Neutral sphingomyelinase inhibitor scyphostatin prevents and ceramide mimics mechanotransduction in vascular endothelium

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Czarny, Malgorzata, and Jan E. Schnitzer. Neutral sphingomyelinase inhibitor scyphostatin prevents and ceramide mimics mechanotransduction in vascular endothelium. Am J Physiol Heart Circ Physiol 287: H1344–H1352, 2004. First published May 13, 2004; 10.1152/ajpheart.00222.2004.—Recently, we showed that neutral sphingomyelinase (N-SMase) is concentrated at the endothelial cell surface in caveolae and is activated to produce ceramide in an acute and transient manner by increase in flow rate and pressure in rat lung vasculature (Czarny M, Liu J, Oh P, and Schnitzer JE, J Biol Chem 278: 4424–4430, 2003). Here, we report further on our investigations of this new acute mechanotransduction pathway. We employed three experimental models to explore the role of N-SMase and ceramides in mechanosignaling: 1) a cell-free, in vitro model using isolated luminal plasma membranes of rat lung endothelium; 2) a fluid shear stress model using monolayers of intact bovine aorta endothelial cell in culture; and 3) an in situ model using controlled perfusion of the rat lung vasculature. Scyphostatin, which specifically inhibited N-SMase but not acid SMase activity, prevented mechanooactivation of N-SMase as well as downstream tyrosine and mitogen-activated protein kinases. Cell-permeable ceramide analogs (N-acetylsphingosine, C2-ceramide, and N-hexanoylsphingosine, C6-ceramide) but not the inactive dihydroderivatives D2-ceramide and D6-ceramide (N-acetylphosphatidylethanolamine and N-hexanoylsphinganine, respectively) mimic rapid mechano-induced tyrosine phosphorylation of cell surface proteins as well as mecanoactivation of Src-like kinases and the extracellular regulated kinase pathway. The responses common to ceramide and mechanical stress were inhibited by genisteen, herbamycin A, and PP2, but not PP3, which suggests an obligate role of Src-like kinases in ceramide-mediating mechanotransduction. Ceramides also induced serine/threonine phosphorylation to activate the Akt/endothelial nitric oxide synthase pathway. Thus N-SMase at the plasma membrane in caveolae may be an upstream initiating mechanosensor, which acutely triggers mechanotransduction by generation of the lipid second messenger ceramide.

caveolae; endothelium; shear stress; mechanosignal transduction

FLUID SHEAR STRESS AND PRESSURE from blood flow maintain blood vessel tone and control tissue homeostasis. The luminal surface of endothelial cells lining blood vessels is directly exposed to the hemodynamic forces of the circulation and reacts rapidly to mechanostimulation from fluid stressors of the circulating blood by inducing signaling cascades, including kinase activation, to cause tyrosine phosphorylation of membrane proteins, as well as stimulation of both the Ras/Raf/MEK/ERK pathway and endothelial nitric oxide (NO) synthase (eNOS) to generate a critical mediator of signal transduction (22, 28, 29, 31). The mechanosignaling molecules such as eNOS (41). Direct mechanotransduction in caveolae has been detected including the activation of eNOS, N-SMase, and tyrosine kinases (9, 41, 43). Subjecting endothelial cells to chronic shear increases the density of caveolae at the cell surface and responsiveness of the cells to changes in shear stress (42).

Caveolae appear to have also a distinct lipid composition consisting of cholesterol, sphingolipids (sphingomyelin and glycosphingolipids), and phosphatidylglycerol. Those lipids and their metabolites are important signaling molecules. N-SMase in caveolae responds acutely and transiently to mechanostimulation by fluid shear stress (9). The activation of N-SMase initiates the sphingomyelin pathway, which leads to the generation of ceramide, an important lipid mediator of signal transduction (22, 28, 29, 31). The mechanism of ceramide biological activity may occur through perturbations of membrane bilayer by formation and maintenance of ceramide-rich microdomains (12, 30). Bacterial SMase (bSMase), which similarly to membrane-bound N-SMase cleaves sphingomyelin at the plasma membrane, induces formation of lipid microdomains (23). Ceramide interaction with other membrane components is an important factor in determining membrane lateral organization and physical properties, such as membrane curvature. Formation of ceramide microdomains facilitates Fas clustering and cap formation in Jurkat T lymphocytes (8) and clustering of CD95 and CD40 in B-lymphocytes to allow signaling to occur (14, 18, 19).

