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Roles and regulation of neutral sphingomyelinase-2 in cellular and pathological processes

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A B S T R A C T

Our understanding of the functions of ceramide signaling has advanced tremendously over the past decade. In this review, we focus on the roles and regulation of neutral sphingomyelinase 2 (nSMase2), an enzyme that generates the bioactive lipid ceramide through the hydrolysis of the membrane lipid sphingomyelin. A large body of work has now implicated nSMase2 in a diverse set of cellular functions, physiological processes, and disease pathologies. We discuss different aspects of this enzyme's regulation from transcriptional, post-translational, and biochemical. Furthermore, we highlight nSMase2 involvement in cellular processes including inflammatory signaling, exosome generation, cell growth, and apoptosis, which in turn play important roles in pathologies such as cancer metastasis, Alzheimer's disease, and other organ systems disorders. Lastly, we examine avenues where targeted nSMase2-inhibition may be clinically beneficial in disease scenarios.

Introduction

Sphingomyelin hydrolysis is catalyzed by a class of enzymes referred to as sphingomyelinases (SMases) to generate ceramide, a bioactive lipid involved in diverse cellular processes (Hannun and

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Obeid, 2011; Ogretmen and Hannun, 2004). SMases are classified based on their pH optima of activity into acid, neutral, and alkaline subtypes. Of the four different mammalian neutral SMases that have been identified; neutral sphingomyelinase-2 (nSMase2) appears to be the predominant nSMase in cellular systems, physiologies, and pathologies (Airola and Hannun, 2013; Clarke et al., 2011b; Goni and Alonso, 2002; Wu et al., 2010). This review will focus on the roles and regulation of this enzyme emphasizing recent findings implicating nSMase2 in disease processes.

Characterization and regulation of nSMase2

Cloning

Cloning of nSMase2 revealed it as part of a protein superfamily that hydrolyzes phosphodiester linkages and requiring Mg^{2+} for activity. Both the human and mouse nSMase2 gene (SMPD3) encode for a 655 amino acid protein with a molecular mass of 71 kDa that contains an N-terminus with 2 hydrophobic segments and a C-terminus consisting of the catalytic site (Hofmann et al., 2000). The mouse and human versions are very similar and share 90% sequence identity.

Basic biochemical properties

NSMase2 specifically hydrolyzes the phosphocholine-headgroup from sphingomyelin and does not exhibit Phospholipase C-type activity against phosphatidylcholine, lyso-phosphatidylcholine, platelet activating factor or lyso-platelet activating factor. Neutral pH and divalent cations (Mg^{2+} or Mn^{2+}) are required for activity, while phosphatidylserine (PS) and unsaturated fatty acids stimulate enzymatic activity in vitro (Hofmann et al., 2000). A later study found that sphingosylphosphocholine, the deacylated form of SM, can be hydrolyzed under detergent-free conditions by nSMase2 (Miura et al., 2004).

Structural features

To date, there has not been a crystal structure reported for nSMase2. Therefore, our current understanding of the structure and mechanism of nSMase2 is based on investigations of related bacterial SMases. Three different structures of bacterial neutral SMases from the pathogenic organisms *Bacillus cereus*, *Listeria ivanovii*, and *Staphylococcus aureus* has confirmed neutral SMases belong to the DNase I type protein superfamily, which also includes inositol phosphatases (Ago et al., 2006; Huseby et al., 2007; Matsuo et al., 1996; Openshaw et al., 2005). NSMase2 shares relatively low sequence identity with the bacterial versions but most likely shares a similar protein fold and catalytic mechanism for SM hydrolysis (Ago et al., 2006; Openshaw et al., 2005). Studies by Tani et al. showed that the enzyme harbors two hydrophobic loops at the amino terminus rather than the sequence-predicted transmembrane segments (Tani and Hannun, 2007a).

Cellular localization

After initially cloning nSMase2, Hoffman et al. showed by antibody staining that nSMase2 localized to the Golgi apparatus in both PC12 and SH-SY5Y cells (Hofmann et al., 2000). Subsequently, overexpressed nSMase2 was shown to localize to the plasma membrane (PM) in the confluence phase of MCF7 cells and in primary hepatocytes (Karakashian et al., 2004; Marchesini et al., 2004). Building on these findings, Tani et al. found that nSMase2 localized to the inner leaflet of the PM in confluent MCF7 cells and was palmitoylated at two Cysteine clusters (Tani and Hannun, 2007a,b). The first cluster encompasses Cys53, Cys54 and Cys59 that are present between the 2 hydrophobic segments of the N-terminus, and the second cluster encompasses Cys395 and Cys396, which are present at the beginning of the catalytic site (Tani and Hannun, 2007b). Studies with cycloheximide in subconfluent MCF7 cells demonstrated that the Golgi localization of overexpressed subconfluent nSMase2 is the result of two protein pools: newly synthesized protein and a second that recycles back from the PM through the endosomal system (Milhas et al., 2010).

Regulation by anionic phospholipids

Prior to the cloning of the different isoforms of neutral SMases, anionic phospholipids (APLs) were found to increase neutral sphingomyelinase activity in rat hepatomas. Delipidated membranes treated with Triton X-100 recovered NSMase activity only upon addition of PS, phosphatidic acid, phosphatidylinositol but not the neutral lipids phosphatidylcholine or phosphatidylethanolamine (Tamiya-Koizumi and Kojima, 1986). These findings were later confirmed in a purified membrane bound neutral sphingomyelinase from rat brain (Liu et al., 1998b). Following the cloning of nSMase2, biochemical characterization showed that the enzyme has a catalytic pH optimum of 7.5, with a strong stimulation by PS or cardiolipin and a requirement for either Mg^{2+} or Mn^{2+} for activity (Hofmann et al., 2000; Marchesini et al., 2003). The activity of the previously cloned neutral SMase1 was shown to be APL-independent (Tomiuk et al., 2000), thus identifying nSMase2 as the previously characterized magnesium dependent neutral SMase that was activated by APLs. A detailed mechanistic study found that APLs bind to the N-terminus of nSMase2 at two distinct positively charged sites. The first site binds both PS and phosphatidic acid and required R33 and the amino acids 45–48 (KRQR). The second site selectively bound PS and required R92 and R93 (Wu et al., 2011). Biochemically, APL binding was found to affect both the substrate affinity (K_m) and rate of hydrolysis (V_{max}) of nSMase2. Mutation of the APL binding sites altered the localization to the endoplasmic reticulum (ER). In yeast strains lacking the nSMase2 homolog Isc1, the wild type protein corrected sensitivity to hydroxyurea, while the mutant protein was unable to do so (Wu et al., 2011) (Fig. 1).

