The vascular endothelium acutely autoregulates blood flow in vivo in part through unknown mechanosensing mechanisms. Here, we report the discovery of a new acute mechanotransduction pathway. Hemodynamic stressors from increased vascular flow and pressure in situ rapidly and transiently induce the activity of neutral sphingomyelinase but not that of acid sphingomyelinase in a time- and flow rate-dependent manner, followed by the generation of ceramides. This acute mechanosactivation occurs directly at the luminal endothelial cell surface primarily in caveolae enriched in sphingomyelin and neutral sphingomyelinase, but not acid sphingomyelinase. Scyphostatin, which specifically blocks neutral but not acid sphingomyelinase, inhibits mecano-neutral sphingomyelinase activity as well as downstream activation of extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) by increased flow in situ. We postulate a novel physiological function for neutral sphingomyelinase as a new mechanosensor initiating the ERK cascade and possibly other mechanotransduction pathways.

Acute vasoregulation of blood flow through the mechanosensitivity of vascular endothelial cells to hemodynamic stressors sets blood vessel tone, maintains normal tissue homeostasis, and influences the pathogenesis of vascular disease (1, 2). Increased hemodynamic forces such as shear stress and pressure from fluid flowing over the luminal surface of vascular endothelial cells lining blood vessels very rapidly induce various cell surface signaling events, including protein phosphorylation, the Ras/Raf/mitogen-activated protein (MAP)4 kinase pathway (3), and, most notably, endothelial nitric oxide synthase (eNOS) to generate the critical compensatory vasodilator nitric oxide (NO) (4, 5). Increasing fluid shear over cultured endothelial cell monolayers stimulates heterotrimeric G-protein-coupled signaling (6–8), MAP kinases (9), NO production (5, 7), focal adhesion kinase (FAK), and ion channels (10). This diversity of the mechanoresponse may be categorized temporally (11). Acute responses, occurring in seconds to minutes, have been detected in most cases both in vitro/in situ and in culture and include the activation of ion channels, eNOS, plasmalemmal tyrosine kinases, G proteins and, minutes later, the cytosolic MAP kinases (3, 4, 6–10). After 15 min to 1 h, FAK and c-Jun N-terminal kinase (JNK) are activated, at least in culture systems (12). Later, transcription factors will be activated to alter gene expression (2, 13, 14) and, with time, to modulate endothelial cell phenotype, cell adhesion, and even atherogenesis (2, 14). The mechanisms mediating these effects, especially the key initiating mechanosensing molecule, remain largely unknown.

Caveolae are small, flask-shaped, plasmalemmal invaginations that are found on the surface of many cell types. In the endothelium, caveolae are thought to function primarily as vesicular carriers transporting molecules into and across the cell (15). Our laboratory has discovered that endothelial caveolae can also act as mechanosensing organelles that respond to mechanical stressors (3, 4, 11, 16). Caveolae are highly concentrated in tyrosine kinase activity, and increased vascular flow and pressure can rapidly induce the tyrosine phosphorylation of cell surface proteins located primarily in caveolae (3). Disassembly of caveolae by the depletion of cholesterol disperses the molecular constituents of this specialized compartment and prevents acute flow activation of cell surface tyrosine kinases as well as downstream cytosolic kinases such as ERK, but not JNK (3, 4, 17). Increased flow also rapidly stimulates eNOS, which is concentrated at the endothelial cell surface in caveolae (4). Many of the newly implicated cell surface molecules (tyrosine kinases (11, 18), caveolin (3, 4, 11), eNOS (4), and G-proteins (19)) exist in caveolae on the cytoplasmic side of the plasma membrane (4, 20, 21) and may respond to an unidentified upstream mechanoregulator.