We (9) have recently shown that N-SMase in caveolae is acutely activated by increased fluid shear stress. This activation resulted in generation of ceramides as well as local and transient increase in ceramide levels at the plasma membrane. Here, we examined the role of the product of N-SMase activity, ceramide, in mechanosignal transduction as well as tested a specific inhibitor of N-SMase, scyphostatin, to further assess the function of N-SMase in mechanotransduction.
MATERIALS AND METHODS

Materials. Reagents and other supplies were obtained from the following sources: [14C]sphingomyelin (55 mCi/mmol) from American (Arlington Heights, IL); sphingomyelinase (Staphylococcus aureus) and diphosteo-p44/42 MAPK E10 monoclonal antibody from Sigma (St. Louis, MO); MEKI/MEK2 phosphospecific polyclonal antibodies, herbasmycin A, genistein, PP2, PP3, protease and phosphatase cocktails from Calbiochem (San Diego, CA); caveolin-1 monoclonal antibody (Z034) from Zymed Laboratories (San Francisco, CA); MEKI, MEK2, and pan ERK monoclonal antibodies, caveolin-1 polyclonal antibody, eNOS monoclonal antibody from BD Pharmingen; phosphotyrosine monoclonal antibody (4G10) from Upstate Biology (Lake Placid, NY); Src[Y418] from BioSource International; phospho-Src (Y416), phospho-eNOS (Ser1177), phospho-Akt (Ser473), and Akt antibodies from Cell Signaling Technology; and C2, C6 ceramides and D2, D6 dihydroceramides from Biomol Research Laboratories (Plymouth Meeting, PA). Scyphostatin was a generous gift from Sankyo (Tokyo, Japan).

Cell culture. Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA) and maintained in culture as described by the provider in DMEM (Invitrogen; Carlsbad, CA) and 10% fetal bovine serum (Omega; Tarzana, CA). BAEC used in this study were between passages 4 and 6. Cells were seeded in 60-mm tissue culture dishes (Falcon) and grown to confluence in the growth medium before exposure to shear stress.

Rat lung perfusion. As described previously (9), the rat vasculature of anesthetized Sprague-Dawley rats (Harlan Sprague Dawley, 170 g) was first flushed in situ at 37°C via the pulmonary artery at a perfusion pressure of 6–8 mmHg and flow rate of 4–5 ml/min for 5 min with mammalian Ringer’s solution composed of (in mM) 140 NaCl, 4.5 KCl, 1 MgSO4, 11 glucose, 1 NaH2PO4, and 25 NaHCO3 and then perfused at pressure ranging from 6 to 20 mmHg (flow rate from 4 to 16 ml/min) for a period of 0–10 min.

Tissue subfractionation to isolate luminal endothelial cell plasma membranes. After the perfusion described above, the luminal cell surface of the endothelium in the rat lung tissue and its caveolae were purified using the in situ silica-coating procedure, as previously described in our past work (39, 45). Briefly, the rat lung vasculature was perfused with ice-cold colloidal silica solution to selectively coat the luminal surface of the endothelium in situ to allow purification of the silica-coated endothelial cell plasma membranes from the tissue homogenate by density gradient centrifugation.

Assay for N- and acid-SMase activity. The activity of N- and acid (A)-SMase was estimated by the method of Wiegmann et al. (48). Briefly, to determine the activity of N-SMase, perfused rat lungs were homogenized in HEPES-buffered sucrose with protease inhibitors and phosphatase inhibitors, and the subcellular fractions (homogenate, silica-isolated endothelial plasma membranes) were isolated as described above. The selected fractions (10 μg protein) were incubated at 37°C for 1 h in 20 mM HEPES buffer (pH 7.4) containing 1 mM MgCl2 and 3.4 μM [14C]sphingomyelin (55 mCi/mmol). The reaction was terminated by the addition of 800 μl chloroform-methanol (1:1) and 200 μl of H2O. [14C]Phosphorylcholine released from [14C]sphingomyelin in the aqueous phase was collected and counted by liquid scintillation counting. To measure the activity of acid A-SMase, the subcellular fractions (10 μg protein) were incubated at 37°C for 1 h with 250 mM sodium acetate (pH 5.0) containing 1 mM EDTA and 3.4 μM [14C]sphingomyelin. [14C]Phosphorylcholine was collected and measured as described above. For inhibition studies, rat lungs were perfused with 50 μM scyphostatin for 5 min before the change in pressure/flow rate occurred or membranes were preincubated with various concentrations of scyphostatin for 15 min at 37°C before the addition of the substrate.

