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Ceramide: A Novel Cell Signaling Mechanism for Vasodilation

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Ceramide is a lipid second messenger generated by membrane hydrolysis of sphingomyelin by sphingomyelinase, but a role for this novel signaling pathway in vascular smooth muscle has not been elucidated. Based upon observations of cytokine-induced increases in sphingomyelinase activity, we hypothesized that ceramide plays a cell signaling role in vasodilation. Here, we demonstrate that ceramide is present at significant basal levels in cultured vascular smooth muscle cells and that these levels may be increased using exogenous sphingomyelinase. We also report that both exogenously added ceramide and sphingomyelinase cause dose-dependent relaxation in phenylephrine-contracted endothelium-denuded rat thoractic aortic rings. We conclude that the ceramide signaling pathway represents a novel signal transduction mechanism for vasodilation.

Ceramide is a sphingolipid second messenger formed by hydrolysis of membrane sphingomyelin by sphingomyelinase (SMase, 1). Membrane-bound neutral SMase is a member of a superfamily of lipid mediator-generating enzymes including phospholipase A, phospholipase C and phospholipase D (2). The A, C, and D forms of phospholipase have well-defined roles in vascular smooth muscle in the context of contraction via generation of lipid second messengers such as arachidonic acid, diacylglycerol, and phosphatidic acid. While ceramide has been shown to mediate intracellular signals of cytokines such as such TNF-α, γ-INF, and IL-1β (1,3), such a role has not yet been defined for sphingomyelinase and the second messenger ceramide in vascular smooth muscle. Because these cytokines are all vasodilators, we hypothesized that ceramide plays a signaling role in vascular smooth muscle relaxation.

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We report that cultured vascular smooth muscle cells contain basal ceramide levels that can be increased by incubation with sphingomyelinase. We also report that treatment of contracted blood vessel segments with both ceramide and sphingomyelinase results in concentration-dependent relaxation.

MATERIALS AND METHODS

Cell culture. Vascular smooth muscle cells (VSMCs) were isolated and cultured by explantation. Briefly, segments of Sprague Dawley rat thoracic aorta cleaned of adherent fat, connective tissue and endothelium were seated in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum. After a 7-10 day period of VSMC proliferation, tissue segments were removed, and VSMCs were passaged upon confluency. Cells were maintained through 10-15 passages in DMEM supplemented with 5% fetal bovine serum.

Ceramide measurement. For extraction of cellular lipids, the methods of Van Veldoven and Bell were used (4). Lipids were extracted VSMCs after treatment with SMase or vehicle for the indicated time points. Ceramide measurement was carried out with the methods of Preiss, et al. (5). Known concentrations of 1,2-dioleoyl-*sn*-glycerol and type III ceramides were used to obtain standard curves. Protein was determined using the Bio-Rad protein assay kit.

Vessel preparation. Male Sprague Dawley rats weighing approximately 200-250 g were used for this study. After anesthetizing with pentobarbital (50 mg / kg), the chest was opened and the thoracic aorta removed and placed in cold physiologic salt solution (PSS)(130 mM NaCl, 4.5 mM KCl, 1.18 mM $KHPO_4$, 1.17 mM $MgSO_4$, 1.6 mM $CaCl_2$-$2H_2O$, 14.9 mM $NaHCO_3$, 5.5 mM dextrose, and 0.03 mM $CaNa_2$ EDTA). Each aorta was cleaned of adherent fat and connective tissue and cut into rings (4 mm long). Endothelium was removed from the rings by gently rubbing the lumen with the tips of a pair of forceps.

