Role for Neutral Sphingomyelinase-2 in Tumor Necrosis Factor α-Stimulated Expression of Vascular Cell Adhesion Molecule-1 (VCAM) and Intercellular Adhesion Molecule-1 (ICAM) in Lung Epithelial Cells

p38 MAPK IS AN UPSTREAM REGULATOR OF nSMase2*

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Neutral sphingomyelinases (N-SMases) are major candidates for stress-induced ceramide production. However, there is little information on the physiological regulation and roles of the cloned N-SMase enzyme, nSMase2. In this study, nSMase2 was found to translocate acutely to the plasma membrane of A549 epithelial cells in response to tumor necrosis factor α (TNF-α) in a time- and dose-dependent manner. Additionally, TNF-α increased N-SMase activity rapidly and transiently both endogenously and in cells overexpressing nSMase2. Furthermore, the translocation of nSMase2 was regulated by p38-α MAPK, but not ERK or JNK, and the increase in endogenous N-SMase activity was abrogated by p38 MAPK inhibition. In addition, both p38-α MAPK and nSMase2 were implicated in the TNF-α-stimulated up-regulation of the adhesion proteins vascular cell adhesion molecule-1 (VCAM) and intercellular adhesion molecule-1 (ICAM), but this was largely independent of NF-κB activation. These data reveal p38 MAPK as an upstream regulator of nSMase2 and indicate a role for nSMase2 in pro-inflammatory responses induced by TNF-α as a regulator of adhesion proteins.

The bioactive role of ceramide in the cellular responses to stress is well established (1). The sphingomyelinase (SMase)2-mediated hydrolysis of sphingomyelin is emerging as a major pathway of stress-induced ceramide production, and, at present, five types of SMases have been identified (reviewed in Ref. 2). Of these, the Mg2+−−dependent neutral SMases (N-SMases) are prime candidates for stress-induced ceramide production. To date, two proteins with both in vitro and in vivo N-SMase activity have been cloned and termed nSMase2 and nSMase3 (3,4). Previous research found that confluence induces translocation of nSMase2 to the plasma membrane (PM), resulting in an increase of ceramide levels (5). H2O2 also induces translocation of nSMase2 to the PM in human airway epithelial cells. Importantly, ceramide generation induced by H2O2 is prevented by nSMase2 siRNA (6). Together, this suggests that translocation to the PM may constitute an important mechanism for nSMase2-mediated functions. However, the regulation of this process has not been investigated.

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine, important in mediating systemic inflammatory and immune responses (7). The binding of TNF-α to two PM receptors (p55 and p75) activates a number of signaling cascades, including mitogen-activated protein kinases (MAPKs) and NF-κB (reviewed in Ref. 8). TNF-α has also been shown to activate SMases, producing ceramide and other downstream signaling lipids such as sphingosine-1-phosphate (S1P) (9, 10). Importantly, TNF-α was found to activate both nSMase2 and nSMase3 (4, 11). Furthermore, N-SMase activation has been implicated in TNF-α-stimulated COX-2 induction in airway epithelial cells (12), and a recent study in TNF-α-stimulated HUVEC cells found that nSMase2 was upstream of endothelial nitric-oxide synthase activation (13). Moreover, in hepatocytes, nSMase2 is found constitutively at the PM, where it has been implicated in the action of the pro-inflammatory cytokine interleukin 1 on JNK phosphorylation and activation (14). As these are pro-inflammatory pathways, this suggests that N-SMase and, specifically nSMase2, may be important for TNF-α-induced inflammatory signaling.

The regulated expression of adhesion proteins such as vascular cell adhesion molecule-1 (VCAM) and intercellular adhesion molecule-1 (ICAM) on the surface of leukocytes, epithelial cells, and vascular endothelial cells is essential for controlling migration of cells, especially in inflammatory diseases (15, 16) and in metastasis of cancer cells (17). Previously, TNF-α was shown to up-regulate VCAM and ICAM in HUVECs and A549 cells (18–21). Although NF-κB activation was reported as crucial for this process, evidence also suggests the existence of additional regulatory pathways. For example, p38 MAPK was implicated in the expression of VCAM, but not ICAM, in TNF-α-stimulated A549 cells (18, 20, 21). Furthermore, there is

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2 The abbreviations used are: SMase, sphingomyelinase; N-SMase, neutral SMase; PM, plasma membrane; siRNA, small interference RNA; TNF, tumor necrosis factor; S1P, sphingosine-1-phosphate; HUVEC, human umbilical vein endothelial cell; VCAM, vascular cell adhesion molecule-1; ICAM, intercellular adhesion molecule-1; PBS, phosphate-buffered saline; Scr, scrambled; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.
evidence implicating sphingolipids in the regulation of adhesion proteins. Studies in TNF-α-stimulated HUVEC cells report a role for sphingosine kinase and S1P in VCAM and ICAM expression and the glycosphingolipid lactosylceramide in ICAM expression (22–24); in all cases, this was through regulation of the NF-κB pathway. However, there is conflicting evidence as to the role of ceramide in expression of adhesion proteins, with reports that ceramide generated by bacterial SMase treatment is able to induce expression of VCAM and ICAM whereas exogenously added ceramide cannot (22, 25). Thus, the involvement of ceramide may be specific to that generated endogenously. However, to date, no study has investigated the role of endogenous ceramide-producing enzymes in regulation of adhesion proteins.