In vitro kinase assay. Protein kinase assay was performed as described previously on rat lung PMF with minor modifications (32). Briefly, 5 μg of proteins from PMF were incubated in kinase buffer (in mM) 25 KCl, 2.5 magnesium acetate, 150 potassium acetate, 5 EGTA, and 25 HEPES, pH 7.4, in the presence or absence of 100 μM ATP and various ceramides at 37°C for 5 min. For inhibition studies, membranes were preincubated before the addition of ATP with 20 μM genistein, 4 μM PP3, 50 mM PP2, 1 μM herbasmycin A, and 1 or 50 μM scyphostatin at 37°C for 15 min. The assay was terminated by adding sample buffer and boiling samples for 5 min, followed by SDS-PAGE and Western blot analysis to determine protein-tyrosine phosphorylation.

Shear stress studies. Confluent BAEC monolayers were assembled with a silicon gasket into a parallel plate shear chamber forming a flow channel (220-μm height, 2.5-cm width, 2.5-cm length) between the monolayer and fabricated polycarbonate plate. Nonpulsatile steady laminar shear stress was controlled by changing the flow rate of the medium delivered to the cells using the constant flow loop and the peristaltic pump with a damping reservoir. Cells were sheared at 20 dyn/cm² for time indicated in figures at 37°C.

Preparation of cell lysates. After experimental treatments, BAEC were washed in ice-cold PBS and lysed in 100 μl lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% glycerol, and 1.5 mM MgCl2 with 1% Triton X-100 and cocktails of protease and phosphatase inhibitors). Cells were disrupted by 10 1-s bursts at 50% cycle setting 5 of the Branson sonicator probe. Cell lysates were clarified by spinning at 14,000 g for 15 min at 4°C.

Western blot analysis. Unless specified otherwise in the figures, aliquots of cell lysates (20 μg protein each) or aliquots of rat lung subfractions (10 μg of protein for homogenate and 2 μg for endothelial plasma membranes) were resolved on a 8–16% precast SDS-PAGE gel (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated with primary antibodies for 2 h at room temperature or overnight at 4°C and then with a secondary antibody conjugated with horseradish peroxidase (1 h at room temperature). Immunoreactivity was detected by chemiluminescence method (Pierce). Intensities of immunoreactive bands in the Western blots were analyzed by scanning densitometry and quantified with the use of NIH Image software (version 6).

Protein assays. Protein concentrations of the subcellular fractions were determined by BioRad Dc protein assay as per manufacturer’s instruction using bovine serum albumin as a standard.

RESULTS

Scyphostatin inhibits mechano-induced N-SMase activity. Our previous work (9) showed that at least one isoform of N-SMase resides in caveolae and is rapidly activated by shear stress. To inhibit ceramide production by N-SMase, we tested scyphostatin, a new inhibitor specific for Mg2+-dependent, membrane-bound N-SMase, isolated from Dasycyphus mollissimus (37, 38). First, to assess the specificity of this compound in our experimental system, we preincubated luminal endothelial cell plasma membranes that were isolated from whole rat lung tissue homogenates using previously described silica coating procedure (39) with scyphostatin for 15 min before measuring SMase activity. Scyphostatin caused a significant, dose-dependent decrease in the activity of N-SMase, but not A-SMase (Fig. 1), consistent with past reports (3–5) defining the specificity of scyphostatin for N-SMase. N-SMase inhibition was also observed when we used endothelial plasma membranes from lungs perfused under high flow and pressure conditions, where the activity of N-SMase is elevated twofold, which also confirms that increased flow rapidly increases the activity of N-SMase but not A-SMase (9) (Fig. 2, and data not shown).