Regulation by phosphorylation

NSMase2 was originally identified as a phosphoprotein in human bronchial epithelial and A549 cells with phosphorylation occurring basally on serine residues (Filosto et al., 2010). The serine/threonine phosphatase Calcineurin (also known also as protein phosphatase 2B) was found to bind a PQIKIY motif between the N-terminus and the C-terminus to dephosphorylate nSMase2 (Filosto et al., 2010). The phosphorylation of nSMase2 under H_2O_2 stimulation in A549 cells occurs on 5 conserved serine residues: S173, S209, S291, S294 and S301, which are located near the calcineurin binding site. Alanine point mutants lacking the phosphorylation sites are not phosphorylated by H_2O_2 and are not activated in response to oxidative stress. In addition protein phosphorylation at these sites regulated the stability of nSMase2 in this cellular model (Filosto et al., 2012). In addition, some of nSMase2 functional effects have been found to be dependent on protein kinases. For instance, p38 and PKCδ regulate the translocation of nSMase2 to the plasma membrane in response to TNF (Clarke et al., 2007). Furthermore PKCδ role in this translocation is independent of effects on the activation of nSMase2 in response to TNF (Clarke et al., 2008). However, it is not known if these kinases phosphorylate nSMase2 directly or affect nSMase2 function through signaling pathways.

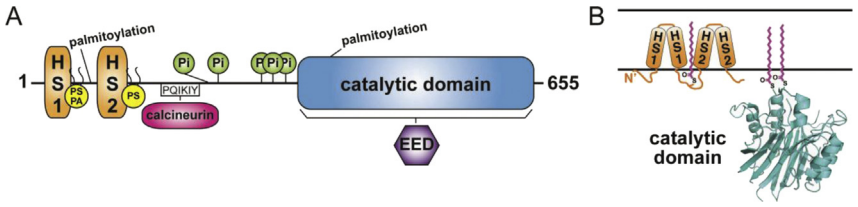

Fig. 1. Domain architecture and membrane topology of nSMase2. (A) Domain architecture of nSMase2. Catalytic domain, blue; Hydrophobic segments 1 (HS1) and 2 (HS2), orange; Anionic phospholipid binding sites, yellow lipids; Phosphorylation sites (P_i), green; Calcineurin binding motif, magenta; EED binding region, purple; and palmitoylation sites are indicated. (B) Membrane topology of nSMase2 with palmitoylation sites, magenta. HS1 and HS2 associate with, but do not transverse, the membrane. A representative structure of the nSMase2 catalytic domain is shown from *B. cereus* sphingomyelinase (PDB ID 2DDT). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Transcriptional regulation of nSMase2

Recent insights into stimuli activating nSMase2 shed light on some aspects of its transcriptional regulation. The chemotherapeutics Daunorubicin and Camptothecin were found to induce nSMase2 transcription in MCF-7 breast cancer cells and K562 leukemia cells via activation of the putative promoter by the transcription factors Sp1 and Sp3. The region of the promoter necessary for this activation was mapped to −147 bp upstream of exon 1 (Ito et al., 2009). All-trans retinoic acid (ATRA) was also found to induce transcriptional activation of nSMase2 via the same mechanism (Ito et al., 2012).

Tools for the study of nSMase2: inhibitors and mouse models

Initial studies of NSMase activity relied heavily on the natural occurring inhibitors Scyphostatin and Manumycin A that were originally identified from fungi and bacteria respectively (Arenz et al., 2001; Nara et al., 1999a; Zeeck et al., 1987). These inhibitors had a broad NSMase inhibitory activity with no selectivity towards different NSMase isoforms. In addition, Scyphostatin had inhibited acid SMase activity but with a higher IC_{50} (Canals et al., 2011; Nara et al., 1999b). A high throughput screen for small molecule inhibitors of nSMase2 identified GW4869 as a selective nSMase2 inhibitor with an IC_{50} of 1 μM. GW4869 is a non-competitive nSMase2 inhibitor and its mechanism of action is thought to be through interference with APL activation of the enzyme (Canals et al., 2011; Luberto et al., 2002). Since its identification, GW4869 has been the most commonly employed pharmacological tool to study nSMase2 function in cellular and animal models.

Mice models to study nSMase2 function were characterized at the beginning of the 21st century. Stoffel et al. generated the first $SMPD3^{-/-}$ mouse and showed that it has decreased neutral SMase activity in many liver and brain while the $SMPD3^{-/-}$ $SMPD2^{-/-}$ double knock out mouse, which deletes both nSMase1 and nSMase2, abolished all neutral SMase activity (Stoffel et al., 2005). The fragilitas osseum (fro/fro) mouse model, which was originally identified in a chemical screen for bone defects, was shown by Aubin et al. to have a catalytically inactive nSMase2 due to deletion of the C-terminal 33 residues and has since been used as an additional model to study nSMase2 function. The original study reported the fro/fro mouse retained residual NSMase activity to almost 10% of the wild type (Aubin et al., 2005). More recent reports have found overexpression of the fro/fro mutation in a cellular model did not increase nSMase2 activity. This is more consistent with the deletion of two catalytically conserved residues, D638 and H639, in the fro/fro mutation (Clarke et al., 2011b; Wu et al., 2010) and it was speculated that residual nSMase activity in the fro/fro mouse is due to the presence of other nSMase isoforms.

NSMase2 as a regulator of biological functions

NSMase2 has been implicated in many physiological processes as well as disease pathologies. In this section, we will review the most important biological functions relating to nSMase2 and highlight potential avenues for future research.

