Chapter 18
Neutral Sphingomyelinase 2: Structure, Function, and Regulation with Emphasis on Nitric Oxide Involvement and Potential Implications for Cancer Therapy

Bei Lei Sun and Bulent Mutus

Abstract Mammalian neutral sphingomyelinase 2 is encoded by the gene *smpd3* and belongs to the family of hydrolases which catalyze the breakdown of sphingomyelin to form ceramide and phosphocholine. The bioactive ceramide can then act as the second messenger molecule capable of mediating an array of cellular events, such as growth arrest and apoptosis. Recent studies have revealed that the expression and activity of neutral sphingomyelinase 2 are selectively regulated and this regulation can take place at the transcriptional level as well as at the posttranslational level. Upon exposure to oxidative stress, endoplasmic reticulum stress, tumour necrosis factor alpha stimulation or anti-cancer drugs, altered neutral sphingomyelinase 2 activity directly translates into changes in ceramide levels which help cells mount an appropriate response. On the other hand, inappropriate activation or inhibition of neutral sphingomyelinase 2 could contribute to the development of pathological conditions such as cancer and endothelial dysfunction. In this chapter, we focus on current knowledge regarding neutral sphingomyelinase 2 structure, the regulation of its activity, its function and potential involvement in stress response and cancer genesis.

Keywords Cancer · Ceramide · Neutral sphingomyelinase 2 · Nitric oxide · Post-translational modifications · Stress response

Abbreviations

APL	Anionic phospholipid
ATRA	All-trans retinoic acid
BAEC	Bovine aortic endothelial cells
EED	Embryonic ectodermal development
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase

B. Mutus (✉) · B. L. Sun
Department of Chemistry & Biochemistry, University of Windsor, 273-1 Essex Hall, 401 Sunset Ave., Windsor, ON N9B 3P4, Canada
e-mail: mutusb@uwindsor.ca

nNOS   Neuronal nitric oxide synthase
ER   Endoplasmic reticulum
HAEC   Human airway epithelial cells
HEK   Human embryonic kidney
HSP   Heat shock protein
MAPK   Mitogen activated protein kinase
PKC   Protein kinase C
ROS   Reactive oxygen species
SM   Sphingomyelin
SMase   Sphingomyelinase
TNF   Tumour necrosis factor

Introduction

Sphingolipids are a major component of the plasma membrane in eukaryotic cells. This class of lipids typically consists of a sphingosine backbone, a long chain fatty acid molecule, and a variable polar head group. Originally considered to serve only structural roles, these lipids are now recognized as important players in a wide range of signal transduction pathways [1–3]. In particular, sphingomyelin (SM)-based pathways have received considerable attention in recent years.

The SM molecule has a polar phosphorylcholine head group and the composition of the long chain fatty acid varies from tissue to tissue and can be either saturated or mono-unsaturated with 14 to 24 carbons [4–5]. Results based on cell fractionation studies and degradation experiments suggest that more than half of the cellular SM mass is confined to the plasma membrane [6]. The exact percentage may vary from one cell type to another, though it has been reported that cells with extensive plasma membrane recycling have a larger fraction of SM in the intracellular compartments [7].

The hydrolysis of SM yields ceramide, which is an important second messenger molecule capable of modulating a variety of cellular events, such as cell cycle arrest, differentiation, inflammation and apoptosis [8–10]. SM hydrolysis is specifically catalyzed by a group of enzymes known as sphingomyelinase (EC.3.1.4.12). SMase can be further classified into three groups (acid, alkaline and neutral) based on their distinct pH optimum. Acid SMase is responsible for the catabolism of SM within the lysosomes and a deficiency of this enzyme leads to the human Niemann-Pick disease [11, 12]. In recent years, acid SMase has also been reported to be an important player in stress-induced ceramide generation and subsequent signaling pathways [13–16]. For more detailed information on acid SMase, we recommend the reviews by Smith and Schuchman [17]; and Zeidan and Hannun [18]. On the other hand, alkaline SMase is found in the intestinal tract, bile and liver, and it plays a crucial role in SM digestion [19, 20]. Recent findings by Zhang et al. also point toward the potential involvement of alkaline SMase in regulating mucosal growth as well as the function of alkaline phosphatase [21].

   Neutral magnesium-dependent SMase activity was first described in 1967 by
Scheider and Kennedy [12]; since then, several mammalian forms have been iden-
tified and characterized. Neutral SMase1 was identified and cloned based on re-
mote sequence similarity to known bacterial sphingomyelinases in 1998 [22]. A
year later, results from overexpression and radiolabeling experiments suggested
that this 423 amino acid integral membrane protein acts as lyso-platelet activating
factor phospholipase C rather than sphingomyelinase in cells [23]. Additional stud-
ies are needed to further determine the physiological roles of neutral SMase1. More
recently, neutral SMase3, a C-tail anchored protein, was identified using peptide
sequence from purified bovine SMases [24]. A study by Cororan et al. suggested
this 97 kDa protein may be linked to tumorigenesis and cellular stress response
[25]. Neutral SMase2 is the most studied member of the neutral SMase family and
has been implicated in a number of pathological conditions. This short chapter will
review current knowledge regarding the structure and function of neutral SMase2,
as well as regulation of its expression and activity.

