Neutral Sphingomyelinase 2 (nSMase2) Is a Phosphoprotein Regulated by Calcineurin (PP2B)*

Simone Filosto1, William Fry1, Anne A. Knowlton, and Tzipora Goldkorn2

From the Department of Internal Medicine, Genome and Biomedical Sciences Facility, University of California School of Medicine, Davis, California 95616

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We previously reported that exposure of human airway epithelial cells to oxidative stress increased ceramide generation via specific activation of neutral sphingomyelinase2 (nSMase2). Here we show that nSMase2 is a phosphoprotein exclusively phosphorylated at serine residues. The level of nSMase2 phosphorylation can be modulated by treatment with anisomycin or phorbol 12-myristate 13-acetate (PMA/12-O-tetradecanoylphorbol-13-acetate), suggesting that p38 mitogen-activated protein kinase (MAPK) and protein kinases Cs are upstream of nSMase2 phosphorylation. Oxidative stress enhances both the activity and phosphorylation of nSMase2. Strikingly, we show here that nSMase2 is bound directly by the phosphatase calcineurin (CaN), which acts as an on/off switch for nSMase2 phosphorylation in the presence or absence of oxidative stress. Specifically, CaN is being inhibited/degraded and therefore does not bind nSMase2 under oxidative stress, and a mutant nSMase2 that lacks the CaN binding site exhibits constitutively elevated phosphorylation and increased activity relative to wild type nSMase2. Importantly, the phosphorylation and activity of the mutant no longer responds to oxidative stress, confirming that CaN is the critical link that allows oxidative stress to modulate nSMase2 phosphorylation and function.

We have shown that ceramide generation coordinates stress responses and is elevated in HAE cells in response to reactive oxygen species (1–8).

Ceramide is synthesized through either a de novo pathway involving serine palmitoyl-CoA transferase and ceramide synthase, or from breakdown of membrane sphingomyelin (N-acylsphingosine-1-phosphocholine) (Fig. 1A), a phospholipid preferentially concentrated in the plasma membrane of mammalian cells (9). Sphingomyelin catabolism occurs via the action of sphingomyelinases (SMases),3 which are sphingomyelin-specific forms of phospholipase C that hydrolyze the phosphodiester bond of sphingomyelin, yielding ceramide and phosphorylcholine. Ceramide then serves as a second messenger, leading to apoptotic DNA degradation.

We suggested that reactive oxidants up-regulate ceramide generation and cause elevated apoptosis in human airway epithelial (HAE) cells, thereby leading to lung injury pathologies. However, the mechanisms and target molecules of reactive oxidants affecting the HAE cells are not fully understood. Therefore, we proposed that increased oxidative stress and elevated ceramide generation are coupled at the molecular level by an unknown SMase that generates ceramide by hydrolysis of sphingomyelin (Fig. 1B). To proceed from the cellular to the molecular level, we searched for the specific SMase that is modulated by reactive oxygen species in lung epithelial cells, which led to our isolation of the novel nSMase2 from monkey lung tissue and HAE cells (1). This nSMase2 was previously found in the brain (10).

We then demonstrated that nSMase2 is the only member of the sphingomyelin phosphodiesterases family that is up-regulated and responsible for ceramide generation in HAE cells exposed to cigarette smoke (CS) or to H2O2 (1, 2). Moreover, we ascertained that CS exposure generates H2O2 in the medium of HAE cells in a dose-dependent manner (2, 11) and that pretreatment with glutathione (GSH) prevented both H2O2 and ceramide generation (2, 8).

Here we show that nSMase2 is a phosphoprotein in which the level of phosphorylation is modulated by oxidative stress, which also controls nSMase2 function. Furthermore, we demonstrate here that nSMase2 phosphorylation is regulated by a specific phosphatase that is modulated by oxidative stress.

Calcineurin (CaN) phosphatase (also known as protein phosphatase 2B (PP2B)) interacts directly with nSMase2, but not under the exposure to H2O2-induced oxidative stress. CaN is a Ca2+/calmodulin-dependent serine/threonine phosphatase, which can be inhibited by H2O2 that modifies 2 Cys residues through the oxidative formation of a disulfide bridge, and eventually leads to CaN degradation (12). This phosphatase is known to bind to its substrates via a PXXIT motif as described for its binding to nuclear factor of activated T cells (13). Indeed, we found that nSMase2 contains a PQQIXIT sequence, and we show here unequivocally that deletion of this 6-amino acid PXXIT-related sequence blocks CaN phosphatase binding to nSMase2. Furthermore, the nSMase2 mutant, which does not bind CaN, is much more phosphorylated and activated than the wild type.

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1 To whom correspondence should be addressed: Signal Transduction, University of California School of Medicine, Genome and Biomedical Sciences Facility, Rm. 6321, 451 E. Health Sciences Dr., Davis, CA 95616. Tel.: 530-752-8632; E-mail: ttgoldkorn@ucdavis.edu.

2 The abbreviations used are: SMase, sphingomyelinase; CaN, calcineurin; CS, cigarette smoke; Cycl-A, cyclosporine A; HAE, human airway epithelial; HBE, human bronchial epithelial; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; MT, mutant; nSMase2, neutral sphingomyelinase 2; NBD, nitro-2,1,3-benzoxadiazol-4-yl; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor α; WT, wild type; TPA,12-O-tetradecanoylphorbol-13-acetate; PP2B, protein phosphatase 2B.