Inflammation

Response to tumor necrosis factor-alpha (TNF-α)

Activation of neutral sphingomyelinase in response to the cytokine TNF-α was initially observed in the HL-60 cell line. In these leukemia cells, TNF-α induces a rapid SM hydrolysis, peaking at 1 h concomitant with an increase in ceramide and cellular differentiation into monocytes (Kim et al., 1991). The initial discovery was replicated in a cell-free system (Dressler et al., 1992) and it required the TNF-α receptor-1 (TNFR-1), commonly known as the p55 receptor (Adam et al., 1996; Adam-Klages et al., 1998; Belka et al., 1995; Yang et al., 1993). Mechanistically, a distinct region of the p55 receptor spanning amino acids 309–319 was found to mediate the increase in NSMase activity and was named the Neutral Sphingomyelinase Activation Domain (NSD) (Adam et al., 1996). The 9 amino acid stretch of the NSD binds a protein termed Factor Associated with Neutral sphingomyelinase (FAN) that influences

NSMase activity in response to TNF-α (Adam-Klages et al., 1996, 1998). FAN is a protein of the WD-40 family and contains 5 WD repeats, which are common scaffolding motifs involved in coordinating multi-protein complex assemblies. The N-terminus of FAN is critical for NSMase activity as a truncation of FAN, which contained only the WD repeats, inhibits NSMase activation following TNF-α in a dominant negative fashion (Adam-Klages et al., 1996, 1998). Following that, Receptor for Activated C-Kinase 1 (RACK1) was identified as a FAN interaction partner using a yeast two hybrid system. The interaction between FAN and RACK1 was verified both in vitro and in cells and is dependent on the C-terminal WD repeats of both proteins. RACK1 was also shown to modulate N-SMase activation following TNF-α treatment (Tcherkasowa et al., 2002). Subsequently, a yeast-two hybrid interaction mapping suggested the polycomb protein embryonic ectoderm development (EED) as a binding partner of nSMase2 via its C-terminus. Further investigation showed that EED also binds to RACK1 via the same region that interacts with nSMase2, and rapidly translocates from the nucleus to the PM after TNF-α treatment. The proposed model is one where EED interacts with RACK-1 and nSMase2 and brings them together in a multi-protein complex with TNFR1 through FAN, hence establishing EED as a critical modulator of nSMase2 activation following TNF-α (Philipp et al., 2010).

In addition to the elucidation of the molecular partners, other investigations have focused on cellular factors that influence TNF-α activation of nSMase2. Liu et al. reported that the levels of glutathione (GSH), which can inhibit neutral SMase activity, are critical for TNF-a activation. Their model suggests that baseline inhibition of nSMase2 activity is due to physiologic levels of GSH and TNF-a treatment activates nSMase2 by decreasing levels of GSH (Liu et al., 1998a). Further studies in A549 lung cells demonstrated that TNF-α induces a translocation of nSMase2 from the Golgi to the PM that was largely dependent on p38 MAPK (Clarke et al., 2007) and Protein Kinase C delta (PKC-δ) (Clarke et al., 2008). In vascular smooth muscle cells and fibroblasts, TNF-α activation of nSMase2 was shown to require the activation of the metallomatrix protease 2 (MMP2) from its inactive precursor form MT1-MMP2 by proteolysis mediated by Furin (Tellier et al., 2007).

Functionally, the activation of nSMase2 has implications in the inflammatory response of TNF-α. Studies in HeLa cells showed that nSMase2 acts on the PI3K/Akt pathway to activate endothelial nitric oxide synthase (eNOS) (Barsacchi et al., 2003). In HUVEC cells, this effect required the conversion of ceramide into sphingosine-1-phosphate (S1P), by the action of Sphingosine Kinase 1 (SK1) and eNOS activation regulated the expression of adhesion molecules like E-selection and VCAM-1 (De Palma et al., 2006). Clarke et al. expanded on this theme and demonstrated that in A549 cells, nSMase2 inhibition attenuated TNF-α stimulation of VCAM-1 and ICAM-1 (Clarke et al., 2007). In addition to the vascular effects of TNF-α, nSMase2 mediates some of the effects of TNF-α on the neural system. TNF-α stimulates nSMase2-dependent ceramide production in primary hippocampal neurons that modulates synaptic plasticity through trafficking of NR1 subunits of N-methyl-D-aspartate receptors to lipid rafts (Wheeler et al., 2009). Another report suggests that activation of nSMase2 in primary cortical neurons precedes reactive oxygen species (ROS) formation and damage to neurons (Barth et al., 2012). It is interesting to note that the TNF-α derived vascular effects seem to require the sequential action of both nSMase2 and SK1, while in contrast the neuronal effects of TNF-α is dependent only on nSMase2 activation. This suggests that nSMase2-derived ceramide can have bioactive action by itself in response to TNF-α or can be metabolized into other lipids to induce differential responses (Fig. 2).

Response to interleukin-1 beta (IL-1β)

The role of nSMase2 in inflammation has been studied extensively in response to another cytokine, IL-1β. IL-1β is a pro-inflammatory cytokine found to be secreted by inflammosomes, which is an early pathogenic feature of many liver and other organ systems diseases (Szabo and Csak, 2012). IL-1β was first reported to induce NSMase activity in rat hepatocytes (Nikolova-Karakashian et al., 1997). Subsequent studies in murine cortices demonstrated that this activation requires the IL1-Receptor 1 (IL1-R1) based on the observation that IL1-R1 KO mice failed to increase NSMase activity after IL-1β stimulation (Nalivaeva et al., 2000). The major isoform activated by IL-1β was demonstrated to be nSMase2 as overexpression of nSMase2 in IL-1β treated HepG2 cells enhanced phosphorylation of Janus Kinase (JNK) acutely (Karakashian et al., 2004). In the same system, nSMase2 overexpression suppressed phosphorylation and increased the stability of the Interleukin-1 receptor-associated kinase (IRAK-1) through activation of the protein phosphatase 2A (PP2A) family (Karakashian et al., 2004).