Neutral SMase2 Structure and Subcellular Localization

In 2000, Hofmann and colleagues identified the mammalian neutral SMase2 based
on remote similarity to bacterial sphingomyelinases using a bioinformatics based
gene discovery approach coupled with phylogenetic analysis [26]. This membrane
protein consists of 655 amino acid residues with an overall predicted molecular
weight of 71 kDa. Neutral SMase2 was reported to be magnesium dependent and
can be activated by unsaturated fatty acids as well as anionic phospholipids, such
as phosphatidylserine [26, 27]. Unlike neutral SMase1, neutral SMase2 exhibits
SMase activity both *in vitro* and *in vivo* with overexpression of this enzyme result-
ing in accelerated SM catabolism and an increase in ceramide levels [27]. The pro-
posed domain structure of neutral SMase2 (Fig. 18.1) consists of two hydrophobic
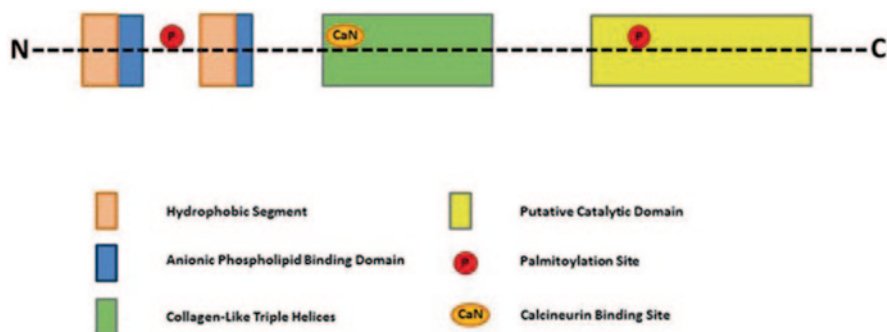segments near the N-terminus, followed by a 200-residue collagen-like triple heli-

Fig. 18.1 Schematic representation of human neutral SMase2 (GenBank accession number
Q9NY59.1). Structural features and putative domains are indicated

ces and a catalytic domain near the C-terminus [26]. Although the two hydrophobic segments were initially proposed to be transmembrane domains, subsequent analysis of neutral SMase2 membrane topology by Tani and Hannun suggested that these segments do not actually span the entire membrane [28]. Two discrete anionic phospholipid (APL) binding domains were identified near the N-terminus which allow neutral SMase2 to interact specifically with certain APLs including phosphatidylserine and phosphatidic acid [29]. The two APL binding domains partially overlay with the two hydrophobic segments and mutagenesis studies revealed that Arg-33, Arg-45 and Arg-48 are essential for interaction with APL in the first domain while Arg-92 and Arg-93 are critical for the second domain [29]. Neutral SMase2 can also be palmitoylated in two cysteine clusters via thioester bonds [30]. Site directed mutagenesis of cysteine to alanine uncovered that this palmitoylation is important for protein stability, as well as its localization with palmitoylation deficient mutants showing rapid degradation and reduced membrane association [30].

The subcellular localization of neutral SMase2 has been reported mainly in two organelles. Hofmann et al. observed localization predominantly at the Golgi in several cell lines derived from the brain [26]. In contrast, Marchesini et al. reported localization at the plasma membrane in confluence arrested MCF7 cells a few years later [31]. Subsequent studies suggest neutral SMase2 is transported between the Golgi and plasma membrane and this intracellular trafficking may be important for its catalytic regulation [32, 33].

Neutral SMase2 Function

During recent years, neutral SMase2 has emerged as an important mediator of cellular stress response, mainly through the production of ceramide. In human airway epithelial cells (HAEC), exposure to oxidative stress (H_2O_2, cigarette smoke) selectively induces the activation of neutral SMase2; and the resultant increase in cellular ceramide leads to HAEC apoptosis and lung injury. This response to oxidative stress is lost upon siRNA silencing of neutral SMase2 [33, 34]. The expression level of neutral SMase2 is also significantly higher in lung tissues obtained from patients with pulmonary emphysema (smokers) as compared to normal control subjects [35]. Together, these studies suggest that neutral SMase2 plays a critical role in ceramide generation following oxidative stress both *in vitro* and *in vivo*. Oxidant exposure has been shown to affect the subcellular localization of neutral SMase2, such that preferential trafficking to the plasma membrane is observed under conditions of oxidative stress; and exposure to the antioxidant glutathione (GSH) leads to the trafficking of neutral SMase2 to the nucleus, where both ceramide generation and apoptosis appear to be attenuated [33]. Clement et al. demonstrated that certain types of neuronal cells could adapt to chronic oxidative stress by down-regulating neutral SMase activity [36]. These cells exhibit increased intracellular cholesterol levels and are resistant to apoptosis. Extracellular treatment of the stress resistant cells with neutral SMase reverses the stress-resistant phenotype; while treatment