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1 Both authors contributed equally to this work.
wild type (WT) nSMase2. This validates that the function of nSMase2 is modulated via its de-phosphorylation by CaN, which does not interact with nSMase2 under oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—We are using immortalized human bronchial epithelial (HBE1) cells (from Dr. Reen Wu, University of California, Davis, CA) and A549 adenocarcinoma cells, which behave similarly to primary airway epithelial cells with respect to reactive oxidant regulation of ceramide generation and apoptosis induction. Therefore, we routinely use HBE1 and A549 cells for most of our studies and then verify key findings in the primary lung epithelial cells. Culture conditions are identical for both HBE1 and primary cells, as described (14, 15): cells are grown in serum-free medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (5 ng/ml), dexamethasone (0.1 μM), cholera toxin (20 ng/ml), and bovine hypothalamus extract (15 μg/ml). A549 cells were grown in F12-K medium (Invitrogen) supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin.

The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and cultured as previously described (6, 16). Tran-

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The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and cultured as previously described (6, 16). Transient transfections of pFLAG-nSMase2 and V5-nSMase2 were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. Transfected cells were treated 24 h post-transfection.

The mutant (MT) that lacks the putative CaN binding site with respect to nSMase2 (SMPD3) residues PQIKIY. Wild type- or MT-nSMase2 were tagged at 3’ with the V5 epitope by using the pEF6/V5-His-TOPO vector (Invitrogen) for transfection; FLAG-nSMase2 was transfected in cells using vector pCMV-Tag2 (Stratagene).

**Immunoprecipitation**—Cells in Triton X-100 lysis buffer were incubated for 30 min at 4 °C in an orbital shaker. Cell lysates were centrifuged at 14,000 x g for 5 min at 4 °C and the postnuclear supernatant was collected. Aliquots of the postnuclear supernatant containing equal amounts of protein (400 μg) were immunoprecipitated either 2 h or overnight at 4 °C with anti-(α)V5 (Invitrogen) and anti-calcineurin (Millipore) antibodies, conjugated with protein A-Sepharose (RepliGen).

**Immunoblotting**—Protein samples were separated on 10% acrylamide SDS-PAGE or 5–15% gradient gels (Bio-Rad) at 80–100 V before transfer to nitrocellulose membrane (20 μM pore, Bio-Rad) in Tris glycine buffer at 100 V for 60 min at 4 °C. Membranes were blocked in 5% milk in 0.1% Tris-buffered saline/Tween 20 (TBST) and probed with primary antibody for 2 h at room temperature (anti-V5 mouse 1:5000 from Invitrogen; anti-calcineurin rabbit 1:500 from Millipore; anti-nSMase2 1:1000, specially generated for us in rabbit (Sigma) against a partially purified nSMase2). Membranes were washed (three times with TBST), probed with horseradish peroxidase-conjugated secondary antibody (1:7500 mouse or rabbit in 5% nonfat dry milk) or protein A-horseradish peroxidase (Invitrogen) for 90 min at room temperature, and washed (three times with TBST). Proteins were visualized by enhanced chemiluminescence (Pierce).

**Alkaline Phosphatase Treatment**—After immunoprecipitation of V5-nSMase2, the treatment was done by incubating the proteins in a mixture containing alkaline phosphatase enzyme for 30 min at 37 °C, according to the manufacturer’s instruction (New England Biolabs).

**32P in Vivo Labeling for Revealing Phosphorylation of nSMase2**—Cells were transfected with V5-nSMase2 for 24 h, then incubated in a phosphate-free Dulbecco’s modified Eagle’s medium (MP Biochemicals) for 4 h in the presence of [γ-32P]orthophosphate (MP Biochemicals) and treated (or not) for the indicated time point with 250 μM H₂O₂, 20–200 nM PMA/TPA, 10–50 μM anisomycin, 10 μM cyclosporine A, 10 μM ionomycin, or 10 μM SB202190. After cell lysis and immunoprecipitation of V5-nSMase2, radioactivity of nSMase2 was assessed by autoradiography and normalized to the amount of V5-nSMase2 measured by immunoblotting.

**Partial Acid Hydrolysis of 32P-labeled V5-nSMase2**—In vivo radiolabeled V5-nSMase2 was immunoprecipitated, discriminated on 9% acrylamide SDS-PAGE, transferred to nitrocellulose membrane, and stained by immunoblotting against the V5 epitope. Then, the relative cut radiolabeled bands were rinsed in water and acid hydrolyzed in 5.8 N HCl at 110 °C for 1.5 h. The samples were dried under vacuum and resuspended in a standard mixture of phosphoamino acids (1 mg/ml of o-phospho-DL-serine, threonine, and tyrosine dissolved in H₂O). Counts per minute were determined by scintillation counting and a sample volume corresponding to 300 counts/min was resolved by thin-layer electrophoresis as described by Hardin and Wolniak (17) at 250 V for 1.5 h. Radiosensitive intensifying screen was used to visualize the radioactive amino acids, and staining with 0.5% ninhydrin solution in acetone was used to detect the standards.
nSMase2 Phosphorylation Regulated by Calcineurin Interaction