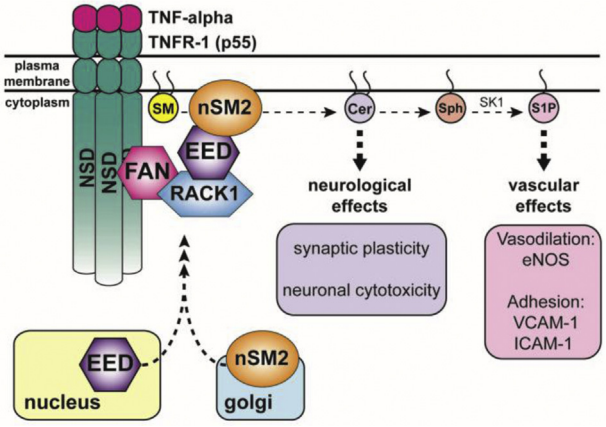

Fig. 2. nSMase2 is a mediator of TNF-α signaling. TNF-α induces nSMase2 activation and ceramide generation. Recruitment of nSMase2 to the plasma membrane occurs through formation of a complex between five proteins: nSMase2, EED, RACK1, FAN, and TNFR-1.

Further studies identified the phosphatase isoform involved as PP2Ac and showed that nSMase-2-dependent dephosphorylation of IRAK-1 by PP2Ac delayed IRAK-1 ubiquitination, which led to its stabilization and amplification of the IL-1β response by nSMase2 (Dobierzewska et al., 2011).

Functionally, the role of nSMase2 in the IL-1β response was elucidated through aging models. Aged hepatocytes display increased sensitivity to IL-1β stimulation that is translated via higher JNK phosphorylation as well as higher IRAK-1 stability (Rutkute et al., 2007a). This is due to an increase in nSMase2 activity in aged hepatocytes that is dependent on decreased levels of GSH (Rutkute et al., 2007a). Attenuation of nSMase2 levels by siRNA or by shRNA decreased IRAK-1 stability and JNK phosphorylation to restore a normal response to IL-1β in aged hepatocytes (Rutkute et al., 2007b). Moreover in normal rat hepatocytes and in HEK293 cells, nSMase2 was shown to stimulate post-transcriptional FOXO1, accumulation in the nucleus through its JNK/ERK activation in response to IL-1β (Dobierzewska et al., 2012). In glial cells, nSMase2 was shown to be important in IL-1β dependent Src activation that mediates IL-6 secretion (Tsakiri et al., 2008). Taken together, these results place nSMase2 as an attractive drug target in inflammatory diseases of the liver and other organ systems.

Response to interferon gamma (IFN-γ)

IFN-γ is another cytokine that was studied in the induction of nSMase2 activity. IFN-γ was shown to initially increase NSMase activity with acute stimulations in HL-60 cells (Kim et al., 1991). Subsequently, nSMase2 was demonstrated to be the major NSMase activated in response to IFN-γ and induced translocation of PKC-δ to the Golgi in HeLa cells in a JAK1/2 dependent manner (Kajimoto et al., 2001). In RAW264.7 macrophages IFN-γ activates an nSMase2 → PP2A → Akt/GSK → Nitric oxide pathway (Tsai et al., 2009); while in rat vascular smooth muscle cells, NSMase inhibition by 3-O-methyl sphingomyelin prevented IFN-γ-mediated nitric oxide production. This is due to the inhibition of translocation of p65 subunit of NF-k κB to the nucleus and hence failure of the NF-κB signaling cascade to activate iNOS at the mRNA level (Hsieh et al., 2011).

Involvement in phagocytosis

NSMase2 has a described role in phagocytes, which are typical cells of acute inflammation that engulf bacterial cells or other small cells. Polymorphonuclear cells, of which the phagocytic neutrophils constitute the major portion, display a plasma membrane NSMase activity that increases during phagocytosis (Hinkovska-Galcheva et al., 1998). Inhibition of nSMase2 by GW4869 causes loss of directional motility and migration in these cells (Sitrin et al., 2011). Finally, peptidoglycan, a major component of bacterial cell wall, was shown to induce nSMase2 activity in human macrophages that

leads to the activation of p38 MAPK and NF-κB to increase cyclooxygenase-2 expression (Chen et al., 2009).

Involvement in pulmonary pathophysiology

Recent findings have demonstrated a functional regulation of nSMase2 in response to lung insults and in certain lung diseases. Initially, Castillo et al. reported that H_2O_2 stimulation and ROS formation activates nSMase2 to mediate apoptosis in human airway epithelium, while glutathione (GSH) pretreatment prevents apoptosis (Castillo et al., 2007). Building on this in a pathophysiological context, it was found that cigarette smoking, a potent ROS-generating lung insult, increased NSMase activity acutely in immortalized human airway epithelium leading to apoptosis (Levy et al., 2009). GSH pretreatment or siRNA to nSMase2 were found to inhibit apoptosis, concordant with the previous study. Consistent with the cellular studies, mouse models of cigarette smoke exposure showed an accumulation of ceramide in lungs of mice 1–3 weeks after exposure, causing apoptosis (Filosto et al., 2011). There was a simultaneous increase in nSMase2 protein expression in both bronchial epithelium and alveolar septae of mice that was prevented by pretreatment with the ROS scavenger N-acetylcysteine (NAC) (Filosto et al., 2011). One major consequence of chronic smoking is the development of chronic obstructive pulmonary disease (COPD), a clinical entity comprising two distinct pathologies, namely emphysema and chronic bronchitis. Conflicting results have been reported on the involvement of nSMase2 in the pathophysiology of emphysema. Poirier et al. showed increased morphological changes that resemble emphysematous changes in lungs of fro/fro mice in the absence of any insult (Poirier et al., 2012). These changes included alveolar distention and increased lung compliance by pulmonary functional testing, which suggests that lack of nSMase2 activity in the fro/fro mice mediates emphysematous changes (Poirier et al., 2012). In contrast, Filosto et al. reported increased nSMase2 activity in the alveolar space of emphysematous patients (Filosto et al., 2011). Tibboel et al. observed in an elastase-treatment model of emphysema that there was an nSMase2-independent increase in ceramide levels in broncheo-alveolar lavage fluid at day 2–5 post-treatment (Goldkorn et al., 2014). NSMase2 has also been implicated in hypoxic pulmonary vasoconstriction, which is a physiologic pulmonary smooth muscle vasoconstriction in response to poor perfusion in order to shunt the blood supply to better-oxygenated alveoli (Cogolludo et al., 2009). Hypoxia induces a GW4869-inhibited acute ceramide increase and pulmonary arterial smooth muscle contraction (Cogolludo et al., 2009). Further elucidation of the mechanism revealed that nSMase2 activation in rat pulmonary arteries in response to hypoxia generated ROS and was prevented by blockage of the mitochondrial NADPH oxidase (Frazziano et al., 2011). Finally, Lin et al. suggested a role for nSMase2 and sphingosine kinase in antagonizing anti-apoptotic properties of lipopolysaccharide during acute lung injury, which improved lung function and survival in these patients (Lin et al., 2011). Taken together, these studies suggest a profound role of nSMase2 in modulation of lung responses to pathologies.