To further assess the role of N-SMase in mechanotransduction, we perfused the rat lung microvasculature with scypho-
Scyphostatin inhibits cell surface and downstream mechanosignaling in situ. Two well-documented cellular responses to mechanical stimulation are rapid tyrosine phosphorylation of select cell surface proteins (43) and the downstream activation of the Ras/Raf/MEK/ERK pathway (26, 43, 46, 47). To determine whether N-SMase inhibition affects these responses, we perfused rat lungs in situ with scyphostatin as above. Western blot analysis of the homogenate and endothelial plasma membranes showed that high pressure/flow rate-induced protein tyrosine phosphorylation was detected much more readily in PMF than homogenate (Fig. 3A; note fivefold greater protein loads for homogenate than endothelial plasma membranes). Scyphostatin specifically inhibited tyrosine phosphorylation of several proteins while leaving others unaffected. Scyphostatin appeared to affect only the proteins whose phosphorylation...
was induced by increased mechanical stress (Fig. 3A). To further ensure that scyphostatin is not acting as a nonspecific kinase inhibitor, we performed an in vitro kinase assay on endothelial plasma membrane in the presence of scyphostatin and found that scyphostatin did not prevent or influence protein tyrosine phosphorylation in vitro (Fig. 4C and data not shown). It appears that specific N-SMase inhibition by scyphostatin prevented acute mechano-induced protein tyrosine phosphorylation at the endothelial cell surface in situ.

Next, we analyzed downstream mechanosignaling through mitogen activated protein kinases (MAP kinases). We used phospho-MEK1/2 antibodies that detect endogenous levels of activated MEK1/2 (phosphorylated at Ser217/221) as well as phospho-ERK1/2 antibodies that recognize ERK1/2 activated through specific diphosphorylation on threonine and tyrosine residues within the regulatory site of active ERK1/2 kinase (9, 49). Scyphostatin inhibited mechano-induced phosphorylation of both MEK1/2 (Fig. 3B) and as we showed previously ERK1/2 (9). Because ERK1/2 is an endogenous substrate of MEK, the increase in ERK1/2 phosphorylation on fluid shear additionally confirmed activation of MEK1/2. We found no evidence that the exposure to scyphostatin alone inhibits MEK1/2 and ERK1/2 activation (Figs. 3B, 8B, and data not shown). Thus scyphostatin, not as a general inhibitor, but

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Fig. 4. Ceramide induces protein tyrosine phosphorylation in vitro that is inhibited by Src-like kinase inhibitors but not scyphostatin. For dose- and time-response studies of ceramide-induced tyrosine phosphorylation, 5 μg of silica-coated plasma membranes from rat lung (P) were incubated with the indicated concentrations of active ceramides (C2- or C6-ceramide) or their inactive analogs (D2- or D6-ceramide) for 5 min (A) or for the indicated time at 10 μM final ceramide concentration (B, inset, a representative blot; arrows indicate bands that were used for densitometry analysis). For inhibition studies, P was pretreated for 10 min with genistein (20 μM), herbamycin A (1 μM), and scyphostatin (1 or 50 μM) (C) or 50 nM PP2 or 4 μM PP3 (D) before the addition of 10 μM ceramide. The assays were followed by Western blot analysis using anti-phosphotyrosine 4G10 monoclonal antibodies and anti-caveolin-1 polyclonal antibody (cav-1) or anti-β-actin monoclonal antibody for load control. Each experiment is representative of at least three experiments.

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rather as a specific inhibitor of N-SMase, was able to abolish mechano-induced tyrosine phosphorylation at the luminal cell surface of rat lung endothelium as well as intracellular downstream MAP kinase activation.