Caveolae may have a distinct lipid composition consisting of cholesterol, sphingolipids (sphingomyelin and glycosphingolipids), and phosphatidylglycerol (20, 22). Depending on the cell type, caveolae may contain up to 95% of total cell sphingomyelin. In addition, ligand-induced sphingomyelin hydrolysis occurs within caveolin-rich membranes (23, 24). The sphingomyelin pathway is initiated by activation of sphingomyelinase membrane fraction; DETAPAC, diethylenetriamine pentacetic acid; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; JNK, c-Jun N-terminal kinase; BLMVEC, bovine lung microvascular endothelial cells; NO, nitric oxide; 5′-NT, 5′ nucleotidase.
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EXPERIMENTAL PROCEDURES

Materials—Reagents and other supplies were obtained from the following sources: [N-methyl-3H]phosphorylcholine (55 mCi/mmol) and Percoll from Amersham Biosciences; imidazole, ceramide, DETAPAC, octyl-β-D-glucopyranoside, sphingomyelinase (Staphylococcus aureus), glycerophosphate, anti-diphosphorylated ERK1 and 2 monoclonal antibody, and anti-par ERK antibody from Sigma; cardiolipectin from Avanti Polar Lipids (Alabama, AL); diacylglycerol kinase from Calbiochem (San Diego, CA); [γ-32P]ATP (3000 Ci/mmol) from PerkinElmer Life Sciences; the bicinechonic acid (BCA) protein assay kit from Pierce; anti-acid sphingomyelinase (A-SMase) antibodies from Santa Cruz Biotechnology; anti-caveolin-1 monoclonal antibody (2234) from BD Biosciences; M-450 Dynabeads from Dynal (New Hyde Park, NY); and Optiprep from InVitrogen. Preimmune serum and serum raised against bovine N-SMase were generously provided by Dr. Martin Kroenke (Cologne University, Germany) (27). Scyphostatin was a generous gift from Dr. Takeshi Ogita (Sankyo Co., Tokyo, Japan).

Rat Lung Perfusion—As described previously (3, 4), the lung vasculature of anesthetized Sprague-Dawley rats (Harlan Sprague-Dawley, 150–170 g) was first flushed via the pulmonary artery at 6–8 mm Hg for 5 min with mammalian Ringer’s solution using a syringe infusion pump and then perfused at pressure ranging from 6 to 20 mm Hg for 0–10 min at 37 °C.

Subfractionation to Isolate Luminal Endothelial Cell Plasma Membrane and Caveolae—After the perfusion described above, the luminal cell plasma membranes and caveolae of the endothelium were purified using the in situ silica coating procedure as described in our past work (28, 29). Briefly, the luminal surface of the endothelium of the rat lung vasculature was coated with an ice-cold colloidal silica solution in situ. After tissue homogenization, the silica-coated endothelial cell plasma membrane (P) was isolated from the tissue homogenate (H) by density centrifugation. Caveolae were separated from P by homogenization in the presence or absence of 1% Triton X-100 at 4 °C and then isolated by flotation in sucrose gradient (V and V

Isolation of Plasma Membrane and Caveolin-enriched Membrane Fractions from Cultured Cells—Bovine lung microvascular endothelial cells (BLMVECs) were grown and used to isolate a plasmalemmal (PM) and caveolin-rich membrane fractions (AC) as described (29–31). Briefly, confluent BLMVEC cells (ten 150 mm dishes) were washed, scraped and homogenized before centrifugation (1000 × g) for 10 min at 4 °C. The post nuclear supernatant (PNS) was overlaid on 30% Percoll in 100:100:1 (v/v) N HCl (100:100:1; v/v), and lipids in organic phase were collected and dried under N2. The reaction was initiated by mixing the lipid film with reaction buffer containing 50 mM imidazole (pH 6.5), 50 mM NaCl, 12.5 mM MgCl2, 1 mM EDTA, 5 mM cardiolipin, and 1.5% octyl-β-D-glucoside, 0.2 mM DETAPAC, 2 mM diithiothreitol, 4 μg of diacylglycerol kinase, and 1 μM [γ-32P]ATP (3000 Ci/mmol). After incubation at 25 °C for 30 min, the reaction was terminated with CHCl3/CH3OH (2:1; v/v). Ceramide-1-phosphate was resolved by thin layer chromatography using CHCl3/CH3OH/acetic acid at the ratio 65:15:5 (v/v). The lipid was identified by γ-32P-labeled ceramide standard and quantified by liquid scintillation counting.

Protein Assays—Protein concentrations were determined by Micro BCA (Pierce) and DC (Bio-Rad) protein assays accordingly to each manufacturer’s instructions using bovine serum albumin as a standard. Western analysis was performed as in past work (3).