In this study, we sought to investigate the regulation of nSMase2 in a TNF-α-stimulated inflammatory context and its role in the up-regulation of adhesion proteins. We found that TNF-α induces acute translocation and activation of nSMase2 in A549 cells and this requires p38 MAPK. Furthermore, both nSMase2 and p38-α MAPK are involved in TNF-α-stimulated VCAM and ICAM induction, and this is independent of NF-κB activation. These data shed light on the role of nSMase2 in a physiological model of inflammation and, for the first time, identify p38 MAPK as an upstream regulator of nSMase2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and siRNA**—A549 cells were maintained in 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium (Invitrogen) at 37 °C in 5% CO₂. For siRNA experiments, cells were seeded in 60-mm dishes (5–7.5 × 10⁴/dish). After 24 h, cells were transfected with scramble or specific siRNA (20 nM) using Oligofectamine according to the manufacturer’s protocol. Cells were allowed to grow for 48 h (p38-α, nSMase3) or 72 h (nSMase2) prior to experiments. siRNA oligonucleotides for Scr and...
nSMase2 were as described previously (5). Predesigned siRNA oligonucleotides for nSMase3 (number 55627) were purchased from Qiagen.

**Immunofluorescence and Confocal Microscopy—**MCF-7 cells were incubated with brefeldin A (2.5 μg/ml) for 1 h prior to fixation. For MAPK inhibitor experiments, A549 cells were preincubated with PD98059, SP600125, or SB202190 (10 μM or varying concentrations as indicated) for 1 h prior to stimulation. Cells were fixed with paraformaldehyde (4%, 10 min), permeabilized with 100% methanol (5 min, −20 °C), and blocked with 2% human serum in PBS (>30 min, room temperature). Cells were probed with anti-V5 antibody (1:200) and anti-giantin (1:200) in 2% serum (2 h, room temperature), washed (3× PBS), probed with fluorescent secondary antibody (1:200 anti-mouse 488; 1:100 anti-rabbit Rhodamine; 30–45 min, room temperature, dark) and washed (3× PBS). Cells were viewed on a Zeiss LSM 510 Meta Confocal Microscope.

**Immunoblotting—**Protein samples were separated on 4–20% gradient gels (Bio-Rad Criterion) at 60–100 V before transfer to nitrocellulose membrane in Tris/glycine buffer (100 V, 30 min, 4 °C). Membranes were blocked (5% milk, 30 min) and probed with primary antibody overnight (4 °C). Membranes were washed (3× 0.1% Tween Tris-buffered saline), probed with horseradish peroxidase-conjugated secondary antibody (1:5000 mouse or rabbit in 5% milk) for 30–45 min at room temperature, and washed (3× 0.1% Tween Tris-buffered saline). Proteins were visualized by enhanced chemiluminescence (Pierce).

**Cell Adhesion Assay—**A549 cells (2 × 10⁴) were seeded in glass-bottomed 6-well trays (Corning). After 24 h, cells were transfected with Scr or nSMase2 siRNA (20 nM, 72 h) before stimulation with TNF-α (50 ng/ml or vehicle for 3 h). Meanwhile, U87 cells were labeled with Green 5-chloromethylfluorescein diacetate (Molecular Probes) according to the manufacturer’s instructions and were trypsinized. Following TNF-α

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**FIGURE 2.** nSMase2 siRNA decreases adhesion of U87 cells to A549 cells. A549 cells (2 × 10⁴) were seeded in glass-bottomed 6-well dishes and after 24 h were transfected with Scr or nSMase2 siRNA (20 nM, 72 h). Cells were stimulated with TNF-α (3 nM) for 3 h and subsequently incubated with fluorescently labeled U87 cells (1 × 10⁵) at 37 °C. After 1 h, cells were fixed with 4% paraformaldehyde and fluorescent cells viewed and quantified by confocal microscopy. A, effect of nSMase2 siRNA on U87 adhesion to A549 cells. Representative of at least 10 fields taken from each of triplicate experiments on two independent occasions. B, quantification of U87 adhesion to A549 cells. Cell number was normalized for protein content of identically treated wells without addition of U87 cells. (*, p < 0.05, n = 2).
stimulation of A549 cells, U87 cells were added in fresh medium (1 × 10^5 cells/well), and cells were incubated at 37 °C for 1 h before fixation with 4% paraformaldehyde. Fluorescent U87 cells were visualized on a Zeiss LSM 510 Meta Confocal Microscope. For quantification, U87 cells in 15 random fields were counted. Numbers were normalized to A549 protein levels, determined by BCA assay (26) from parallel trays without U87 cells.