Determination of Cellular Ceramide Levels by Diacylglycerol Kinase Assay—Ceramide was quantified by the diacylglycerol kinase assay as previously described (7, 16). Briefly, lipids were extracted with methanol, chloroform, 1 n HCl (100:100:1, v/v/v). The lipids in the organic phase were dried under vacuum and resuspended in 100 μl of reaction mixture containing [γ-32P]ATP and incubated at room temperature for 1 h. The reactions were terminated by extraction of lipids with 1 ml of methanol, chloroform, 1 n HCl, 170 μl of buffered saline solution, and 30 μl of 0.1 M EDTA. The lower organic phase was dried under vacuum, and the lipids resolved by thin layer chromatography on Silica Gel 60 plates (Whatman) using a solvent of chloroform:methanol:acetic acid (65:15:5, v/v/v). Ceramide 1-phosphate was detected by autoradiography, and incorporated 32P was quantified by densitometry scanning using a Molecular Dynamics Gel Scanner.

Assays for nSMase Activity—The enzyme activity of nSMase was determined as described (18, 19). Briefly, an enzyme preparation of 10 μg of total protein (from V5-nSMase2-transfected cells) in 20 mM Tris-HCl, pH 7.4, was mixed with [14C]sphingomyelin (SM) (10 nmol/1,000,000 disintegrations/min) in 1 mM MgCl2, 10 mM dithiothreitol, 10 nM phosphatidylserine, and 1 mg/ml of bovine serum albumin. The incubation time was 30 min at 37 °C. The reaction was terminated by the addition of 1 ml of chloroform:methanol (2:1) followed by 0.2 ml of distilled water. After phase separation, the upper phase was removed and the radioactivity determined by liquid scintillation counting. Alternatively the mixture was incubated with fluorescent C6-NBD-labeled-SM, N-hexanoyl-NBD-sphin-gosylphosphorylcholine (Matreya). The NBD-ceramide generated in the reaction was solved by thin layer chromatography and quantified by densitometry fluorescence scanning.

Yeast Two-hybrid—A library-scale screen was carried out using full-length hnSMase2-pGBK7 bait vector to interrogate a human heart Matchmaker cDNA library (Clontech) in the pAct2 prey vector. The initial screen was done at medium stringency and used the activation of just one of the three reporter genes as the basis for candidate selection. Our initial screen gave ~400 candidate colonies of a total of 500,000 colonies screened. These (400) colonies were then re-streaked onto agar plates lacking leucine and tryptophan (to maintain selection on both prey and bait plasmids) and lacking histidine, adenine, and supplied with α-X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Of 400 candidate colonies obtained from the initial medium stringency screen less than 20 clones were able to grow on the full selection plates (−LEU/−TRP/−HIS/−Ade + X-gal). Calcineurin (CaN) phosphatase showed up repeatedly among these clones. All reagents, unless stated otherwise, were from Sigma.

Statistical Analysis—Each time point or dose was analyzed at least in duplicate. Each group of studies was repeated and reproduced, at least three times. The data are reported as mean ± S.D. Statistical significance was determined by Student’s t test and p value < 0.05 was considered statistically significant; n = number of experiments, as indicated.

RESULTS

nSMase2 Is a Phosphoprotein—In an attempt to understand the link between H2O2 oxidative stress generation and nSMase2 activation, we investigated the molecular mechanisms involved in modulation of the nSMase2 function. By using HBE1, human bronchial epithelial cells, and A549 adenocarcinoma cells we showed that nSMase2 is a phosphoprotein (Fig. 2). First, the FLAG-tagged nSMase2 was transiently transfected in HBE1 cells, immunoprecipitated, and then treated (or not) with alkaline phosphatase. A gel shift analysis of a high resolution SDS-PAGE indicated that
nSMase2 was phosphorylated (Fig. 2A). In vivo labeling with [32P]orthophosphate of HBE1 cells transiently transfected with V5-tagged nSMase2 also confirmed that the enzyme was basally phosphorylated (Fig. 2B). Finally, immunoprecipitated nSMase2 from 32P-labeled cells was hydrolyzed and resolved by thin layer electrophoresis (Fig. 2C), which demonstrated that nSMase2 was phosphorylated on serine residues only.

Studies by others reported that p38 MAPK is upstream of nSMase2 in augmenting its activity during TNF-α/H9251 and PMA treatment of A549 cells. In addition, PKC/H9254 was implicated in the translocation of nSMase2 from the Golgi to the plasma membrane during such treatments (20, 21). Here we show that treatments with either PMA or anisomycin, the respective stimulators of the “conventional” PKC(s) and p38 MAPK (22, 23) elevate nSMase2 phosphorylation (Fig. 3). Anisomycin enhanced the phosphorylation of nSMase2 in a dose-dependent manner, suggesting the involvement of p38 MAPK upstream of nSMase2 phosphorylation (Fig. 3A). At the same time, as shown in Fig. 3B, the p38 inhibitor, SB202190, reduced the phosphorylation of nSMase2.