RESULTS

Mechanoactivation of N-SMase but Not A-SMase to Generate Ceramide at the Endothelial Cell Surface—To determine whether elevated hemodynamic stressors in situ could activate SMase activity, we measured the activity of N- and A-SMase in rat lungs under elevated pulmonary artery pressure. The blood was flushed at 37 °C from the lung vasculature at pulmonary artery pressures of 6–8 mm Hg for 5 min. Then the vascular pressure was either maintained at 6–8 mm Hg (basal flow) or elevated to 14–16 mm Hg, which more than doubles the flow rate (high flow). The lungs were subfractionated in order to measure the activity of both N- and A-SMase in the total lung H and in the isolated P. P relative to H is enriched 15–20-fold in endothelial cell surface markers (i.e. caveolin, 5′-nucleotidase (5′-NT), and angiotensin converting enzyme) while being markedly depleted of proteins localized elsewhere in the cell or tissue (i.e. fibroblast surface antigen, e-COP, ERK1/2) (3, 4, 20, 29) (Fig 3D). Our previous studies using this perfusion model and isolation system detected rapid activation of eNOS and tyrosine kinases at the luminal endothelial cell plasma membrane (3, 4). Here, N-SMase activity in P increased rapidly with greater vascular pressure/flow rate (Fig 1). This response was not detected in H, consistent with the small proportion of luminal endothelial cell plasma membranes in the tissue. A-SMase activity was detected, but did not change in either fraction over the 5 min of increased vascular pressure/flow.

We next focused on the mechanoactivation of N-SMase at the
endothelial cell surface in greater detail. Fig. 2A shows that the increase in the specific activity of N-SMase in P began to occur within 30 s of elevated perfusion pressure/flow. The activity reached a maximum of 2.2-fold over baseline at 2 min (1.53 ± 0.1 nmol/mg/h (n = 4) versus 0.68 ± 0.017 nmol/mg/h (n = 4)). The N-SMase activity began to decrease after 3 min and had returned to baseline levels by 10 min. The N-SMase activity appeared constant in controls subjected only to the baseline pressure/flow. We also tested whether this transient mechanoresponse could be repetitively induced. Lungs perfused at high pressures for 2 min and then at baseline pressure for 3 min followed by a second high pressure/flow rate perfusion for 2 min showed a second rapid increase in N-SMase activity in the P (data not shown). Thus, the activity of N- but not A-SMase was acutely responsive to, and rapidly activated by, increased hemodynamic stressor in situ. This induction was transient but not refractory after only 3 min.

Because SMase catalyzes the hydrolysis of sphingomyelin to produce ceramide, the activation of this enzyme should result in the transient accumulation of the product in the membranes. We measured ceramide levels in P under similar perfusion conditions and found that ceramide levels closely followed N-SMase activity. Just 1 min of vascular perfusion at the elevated pressure/flow rate increased ceramide level detected in P by 30% over the control (Fig. 2B). This response reached a maximum of 80% after 3 min of perfusion, maintained a plateau through 5 min, and decreased significantly by 10 min. In the controls where the flow rate/pressure was kept constant over the same time, the ceramide levels did not change.

Next, we studied the sensitivity of N-SMase and ceramide formation to different flow rates and vascular pressures. Because maximal activation was observed after 2 min stimulation (Fig. 2A), we perfused the vasculature for 2 min at pressures varying from 6 to 18 mm Hg, which produced flow rates in this system varying from 4 to 14 ml/min, respectively. As shown in Fig. 2C, even a small increase to 8 mm Hg (6 ml/min) from a baseline of 6 mm Hg increased N-SMase activity in P by 30%. A maximum of nearly 2-fold over baseline activity was reached at 12–14 mm Hg (10–12 ml/min). Under the same conditions, ceramide levels in P also increased progressively to an apparent maximum of 1.7-fold over baseline at 12–14 mm Hg (10–12 ml/min) (Fig. 2D). These cumulative results demonstrated that the acute increase in vascular pressure/flow rapidly stimulates N-SMase activity to induce formation and accumulation of ceramide in the endothelial cell plasma membrane in a time- and flow rate-dependent manner.