In Vitro Sphingomyelinase Assay—Neutral sphingomyelinase assay was assessed in vitro using [choline-methyl-14C]sphingomyelin as described previously (5, 11).

Immunoprecipitation—Following stimulation with TNF-α (50 ng/ml; 0, 10, 30 min), mock- or V5-transfected cells were lysed in immunoprecipitation buffer by sonication (3 × 10 s, 2 intensities), and unbroken cells were pelleted by centrifugation at 3000 rpm for 5 min. Following protein estimation by Bradford assay (27), equal protein from each sample was precleared with 30 μl of Protein A/G-agarose-conjugated beads (Santa Cruz Biotechnology) for 1 h at 4 °C. Samples were then rotated overnight with primary antibody (1 μg) at 4 °C. The following day, 50 μl of beads were added and samples rotated for at least 3 h at 4 °C. Beads were washed (2× PBS) and vortexed well with 2× sample buffer to obtain immunoprecipitates. Both supernatants and immunoprecipitates were boiled for 5–10 min before analysis by SDS-PAGE and immunoblotting as described above.

Real-time Reverse Transcription PCR—mRNA was extracted from A549 cells treated with Scr or nSMase3 siRNA, and nSMase3 mRNA levels were quantified by real-time reverse transcription PCR as described previously (5).

Statistical Analysis—Comparisons between two groups were analyzed by Student’s t test. p < 0.05 was considered statistically significant with n = number of experiments as indicated.

Materials—Monoclonal anti-V5 antibody was from Invitrogen. Polyclonal giantin was from Covance. Polyclonal phospho-p38 was from Promega (Madison, WI). Polyclonal GM130 (p-20), polyclonal anti-p38 MAPK (clone C-20), polyclonal anti-IκB (C-21), monoclonal anti-actin (clone C-2), monoclonal ICAM (G-5), and polyclonal VCAM (H-276) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent secondary antibodies were from Jackson Laboratories. Predesigned nSMase2 siRNA (5) and unvalidated nSMase3 siRNA (number 55627) were from Qiagen. Prevalidated p38-α MAPK siRNA (number 1312) was from Ambion Inc. (Austin, TX). Human recombinant TNF-α was purchased from Preprotech Inc. [choline-methyl-14C]Sphingomyelin was provided by Dr. Alicja Bielawska (Medical University of South Carolina, Charleston, SC). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Scintillation mixture Safety Solve was from Research Products International.

RESULTS

Role for nSMase2 in TNF-α-stimulated Induction of VCAM and ICAM—Evidence suggests that nSMase2 may be important for TNF-α-stimulated inflammatory signaling (13). Therefore, the role of nSMase2 in TNF-α-stimulated induction of the adhesion proteins VCAM and ICAM in lung epithelial cells was investigated. Using siRNA as previously reported (5), the expression of nSMase2 was down-regulated. A549 cells were treated with scrambled RNA (Scr) or nSMase2 siRNA (20 nM, 72 h) and stimulated with TNF-α (50 ng/ml) or vehicle (PBS) for 0, 3, 6, or 12 h, and levels of VCAM and ICAM were analyzed.
FIGURE 4. Localization of V5-tagged nSMase2. Cells were seeded in 35-mm confocal dishes and 48 h later were transfected with 3’-V5-tagged nSMase2 (0.25 μg/dish) for 12 h. Medium was changed, and cells were allowed to grow for 6–12 h. Cells were fixed and stained with anti-V5 (green) and anti-giantin (red) or anti-V5 (red) and anti-GM130 (green) as described under “Experimental Procedures.” A, localization of nSMase2 in subconfluent (2.5 × 10^4/dish) and confluent (7–9 × 10^4/dish) MCF-7 cells. B, localization of nSMase2 in subconfluent (2.5 × 10^4/dish) MCF-7, HeLa, human embryonic kidney, and A549 cells. C, effect of brefeldin pretreatment (2.5 μg/ml, 1 h prior to fixation) on localization of nSMase2 and giantin. (Pictures are representative of 5 fields taken from at least four independent experiments).
p38 MAPK Regulates nSMase2 in Response to TNF-α

By immunoblotting, down-regulation of nSMase2 significantly attenuated the TNF-α-stimulated induction of VCAM and ICAM at all time points. In the presence of nSMase2 siRNA, VCAM levels were reduced by 56.1% at 3 h, 49.6% at 6 h, and 32.7% at 12 h (Fig. 1, A and B) whereas ICAM levels were reduced by 51.8% at 3 h, 39.6% at 6 h, and 34.3% at 12 h when compared with Scr controls (Fig. 1, A and C). Thus, nSMase2 is important for TNF-α-stimulated induction of VCAM and ICAM in A549 cells.