Interestingly, a 30-min treatment with 20 nM PMA induced a modest increase in nSMase2 phosphorylation, whereas a 4-h treatment with 200 nM PMA caused a substantial decrease in the level of nSMase2 phosphorylation (Fig. 3C). Because, it is well known that PKC activity is stimulated by a short/low concentration PMA treatment, but inhibited after a long/strong treatment (24), these data suggest that PKC(s) may also be involved in maintaining nSMase2 phosphorylation.

nSMase2 Phosphorylation and Activity Are Up-regulated by Oxidative Stress—Next, we carried out experiments to find out whether H2O2-induced oxidative stress affects the level of nSMase2 phosphorylation. Overexpression of V5-nSMase2 in A549 cells was followed by 32P in vivo labeling and exposure to 250 μM H2O2 for the last 30 min of labeling. After immunoprecipitation the level of nSMase2 phosphorylation was determined by autoradiography and quantified/standardized by immunoblotting the tagged V5 epitope with anti-V5 antibody. As shown in Fig. 4A, H2O2-induced oxidative stress stimulated a 1.5-fold increase in nSMase2 phosphorylation. As previously reported, we show here again (Fig. 4B) that such exposure to oxidative stress enhances nSMase2 activity, suggesting that nSMase2 phosphorylation may affect its function.

Novel Protein-Protein Interaction: CaN Phosphatase Interacts with nSMase2 and This Interaction Is Down-regulated under Exposure to Oxidative Stress—We used yeast two-hybrid screening to identify potential candidates that may interact physically with nSMase2. Using human nSMase2 as a bait to interrogate a human cDNA library, we searched for candidates that bind nSMase2. In parallel, we ran global mass spectrometry analyses after immunoprecipitation of nSMase2 from primary lung epithelial cells, exposed (or not) to various oxidative stress conditions. Of several possible candidates we report here that CaN phosphatase interacts with nSMase2, but not under H2O2-induced oxidative stress.

CaN is a Ca2+/calmodulin-dependent serine/threonine phosphatase also known as protein phosphatase-2B. It can be inhibited by H2O2, which modifies 2 Cys residues through the oxidative formation of a disulfide bridge (12). Co-immunoprecipitation analysis of endogenous CaN with overexpressed V5-nSMase2 confirmed the above screenings. The V5 construct was immunoprecipitated from lysates of transiently transfected
We then analyzed samples of cell lysate (50 μg of total protein) by Western blotting with a specific antibody against nSMase2. Because it has been reported (25) that oxidative stress can induce degradation and inactivation of CaN, we treated the HBE1 cells with 5 mM GSH or 250 μM H₂O₂ for the last 30 min of labeling. V5-nSMase2 was then immunoprecipitated by αV5 antibody and examined by immunoblotting (IB) and autoradiography. B, same transfection and treatments were done prior to assessing the total nSMase activity of 10 μg of cell homogenate using [³²P]sphingomyelin as substrate as described under “Experimental Procedures.” Standard deviations are indicated; *, p < 0.05, n = 5.

HBE1 cells, treated (or not) for 30 min with 250 μM H₂O₂ or 5 mM GSH, and analyzed by immunoblotting, using specific antibodies against the V5 epitope and against CaN. As shown in Fig. 5A, CaN indeed interacts with nSMase2 but not when the cells were exposed to H₂O₂. Furthermore, when endogenous CaN was immunoprecipitated with anti-CaN (Fig. 5B) the overexpressed nSMase2 could also be pooled down and immunoblotted. But, under H₂O₂ exposure nSMase2 could not be pulled down and detected by immunoblotting. Because it has been reported (25) that oxidative stress can induce degradation and inactivation of CaN, we treated the HBE1 cells with 5 mM GSH or 250 μM H₂O₂ for the time points indicated in Fig. 5C. We then analyzed samples of cell lysate (50 μg of total protein extract) by Western blotting with a specific antibody against CaN. Fig. 5C indeed confirms that H₂O₂ treatment resulted in a time-dependent down-regulation of CaN. Additionally, Fig. 5D demonstrates that when the endogenous CaN was immunoprecipitated, the endogenous nSMase2 could be clearly pulled down and detected by immunoblotting with the anti-endogenous nSMase2 antibody.

Cyclosporine A (Cycl-A) is a well known inhibitor of CaN (26, 27). Therefore, A549 cells transiently transfected with nSMase2 were [³²P]in vivo labeled as described above, but exposed to Cycl-A for the last 30 min of labeling. Fig. 6A demonstrates that the Cycl-A treatment indeed triggered an increase of nSMase2 phosphorylation. On the other hand, stimulation of CaN activity with ionomycin, a calcium ionophore (28), caused de-phosphorylation of nSMase2 (Fig. 6B). Therefore, we propose that CaN plays a key role in the regulation of nSMase2 phosphorylation: when CaN is inhibited by Cycl-A or activated by ionomycin the phosphorylation of nSMase2 is increased or reduced, respectively. Therefore, CaN may indeed have a critical function in the regulation of nSMase2 phosphorylation under different oxidative stress conditions: CaN is degraded and therefore does not bind nSMase2 during H₂O₂ treatment, thus allowing nSMase2 to be fully phosphorylated downstream of p38 MAPK and PKCs.