Ceramide mimics mechanotransduction in rat lungs. Ceramide is the product of N-SMase activity and can act as a second messenger (28, 31). Because increasing vascular pressure/flow rate can rapidly activate tyrosine kinases (43) and increase the level of ceramides at the plasma membrane (9), we wondered whether ceramide on its own can induce and even mimic mechano-induced plasmalemmal protein tyrosine phosphorylation. First, with the use of an in vitro, cell-free kinase assay on isolated luminal endothelial plasma membrane, we tested various concentrations of synthetic analogs of ceramide (N-acetylsphingosine and N-hexanoylsphingosine; C2-ceramide and C6-ceramide, respectively), which were shown to mimic the biological effects of sphingomyelinase pathway (22) versus their corresponding relatively biologically inactive dihydro derivatives (D2- and D6-ceramide). Western blot analysis with phosphotyrosine antibodies (4G10), followed by densitometry measurements, revealed that active ceramides induced a dose-dependent increase in protein tyrosine phosphorylation (Fig. 4A), whereas the inactive ceramide analogs exhibited significantly less activity. Tyrosine phosphorylation on addition of active ceramide was also time dependent, reaching maximal activity at 3 min and maintained through 10 min posttreatment (Fig. 4B). This ceramide-induced tyrosine phosphorylation was inhibited by pretreatment of endothelial plasma membranes with a general tyrosine kinase inhibitor, genistein (20 μM), and inhibitors more specific for Src-like protein kinases, herbinycin A (1 μM) and PP2 (50 nM) (Fig. 4, C and D). Sphingostatin (1 and 50 μM) did not affect in vitro phosphorylation of proteins on endothelial plasma membranes nor on ceramide-induced phosphorylation (Fig. 4C). A specific inhibitor of Src family tyrosine kinases, PP2 (20), blocked tyrosine phosphorylation induced by ceramides (C2 and C6) and had no influence on basal tyrosine phosphorylation nor on the levels of phosphorylation after treatment with inactive ceramides (D2 and D6) (Fig. 4D). Pretreatment with PP3 (a noninhibitory chemical analog of PP2) under identical experimental conditions did not modify either basal or ceramide-stimulated tyrosine phosphorylation (Fig. 4D), demonstrating the specificity of PP2. Thus ceramide in this model system can act as a second messenger to activate tyrosine kinases in endothelial plasma membranes and scyphostatin per se is not a direct general inhibitor of tyrosine kinases. Moreover, the sphingostatin inhibitory effects must occur upstream of ceramide, consistent with its specific inhibition of N-SMase.

Next, we perfused rat lungs at high pressure/flow or at baseline pressure/flow in the presence or absence of C6-ceramide. Western blot analysis of isolated luminal endothelial plasma membranes from these lungs revealed that at baseline pressure flow, C6-ceramide induced tyrosine phosphorylation of many of the same protein bands induced by high vascular pressure/flow (Fig. 5A, marked with arrows). The similarities were also found when we analyzed isolated caveolae (data not shown). Moreover, the levels of ERK1/2 phosphorylation were significantly enhanced in total rat lung homogenates from animals perfused at baseline pressure/flow with active ceramides or high-flow conditions for 2 min (Fig. 5B), whereas the levels of phosphorylated JNK or p38 MAP kinases remained unchanged over this period (data not shown). It appears that ceramide can induce tyrosine phosphorylation in vitro directly in endothelial plasma membranes as well as mimic mechano-induced tyrosine phosphorylation in tissue in situ. Thus ceramide may be an important second messenger for acute mechanotransduction.

Ceramide can stimulate protein phosphorylation in intact cultured cells. To assess the effect of ceramide in living cells, we used early passages (4–6) of BAEC for shear flow experiments in parallel flow chambers. Cells exposed to shear stress of 20 dyn/cm² for 0 to 10 min, showed enhanced protein tyrosine phosphorylation within 1 min (Fig. 6A) that continued to increase reaching maximum at 5 min that was maintained through 10 min. Activation of ERK1/2 increased steadily throughout the 10 min of shear (Fig. 6B). To ensure equal loading of protein per gel lane, we routinely reprobed membranes with pan ERK, caveolin-1, and/or β-actin antibodies (Fig. 6B and data not shown). Thus this cell culture system shows the expected mechanoactivation of tyrosine and MAP kinases in intact cultured BAEC monolayers. To compare signals evoked by fluid shear stress with possible effects of ceramide, we treated confluent monolayers of BAEC with bSMase (0.125 U/ml) or the cell-permeable, synthetic active (C2, C6) and inactive (D2, D6) ceramides (10 μM final concentration) (Fig. 7). Both physiologically active ceramides as well as bSMase enhanced tyrosine phosphorylation of proteins in a time-dependent manner (Fig. 7, A and B, and data not shown). Inactive dihydro-ceramides had little to no effect. Activated phosphorylation of Src-like kinases detected using PY418 antibodies was also observed within 5 min of shear flow or ceramide treatment (Fig. 7C) and, in this case, only active