To confirm the functional importance of nSMase2 as a regulator of adhesion proteins, cell adhesion assays were performed. Briefly, Scr or nSMase2 siRNA treated A549 cells were incubated with fluorescently labeled U87 cells for 1 h at 37 °C, washed three times with PBS, and fixed with paraformaldehyde for quantification by confocal microscopy (Fig. 2). Results showed that TNF-α (50 ng/ml, 3 h) more than doubled the adhesion of U87 cells to A549 cells, consistent with the up-regulation of VCAM and ICAM. Furthermore, whereas nSMase2 siRNA had no significant difference on basal cell-cell adhesion, the increase in cell-cell adhesion induced by TNF-α was markedly reduced, consistent with nSMase2 siRNA effects on adhesion proteins. Thus, nSMase2 plays a role in cell-cell adhesion and as a regulator of the adhesion proteins VCAM and ICAM.

To determine whether other N-SMases may also play a role in VCAM and ICAM induction, siRNA against the recently cloned nSMase3 (4) was utilized. To validate the siRNA, A549 cells were transfected with Scr or nSMase3 siRNA (20 nM, 48 h) and nSMase3 mRNA levels were analyzed by realtime reverse transcription PCR. Treatment of A549 cells with nSMase3 siRNA (20 nM, 48 h) dramatically reduced nSMase3 mRNA to 17.2 ± 10.0% of Scr-treated controls (Fig. 3A; p < 0.01, n = 2). Utilizing this siRNA, the role of nSMase3 in VCAM and ICAM induction was investigated. Down-regulation of nSMase3 showed a significant effect on VCAM and ICAM induction only at 3 h of TNF stimulation compared with Scr controls (p < 0.05, n = 4), and this effect was less than that observed with nSMase2 siRNA (Fig. 3B). Furthermore, although levels of both ICAM and VCAM were still slightly reduced at 6 and 12 h of stimulation, this was not statistically significant when compared with Scr controls (Fig. 3B). This suggests that nSMase3 may also play a role in induction of VCAM and ICAM, albeit comparatively minor to that of nSMase2.

Localization of a V5-tagged nSMase2—Next, studies were conducted to establish the subcellular localization of nSMase2 and to examine possible translocation of the enzyme in response to TNF-α. Although, a FLAG-tagged nSMase2 was previously used (5), a high background signal was observed with anti-FLAG antibodies. Accordingly, a construct of nSMase2 tagged at the C terminus with a V5 epitope, a short peptide sequence from Paramyxovirus SV5 (28), was constructed. To confirm that the V5 tag had no effects on nSMase2 localization, sequencing from Paramyxovirus SV5 (28), was constructed. To confirm that the V5 tag had no effects on nSMase2 localization, a high background signal was observed with anti-FLAG antibodies. Accordingly, a construct of nSMase2 tagged at the C terminus with a V5 epitope, a short peptide sequence from Paramyxovirus SV5 (28), was constructed. To confirm that the V5 tag had no effects on nSMase2 localization, sequencing from Paramyxovirus SV5 (28), was constructed.
thermore, treatment of subconfluent MCF-7 cells with brefeldin A for 1 h disrupted the Golgi, resulting in a diffuse distribution of both giantin and V5-nSMase2. This confirms localization of V5-nSMase2 to the Golgi (Fig. 4C) and agrees with previous studies on nSMase2 localization (3). Importantly, in confluent MCF-7 cells, V5-nSMase2 localized at the plasma membrane (Fig. 4A) in agreement with our previous study in MCF-7 cells utilizing a FLAG-tagged nSMase2 (5). Finally, overexpression of V5-nSMase2 significantly increased N-SMase activity of MCF-7 cells, confirming that V5-nSMase2 is active (data not shown).

nSMase2 Is a TNF-α-responsive Enzyme in A549 Cells—Previously it was shown that nSMase2 is TNF-α-responsive in MCF-7 cells by measuring enzyme activity (11). Therefore, it became important to determine whether TNF-α regulated nSMase2 in A549 lung epithelial carcinoma cells. Moreover, we were specifically interested to determine whether TNF-α regulated the subcellular localization of the enzyme. In unstimulated A549 cells, V5-nSMase2 strongly co-localized with the Golgi marker giantin, consistent with results from MCF-7, HeLa, and human embryonic kidney cells (Fig. 4B). Stimulation with TNF-α (50 ng/ml) induced a rapid (5–10 min) translocation to the PM that was sustained up to 60 min (Fig. 5A). To further characterize the TNF-α-induced translocation of nSMase2, a dose response at 30 min of stimulation was performed. At concentrations of 1 and 10 ng/ml TNF-α nSMase2 was intracellular, mainly localizing to the Golgi. However, concentrations of 25 ng/ml TNF-α and higher were sufficient to induce PM translocation of nSMase2 (Fig. 5B).