of oxidative stress sensitive neuronal cells with neutral SMase2 inhibitors elevated cellular cholesterol and made the cells more resistant to oxidative stress [36].

Endoplasmic reticulum (ER) stress has been shown to inhibit the activity of neutral SMase2 in bovine aortic endothelial cells (BAEC) [37]. When treated with the ER stressor palmitate or tunicamycin, reduced neutral SMase2 activity in BAEC leads to less ceramide generation, which results in a decrease in NO production as endothelial nitric oxide synthase (eNOS) activation is ceramide-dependent [38]. Similarly, siRNA mediated knock-down of neutral SMase2 also results in a decrease in NO generation [37]. This reduced bioavailability of NO promotes the dominance of vasoconstriction over vasodilation. In this way, decreased neutral SMase2 activity could be a contributing factor in the induction of endothelial dysfunction. Neutral SMase2 is involved in the activation of inducible nitric oxide synthase (iNOS). In C6 rat glioma cells, inhibition of neutral SMase2 prevents the induction of iNOS by lipopolysaccharide, whereas inhibition of acid SMase or ceramide *de novo* synthesis had no effect, suggesting that the ceramide produced by neutral SMase2 is critical in the regulation of iNOS expression [39]. Similarly, treatment with GW4869, a specific neutral SMase inhibitor, decreases iNOS expression in cultured human retinal pigment epithelial cells and protects these cells from ER stress-induced apoptosis [40].

Neutral SMase2 also participates in the regulation of cell growth and cancer genesis. Marchesini and colleagues demonstrated that neutral SMase2 is involved in confluence-induced growth arrest in MCF7 cells [31]. Specifically, endogenous neutral SMase2 mRNA is up-regulated when cells become confluent and this up-regulation is associated with G_0/G_1 cell cycle arrest as well as an increase in the level of ceramide. While neutral SMase2 is distributed throughout the cells in sub-confluent, proliferating cultures, its localization is limited to the plasma membrane in growth arrested cultures at confluence [31]. Nucleotide sequencing in a panel of human cancers revealed mutations in the *smpd3* gene, which encodes neutral SMase2, were present in a subset of human leukemia [41], suggesting that neutral SMase2 may have a functional role in cancer initiation or progression.

A number of anti-cancer drugs can act through neutral SMase2. Ito et al. showed that treatment with daunorubicin increases neutral SMase2 mRNA and protein levels in MCF7 cells, placing neutral SMase2 in an important role in daunorubicin-induced cell death [42]. In oligodendrocytes, neutral SMase2 over-expression leads to increased ceramide generation and enhances apoptosis induced by staurosporine or C(2) ceramide [43]. Furthermore, the Sonic-hedgehog inhibitor cyclopamine has been shown to induce apoptosis in Daoy human medulloblastoma cells by selectively activating neutral SMase2 in a nNOS/NO-dependent fashion. siRNA knock-down of neutral SMase2 protected these cells from drug induced apoptosis [44]. Protopanaxadiol, from the root extract of Panax ginseng, can exert cytotoxicity against 5 different cancer cell lines through neutral SMase2 activation and disruption of membrane lipid rafts [45]. Although much more work is needed to deduce all relevant mechanisms, neutral SMase2 could potentially be used to improve the efficiency and selectivity of chemotherapeutic treatments.

Regulation of Neutral SMase2

Neutral SMase2 is a redox sensitive enzyme and the antioxidant GSH inhibits its upregulation [33, 46]. Pre-treatment of MCF7 cells with GSH has been shown to prevent diamide-induced neutral SMase activation [47]. Similarly, treatment with GSH can also protect HAEC from oxidative stress-induced ceramide generation and apoptosis [33, 34]. Further experiments are needed to deduce the specific mechanisms of this inhibition.

All-trans retinoic acid causes G_0/G_1 growth arrest in many cell types. Using MCF7 cells as a model system, Clarke et al. showed that this growth arrest was mediated by an increase in neutral SMase2 activity [48]. This increase in activity was later found to be mostly due to enhanced transcription [49]. Promoter analysis revealed the importance of the 5' promoter region of neutral SMase2 which contains 3 Sp1 sites. Mechanistically, Ito et al. suggested that ATRA treatment activates PKCδ which then phosphorylates Sp1. The phosphorylated Sp1 transcription factor binds to the neutral SMase2 promoter, resulting in increased level of transcription [49].