Phosphorylation and Activity of nSMase2 Are Related and Down-regulated by Calcineurin Phosphatase—To further substantiate our findings and in an attempt to define the role of CaN in nSMase2 function we found that the nSMase2 primary sequence presents a region, which may provide a docking site for CaN, PXIXIT (13, 29). Therefore, we deleted that putative binding site of CaN, PQIKIY, and generated a MT of nSMase2. Fig. 7A shows that indeed the MT nSMase2 does not bind CaN. In addition, Fig. 7B demonstrates that when endogenous CaN is immunoprecipitated, the MT nSMase2 is hardly pulled down...
compared with the WT nSMase2. Moreover, Fig. 7C demonstrates that such a mutant is much more phosphorylated in comparison to the WT nSMase2. In fact, it is phosphorylated as much as the WT nSMase2 exposed to H2O2.

Notably, the nSMase2 mutant that does not bind CaN turns out to be much more active constitutively than the WT nSMase2. As shown in Fig. 8A the total nSMase enzymatic activity in cells transiently transfected with empty vector, WT, and MT showed that the MT is about 40% more active than the WT. Furthermore, Fig. 8B confirms that the MT is more active than the WT nSMase2 because when the MT nSMase2 (that misses CaN binding) was transfected into A549 cells that were not exposed to oxidative stress it triggered an increase in the levels of endogenous ceramide that were compatible with the levels of ceramide induced by the transfected WT nSMase2 but only after treatment with H2O2. Importantly, as shown by the lower panel of Fig. 8B, all the various constructs were transfected to comparable levels.
Most importantly, as shown in Figs. 7 and 8, H$_2$O$_2$ could not enhance the phosphorylation or the activity of the mutant, further confirming that interaction between CaN and nSMase2 is not only affecting the phosphorylation of nSMase2 but also affecting its activity. Moreover, this also suggests that the target for oxidative stress modulation of nSMase2 phosphorylation is solely the phosphatase CaN and not any kinase, as discussed below.

All together, and as shown in the model proposed in Fig. 9, these studies demonstrate that nSMase2 phosphorylation and function are inter-connected and that CaN down-regulates nSMase2 activity by de-phosphorylation, whereas in turn the oxidative stress exerts an up-regulation of nSMase2 phosphorylation and activity by abolishing CaN-nSMase2 interaction.

**DISCUSSION**

The results presented in this work suggest a critical role for PP2B, calcineurin, in the modulation of the phosphorylation and function of nSMase2 during exposure to oxidative stress of human lung epithelial cells, resulting in the increase of cellular ceramide levels.

Ceramide formation in mammalian systems under stress derives mainly from hydrolysis of membrane SM. The break of SM is driven by a single class of enzymes, the SMases. The main forms of SMases are distinguished by their pH optima (18, 19, 30–33). Human and murine acid sphingomyelinase (aSMase; pH optimum 4.5–5.0) as well as Mg$^{2+}$/H$^{+}$-dependent or -independent neutral SMases (nSMase; pH optimum 7.4) have been cloned and determined to be the products of conserved genes (10, 34, 35). Interestingly, membrane nSMase does not gain access to the signaling events activated by the lysosomal aSMase and vice versa, indicating that ceramide action may be determined by the subcellular site of its production.

Airway epithelial cells are the first line of defense of the lungs and are thus extensively exposed to reactive oxidants. Over the last few years we initiated studies to address whether these cells
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are capable of entering apoptosis when exposed to micromolar concentrations of H₂O₂, and whether the process is mediated by ceramide as a second messenger (7, 16). The range of 50–250 μM H₂O₂ is considered to be the physiological range in which apoptosis can occur depending on the length of exposure to H₂O₂. As shown before (2, 36), exposure to CS can generate between 100 and 800 μM H₂O₂. Any concentration above 400 μM would be considered pathological (2, 8).

Our previous studies in HAE cells showed that nSMase2 is activated by both H₂O₂-induced oxidative stress and CS exposures (1, 2). This elevates the levels of cellular ceramide and augments apoptosis in the exposed lung epithelial cells. Loss of function experiments demonstrated that nSMase2 is the only specific target of H₂O₂ and CS among the nSMase family members that are activated by oxidative stress and are essential for oxidative stress-induced apoptosis in lung epithelial cells (1, 2). Here we show that oxidative stress not only controls nSMase2 function but that nSMase2 is a phosphoprotein in which oxidative stress modulates the level of phosphorylation.

In our yeast two-hybrid screening, CaN showed up repeatedly with multiple positive clones, whereas the number of other positive clones was limited and were viewed as weak candidates. We identified CaN, which is a Ca²⁺/calmodulin-dependent serine/threonine phosphatase (also known as PP2B) to directly interact with nSMase2. Moreover, CaN interaction with nSMase2 disappeared under oxidative stress, as confirmed by direct co-IP of endogenous CaN with an overexpressed V5-nSMase2 in HBE1 cells. We found that underexposure to H₂O₂ CaN is degraded and therefore not available for binding to nSMase2. Moreover, a mutant nSMase2 that lacks the binding site to CaN was found to be constitutively overphosphorylated and activated when compared with the WT nSMase2 (see Figs. 7 and 8). Because CaN is able to down-regulate p38 MAPK signaling (37), whereas PMA can inhibit the activity of CaN (38), it is possible that phosphorylation of nSMase2 is mediated via the p38 and PKC serine/threonine kinases and down-modulated via CaN phosphatase.