N-SMase and Its Activity in Caveolae—The mechanosensitive SMase existing at the luminal endothelial cell surface may be associated with caveolae, because endothelial caveolae are primary sites for rapid mechano-induced tyrosine phosphorylation of proteins (3) and may be mechanosensing organelles (3, 4, 11, 16) containing many signaling molecules including sphingomyelin and nonreceptor tyrosine kinases (20, 22). Enzyme activities were measured in caveolae isolated from P in the presence or absence of Triton X-100 (V and V' fraction, respectively). With detergent, the major activity was detected in the Triton-soluble fractions at levels nearly 5-fold enriched over P (Fig. 3A). This is consistent with the known detergent solubility of N-SMase as well as sphingomyelin extractability (34). Without detergent, substantial N-SMase activity was found in caveolae with nearly 6-fold enrichment over P (Fig. 3B). By contrast, the major activity of A-SMase was found in lung homogenates with >10-fold depletion in P and even more so in caveolae (Fig. 3C). As shown in Fig. 3D and in past studies (4,
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20, 28, 31), the isolated caveolae were enriched >15-fold in caveolar markers, such as caveolin, while being markedly depleted (>15-fold) in noncaveolar markers such as β-actin, 5'-NT, and ε-COP. These results indicated that the luminal endothelial cell plasma membranes and their caveolae are enriched in functional N- but not A-SMase and that this N-SMase is at least partially detergent-soluble. These results are consistent with past data showing A-SMase to be an intracellular enzyme primarily localized to the cytoplasm, lysosomes, and endosomes (26). Although one report found A-SMase activity in caveolin-rich membrane subfractions (35), a more detailed examination of these fractions by Western analysis and immunoisolation with caveolin antibodies demonstrated that these isolates contain not only caveolae but also lipid rafts as well as possible microdomains of endosomal, Golgi, nuclear, and possibly other cell membranes (28, 31, 36).

To determine the protein localization of N- and A-SMase, we examined their distribution in plasma membranes and a caveole fraction isolated from BLMVEC using our immunoisolation procedure (31). Western analysis detected significantly more N-SMase in AC over the crude PM, whereas A-SMase resided primarily in the PNS (Fig. 4). Because AC contains nuclear and other membrane microdomains in addition to cell surface lipid rafts and caveolae (31, 36), we immunoisolated caveolae from AC using anti-caveolin-1 antibodies (31). The bound fraction of caveolae contained readily detectable N-SMase not found in the unbound fraction. We used bovine cells because the N-SMase antibodies were generated against a peptide derived from bovine enzyme (27) and did not cross-react with N-SMase from rat tissues (data not shown). Western analysis of H and P from BLMVEC detected a single immunoreactive band of a 95–100-kDa protein, which was not immunoreactive to the preimmune serum (data not shown). Western analysis using a commercially available A-SMase antibody showed enrichment in PNS but not in PM or AC (Fig. 4). Thus, N-SMase resides primarily in caveolae.

Increased Pressure/Flow Stimulates Caveolar N-SMase—To determine whether increased pressure/flow stimulates N-SMase located in caveolae, we subfractionated rat lungs subjected to baseline versus high pressure/flow conditions before measuring N-SMase activity. Consistent with the experiments described above (Fig. 2A), the N-SMase activity in P nearly doubled when the pressure/flow rate was elevated. This effect was even more pronounced in V' (Fig. 5), with a 2.6-fold increase over baseline conditions. With increased pressure/flow, the N-SMase-specific activity in caveolae was 6.4-fold higher than that in P fraction. Under high flow conditions, a modest but significant (56% over the control) increase in the N-SMase activity in plasma membranes stripped of caveolae (P-V') was observed. This effect can be explained by some residual caveolae left attached to P-V' as detected by Western analysis for caveolin-1 (Fig. 3D). Because the caveolin membrane constitutes about 50% of the lung luminal endothelial plasma membrane and assuming that V' is indeed representative of those caveolae, then >85% of the baseline as well as mechano-induced N-SMase activity at the plasma membrane occurs in the caveolae. These results cumulatively indicate that the N-SMase activity associated with caveolae is sensitive to mechanical shear forces and can be rapidly activated by increased vascular pressure/flow in situ.