HSP60 has been shown to interact with neutral SMase2 using proximity ligation assay and immunoprecipitation. Treatment with HSP60 siRNA leads to an increase in neutral SMase2 protein levels in neutral SMase2 overexpressing HEK293 cells, suggesting that HSP60 could be a negative regulator of neutral SMase2 [50].

Long-term as well as acute stimulation with the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) can activate neutral SMase2 in a number of cell lines including MCF7, A549, HUVEC and smooth muscle cells [27, 38, 51, 52]. In A549 cells, exposure to TNF-α results in the translocation of neutral SMase2 to the plasma membrane in a time- and dose-dependent manner [51]. Interestingly, both the activation and translocation of neutral SMase2 following TNF-α stimulation appear to be dependent upon p38 MAPK [51]. These results suggest that neutral SMase2 activity could be modulated by intracellular trafficking and the major site of action is likely at the plasma membrane. In 2012, Barth and colleagues have also shown that TNF-α activates neutral SMase2 in both neurons and non-neuron cells, causing ceramide accumulation, ROS formation and apoptosis [53]. The polycomb group protein EED has been identified as an interaction partner for neutral SMase2 and physically couples neutral SMase2 to the [RACK1-FAN-TNF receptor] complex allowing the transduction of signals initiated by TNF [54].

Recently, Filosto and colleagues reported that neutral SMase2 is a phosphoprotein with phosphorylation occurring exclusively at serine residues [55]. This phosphorylation event has been suggested to occur downstream of p38 MAPK and PKC [55]. In human airway epithelial cells, exposure to oxidative stress enhances the phosphorylation of neutral SMase2, which leads to increased activity [55, 56]. In addition, the phosphatase calcineurin has been reported to bind directly to neutral SMase2, and when the binding site was mutated away, neutral SMase2 would exhibit constitutively elevated phosphorylation and activity [55]. A subsequent publication by the same research group identified five serine residues which were phosphorylated. Three of those residues (Ser-289, Ser-292 and Ser-299) are positioned

near the catalytic domain, while the other two (Ser-173 and Ser-208) are next to the calcineurin binding site [56]. Overall, the phosphorylation of these five serine residues plays a critical role in neutral SMase2 activation under oxidative stress. In addition, neutral SMase2 protein stability could also be regulated post-translationally by phosphorylation; specifically, the phosphorylation of Ser-208 leads to increased protein stability [56].

Another reported post-translational modification of neutral SMase2 is palmitoylation. Two palmitoylated Cys. clusters were identified by Tani and Hannun based on site directed mutagenesis [30]. One of those two clusters is located between the hydrophobic segments, while the other one is found within the catalytic domain. This modification is important for the plasma membrane localization of neutral SMase2 as well as its stability with the palmitoylation deficient mutants being directed to lysosomes and rapidly degraded [30].

Unpublished work in the Mutus lab points toward the possibility that protein S-nitrosylation could be an additional type of post-translational modification capable of down-regulating the activity of neutral SMase2. With reduced activity, the corresponding decrease in ceramide generation can lead to evasion of apoptosis by cancer cells in order to exhibit continued survival and proliferation following exposure to stressors such as oxidative stress and chemotherapeutic drugs. Investigation is currently ongoing to determine whether inappropriate S-nitrosylation of neutral SMase2 could confer a survival advantage to cancer cells.

Conclusion

Since its identification and cloning in 2000, neutral SMase2 has emerged as an important regulator of ceramide generation and sphingolipid signaling. Upon exposure to oxidative stress, anti-cancer drugs or TNF-α stimulation, the activity of neutral SMase2 is selectively up-regulated, resulting in elevated levels of cellular ceramide, which then activate pathways leading to programmed cell death. This increase in neutral SMase2 activity could be attributed to an up-regulation of transcription and/ or post-translational modifications. The subcellular localization of neutral SMase2 also affects its activity. Certain types of stimuli, such as hydrogen peroxide and TNF-α, cause the preferential trafficking of neutral SMase2 to the plasma membrane, where there is an enrichment of the substrate SM and the neutral SMase2 activating lipid phosphatidylserine [57]. Determination of trafficking mechanisms will allow us to gain a deeper understanding of neutral SMase2 physiology. In addition to stress response, neutral SMase2 is also implicated in cell growth and cancer genesis. Elucidation of relevant pathways will determine if neutral SMase2 could be used as a therapeutic target for cancer treatments.

Conflict of interest No potential conflicts of interest are disclosed.

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