Involvement in circulatory and cardiac pathophysiology

nSMase2 in circulatory conditions

NSMase2 has been implicated in the development of hypertension. Initial findings pointed to activation of a membrane NSMase in response to acute changes in vascular pressure associated with NSMase-dependent ERK1/2 and MEK1/2 phosphorylation (Czarny et al., 2003; Czarny and Schnitzer, 2004). Applying this finding to animal models, young and old rat aortas and mesenteric vessels were studied. Old aortas lost acetylcholine mediated vascular relaxation, which was associated with inhibitory phosphorylation on eNOS (Smith et al., 2006). NSMase activity was also increased, and GW4869 treatment improved the vasomotor action of aged vessels (Ohanian et al., 2014; Smith et al., 2006). Furthermore, the inhibitory effect on eNOS was recapitulated in a mouse model of ER stress that showed increased nSMase2 activity associated with inactivation of eNOS. NSMase2 overexpression mimicked ER stress and resulted in decreased NO production, a potent vasodilator (Chaube et al., 2012; Mogami et al., 2005). These effects suggest that activation of nSMase2 is associated with a vasoconstrictor phenotype. In that context, the effect of the physiologic vasoconstrictors endothelin-1 (ET-1) on nSMase2 was studied. ET-1 activates nSMase2 to induce VCAM-1 expression in rat small mesenteric

artery (Ohanian et al., 2012). Probing the system further, the effect of dietary Mg^{2+} deficiency was studied. Mg^{2+} deficiency has been demonstrated experimentally and is clinically associated with hypertension. In rats, a Mg^{2+} deficient diet for 21 days increased NSMase activity in both aortic smooth muscle and the left ventricle (Altura et al., 2013). This was reversed by supplementing the diet with magnesium and resulted in alterations of telomerase activity that correlated with NSMase inhibition (Altura et al., 2013; Shah et al., 2014). Taken together, this presents nSMase2 as a potential target for pharmacological inhibition in clinical hypertension.

Studies have implicated nSMase2 in various other circulatory pathologies. Transplant vasculopathy is a major cause of transplant rejection and is mediated by anti-HLA antibodies of the host. Anti-HLA activates nSMase2 via MMP2 in smooth muscle cells and produces a mitogenic signaling that leads to thickening of the intima of the vessels (Galvani et al., 2011). The MMP2 → nSMase2 → mitogenic activation cascade is also found in endothelial ECV304 cells in response to urokinase. Here, integrin $\alpha_5\beta_3$ activation of MMP2 is a critical step for nSMase2 activation (Maupas-Schwalm et al., 2009). Finally, nSMase2 activity has implications in circulatory changes in dyslipidemia. Reports suggest that ApoC-1 enriched HDL particles, which are HDL particles with limited peripheral absorption, leads to an nSMase2-dependent apoptosis, when exogenously added to aortic smooth muscle cells (Kolmakova et al., 2004).

nSMase2 in cardiac conditions

The literature on nSMase2 in cardiac pathologies is not thoroughly developed, but seems to suggest that activation of nSMase2 occurs in cardiac conditions and is, at least partly, responsible for cytotoxic outcomes. Early studies reported that following ischemia, reperfusion caused acute activation of a Mg^{2+}-dependent NSMase in rat cardiac myocytes (Hernandez et al., 2000), which was prevented by pre-treatment with NAC, and NSMase-derived ceramide activated the JNK pathway (Hernandez et al., 2000). Furthermore, post-myocardial infarction rat hearts displayed reduced GSH and its repletion using NAC resulted in inhibition of a nSMase2/Bcl-2/caspase3 axis and decreased oxidative stress resulting improved left ventricular function (Adamy et al., 2007). Another report suggested that obese type II diabetic patients have increased serum NSMase activity but that serum ceramide levels were unaffected (Baranowski et al., 2010).

Involvement in neurobiology and neuropathology

Response to neurotrophins

Neurotrophins are a family of four mammalian proteins that promote neuronal survival and function through 2 classes of receptor mediated signaling; the p75 neurotrophin receptor, which is a common receptor for all neurotrophins and the Tropomyosin-receptor kinase (Trk) receptors that selectively bind specific neurotrophins. Initial studies demonstrated activation of the sphingomyelin cycle following neurotrophin treatment in T9 gliomas and PC12 cells (Dobrowsky et al., 1994, 1995). Application of the neurotrophin Nerve Growth Factor (NGF) to cultured hippocampal neurons produces ceramide via nSMase2 activation to engage apoptosis (Brann et al., 2002). NSMase2 activation requires the p75 receptor and increases phosphorylation of JNK (Brann et al., 2002). This effect is recapitulated in adult motor neurons as NGF-induced apoptosis is blocked by the nSMase inhibitors manumycin A and GW4869 (Pehar et al., 2007). p75 activation of nSMase2 also increases action potentials in capsaicin sensitive sensory neurons through increased current in tetrodoxin resistant sodium channels and suppression of a delayed rectifier outward potassium current (Zhang et al., 2002). Other data suggest that nSMase2-derived ceramide may also act upstream of p75 receptors. Peng et al. showed that penta-acetyl geniposide induces NSMase activity in C6 gliomas with ceramide generation that is inhibited by GW4869 (Peng et al., 2006). NGF and p75 are upregulated in that setting in an nSMase2-dependent manner (Peng et al., 2006). Moreover, the role of nSMase2-derived ceramide in apoptosis/survival in response to neurotrophins has recently been clarified. Candalija et al. reported a protective, cell survival-promoting role of nSMase2 in response to the neurotrophin brain-derived neurotrophic factor in granule neurons and NGF in PC12 cells (Candalija et al., 2014). However, unlike previous studies, the increase in cell survival stemmed from activation of the Trk receptors (Candalija et al., 2014). This leads to a possible model where activation of the p75 low affinity receptor

in response to NGF promotes an apoptotic nSMase2-dependent signal, while neurotrophin activation of the Trk receptors promotes a pro-survival nSMase2-dependent signal.

