A-SMase-generated ceramide, when the enzyme is activated by bFGF, was demonstrated to activate NOS III, however, with a mechanism completely different from that involving N-SMase, since it is independent of PI3K/Akt activation (30). The different pathways of NOS III activation by TNF-α and bFGF may explain the differences in biological effects NO generated. A-SMase-dependent NO generation cooperates with the mitogenic effect of bFGF (30), whereas N-SMase/SK1/Sph1P-dependent activation of NOS III is an autocrine loop switched on by TNF-α to regulate its own effects in an inhibitory fashion. In cells in which TNF-α is proapoptotic, NOS III activation reduces apoptosis (14, 22). In contexts in which the proinflammatory effects of TNF-α prevail, such as when TNF-α induces expression of proinflammatory adhesion molecules by endothelial cells, NOS III acts as an anti-inflammatory agent, inhibiting the expression of adhesion molecules and thus reducing adhesion of dendritic cells to the endothelium (23). NO can also act as a pro-inflammatory agent by upregulating phospholipase A2 and prostaglandin formation in various cell types. This occurs especially when NO is persistently generated at high concentrations by NOS II (68). How the anti-inflammatory effects of low NO concentrations generated by constitutive NOS III integrate with the pro-inflammatory effects of high NO concentrations remains to be investigated.

Overview of the Interactions Between the NO and SMases Signaling Pathways

Many different stimuli, including agonists of receptors of the TNF-α superfamily, such as TNF-α, LPS, CD95 ligand, chemotherapeutic drugs, and radiation, lead to early transient activation of A- and/or N-SMases. In the case of A-SMase, it has been found that this process requires intracellular trafficking of the enzyme from intracellular compartments (lysosomes and possibly other as yet unidentified organelles) to the outer leaflet PM in a process that appears to involve secretory pathways (for a review, see Ref. 37). This translocation triggers ceramide generation and further ceramide-dependent clustering of A-SMase-activating receptors in a feed-forward loop. This mode of activation of A-SMase opens the question as to how cGMP generated by NO since an intracellular messenger can inhibit A-SMase. NO is known to regulate vesicle trafficking and secretion, and it may inhibit A-SMase in a cGMP-dependent manner in several cell types (9, 15, 93). We have preliminary evidence that cGMP inhibits A-SMase activity by inhibiting its translocation to the PM, driven by some as yet undefined type of secretory vesicles. The mechanism of activation of N-SMases, which resides on the inner leaflet of the PM and possibly of multivesicular bodies (91), needs the involvement of intermediate protein, such as FAN (88); it is therefore conceivable that cGMP, through activation of its downstream effector protein kinase G, inhibits the interaction of N-SMase with this interactor. This hypothesis is supported by data showing that N-SMase is regulated by PKC, indicating that phosphorylation is a mechanism of inhibition of N-SMase activity (6).

Ceramide generated by A-SMase or N-SMase appears to influence cell fate in different ways. A-SMase-generated ceramide is in general involved in the induction and progress of apoptosis, whereas N-SMase-generated ceramide is more often not apoptogenic. In the context of NO signaling, N-SMase-generated ceramide is metabolized to sphingosine to become Sph1P that stimulates NOS III to generate NO; NO thus generated inhibits activity and/or activation of both SMases (FIGURE 1). This loop might thus regulate apoptosis in an inhibitory fashion, in concert with Sph1P, derived from N-SMase-generated ceramide, which is per se important in regulating cell growth, differentiation, and response to inflammatory cues (23, 50, 57, 69, 79). Whether the stimulation of NOS III by receptors of the TNF-α superfamily persists or whether this signaling also leads to inflammation and expression of NO II, the high NO concentrations generated would turn the SMase inhibitory loop described above into a stimulatory one (FIGURE 1). The high cellular levels of NO and ceramide reached under these conditions could then act on a variety of apoptogenic signaling events (13, 40) and ultimately synergise to stimulate apoptosis.

Conclusions

The cross talk among the NO/NOS and ceramide/SMases signaling pathways with its multiple feedback controls shares properties common to most modulatory systems of cellular signaling, e.g., the control of calcium homeostasis, and extends its relevance to many complex pathophysiological cellular processes. In such complex settings, interactions with other signaling cascade may be pos-
sible. A thorough characterization of the molecular mechanisms at the basis of the cross talk among the NO/NOS and ceramide/SMase signaling pathways, which is still lacking, should provide missing links and lead to a better understanding of the networks involving NO and ceramide in the more general context of cellular signaling and elucidate in further detail how processes as relevant as apoptosis, differentiation, and inflammation are controlled.

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