![Fig. 5. Ceramide-induced protein tyrosine phosphorylation mimics that from high flow in situ. Rat lungs were perfused for 2 min at high pressure/flow or at baseline pressure/flow in the presence of 20 μM C6-ceramide (C6), C2-ceramide (C2) and their inactive analogs (D2 and D6), or vehicle control, followed by isolation of homogenate and plasma membrane fractions for Western blot analysis. Silica-coated plasma membranes (P) fractions (2 μg of protein) were analyzed using anti-phosphotyrosine antibodies (A) and total lung homogenates 20 μg proteins using anti-diphospho-ERK1/2 and anti-panERK antibodies (B). Arrows indicate similarities in phosphorylation patterns. Blots are representative of three experiments.](http://ajpheart.physiology.org/)

![Fig. 6. BAEC for shear flow experiments in parallel flow chambers. Cells exposed to shear stress of 20 dyn/cm² for 0 to 10 min, showed enhanced protein tyrosine phosphorylation within 1 min (Fig. 6A) that continued to increase reaching maximum at 5 min that was maintained through 10 min. Activation of ERK1/2 increased steadily throughout the 10 min of shear (Fig. 6B). To ensure equal loading of protein per gel lane, we routinely reprobed membranes with pan ERK, caveolin-1, and/or β-actin antibodies (Fig. 6B and data not shown). Thus this cell culture system shows the expected mechanoactivation of tyrosine and MAP kinases in intact cultured BAEC monolayers. To compare signals evoked by fluid shear stress with possible effects of ceramide, we treated confluent monolayers of BAEC with bSMase (0.125 U/ml) or the cell-permeable, synthetic active (C2, C6) and inactive (D2, D6) ceramides (10 μM final concentration) (Fig. 7). Both physiologically active ceramides as well as bSMase enhanced tyrosine phosphorylation of proteins in a time-dependent manner (Fig. 7, A and B, and data not shown). Inactive dihydro-ceramides had little to no effect. Activated phosphorylation of Src-like kinases detected using PY418 antibodies was also observed within 5 min of shear flow or ceramide treatment (Fig. 7C) and, in this case, only active phosphorylation was detected.](http://ajpheart.physiology.org/)
C2 and C6 ceramides increased Src-like kinase phosphorylation similarly to the fluid shear forces (Fig. 7C). Consistent with past reports showing ceramide activation of MAP kinase signaling cascades (36), treatment of the cells with active ceramides also caused time-dependent activation of ERK1/2 (Fig. 7D). Acute activation of other MAP kinase pathways, including p38 and JNK, which were shown to be a part of ceramide signaling in other systems, was not observed for the times tested (data not shown). Moreover, in BAEC monolayers, active ceramides mimicked high flow activation of the Akt/eNOS pathway increasing phosphorylation of Akt on Ser473 and eNOS on Ser1177 (Fig. 7E). Thus ceramides alone, added externally as cell-permeable analogs or generated by the addition of bSMase, stimulated tyrosine phosphorylation of...

Fig. 6. Shear stress-induced signaling in vivo. Confluent bovine aortic endothelial cells (BAEC) were maintained in culture medium for at least 2 days before exposure to laminar shear stress for the indicated times. Aliquots of cell lysates were analyzed for protein phosphorylation on tyrosine with 4G10 monoclonal antibody (A) and for activation of ERK1/2 with anti-diphospho-ERK1/2 monoclonal antibody (B). β-Actin and caveolin-1 were used as load controls. Densitometry data are quantified from Western blots from two independent experiments.

Fig. 7. Ceramide-induced signaling in vivo in cultured endothelial cell monolayers. Confluent BAEC were maintained in culture medium for at least 2 days before treatment with 0.125 U/ml bacterial SMase (bSMase) or 10 μM C2, C6 ceramide or D2, D6 dihydro-ceramide (in BSA complex) for the indicated times (A, B, and D) or 10 min (C and E). Aliquots of cell lysates were analyzed for protein tyrosine phosphorylation with 4G10 monoclonal antibody (A) and densitometry analysis of Western blots (B). Western blot analysis for activation of Src-like kinase phosphorylation with phosphospecific anti-PY418Src antibody (C), activation of ERK1/2 with anti-diphospho-ERK1/2 monoclonal antibody (D), and activation of Akt/endothelial nitric oxide synthase (eNOS) pathway using phospho-eNOS (Ser1177) and phospho-Akt (Ser473) polyclonal antibodies (E). Anti-caveolin-1 antibody was used as a load control. Densitometric analysis of the Western blots was averaged from two experiments.
membrane proteins and activation of Src-like kinases and activated both ERK and Akt/eNOS pathways in BAECs.