Relaxation response to ceramide/SMase. Aortic rings were mounted between a force transducer (Grass F&03, Quincy, MA) and a displacement device in a jacketed organ bath filled with PSS, gassed with 95% O_2 and 5% CO_2, and maintained at 37°C. Isometric force was measured throughout the experiment. A passive force of 3 g was applied to the rings for an equilibration period of 1 hr, during which indomethacin (10^{-5} M) was added to inhibit cyclooxygenase. A concentration response curve to phenylephrine (10^{-9} to 10^{-5} M) was constructed for each ring to determine the concentration producing a half-maximal (EC_{50}) contractile response to phenylephrine. After the effects of phenylephrine were washed out, vessel segments were

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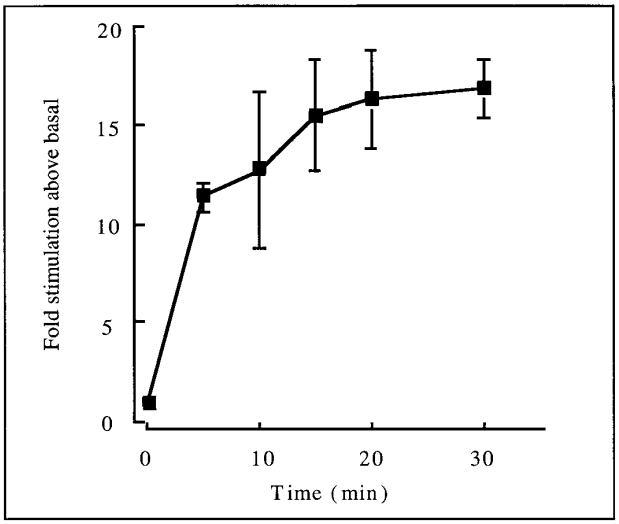

FIG. 1. Incubation of vascular smooth muscle cells (VSMCs) with 0.1 U/ml SMase increases intracellular ceramide. Cultured VSMCs were treated with 0.1 U/ml SMase for the indicated time points. Basal ceramide levels were determined in cells not treated with SMase. Intracellular ceramide was measured using the methods of Van Veldoven, et al., and Preiss, et al. (4,5). Stimulated levels measured at 5 min and longer were significantly greater than basal ($p < 0.05$).

contracted with an EC_{50} dose of phenylephrine. When the contraction reached a plateau phase, ceramide (N-acetyl-D-sphingosine, 10^{-9} to 10^{-5} M) or bacterial sphingomyelinase (0.001 to 0.1 U/ml) was added cumulatively to the bath. The relaxation response was normalized to the percent of the contraction induced by EC_{50} phenylephrine.

Pharmacological agents. The following compounds were used in this experiment: Type III ceramides from bovine brain, N-acetyl-D-sphingosine, sphingomyelinase (from *B cereus*), phenylephrine HCl, indomethacin were purchased from Sigma Chemical Co., St. Louis, MO. N-Acetyl-D-sphingosine was prepared as 2×10^{-2} M a stock solution in 95% ethanol. The concentration of ethanol in the organ bath did not exceed 0.1%. sn-1,2-Diacylglycerol assay kit was purchased from Amersham Life Sciences.

Statistics. Data are presented as means ± standard error of the mean. For two-group comparisons, Student's t test was used, with a p-value less than 0.05 being considered significant.

RESULTS AND DISCUSSION

Cultured VSMCs were used between passages 10 and 15 for this experiment. In the absence of SMase incubation, VSMCs contained basal levels of ceramide (0.3 nmol/mg protein). Treatment of VSMCs with exogenous SMase (0.1 U/ml) resulted in a time-dependent increase in intracellular ceramide, ranging from a 12-fold increase at 5 min, to 17-fold at 30 min (Figure 1). The basal levels of ceramide determined in this experiment fall within the range of basal values found in other cell types such as vascular endothelial cells (approximately 0.5 nmol/mg prot.), NIH 3T3 fibroblasts (6), and human polymorphonuclear leukocytes (7). Previous groups have shown that treatment of various cell types with SMase results in significant increases in intracellular ceramide levels , ranging from 3 to 17-fold increase above baseline values at similar time points (8, 9). Therefore, our results are similar to those reported for other cell systems.