It was previously reported that nSMase2 traffics to the plasma membrane in confluent MCF7 human breast adenocarcinoma cells, causing an increase in ceramide levels (39). Moreover, both H₂O₂ and TNF-α exposures could induce nSMase2 translocation to the plasma membrane in A549 cells, suggesting that such a mechanism may be important for regulation of its activity (1, 21). Clarke et al. (20) reported that nSMase2 activity is rapidly and transiently up-regulated in A549 cells by TNF-α via a p38 MAPK-dependent mechanism. The same group demonstrated that the novel PKC-δ is upstream of the nSMase2 translocation from the Golgi to the plasma membrane during stimulation with either TNF-α or PMA, although PMA did not increase nSMase activity and PKC-δ did not regulate the TNF-α-induced increased activity (21). Moreover, PKC-δ was not found to interact (co-immunoprecipitate) with nSMase2 (20, 21). Clearly, additional studies are needed to provide full insight into the modulation of the nSMase2 structure function.

Indeed, we found that both anisomycin and PMA/TPA (low concentration of 20 nM) enhanced nSMase2 phosphorylation, whereas the p38 inhibitor, SB202190, reduced it, strongly suggesting that nSMase2 may be phosphorylated downstream of p38 αMAPK and PKCs. In addition, we observed a 60% decrease of nSMase2 phosphorylation after a prolonged PMA stimulation of lung epithelial cells, a treatment well known to typically inactivate PKCs. However, it seems unlikely that oxidative stress enhances nSMase2 phosphorylation via activation of a PKC or p38 MAPK. Even though others have shown that PKC-δ and p38 can be activated by exposure to H₂O₂-induced oxidative stress (40–42), our data presented in Figs. 8 and 9 demonstrate unequivocally that once nSMase2 cannot bind CaN its phosphorylation (and function) could not be further enhanced by exposure to H₂O₂-induced oxidative stress. Therefore, we suggest (see also model in Fig. 9, below) that only the inhibition of CaN by H₂O₂ oxidative stress shuts off the phosphatase and enables nSMase2 to be fully phosphorylated and activated downstream of PKC and/or p38 MAPK (38, 43, 44). Our studies also demonstrated (Fig. 6) that treatment with Cycl-A, a well known inhibitor of CaN (26, 27), triggers an increase of nSMase2 phosphorylation, whereas stimulation of CaN activity with ionomycin, a calcium ionophore (28), causes de-phosphorylation of nSMase2, confirming the regulation exerted by CaN on nSMase2 phosphorylation.

Of note is the down-regulation of CaN by Cycl-A. This drug is widely used in post-transplantation procedures because it prevents inflammatory responses generated by cytokine release, which are, in turn, generated by CaN-dependent activation of the NFAT cells (46). At the same time, this drug may also trigger severe adverse symptoms and systemic complications such as renal and neurotoxicity (47). This toxicity of Cycl-A is not well understood. One direction that should be further explored is whether cyclosporine affects sphingo-lipid metabolism through enhancing nSMase2 function, elevating cellular ceramide levels and thus enhancing cell death (26, 48, 49).

Recent studies provide a very strong case for cell death having a major role in lung injury in several pulmonary diseases (50–61). In simple terms, loss of cells by augmented apoptosis would be expected to be involved, or perhaps initiate, the overall tissue destruction responsible for lung injury (53–61). Although a link between reactive oxidants and epithelial injury in the lung has been established (62), the cellular and molecular mechanisms leading to epithelial dysfunction were poorly defined.

Progress has recently evolved in the understanding of the underlying mechanisms of these destructive lung processes. For example, Gulbins et al. (63) and Worgall et al. (64) have recently reported that ceramide accumulation mediates inflammation, cell death, and infection susceptibility in cystic fibrosis. Several other studies implicate sphingomyelin hydrolysis in acute lung injury (56) and pulmonary edema (65). It was reported that ceramide may be a critical mediator of endothelial and alveolar cell apoptosis in the vascular endothelial growth factor mouse model of chronic obstructive pulmonary disease (56). Also, it has been shown (66) that superoxide dismutase protects against apoptosis and alveolar enlargement induced by ceramide. However, multiple questions still emerge (67), and additional characterization of the ceramide pathway that leads to lung injury is still needed, given that cigarette smoke is a predominant cause of chronic obstructive pulmo-
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Aramburu, J., García-Cózar, F., Raghavan, A., Okamura, H., Rao, A., and Hogan, P. G. (1998) Mol. Cell 1, 627–637

Chen, Y., Zhao, Y. H., and Wu, R. (2001) Am. J. Respir. Cell Mol. Biol. 25, 409–417

Robinson, C. B., and Wu, R. (1991) Methods in Cell Science 13, 95–102

Chan, C., and Goldkorn, T. (2000) Am. J. Respir. Cell Mol. Biol. 22, 460–468

Hardin, S. C., and Wolniak, S. M. (1998) BioTechniques 24, 344–346

Okazaki, T., Bielawska, A., Domae, N., Bell, R. M., and Hannun, Y. A. (1994) J. Biol. Chem. 269, 4070–4077