As confirmation that nSMase2 is a TNF-responsive enzyme, N-SMase activity was assayed in A549 cells with and without nSMase2 overexpression. In both cases, TNF-α rapidly and transiently increased N-SMase activity, with peak activity observed at 5 min (Fig. 5C). Furthermore, the remarkably similar effects of TNF-α on endogenous and overexpressed N-SMase activity suggests that nSMase2 is a major contributor to TNF-α-stimulated N-SMase activity in A549 cells.

Role of p38-MAPK in TNF-α-induced Induction of VCAM and ICAM—A previous study implicated p38 MAPK in the regulation of VCAM, but not ICAM, in response to TNF-α (18). Therefore, it became important to determine the role of p38 MAPK with respect to nSMase2 in mediating the effects of TNF-α. Initially, studies were conducted to investigate the role of specific MAPKs in mediating the actions of TNF-α. Therefore, the effects of inhibitors of ERK-1/2, JNK, or p38 MAPK on induction of ICAM and VCAM were investigated. A549 cells were treated with 10 μM PD98059, SP600125, or SB202190 for 1 h prior to stimulation with TNF-α (50 ng/ml) for 3 h. Neither ERK nor JNK inhibition significantly reduced VCAM or ICAM induction; levels of both proteins were significantly increased compared with controls. However, p38-MAPK inhibition significantly attenuated the induction of VCAM (52.3% reduction) and modestly attenuated the induction of ICAM (21.2% reduction) (Fig. 6A).

To corroborate these results and to further explore the role of p38 MAPK in induction of adhesion proteins, siRNA specific to p38-α, a ubiquitous p38 isoform, was utilized. Transfection of A549 cells with p38-α siRNA (20 nm, 48 h) significantly down-regulated p38 MAPK protein levels to 30.1 ± 4.8% of Scr controls, confirming its effectiveness (Fig. 6B). Importantly, down-regulation of p38-α MAPK significantly reduced VCAM expression at all time points (Fig. 6, B and C), consistent with the effect of p38 MAPK inhibitors with levels reduced by 61.2% at 3 h, 58.4% at 6 h, and 29.7% at 12 h. Furthermore,
induction of ICAM was also inhibited with levels reduced by 31.6% at 3 h, 29.5% at 6 h, and 24.1% at 12 h (Fig. 6, B and D). Thus, p38-α MAPK, but not other MAPKs, is an upstream regulator of adhesion proteins in TNF-α-stimulated A549 cells.

nSMase2 and p38 Do Not Act Upstream of NF-κB Activation—Considerable evidence in the literature has implicated NF-κB transcriptional regulation of VCAM and ICAM in response to TNF-α (18–21). To determine whether nSMase2 and p38-α MAPK act upstream of this pathway, the effects of nSMase2 or p38-α MAPK down-regulation on activation of the NF-κB pathway by TNF-α were examined. Because the primary mechanism of action of TNF-α involves the induction of loss of IκB-α, the inhibitory protein for NF-κB, the levels of IκB-α in response to TNF-α stimulation between 0–60 min were determined. TNF-α induced a rapid and transient decrease in IκB-α protein levels (Fig. 7) consistent with previous reports (29). However, down-regulation of either nSMase2 (Fig. 7A) or p38-α MAPK (Fig. 7B) had no significant effects on either basal IκB-α levels or IκB-α degradation. These results suggest that both nSMase2 and p38-α MAPK regulate induction of VCAM and ICAM either independently of or downstream of NF-κB activation in regulating adhesion proteins.

TNF-α-stimulated nSMase2 Translocation Is Mediated by p38 MAPK but Not ERK or JNK—Taken together, the above results suggested that p38 MAPK and nSMase2 may act within the same pathway. Accordingly, connections between p38 and nSMase2 were explored. Preincubation of A549 cells with inhibitors of ERK, JNK, or p38 MAPK for 1 h had no significant effect on basal localization of nSMase2 (Fig. 8A). Also, neither ERK (PD98059) nor JNK (SP600125) inhibition had an effect on the TNF-α-stimulated nSMase2 translocation to the PM (50 ng/ml, 30 min). However, inhibition of p38 MAPK (SB202190) prevented this translocation with nSMase2, remaining localized predominately to the Golgi (Fig. 8A). To confirm that this was not due to nonspecific effects of the inhibitor, a dose response of SB202190 on TNF-α-stimulated V5-nSMase2 translocation was established (Fig. 8B). Results indicated that a concentration of SB202190 as low as 100 nM was sufficient to inhibit nSMase2 translocation, further suggesting that the observed effect was due to p38 MAPK inhibition and was not a result of nonspecific effects.

