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

Malgorzata Czarny and Jan E. Schnitzer

Division of Vascular Biology and Angiogenesis, Sidney Kimmel Cancer Center, San Diego, California 92121

Submitted 4 March 2004; accepted in final form 11 May 2004

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 mechanocuactivity of N-SMase as well as downstream tyrosine and mitogen-activated protein kinases. Cell-permeable ceramide analogs (N-acetylphosphosine, C2-ceramide, and N-hexanoylsphingosine, C6-ceramide) but not the inactive dihydroderivatives D2-ceramide and D6-ceramide (N-acetylphosphosine and N-hexanoylsphingosine, respectively) mimic rapid mechanon-induced tyrosine phosphorylation of cell surface proteins as well as mechanoactivation of Src-like kinases and the extracellular regulated kinase pathway. The responses common to ceramides and mechanical stress were inhibited by genistein, herbamycin A, and PP2, but not PP3, which suggests an obligate role of Src-like kinases in ceramide-mediated 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 stimulator of both the Ras/Raf/MEK/ERK pathway and endothelial cell surface. They concentrate key mechanosignaling molecules such as eNOS, Gq (40, 41), and, as recently reported (9), neutral sphingomyelinase (N-SMase). The caveolin oligomeric coat of caveolae appears to respond to acute changes in mechanical forces to release and control the activity of key signaling molecules, such as eNOS (41). Direct mechanotransduction in caveolae has been observed 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 phosphatidylinositol (32, 33). 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 ceramides, 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 mechanosensory transduction as well as a specific inhibitor of N-SMase, scyphostatin, to further assess the function of N-SMase in mechanotransduction.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Materials. Reagents and other supplies were obtained from the following sources: [14C]sphingomyelin (55 mCi/mmol) from Amer-shares (Arlington Heights, IL); sphingomyelinase (Staphylococcus aureus) and diphaspho-p44/42 MAPK E10 monoclonal antibody from Sigma (St. Louis, MO); MEK1/MEK2 phosphospecific polyclonal antibodies, herbasmycin A, genistein, P2, P3, protase and phospho-tyrosine antibodies, caveolin-1 monoclonal antibody (Z034) from Zymed Laboratories (San Francisco, CA); MEK1, MEK2, and pan ERK monoclonal antibodies, caveolin-1 polyclonal antibody, eNOS monoclonal antibody from BD Pharmingen; phosphotyrosine monoclonal antibody (4G10) from Up-state Biology (Lake Placid, NY); Src[pY418] from BioSource International; phospho-Src (Y416), phospho-eNOS (Ser1177), phospho-Akt (Ser473), and Akt antibodies from Cell Signaling Technology; and C2, C6 cereamides 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 growth 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, 10% 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 (Branson). 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 D₆ protein assay as per manufacturer’s instruction using bovine serum albumin as a standard.

RESULTS

Scyphostatin inhibits mecano-induced N-SMase activity. Our previous work (9) showed that at least one isosform of N-SMase resides in caveola 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 Dasycyclus mol-lissim (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 silicon 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 neutral sphingomyelinase (N-SMase) activity in vitro. Ten micrograms of silica-coated plasma membranes isolated from rat lungs (P) was preincubated with the indicated concentrations of scyphostatin for 15 min at 37°C before determination of N-SMase (open circles) or acid (A)-SMase (solid squares) activity. Data are means ± SD from two independent experiments performed in triplicate.

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 decreased by increased vascular pressure/flow.

Fig. 1. Scyphostatin inhibits neutral sphingomyelinase (N-SMase) activity in vitro. Ten micrograms of silica-coated plasma membranes isolated from rat lungs (P) was preincubated with the indicated concentrations of scyphostatin for 15 min at 37°C before determination of N-SMase (open circles) or acid (A)-SMase (solid squares) activity. Data are means ± SD from two independent experiments performed in triplicate.

Fig. 2. Scyphostatin inhibits mecano-induced activity of N-SMase in situ. The rat lung vasculature was perfused with 50 µM scyphostatin (SCY) or a vehicle control for 5 min at a baseline pressure of 6–8 mmHg. The pressure was then either maintained or raised to 18 mmHg for 2 min, followed by silica coating and tissue subfractionation to isolate luminal endothelial plasma membranes from homogenate. At high pressure/flow, N-SMase activity was significantly increased in endothelial plasma membranes but not after scyphostatin pretreatment (Fig. 2). No changes in activity were observed in homogenate. A-SMase activity was highly enriched in homogenate relative to plasma membranes and did not respond in either fraction to the higher pressure/flow or to scyphostatin (data not shown). Thus scyphostatin inhibits N-SMase activity and prevents stimulation of N-SMase activity by increased vascular pressure/flow.

Fig. 3. Scyphostatin inhibits shear stress-induced signaling in situ. The rat lung vasculature was perfused at baseline pressure/flow for 5 min in the presence of 50 µM scyphostatin or vehicle control before 2-min perfusion at either baseline (BF) or high pressure/flow (HF). A: Western blot analysis of homogenate (H; 10 µg protein) and plasma membrane fractions (P; 2 µg protein) using anti-phosphotyrosine antibody 4G10. Arrows indicate significant decrease in tyrosine phosphorylation levels of plasma membrane proteins, and stars show partial inhibition of tyrosine phosphorylation. To ensure even loads per lane, the membranes were reblotted for β-actin. B: Western blot analysis of 20 µg of total lung homogenate protein using antibodies to phospho-MEK1/2 (activated MEK1/2). Equal protein loading was confirmed by immunoblotting with anti-MEK1/2 antibodies. The immunoblots are representative of at least two independent experiments.
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

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.
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, herbimycin A (1 μM) and PP2 (50 nM) (Fig. 4C and D). Scyphostatin (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 scyphostatin 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
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,
short-chain ceramides (C2 and C6) as well as bSMase, which mimics 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-derivatives have no or little effect. The inhibition of this ceramide-induced tyrosine phosphorylation by genistein, a key role in mechanosignaling of N-SMase and its downstream effectors, potentially are ef

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 mes

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.

ACKNOWLEDGMENTS

We acknowledge Dr. Futoshi Nara from Sankyo (Tokyo, Japan) for the gift of scyphostatin and Dr. Victor Rizzo for help with shear chamber. We thank Phil Oh for an insightful critique and helpful discussion, Dr. Sylvie Christope for excellent assistance, and Dr. Lucy Carver for editorial work on this paper. This work was presented in part at the ASCB Meeting Molecular Biology of the Cell, vol. 13, November 2002, p. 232a.

REFERENCES