Lawler, J. F., Jr., Yin, M., Diehl, A. M., Roberts, E., and Chatterjee, S. (1998) J. Biol. Chem. 273, 5053–5059

Clarke, C. J., Truong, T. G., and Hannun, Y. A. (2007) J. Biol. Chem. 282, 1384–1396

Clarke, C. J., Guthrie, J. M., and Hannun, Y. A. (2008) Mol. Pharmacol. 74, 1022–1032

Xiong, W., Lojic, L. Z., Zhang, L., Prasad, S. S., Douglas, R., Wang, Y., and Cytnader, M. S. (2006) Brain Res. 1085, 68–76

Ikeda, T., Kajita, K., Zhiliang, W., Hamamoto, T., Mori, I., Fujikoa, K., Okada, H., Fujikake, T., Uno, Y., Morita, H., Nagano, I., Takahashi, Y., and Ishizu, T. (2009) JIBMB Life 61, 644–650

Heit, I., Wieser, R. J., Herget, T., Faust, D., Borchert-Stuhlräger, M., Oesch, F., and Dietrich, C. (2001) Oncogene 20, 5143–5154

Lee, J. E., Kim, H., Jang, H., Cho, E. J., and Youn, H. D. (2007) J. Neurochem. 100, 1703–1712

Eckstein, L. A., Van Quill, K. R., Bui, S. K., Uusitalo, M. S., and O'Brien, J. M. (2005) Invest. Ophthalmol. Vis. Sci. 46, 782–790

Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) Cell 66, 807–815

Davé, V., Childs, T., and Whitsett, J. A. (2004) J. Biol. Chem. 279, 34578–34588

Rodriguez, A., Roy, J., Martínez-Martínez, S., López-Maderuelo, M. D., Niño-Morenno, P., Ortí, L., Pantajo-Uceda, D., Pineda-Lucena, A., Cyert, M. S., and Redondo, J. M. (2009) Mol. Cell 33, 616–626

Cifone, M. G., De Maria, R., RoncaI, P., Rippo, M. R., Azuma, M., Lanier, L. L., Santoni, A., and Testi, R. (1994) J. Exp. Med. 180, 1547–1552

Jayadve, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Yu, Obeid, L. M., and Hannun, Y. A. (1995) J. Biol. Chem. 270, 2047–2052

Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krönke, M. (1992) Cell 71, 765–776

Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krönke, M. (1994) Cell 78, 1005–1015

Zeidan, Y. H., Wu, B. X., Jenkins, R. W., Obeid, L. M., and Hannun, Y. A. (2008) FASEB J. 22, 183–193

Zeidan, Y. H., and Hannun, Y. A. (2007) J. Biol. Chem. 282, 11549–11561

Chan, E. M., Lanir, R., Danielson, A. R., and Goldkorn, T. (2008) FASEB J. 22, 910–917

Lim, H. W., New, L., and Molkentin, J. D. (2001) J. Biol. Chem. 276, 15913–15919

Szügyáró, Z., Szucs, K., Kovács, L., Zákány, R., Sipka, S., and Gergely, P. (2007) Int. J. Mol. Med. 20, 359–364

Marchesini, N., Osta, W., Bielawska, J., Luberto, C., Obeid, L. M., and Hannun, Y. A. (2004) J. Biol. Chem. 279, 25101–25111

Halliwell, B., and Gutteridge, J. M. (1990) Methods Enzymol. 186, 1–85

Rahman, I. (2005) Mutat. Res. 579, 58–80

Henson, P. M., and Tudor, R. M. (2008) Am. J. Physiol. Lung Cell Mol. Physiol. 294, 601–611

Braz, J. C., Bueno, O. F., Liang, Q., Wilkins, B. J., Dai, Y. S., Parsons, S., and Hannun, Y. A. (2008) Curr. Clin. Pharmacol. 3, 166–173

Jung, Y. J., Jeong, Y. J., Jeong, T. S., Chung, H. J., and Kim, W. J. (2008) Arch. Oral Biol. 53, 1042–1049

REFERENCES

1. Levy, M., Castillo, S. S., and Goldkorn, T. (2006) Biochem. Biophys. Res. Commun. 344, 900–905

2. Levy, M., Khan, E., Careaga, M., and Goldkorn, T. (2009) Am. J. Physiol. Lung Cell Mol. Physiol. 297, 125–133

3. Castillo, S. S., Levy, M., Thaikoottathil, J. V., and Goldkorn, T. (2007) Exp. Cell Res. 313, 2680–2686

4. Castillo, S. S., Levy, M., Wang, C., Thaikoottathil, J. V., Khan, E., and Goldkorn, T. (2007) Exp. Cell Res. 313, 816–823

5. Goldkorn, T., Ravid, T., and Khan, E. M. (2005) Antioxid. Redox Signal 7, 119–128

6. Ravid, T., Tsaba, A., Gee, P., Rasooly, R., Medina, E. A., and Goldkorn, T. (2003) Am. J. Physiol. Lung Cell Mol. Physiol. 284, L1082–L1092