To further implicate activation of p38 MAPK in nSMase2 translocation, A549 cells were stimulated with anisomycin (50 μM), previously shown to activate p38 MAPK, ERK, and JNK in epithelial cells (30). Consistent with this, stimulation with anisomycin for 60 min was sufficient to induce V5-nSMase2 translocation to the plasma membrane (Fig. 8C). Furthermore, the down-regulation of p38-α MAPK with siRNA prevented nSMase2 translocation to the PM (Fig. 8D), consistent with the effects of the pharmacological inhibitor. Finally, inhibition of p38 MAPK significantly reduced the TNF-α-stimulated increase in endogenous N-SMase activity observed at 5 min (Fig. 8E). Taken together, these results...
demonstrate that p38 MAPK functions as an upstream regulator of nSMase2.

Co-immunoprecipitation Studies on p38 MAPK and nSMase2—Translocation of proteins to the PM can often occur through direct interactions with other proteins, for example with phospholipase C-γ and growth factor receptors (31). To determine whether p38 MAPK and nSMase2 directly interact with each other, co-immunoprecipitation studies were carried out. Mock- or V5-nSMase2-transfected A549 cells were stimulated with TNF-α (3 nM) from 0–60 min. Protein samples were prepared and IκB analyzed by SDS-PAGE and immunoblotting. A, upper, quantification of IκB levels in the presence of Scr or nSMase2 siRNA (n = 4); lower, immunoblot is representative of four experiments. B, upper, quantification of IκB levels in the presence of Scr or p38-α siRNA (n = 4); lower, immunoblot is representative of four experiments.

FIGURE 7. nSMase2 and p38-α do not act upstream of NF-κB. A549 cells (4–6 × 10⁴) were seeded in 60-mm dishes and transfected with Scr or nSMase2 siRNA (20 nM, 72 h) (A) or Scr or p38-α siRNA (20 nM, 48 h) (B). Following siRNA treatment, cells were stimulated with TNF-α (3 nM) from 0–60 min. Protein samples were prepared and IκB analyzed by SDS-PAGE and immunoblotting. A, upper, quantification of IκB levels in the presence of Scr or nSMase2 siRNA (n = 4); lower, immunoblot is representative of four experiments. B, upper, quantification of IκB levels in the presence of Scr or p38-α siRNA (n = 4); lower, immunoblot is representative of four experiments.
increased the activity of both endogenous N-SMase and overexpressed nSMase2, providing further evidence that nSMase2 is TNF-α responsive in A549 cells. Moreover, the similarity in activity profiles between endogenous and nSMase2-overexpressing cells suggests that nSMase2 is a major contributor to TNF-stimulated N-SMase activity in this cell line. These results agree with the TNF-induced activation of nSMase2 in HUVEC cells (13) and are consistent with a role for nSMase2 in pro-inflammatory TNF-α signaling in lung epithelial cells.

Supporting this, results indicated a role for nSMase2 in up-regulation of the adhesion proteins VCAM and ICAM. The functional importance of nSMase2 in this pathway was further highlighted by the decrease in cell-cell adhesion observed between U87 glioma cells and nSMase2 siRNA-treated A549 cells. This is somewhat consistent with previous research in HUVEC cells implicating sphingolipids in expression of adhesion proteins although, rather than ceramide per se, sphingosine kinase and S1P were implicated in VCAM and ICAM expression and lactosylceramide in ICAM expression. (22–24).

However, as nSMase2 was recently reported to be upstream of S1P production in HUVEC cells (13), this suggests that nSMase2 would also play a role in expression of both VCAM and ICAM in endothelial cells. Interestingly, siRNA to the recently cloned nSMase3 (4) also had an effect on TNF-stimulated induction of VCAM and ICAM. The observed effects of nSMase3 siRNA at 3 h of TNF stimulation suggest possible redundancy of N-SMases in A549 cells. However, as the effects of nSMase3 siRNA on VCAM and ICAM induction at later time points are not statistically different and appear to be minor at best, it is equally plausible that nSMase3 plays an independent role as a regulator of VCAM and ICAM, but only in the initial phase of induction. Thus, this suggests that the regulation of VCAM and ICAM by sphingolipids could be more complex than anticipated. Further research on the stimulation and regulation of nSMase3 in A549 cells is being undertaken in our laboratory.