N-SMase Activity is Required for MAP Kinase Mechanostimulation—One of the best described signaling events caused by mechanical stressors is activation of cytosolic MAP kinases, i.e. ERK1 and 2 (3, 9). To assess whether N-SMase activity is upstream and required for MAP kinase mechanoactivation, we used scyphostatin, a specific N-SMase inhibitor (27, 37). Using a monoclonal antibody specific for activated ERK1 and 2, we found that (as in our past work; see Ref. 3) high pressure/flow dramatically activate both kinases (Fig. 6). Preexposure to scyphostatin (50 μM) totally blocks this MAP kinase mechanoactivation. We also confirmed past work (27, 37) in in vitro experiments, showing that scyphostatin, at 50 μM, inhibited N-SMase activity in P by 75% ± 2, whereas A-SMase activity was not affected (0% ± 5). We also found that scyphostatin does not in itself inhibit kinase activity as assessed using in vitro

![Graphs showing enrichment of N- and A-SMase in the endothelial luminal surface plasma membranes and their caveolae.](image-url)
assays (Ref. 20, and data not shown). Because our in situ system does not discriminate between pressure and shear effects, we subjected bovine aortic endothelial cell monolayers to increased shear stress and found scyphostatin inhibited shear stress-activation of MAP kinases (data not shown). Thus, this inhibition of N-SMase prevents the downstream mechanoactivation of MAP kinase signaling pathways, further supporting the key role of N-SMase and ceramides as initiators of mechanotransduction.

**DISCUSSION**

Sphingomyelin is a structural lipid component of the plasma membrane, and its metabolites play important roles in signal transduction. The sphingomyelin turnover is initiated by the activation of SMase by cytokines and cellular stressors such as UV and ionizing radiation and heat shock, leading to the generation of ceramide, which acts as a second messenger in activating a variety of cellular functions (26, 38, 39). It is most likely that different isoenzymes, localized to different subcellular compartments, contribute to sphingomyelin turnover and sphingolipid signaling (26). Here, we report the discovery that N-SMase functional activity responds to important cardiovascular mechanical stressors, specifically from elevated intravascular pressure and fluid flow shearing. These hemodynamic stressors rapidly (within less then a minute) but transiently induced N-SMase, but not A-SMase, activity at the luminal endothelial cell surface to generate a transient increase in membrane ceramides. These stressors represent a novel physiological pathway for N-SMase stimulation. Although hemodynamic activation of N-SMase could occur generally over the plasma membrane, it appears to be concentrated and more greatly stimulated in the caveolae. It remains to be seen whether some N-SMase resides at or near the neck region of caveolae for more direct exposure to fluid shear and/or whether more than one pool of N-SMase can be activated to a different extent. The availability of the substrate, sphingomyelin (20, 22, 40), may also contribute to the high activity and rapid stimulation observed in caveolae.

There is no consensus yet on the number, localization, and function of N-SMase isoforms. To date, several N-SMase isoforms have been cloned (41–45) or purified (34, 46–50) from various mammalian tissues, and these isoforms may exhibit distinct tissue and intracellular distribution (43, 51, 52). Ligand-induced sphingomyelin hydrolysis and the generation of ceramide within caveolin-rich fractions prepared by a nondenaturing method was reported previously in fibroblasts (23) and PC12 cells (24). More recently, two independent groups have reported N-SMase activity in low buoyant density, detergent-resistant, caveolin-rich membrane fractions (35, 53). These N-SMase activities, however, were Triton X-100 insoluble, whereas the mechano-induced N-SMase activity detected in our rat lung endothelial caveolae isolates was Triton soluble (detected in V′ and T but not in V; see Fig. 3A), suggesting that the N-SMase activities in the two systems correspond to different forms. Furthermore, whether the previously noted SMase activities reside in caveolae remains to be established because of the heterogeneity of the isolated membrane fractions (35, 53).

The detection of distinct isoforms may be due to differences in cell type (cultured murine endothelial cells (35), human fibroblasts (53) or rat lung endothelial cells in situ (this study)) and isolation methods used in the present and past (35, 53) studies. Note that similarly prepared caveolin-enriched membrane fractions from whole cell lysates (35, 53) have been shown to contain membrane microdomains from plasmalemmal intercellular junction complexes and various intracellular organelles (Golgi, nucleus) in addition to caveolae, caveolin-coated vesicles, and lipid rafts rich in GPI-anchored proteins (28, 31, 54). Here, we used the silica-coating procedure in rat lungs in situ as well as the immunoisolation of caveolae from caveolin-enriched bovine endothelial cell membranes to obtain highly pure preparations of plasma membrane caveolae essentially free of intracellular membranes. Although various isoforms of N-SMase may exist in caveolae or lipid rafts, the caveolar isoform identified here by using specific antibodies corresponds to the isoform cloned by Bernardo et al. (27), which
may be the Triton-soluble isoenzyme that responds to mechanical stimulation in the rat lung.