7. Goldkorn, T., Balaban, N., Shannon, M., Chea, V., Matsukuma, K., Gilchrist, D., Wang, H., and Chan, C. (1998) J. Cell Sci. 111, 3209–3220

8. Lavrentiadou, S. N., Chan, C., Kawcak, T., Ravid, T., Tsaba, A., van der Vliet, A., Rasooly, R., and Goldkorn, T. (2001) Am. J. Respir. Cell Mol. Biol. 25, 676–684

9. Merrill, A. H., Jr., and Jones, D. D. (1990) Biochim. Biophys. Acta 1044, 1–12

10. Hofmann, K., Tomiak, S., Wolff, G., and Stoffel, W. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 5895–5900

11. Khan, E. M., Lanir, R., Danielson, A. R., and Goldkorn, T. (2008) FASEB J. 22, 910–917

12. Bogumil, R., Namgaladze, D., Scharschmidt, D., Schmachtel, T., Hellstern, S., Mutzel, R., and Ullrich, V. (2000) Eur. J. Biochem. 267, 1407–1415

4 S. Filosto, S. S. Castillo, A. Danielson, I. Franzl, E. Khan, N. Kenyon, J. Last, K. Pinkerton, R. Tudor, and T. Goldkorn, submitted for publication.
49. Bezombes, C., Maestre, N., Laurent, G., Levade, T., Bettaieb, A., and Jaffrézou, J. P. (1998) *FASEB J.* **12**, 101–109
50. Kang, H. R., Cho, S. J., Lee, C. G., Homer, R. J., and Elias, J. A. (2007) *J. Biol. Chem.* **282**, 7723–7732
51. Park, I. W., Ryter, S. W., and Choi, A. M. (2007) *COPD* **4**, 347–353
52. Yoshida, T., and Tuder, R. M. (2007) *Physiol. Rev.* **87**, 1047–1082
53. Elias, J. A., and Lee, C. G. (2005) *Nat. Med.* **11**, 471–472
54. Henson, P. M., Vandivier, R. W., and Douglas, I. S. (2006) *Proc. Am. Thorac. Soc.* **3**, 713–717
55. Kasahara, Y., Tuder, R. M., Taraseviciene-Stewart, L., Le Cras, T. D., Abman, S., Hirth, P. K., Waltenberger, J., and Voelkel, N. F. (2000) *J. Clin. Invest.* **106**, 1311–1319
56. Petrache, I., Natarajan, V., Zhen, L., Medler, T. R., Richter, A. T., Cho, C., Hubbard, W. C., Berdyshhev, E. V., and Tuder, R. M. (2005) *Nat. Med.* **11**, 491–498
57. Melgert, B. N., Timens, W., Kerstjens, H. A., Geerlings, M., Luinge, M. A., Schouten, J. P., Postma, D. S., and Hylkema, M. N. (2007) *Clin. Exp. Allergy* **37**, 1798–1808
58. Rennard, S. I., Togo, S., and Holz, O. (2006) *Proc. Am. Thorac. Soc.* **3**, 703–708
59. Segura-Valdez, L., Pardo, A., Gaxiola, M., Uhal, B. D., Becerril, C., and Selman, M. (2000) *Chest* **117**, 684–694
60. Spurzem, J. R., and Rennard, S. I. (2005) *Semin. Respir. Crit. Care Med.* **26**, 142–153
61. Elias, J. A., Kang, M. J., Crothers, K., Homer, R., and Lee, C. G. (2006) *Proc. Am. Thorac. Soc.* **3**, 494–498
62. Rahman, I. (2008) *Ther. Adv. Respir. Dis.* **2**, 351–374
63. Teichgräber, V., Ulrich, M., Endlich, N., Riethmüller, J., Wilker, B., De Oliveira-Munding, C. C., van Heekeren, A. M., Barr, M. L., von Kürthy, G., Schmid, K. W., Weller, M., Tümmler, B., Lang, F., Grassme, H., Döring, G., and Gulbins, E. (2008) *Nat. Med.* **14**, 382–391
64. Hamai, H., Keyserman, F., Quittell, L. M., and Worgall, T. S. (2009) *J. Lipid Res.* **50**, 1101–1118
65. Göggel, R., Winoto-Morbach, S., Vielhaber, G., Imai, Y., Lindner, K., Brade, L., Brade, H., Ehlers, S., Slutsky, A. S., Schütze, S., Gulbins, E., and Uhlig, S. (2004) *Nat. Med.* **10**, 155–160
66. Petrache, I., Medler, T. R., Richter, A. T., Kamocki, K., Chukwueke, U., Zhen, L., Gu, Y., Adamowicz, J., Schweitzer, K. S., Hubbard, W. C., Berdyshhev, E. V., Lungarella, G., and Tuder, R. M. (2008) *Am. J. Physiol. Lung Cell Mol. Physiol.* **295**, L144–L153
67. Zimmerman, G. A., and McIntyre, T. M. (2004) *Trends Mol. Med.* **10**, 245–248
68. Wright, J. L., Cosio, M., and Churg, A. (2008) *Am. J. Physiol. Lung Cell Mol. Physiol.* **295**, L1–L15