Previous research has indicated that the NF-κB pathway is a major regulator of both VCAM and ICAM expression in TNF-α-stimulated A549 cells (18–21). Interestingly, down-regulation of nSMase2 had no effect on TNF-α-stimulated degradation of IκB-α, the major inhibitory protein of NF-κB, suggesting that nSMase2 acts independently of NF-κB in regulating VCAM and ICAM. Moreover, as previous research in both TNF-α-stimulated A549 and HUVEC cells found that the sphingosine kinase-S1P pathway was upstream of NF-κB-mediated transcription and was a regulator of IκB-α phosphorylation and degradation (18, 22, 24), this suggests that the role of nSMase2 is also independent of sphingosine kinase and S1P in A549 cells. Thus, the observed effects may be due to cer-

**FIGURE 8.** p38 MAPK is an upstream regulator of nSMase2. A549 cells (2 × 10⁴) were seeded in 35-mm confocal dishes and 48 h later were transiently transfected with 3'-VS-tagged nSMase2 (0.25 µg/dish) for 12 h. Medium was changed, and cells were allowed to grow for 6–12 h and treated as follows. Cells were fixed and stained with anti-V5 (green) and anti-giantin (red) as described under "Experimental Procedures." A, cells were preincubated with PD98059, SP600125, or SB202190 (10 µM, 1 h) prior to stimulation with TNF-α (3 nM, 30 min). B, cells were preincubated with SB202190 (10–100 nM, 1 h) prior to stimulation with TNF-α (3 nM, 30 min). C, cells were stimulated with anisomycin (50 µM, 1 h), D, 24 h following seeding, and prior to transfection with 3'-VS nSMase2, cells were transfected with scr or p38α siRNA (20 nM) for 48 h. Subsequent to transfection with 3'-VS nSMase2, cells were stimulated with TNF-α (3 nM) for 30 min. Pictures are representative of 5 fields taken from four independent experiments. E, untransfected A549 cells were incubated with SB202190 (10 µM, 1 h) prior to stimulation with TNF-α (3 nM, 5 min) and endogenous N-SMase activity determined (*, p < 0.05, SB + TNF-α compared with TNF-α, n = 4).
amide or other downstream metabolites. Although this appears to conflict with the report of nSMase2 as upstream of sphingosine kinase and S1P (13), this could be due to differences between endothelial and epithelial cells.

The down-regulation of nSmase2 resulted in a marked decrease of VCAM and ICAM expression (40–50% at 3–6 h). This implies that NF-κB-independent pathways play a larger role in regulating adhesion proteins in A549 cells than previously thought. This may be dependent on TNF-α concentration as previous studies utilized 10 ng/ml TNF-α; significantly, this is below the level required for nSmase2 translocation (>25 ng/ml). Thus, at lower concentrations, NF-κB-dependent pathways play a larger role in VCAM and ICAM regulation. However, if TNF-α concentration increases and nSmase2 is activated, there is subsequent nSmase2-dependent activation of NF-κB-independent pathways. The nature of the NF-κB-independent pathway is currently under investigation.

To further define the pathway from TNF-α receptors to nSmase2 to the up-regulation of adhesion proteins, attention was focused on MAPKs as they are known to be involved in TNF-α-stimulated signaling (8). The inhibition of p38 MAPK, but not ERK or JNK, significantly reduced TNF-α-stimulated VCAM expression, an effect also confirmed utilizing specific p38-α MAPK siRNA and in agreement with a previous study in A549 cells (18). However, in contrast to this and other studies (18, 20, 21), inhibition of p38 MAPK also modestly attenuated TNF-α-stimulated ICAM expression. This was specific to p38 MAPK; indeed, JNK and ERK inhibition appeared to enhance ICAM (and VCAM) expression. More importantly, this was also confirmed by siRNA down-regulation of p38-α MAPK. As earlier studies utilized 10 ng/ml TNF-α in contrast to the 50 ng/ml used here, this suggests that p38 MAPK may play a more significant role in ICAM expression at higher concentrations of TNF-α. Certainly, as both pharmacological inhibition and siRNA down-regulation of p38 MAPK produced similar effects, the evidence for a role of p38 MAPK in both VCAM and ICAM expression in A549 cells is convincing. Given the discrepancy between this and earlier research, the effects of p38 MAPK on activation of the NF-κB pathway were investigated. In this case, there was no difference from previous work (18, 20, 21) as siRNA down-regulation of p38-α MAPK had no significant effect on IκB-α degradation, thus confirming that it functions independently of NF-κB activation.