In this report, we describe the localization of N-SMase activity in rat lung endothelial cells to caveolae, which are putative "signaling centers" and "mechanosensing organelles" (16). We also demonstrate by Western analysis the presence of one N-SMase isoform (27) in caveolae isolated from bovine endothelial cells. Sphingomyelin, the major sphingolipid of mammalian plasma membrane, is localized to the outer leaflet of lipid bilayer, which is oriented externally on the plasma membrane (55–57). Interestingly, the N-SMase isoform described by Veldman et al. (53) can be inhibited by interaction with a peptide corresponding to the scaffolding domain of caveolin-1, suggesting an intracellular orientation, contrary to past reports (55, 58). N-SMase also localizes to the plasma membrane and likely exhibits extracellular orientation (55, 58). Thus, the enzyme might be directly exposed to hemodynamic stressors, including fluid shear and vascular pressure, and serve as a mechanosensor on the luminal endothelial cell surface to initiate signal transduction into the cell. If N-SMase is a cell surface mechanosensor acutely initiating the transduction of hemodynamic forces through its direct exposure to these forces that increase activity, then the product of its increased activity, ceramide, will stimulate diverse downstream signaling pathways. Here, we show that inhibition of the N-SMase activity by scyphostatin leads to the inhibition of downstream cytosolic elements of mechanism signaling, namely ERK1 and ERK2 kinases.

The elucidation of upstream and downstream molecules and the mechanism of their involvement in endothelial mechantoresponsiveness is currently underway in our laboratory. Ceramide can activate several intracellular signaling molecules, including JNK (59) through unknown mechanisms and MAP kinase (60) through the interaction of ceramide-activated protein kinase with Raf (61). Ceramide can also regulate the activity of ion channels (62, 63), NRTK and Ras (64), eNOS (65), and, ultimately, transcription factors, such as NFκB (66). Interestingly, all of these downstream targets of ceramide also respond to hemodynamic stressors. Increased flow, pressure, and/or shear can rapidly activate eNOS (4), MAP kinase (3, 9), K⁺ channels (10), and, later, JNK (12). Increased vascular pressure and flow in situ rapidly activates both plasmalemmal tyrosine kinases, causing protein phosphorylation primarily in caveolae and the Ras/Raf/ERK kinase pathway with rapid Raf translocation to caveolae (3). Disassembly of caveolae with dispersion of their molecular constituents over the membrane surface prevents both rapid flow-induced protein phosphorylation and MAP kinase activation (3). Lastly, eNOS plays a very important role modulating vascular tone and maintaining systemic blood pressure, vascular remodeling, and angiogenesis. We have shown in the same system used in this study that eNOS resides quite concentrated in the luminal endothelial caveolae (4) and can be rapidly activated by increased vascular flow/pressure (3, 4). Others have shown calcium-independent activation of eNOS by exogenous ceramide (65) as well as another pathway of eNOS regulation through phosphorylation by the protein kinase Akt (67). Shear activates phosphoinositol-3-kinase (PI-3-kinase) and ceramide can act upstream of Akt by calcium-sensitive activation of PI-3-kinase, possibly via Src-like kinases (68). Increased hemodynamic stressors and exogenous ceramide can induce acute NO-dependent vasodilation (69) as well as ERK (9) and plasmalemmal Src-like tyrosine kinases (9, 11). Moreover, we have observed the activation of Akt in response to both exogenous ceramide administration and high pressure/flow in situ.²

Caveolae may play a central role in acute mechanotransduction, acting as mechanosensing organelles by forming a distinct microdomain or compartment that concentrates N-SMase and its substrate, sphingomyelin, as well as key downstream effectors such as eNOS, NRTK, and PI-3-kinase (20), which respond to the localized generation of ceramide. It appears likely that this specialized compartment is critical for the efficient propagation into the cell of one or more signals induced mechanically. N-SMase and caveolae may be fruitful targets for future investigations for many reasons, including the importance of endothelial dysfunction and disrupted vasoregulation in the pathogenesis of vascular disease.

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