Scyphostatin prevented mechanoactivation of cell surface kinases and downstream MAP kinase pathway. We tested whether scyphostatin is also an effective inhibitor of mechano-transduction in vivo, in this case, induced directly by shear stressors on intact living endothelial cell monolayer grown in culture. Pretreatment of living BAEC monolayers with scyphostatin (50 μM for 10 min) prevented shear stress stimulation of protein tyrosine kinases as well as Src-like kinases (Fig. 8). Figure 8 shows also that both MEK1/2 and ERK1/2 were activated in response to high flow, but not when the cells were pretreated with scyphostatin. The level of phosphorylation did not change on addition of scyphostatin compared with control conditions. Scyphostatin failed to inhibit shear stress-induced activation of Akt/eNOS pathway but rather seemed to be stimulatory on its own (data not shown). We were surprised by this finding and are dissecting the molecular basis of this action. So far, it appears to be a direct effect on phosphatidylinositol 3-kinase (PI3-kinase) (our unpublished data). Thus N-SMase at the luminal endothelial cell surface activated directly by mechanical stimulation to generate ceramide (a second messenger at the plasma membrane) can initiate downstream signaling, including tyrosine kinases and the MAP kinase pathway. Scyphostatin blocked the shear-induced increase in total tyrosine phosphorylation as well as tyrosine phosphorylation of Src-like kinases and activation of MEK1/2 and ERK1/2 in intact living endothelial cells.

DISCUSSION

Work performed in our laboratory (9) has shown that N-SMase concentrated in caveolae at the luminal surface of rat lung vascular endothelium is activated by mechanical stimulation in an acute and transient way with a subsequent generation and accumulation of ceramide in the membrane. We postulated that N-SMase might be a mechanosensor, which responds to shear stress or other mechanical stressors to hydrolyze sphingomyelin and generate ceramide, a well-established lipid second messenger that changes the lipid microenvironment at the plasma membrane and directly propagates downstream mechanosignaling inside the cell (Fig. 9). Activation of N-SMase may occur upstream from known intracellular mechanosignaling events. In this study, we investigated further signal transduction possibly initiated by N-SMase as well as by fluid laminar shear stress and pressure using in vitro (cell-free, subcellular membrane fractions), intact cultured, live BAEC monolayers and in situ (vascular perfusion of rat lung tissue) systems. We examined the effects of synthetic, cell-permeable,

**Fig. 8.** Scyphostatin inhibits shear stress-induced signaling in vivo. Confluent BAEC maintained in culture medium for 2 days before shear experiment. Scyphostatin (50 μM) was added in ethanol 15 min before shear stress experiment. Cell lysates (20 μM) were analyzed by Western immunoblot analysis using antibodies recognizing total tyrosine phosphorylation 4G10 and phosphorylated, active Src kinase (PY418Src) or phosphorylated MEK1/2, diphosphorylated ERK1/2, or total MEK for load controls. Representative of three experiments.

**Fig. 9.** Ceramide mimics and scyphostatin inhibits mechanosignaling. Proposed mechanosignaling pathways through ceramide generated via activation of N-SMase in the plasma membrane caveola. Inhibitory effects of scyphostatin on downstream signaling from N-SMase marked by dark gray shadowing. PI3K, phosphatidylinositol 3-kinase.
short-chain ceramides (C2 and C6) as well as bSMase, which mimic the activity of membrane N-SMase by cleaving sphingomyelin to produce natural ceramide at the plasma membrane (30). In addition, we employed a new, specific inhibitor of N-SMase, scyphostatin (37, 38) to further evaluate the role of this enzyme in mechanosignaling.

Various sphingomyelinases are present in mammalian cells that can be distinguished by their subcellular localization and biochemical properties, such as pH optimum and ion requirements (28). Thus, ceramides can be generated at different subcellular sites through activation of various SMase isoforms, which, in turn, can cause a wide variety of cellular responses. The best described are cytotoxic effects of ceramides leading to cell death through apoptosis, but the increase in ceramide levels may also, in some cases, result in a generation of signal for survival (28, 31). Exogenously supplied synthetic ceramides or ceramide formed at the cell surface after treatment with bSMase can activate multiple signaling pathways, such as kinase suppressor of Ras (50), protein kinase C-ζ (34), and stress kinases (35) as well as protein phosphatases, such as protein phosphatase 1 (21) and protein phosphatase P2A (13).