Activation of neuronal death

A body of literature suggests the involvement of nSMase2-derived ceramide in neuronal death. Ethanol activates nSMase2 to produce glial cell death and signals through the ERK, JNK and p38 MAPK pathways in astrocytes (Pascual et al., 2003). On the other hand H_2O_2 causes apoptotic cell death via activation of nSMase2 in primary oligodendrocyte, a finding that sheds light on one of the contributing factors to the exacerbation of multiple sclerosis, a demyelinating disease involving oligodendrocytes (Jana and Pahan, 2007). Kainic acid-induced status epilepticus increases pro-apototic Bax and decreases anti-apoptotic Bcl-2 levels simultaneous with nSMase2 activation (Mikati et al., 2008). Finally, oxidized phosphatidylcholine increases nSMase2 at the mRNA level in rat oligodendrocytes and leads to the activation of caspase-3 and caspase-8 (Qin et al., 2009).

Alzheimer's disease

There is growing interest in the involvement of nSMase2 in the pathogenesis of Alzheimer's disease. Initial reports showed that $A\beta_{25\text{-}35}$ peptide upregulated NSMase activity in rat primary astrocytes and neurons causing cell death (Ayasolla et al., 2004; Ju et al., 2005). Further studies suggested that $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides activated nSMase2 to negatively control γ secretase activity (Grimm et al., 2005). Recently, inhibition of phospholipase D2 was shown to abrogate Aβ-nSMase2 activation in SH-SY5Y cells (Tanabe et al., 2014). The importance of exosomal secretion of Aβ peptide mediated by nSMase2 is beginning to gain appreciation (Dinkins et al., 2014; Wang et al., 2012; Yuyama et al., 2012). It is thought that exosomes released from microglia prevent Aβ oligomerization, through unknown mechanisms and that the absence of exosomes leads to a soluble Aβ peptide that produces neuronal death (Yuyama et al., 2012).

Other neurological functions

Effects of nSMase2 on normal synaptic regulation was studied in models of dopamine uptake where it was shown that nSMase2 inhibition or downregulation by siRNA decreased dopamine uptake in PC12 cells (Kim et al., 2010) with Hsp60 acting as an interaction partner that negatively regulates nSMase2 in that system (Ahn et al., 2013). This paved the way to more in depth studies of involvement of nSMase2 in dopaminergic pathologies such as Parkinson's disease. Physiologically, nSMase2 inhibition by GW4869 delayed formation of spatial reference memory in mice without affecting episodic-like memory; the likely mechanism is through the modification of the subunit composition of N-methyl-D-aspartate receptors (Tabatadze et al., 2010). Also, a role for nSMase2 was found in inflammation following ischemia/reperfusion injuries in the brain. Astrocytes, but not neurons, displayed increased nSMase2 activity following ischemia/reperfusion with nSMase2-dependent generation of the pro-inflammatory cytokines TNF-α, Interleukin-1 and Interleukin-6 (Gu et al., 2013). Finally, a potential role for nSMase2-derived ceramide was recently described in embryonic stem cells and embryonic stem cell-derived neural progenitors. In these cells, ceramide induce ciliogenesis, a critical step in differentiation that was inhibited by GW4869 and fumonisin B1, an inhibitor of ceramide synthase. NSMase2-derived ceramide prevented the activation of histone deacetylase-6, Aurora A, and sequestered protein kinase C into apicolateral domains preventing its activation (He et al., 2014). Taken together, these findings suggest a profound role of nSMase2 in neuronal pathologies. This will pave way for research understanding more in depth mechanisms of neuronal regulation by nSMase2, but also for the development of specific inhibitors of nSMase2 as novel strategies of treatment of neurological diseases.

NSMase2-mediated release of exosomes

Recently, a body of literature emerged involving nSMase2 in exosome release through a non-canonical pathway independent of ESCRT protein complexes. Exosomes are 40–100 nm vesicles that are released from the cell by the fusion of multivesicular endosomes to the plasma membrane. The resulting secreted exosome carries cellular contents such as protein, RNA, and lipids and serves to

influence biological functions of neighboring cells through the transfer of these contents (Thery et al., 2002). In tumors, nSMase2 was initially shown to mediate exosomal release from HEK293 containing mir16 and mir146 (Kosaka et al., 2010). The functional significance of this was demonstrated by showing that blocking nSMase-2 mediated exosomal release from 4T1 breast xenografts reduced lung metastasis through perturbation of endothelial function (Kosaka et al., 2013). Isolated exosomes from these tumors increased HUVEC tube formation and migration through exosomal transfer of mir-210 (Kosaka et al., 2013). However, this function seems to be tumor specific as another reports suggested that in androgen-resistant prostatic cell line PC-3, nSMase2 does not play a role in exosome release and instead suggested a role of glycolipids in that release (Phuyal et al., 2014).

The role of nSMase2 in Alzheimer's disease is mediated at least partly through exosomes. Primary astrocytes from murine cortices treated with $A\beta_{25-35}$ or $A\beta_{1-42}$ died along with induction of PAR-4, production of ceramide and caspase3 activation (Wang et al., 2012). The PAR4 along with the ceramide was found to localize to exosomes, and that finding was not present in the fro/fro mice (Wang et al., 2012). Treating wild-type mice and 5XFAD Alzheimer's disease mouse model with GW4869 resulted in lower exosomes in the brain, with the 5XFAD mice displaying lower whole brain concentration of $A\beta_{1-42}$ (Dinkins et al., 2014). Finally, nSMase2 mediated exosomal transfer was found to be important in antigen presentation. Antigen presenting cells secrete nSMase2-dependent exosomes, enriched in small RNA species, which regulate antigen presentation (Mittelbrunn et al., 2011).