The correlation between results with p38-α MAPK and nSmase2 siRNA led us to hypothesize that they functioned within the same pathway. Results showed that p38 MAPK is important for TNF-α-stimulated nSmase2 translocation to the PM, and this was indicated by several lines of evidence. 1) Pharmacological inhibition of p38 MAPK, but not ERK or JNK, prevented nSmase2 translocation to the PM. 2) This was not due to nonspecific inhibitor effects, as 100-fold lower inhibitor concentrations were still effective. 3) siRNA down-regulation of p38-α MAPK had similar inhibitory effects. 4) Anisomycin, previously shown to rapidly activate p38 MAPK in kidney epithelial cells (30), also induced nSmase2 translocation to the PM. Taken together, there is compelling evidence that p38 MAPK functions as an upstream regulator of nSmase2 in TNF-α-stimulated A549 cells. Furthermore, although nSmase2 localization could only be investigated utilizing an overexpressed tagged construct, the fact that inhibition of p38 MAPK
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FIGURE 9. Co-immunoprecipitation studies of p38 MAPK and nSMase2. A549 cells (2–4 × 10⁶) were seeded in 100-mm dishes and 48 h later were either mock-transfected or transiently transfected with 3'-V5-tagged nSMase2 (5 μg/dish) for 12 h. Medium was changed, and cells were allowed to grow for 6–12 h before TNF-α stimulation (0, 10, 30 min). V5 was immunoprecipitated from cell lysates as described under “Experimental Procedures.” Immunoprecipitates and supernatants were analyzed for V5-nSMase2 and p38 MAPK content by SDS-PAGE and immunoblotting. Shown is an immunoblot of V5 immunoprecipitates and supernatants, representative of at least four independent experiments. V5 is shown to confirm equality of immunoprecipitation.

FIGURE 10. nSMase2 does not regulate p38 MAPK phosphorylation. A549 cells (4–6 × 10⁶) were seeded in 60-mm dishes. A, after 24 h, dishes were stimulated with TNF-α (3 nm) for 0–60 min. Protein samples were prepared and both phospho-p38 and p38 MAPK levels analyzed by SDS-PAGE and immunoblotting. TNF-α induces rapid and transient phosphorylation of p38 MAPK in A549 cells. Immunoblot is representative of four separate experiments. B, after 24 h, dishes were transfected with Scr or nSMase2 siRNA (20 nm, 72 h). Following siRNA treatment, cells were stimulated with TNF-α (3 nm) for 10 min. Protein samples were prepared and both phospho-p38 and p38 MAPK levels analyzed by SDS-PAGE and immunoblotting. Upper, quantification of phospho-MAPK phosphorylation. Data are expressed as phospho-p38:p38 MAPK ratio, n = 4. Lower, Scr and nSMase2 siRNA on phospho-p38 MAPK; immunoblot is representative of at least four experiments.

also significantly reduced the TNF-α-stimulated increase in endogenous N-Smase activity at 5 min provides further supporting evidence that p38 MAPK regulates nSMase2. However, this regulation appears to be indirect as nSMase2 and p38 MAPK do not directly associate with each other. The signaling pathway leading from p38 MAPK to nSMase2 is the subject of further study. Furthermore, as nSMase2 siRNA does not affect the activation of p38 MAPK as assessed by phosphorylation, this also indicates that there is no feedback regulation of nSMase2 on p38 MAPK. Although this does not preclude a role for ceramide as a regulator of p38 MAPK phosphorylation/activation as reported in other studies (28, 29), it appears that nSMase2 is not involved, at least in A549 cells.

Taking these results together, a pathway may be proposed whereby TNF-α activates p38 MAPK, which induces activation of nSMase2 and this, in turn, leads to the up-regulation of VCAM and ICAM. Although our results do not preclude the involvement of other downstream metabolites of ceramide, they do suggest that nSMase2 is acting independently of sphingosine kinase and S1P in A549 cells as this pathway has previously been implicated in regulation of NF-κB, partly through regulating phosphorylation and degradation of IκB (21, 24), whereas nSMase2 functions independently of NF-κB activation. Furthermore, it is also unclear if the regulation of ICAM and VCAM downstream of nSMase2 occurs through the same signaling pathways. This is the subject of further study. It should also be noted that this study has been performed in an immortalized cell line model of inflammation of airway epithelium. Thus, a necessary continuation of this work is a confirmation of these results in an in vivo animal model or primary cultured cells. These steps are currently underway in our laboratory.

In summary, this study revealed acute translocation and activation of nSMase2 in response to TNF-α, has identified p38 MAPK as an upstream regulator of nSMase2, and has revealed a role for nSMase2 in the TNF-α-stimulated up-regulation of adhesion proteins. Given the involvement of VCAM and ICAM in the recruitment and migration of circulating cells to the alveolar compartments and extravascular spaces (15, 16), this suggests that nSMase2 could play a role in the development of several inflammatory lung diseases and may constitute a useful therapeutic target in the future.

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