We recently demonstrated that increased ceramide levels at the plasma membrane on mechanical stimulation is transient, returning to baseline within 10 min of stimulation (9). Here we show that ceramide induces tyrosine phosphorylation of select membrane proteins. The pattern of phosphorylated proteins mimics the one evoked by shear stress. This effect appears specific for ceramides because synthetic, inactive, dihydro-mimics the one evoked by shear stress. This effect appears transient, membrane proteins. The pattern of phosphorylated proteins mimics the one evoked by shear stress. This effect appears specific for ceramides because synthetic, inactive, dihydro-derivatives have no or little effect. The inhibition of this ceramide-induced tyrosine phosphorylation by genistein, a c inhibitor of N-SMase, scyphostatin, inhibits only shear stress stimulation (28). Antibodies that recognize an autophosphorylation site on Src-like kinases. Western blot analysis, using phosphospecific anti-Src (PY418) antibodies that recognize an autophosphorylation site on Src-like kinases, reveals that both ceramides and mechanical stressors increase Src-like kinase phosphorylation within minutes. Short chain synthetic ceramides can induce rapid phosphorylation and activation of Src in smooth muscle cells (25). A specific inhibitor of N-SMase, scyphostatin, inhibits only shear stress-induced but not ceramide-induced activation of Src-like kinase and concomitant tyrosine phosphorylation of plasma membrane protein, thereby confirming our hypothesis on the key role in mechanosignaling of N-SMase and its downstream product, ceramide.

We also find that ceramide affects another known mechanotransduction response: downstream signaling through MAP kinases (26, 43, 46, 47). In our system, ceramide, similar to shear stress, induces the ERK cascade but not stress-activated kinases (p38 and JNK) pathway. Short exposure to low concentrations of ceramides, which mimics the transient increase in ceramides on shear stress in situ, induces phosphorylation of MEK1/2. This increase in MEK phosphorylation is also a measure of its activity, because the phosphorylation of its immediate substrate p42/p44 ERK1/2 is enhanced as well. Activation of ERK1/2 by ceramides has been shown previously for other cell types (7, 25). N-SMase is upstream of the ERK signaling cascade because scyphostatin totally abolishes shear stress activation of MEK1/2 and ERK1/2. Interestingly, it was reported (2, 16) for ceramides and shear stress that longer exposure can activate yet another common signaling pathway via MAP kinases, namely the stress activated protein kinase JNK.

We have shown that ceramide induces phosphorylation of Akt (Ser473) and eNOS (Ser1177) (Fig. 7E) in BAEcs. Cells exposed to C6 ceramide had a higher activity of PI3-kinase (Czarny unpublished observation). These results show that in endothelium shear stress and ceramides lead to the production of NO through the activation of the PI3-kinase/Akt/eNOS pathway. It has been shown that Akt activation by PI3-kinase as well as phosphorylation of Akt on Thr308 and Ser473 is essential in shear stress-dependent NO production (1, 16). The phosphorylation of eNOS by the PI3-kinase/Akt pathway has been suggested as the underlying mechanism whereby shear stress stimulates NO production (11, 15). PI3-kinase activation by ceramide, sphingomyelinase, and TNF-α was first described in rat2 fibroblast (21). Recently, short-term exposure to TNF-α has been shown to lead to N-SMase-dependent and ceramide-dependent activation of eNOS via PI3-kinase and Akt (6). In preconstructed aortic rings, bSMase and cell permeable ceramide caused vasorelaxation in a concentration and endothelium-dependent manner (27). Thus N-SMase might be a key signaling enzyme, activated by shear stress to transduce the signal across the plasma membrane through lipid second messenger ceramide.

In summary, endothelial caveolae, by concentrating a variety of signaling molecules, lipids and proteins, and their immediate effectors, potentially are efficient microdomains acting as mechanosensors regulating cell surface signaling. Activation of N-SMase in caveolae leads to generation of ceramide, which may act directly to affect the plasma membrane environment (12, 30). Our cumulative data show that onset of acute shear stress signaling can be attributed to the activation of N-SMase with sequential generation of ceramides in endothelial caveolae, which, in turn, activates protein phosphorylation cascades generating an acute response to mechanical stimuli.

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