Role of nSMase2 in cancer

The role of sphingolipids in cancer is a subject that has gained attention recently. While the involvement of nSMase2 in chemotherapeutic-mediated cytotoxicity has been the subject of intense examination, the contribution of nSMase2 in the pathogenesis and perpetuation of human tumors is less well understood (Henry et al., 2013). In leukemia, mutations of the nSMase2 gene, SMPD3, were identified in 5% of acute myeloid leukemia and 6% of acute lymphoid leukemia. Some of these mutations perturbed the stability or localization of the enzyme (Kim et al., 2008). In breast cancer, SMPD3 was one of 55 genes with more than 4 fold upregulation in tumor vasculature of luminal A type breast cancer versus control matched breast tissue (Bhati et al., 2008). Moreover, the metastasis of 4T1 murine breast tumors was found to be dependent on nSMase2-mediated exosome transfer of miR-210 (Kosaka et al., 2013). Moreover, a recent study identified nSMase2 as a possible tumor suppressor in hepatocellular carcinoma as genome-wide analysis revealed hypermethylation of the gene (SMPD3) in human samples. Further experiments demonstrated that overexpression of nSMase2 in these cells slowed growth while its downregulation promoted cell invasion and migration (Revill et al., 2013).

From a therapeutic standpoint, the early hints at a role for nSMase2 mediating effects from tumor therapy came from studies that found stimulation of NSMase activity following 1-β-D-Arabinofuranosylcytosine (Ara-C) peaking at 30 min with increase in ceramide in HL-60 cells (Strum et al., 1994). A similar effect was seen with ionizing radiation (IR) (Haimovitz-Friedman et al., 1994). Erythromyeloblastic cell lines that are resistant to IR fail to activate NSMase and do not undergo apoptosis (Bruno et al., 1998) while c-kit activation, which confers resistant to IR, prevents nSMase2 activation (Bhati et al., 2008; Maddens et al., 2002). Following that, daunorubicin, etoposide, paclitaxel and ara-C were found to activate nSMase2 in different cell lines (Henry et al., 2013; Plo et al., 2001; Sumitomo et al., 2002) via a PKC-dependent mechanism, to mediate the cytotoxic effects of these chemotherapeutics (Plo et al., 2001; Sumitomo et al., 2002). Compounds with anti-tumor properties that are not used in clinics have also been studied. Polyphenols (t-PER and QUER) activate nSMase2 (Ferrer et al., 2007), while nSMase2-dependent apoptosis was described in K562 and MOLT-4 in response to Withanolide D (Mondal et al., 2010), and in K562 and HT29 in response to Protopanaxadiol (Park et al., 2013). Finally, compounds derivative of p53 reactivation and induction of massive apoptosis (PRIMA-1), possess nSMase2-mediated cytotoxic properties against lung cells (Soans et al., 2014). The cytotoxic effect of nSMase2 activation can also mediate some side effects of chemotherapeutics. Gentamicin-induced ototoxicity is prevented by GW4869, as pre-treatment with this inhibitor decreased outer hair loss in organs of Corti explants (Chi et al., 2014).

Role of nSMase2 in cell death

One of the earliest described biological consequences of ceramide formation is cell death. NSMase2 activation to engage cell death machinery has been described both in acute (minutes to a few hours) and in prolonged (>24 h) stimulations. Early on NSMase-derived ceramide action on cell death was defined in TNF-α mediated apoptosis. Activation of NSMase was shown initially in response to TNF-α (Kim et al., 1991), then Obeid et al. demonstrated that TNF-α induced apoptosis and exogenous addition of C2 ceramide mimicked that effect (Obeid et al., 1993), and similar results were shown using exogenous sphingomyelinases (Jarvis et al., 1994). Further characterization was done using bacterial sphingomyelinase as a tool mimicking NSMase activation in cells. Zhang et al. showed that exogenous application of *B. cereus* cloned sphingomyelinase resulted in cell death with activation of PARP (Zhang et al., 1997). Other members of the TNF receptor family such as FAS have been also shown to mediate activation of NSMase to cause cellular apoptosis. Agonist of Fas induced accumulation of ceramide with an increase in membrane bound SMase activity and DNA fragmentation in Fas sensitive cells, a finding that was mimicked with exogenous addition of ceramide in Fas resistant cells (Skowronski et al., 1996; Tepper et al., 1995). Many stimuli were subsequently shown to activate NSMase. In WEHI231 cells, cross-linking of IgM increases membrane sphingomyelinase activity and ceramide production causing apoptosis, preventable by overexpression of Bcl-xL (Wiesner et al., 1997). PC12 cells activate sphingomyelinase in response to hypoxia to produce apoptosis peaking at 24 h. Similar to WEHI231s, this is blocked by overexpression of the anti-apoptotic Bcl2 (Yoshimura et al., 1998), or by pre-treatment with NAC or GSH (Yoshimura et al., 1999). This observation was consolidated in C6 glioma cells where Bcl-2 overexpression prevented etoposide-induced nSMase2 activation (Sawada et al., 2000). Work by Chipuk et al. studied how NSMase-derived ceramide cooperate with Bax and Bcl-2 to promote apoptosis. Isolated mitochondria where found to be resistant to release of cytochrome *c* following cleaved caspase 8 (C8-BID) treatment. A restoration of the sensitivity occurred only when the microsomal fraction (containing NSMase activity) was added to the mitochondrial fraction, and the resensitization was blocked by GW4869 (Chipuk et al., 2012). While the use of GW4869 in that study suggests the involvement of nSMase2 as the NSMase isoform responsible for the ceramide generation, the lack of data on microsomal nSMase2 interacting with mitochondria coupled with the unavailability of data relating to GW4869 inhibition of the recently cloned mitochondrial-associated NSMase lead to the conclusion that further studies are required to determine the NSMase isoform responsible for the biology described.

The effector pathway of apoptotic cell death is through activation of caspases. The role of caspase activation in nSMase2-mediated cell death has been reported with conflicting results. While most studies suggest caspase activation follows the activation of nSMase2 (Meyers-Needham et al., 2012), some define caspase activation as a pre-requisite for nSMase2 activation. For example, inhibition of caspase-3 prevented nSMase2 activation in HL-60 cells following sodium nitroprusside treatment and addition of recombinant caspase-3 activates NSMase activity in a cell free system (Takeda et al., 1999).