Treatment of phenylephrine-contracted endothelium-denuded rat thoracic aortic rings with ceramide resulted in a dose-dependent relaxation, with a maximum relaxation of 40% at 10 μM (Figure 2A). For these experiments, a more cell permeant form of ceramide, N-acetyl-sphingosine (C_2-ceramide), dissolved in absolute ethanol was used. Ethanol concentrations in the muscle bath did not exceed 0.1%. In a similar set of experi-

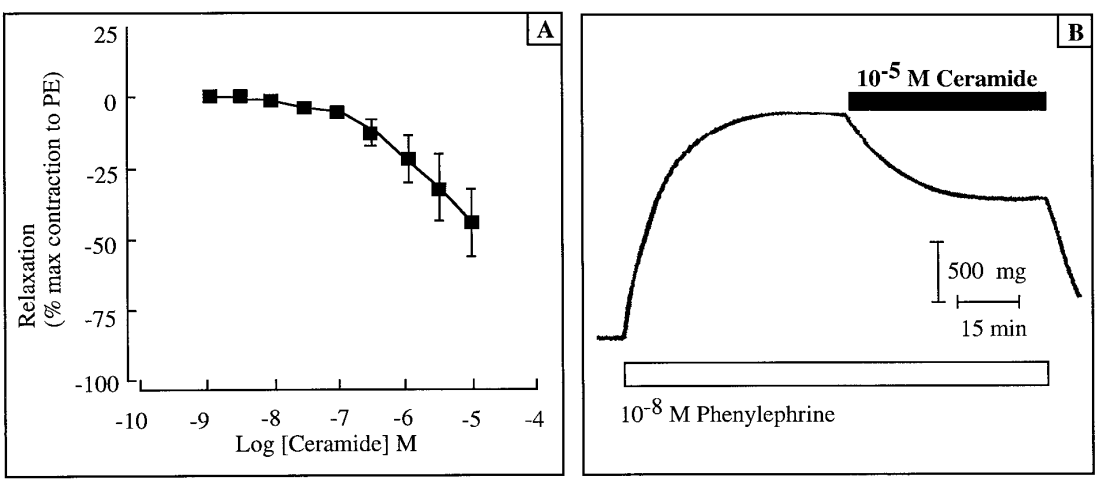

FIG. 2. Ceramide causes concentration-dependent relaxation of endothelium-denuded phenylephrine-contracted rat thoracic aortic rings. Sprague-Dawley rat thoracic aortic rings were contracted with an EC50 of phenylephrine. C_2-ceramide (0 - 10^{-5} M) was added, and the resulting changes in force were recorded as described in Methods. (A) Dose-response curve for ceramide-induced relaxation. (B) Tracing of relaxation response to ceramide (10^{-5} M) in rat thoracic aortic ring. Relaxation occurring at ceramide concentrations greater than 3×10^{-7} Mwas significantly different from EC50 phenylephrine contraction ($p < 0.05$).