One disease process in which nSMase2-mediated apoptosis seems to be relevant is diabetes mellitus (DM). One of the proposed mechanisms of pathogenesis of DM is ER stress and the activation of the unfolded protein response. Initial findings showed that basal nSMase2 was activated in Akita mice, a mouse model of ER stress (Lei et al., 2010). Subsequently pancreatic β islets treated with thapsigargin were shown to undergo apoptosis with activation of nSMase2 at the message level (Lei et al., 2012) and that nSMase2 upregulation is mediated by iPLA2 (Lei et al., 2012, 2013). Finally, phosphatidylinositol ether lipid analogs (PIAs) induce secretion of pro-apoptotic factors in non-exosomes nanovesicles in an nSMase2-dependent manner (Gills et al., 2012).

Role of nSMase2 in cell differentiation and growth arrest

The role of NSMase activity in cell differentiation was examined prior to the cloning of the different isoforms of NSMase. Vitamin D-induced cell differentiation in HL60 leukemia cells was accompanied by activation of a neutral sphingomyelinase and ceramide generation, while the addition of an exogenous SMase potentiated the differentiation effect of subthreshold Vitamin D (Okazaki et al., 1989) with ceramide being identified as the sphingolipid responsible for that effect (Okazaki et al., 1990). The

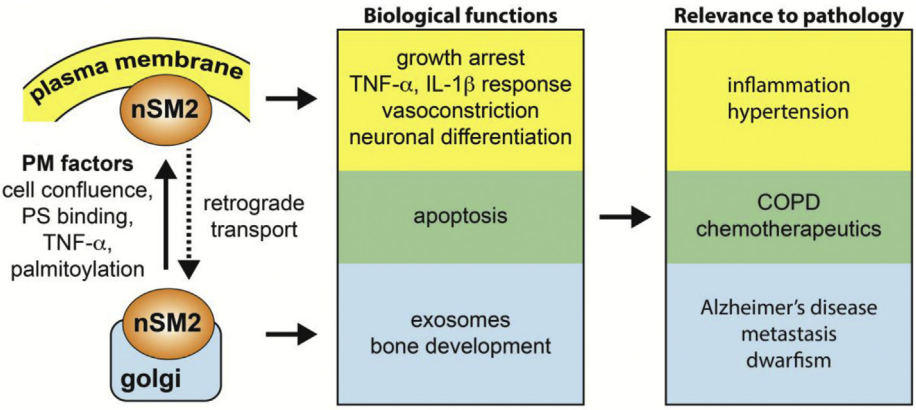

Fig. 3. Summary of nSMase2 functions and relevance to pathology. Association of nSMase2 function in different cellular compartments. Plasma membrane, yellow; Golgi, blue; Unknown, green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effect of NSMase activation on HL60 monocytic differentiation was also shown in response to TNF (Kim et al., 1991) and the later characterization of nSMase2 as the major NSMase activated by TNF suggest this effect is nSMase2 specific. Later studies expanded on the role of NSMase in cell differentiation, with the effect of neurotrophins on the activation of nSMase2 (refer to section Involvement in neurobiology and neuropathology) and the potential role of that in neuronal differentiation being the most characterized of these functions.

The study of nSMase2 in modulation of the cell cycle has gained momentum recently with the discovery of stimuli that activate nSMase2 to produce growth arrest. While ceramide has been described to produce a growth arrest phenotype, among its many biological functions, studies of nSMase2-derived ceramide have lagged behind (Hannun and Obeid, 2011). MCF7 breast cancer cells overexpressing nSMase2 have a similar growth phenotype to control cells in the exponential phase. However, upon serum starvation they get retained in G0/G1 to a greater extent than cells overexpressing control plasmid. Moreover, cell confluence upregulates nSMase2 to arrest cells in G0/G1 with hypophosphorylation of the retinoblastoma protein and induction of p21. Downregulation of nSMase2 by siRNA bypasses this phenotype (Marchesini et al., 2004). Later studies demonstrated nSMase2-dependent dephosphorylation of β catenin on T41/S45 via activation of PPC1γ in confluent cells (Marchesini et al., 2007). Another stimulus that induces nSMase2-dependent growth arrest is All-Trans Retinoic Acid (ATRA). ATRA induces activation of nSMase2 at the transcriptional level in cells with functional retinoic acid receptor-α to arrest them in G1 phase of cell cycle (Somenzi et al., 2007). The mechanism is thought to be through the dephosphorylation of S6K, independent of PP2A (Clarke et al., 2011a).

Role of nSMase2 in post-natal growth and bone development

Stoffel et al. initially described the severe growth retardation in the SMPD3$^{-/-}$ mice starting at embryonic day 14 and accompanied with hypoplasia of all organs except the brain. They also described delayed bone ossification present in the mice (Stoffel et al., 2005). Later, studies on the fro/fro mice showed a reduction in mineralized tissue in long and flat bones. The hypertrophic chondrocytes in long bones persist and do not undergo either apoptosis or mineralization in long bones. The localized expression of SMPD3 corrected this phenotype (Khavandgar et al., 2011). The mechanism by which nSMase2 acts to control chondrocyte maturation and mineralization was further studied to describe two key pathways. The first pertained to hyaluronan synthesis, an essential process in normal bone maturation. Fro/fro fibroblasts were found to have increased hyaluronan with upregulation of hyaluronan synthase-2 (HAS-2) (Qin et al., 2012). In ATDC5 cells, a common cellular model of chondrocyte

maturation, HAS-2 was found to be downregulated during maturation and siRNA to nSMase2 prevented HAS-2 downregulation (Kakoi et al., 2014). Another mechanism thought to partially contribute to the phenotype is the effect on Pi3K/Akt pathway. Fro/fro mice were found to have higher levels of phosphor-Akt and phosphp-S6K and knockdown of nSMase2 in ATDC5 cells during differentiation further potentiated this increase (Kakoi et al., 2014; Qin et al., 2012).

Conclusion

A number of tools developed over the past two decades have enlarged our understanding of the regulation, enzymology and biologic functions of nSMase2. However, many questions still remain (Fig. 3). The determination of a crystal structure of this enzyme may answer basic questions regarding the enzymology and the mechanism of activation. There is a need to understand cellular mechanisms of regulation of nSMase2 and the specific mechanisms by which ceramide and other down stream lipid metabolites mediate the various actions of this critical enzyme. More in depth studies are also needed to transition biological findings into clinical studies with the anticipation that this enzyme could be a drug target for the treatment of several disease processes.

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