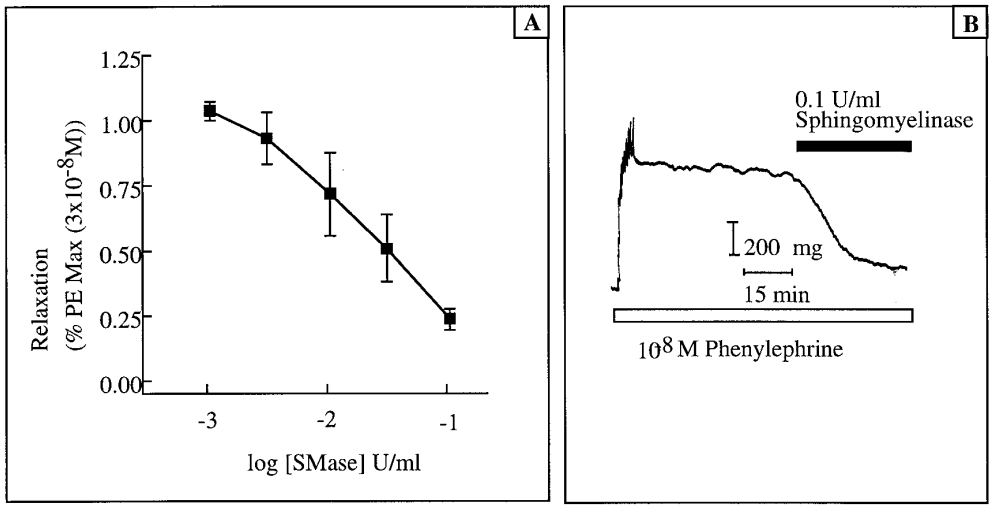

FIG. 3. Sphingomyelinase (SMase) causes dose-dependent relaxation of endothelium-denuded phenylephrine-contracted rat thoracic aortic rings. Sprague-Dawley rat thoracic aortic rings were contracted with an EC50 of phenylephrine. Bacterial SMase (0 - 0.1 U/ml) was added to the muscle baths, and the resulting changes in force were recorded as described in Methods. (A) Concentration-response curve for SMase-induced relaxation. (B) Tracing of relaxation response to SMase (0.1 u/ml) in rat thoracic aortic ring. Relaxation occurring at SMase concentrations greater than 3×10^{-3} U/ml was significantly different from EC50 phenylephrine contraction ($p < 0.05$).

ments, exogenous SMase was added to phenylephrine-contracted endothelium-denuded rat thoracic aortic rings resulting in a dose-dependent relaxation (Figure 2B). Despite the use of C_2-ceramide we were unable to use concentrations higher than 10 μM, due to poor solubility. Therefore, the maximum relaxation response to C_2-ceramide achievable in these experiments was 40%, whereas the rings incubated with 0.1 U/ml SMase relaxed to baseline (Figure 3). It is important to note that the time to relaxation after SMase addition in the aortic ring preparations is similar to the time-course of ceramide generation in cultured VSMCs. That is, relaxation occurs within five minutes of addition of SMase to the muscle bath, parallel to the 12-fold increase in intracellular ceramide after five minutes as seen in Figure 1.

We propose that ceramide plays a signaling role in cytokine-induced vasodilation. TNF-α has been shown to stimulate SMase and elicit increases in intracellular ceramide in other cell systems, and to cause endothelium-independent relaxation of contracted vessel segments (10, 11). Ceramide has been shown to stimulate both a ceramide-activated protein kinase, a ceramide-activated protein phosphatase, as well as to inhibit protein kinase C in other cell systems (9, 12, 13). Protein kinase C is crucial in mediating the contractile effects of g-protein coupled physiologic agonists such as norepinephrine and angiotensin II. Given the importance of PKC in vascular smooth muscle contraction, the vasodilatory effects of TNF-α, and the reports of inhibition of PKC by ceramide, we believe that our results suggest a role for ceramide-mediated relaxation in the vasculature.

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REFERENCES

1. Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 3125–3128.
2. Serhan, C. N., Haeggstrom, J. Z., and Leslie, C. C. (1996) *FASEB J.* **10,** 1147–58.
3. Obeid, L. M., and Hannun, Y. A. (1995) *J. Cell Biochem.* **58,** 191–198.
4. Van Veldoven, P. P., and Bell, R. M. (1988) *Biochim. Biophys. Acta.* **959,** 185–196.
5. Preiss, J. E., Loomis, C. R., Bell, R. M., and Neidel, J. E. (1987) *Meth. Enzymol.* **141,** 294–299.
6. Olivera, A., Buckley, N. E., and Spiegel, S. (1992) *J. Biol. Chem.* **267,** 26121–26127.
7. Nakamura, T., Abe, A., Balazovich, K. J., Wu, D., Suchard, S. J., Boxer, L. A., and Shayman, J. A. (1994) *J. Biol. Chem.* **269,** 18384–18389.
8. Dressler, K. A., and Kolesnick, R. N. (1990) *J. Biol. Chem.* **265,** 14917–14921.
9. Jones, M. J., and Murray, A. W. (1995) *J. Biol. Chem.* **270,** 5007–5013.
10. Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 3125–3128.
11. Hollenberg, S. M., Cunnion, R. E., and Parillo, J. E. (1991) *Chest* **100,** 1133–1137.
12. Mathias, S., Dressler, K. A., and Kolesnick, R. N. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10009–10013.
13. Dobrowsky, R. T., Kamibayashi, C., Mumby, M. C., and Hannun, Y. A. (1993) *J. Biol. Chem.* **268,** 15523–15530.