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Short Communication

Endothelium Dependent and Independent Relaxations Induced by Ceramide in Vascular Smooth Muscles

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Abstract

The second messenger of sphingomyelin signaling, ceramide, acts as an intracellular signal via phosphatase activation and protein kinase C (PKC) inhibition. We tested the hypothesis that ceramide may have an regulatory role in determining vascular tone. Natural ceramide was applied to phenylephrine precontracted aortic rings from Sprague-Dawley rats in an organ bath. In endothelium-intact aortic rings, concentrations of ceramide at 10^{-6} and 10^{-5} mole/L induced 24 ± 6 and 52 ± 7% relaxation, respectively. Removal of the endothelium significantly inhibited ceramide-induced relaxation to 13 ± 5% (10^{-6} mole/L) and 29 ± 5% (10^{-5} mole/L). Similar inhibition was observed in endothelium-intact aortic rings pretreated with N^{ω}-nitro-L-arginine (10^{-4} mole/L) or methylene blue (10^{-5} mole/L), suggesting that endothelium-derived nitric oxide is involved in ceramide-induced relaxation. N-acetylsphingosine (C_2-ceramide), N-hexanoylsphingosine (C_6-ceramide), N-palmitoylsphingosine (C_{16}-ceramide) and D-sphingosine all demonstrated dose-dependent relaxation responses in endothelium-intact vessels. Sphingomyelin signaling through the nitric oxide-dependent mechanism may have an important role in regulating vascular tone.

Key Words: sphingomyelin, ceramide, vascular smooth muscles

Introduction

The sphingomyelin pathway is a ubiquitous signaling system present in mammalian cells (12). This pathway is initiated when sphingomyelin (N-acylsphingosine-1-phosphocholine), a phospholipid concentrated in the plasma membrane, is hydrolyzed (10, 11) to release the second messenger ceramide. Previous studies of ceramide have focused largely on its activation of a cellular kinase (8), a protein phosphatase (1, 13, 20), and on its inhibition of protein kinase C (7).

It has been shown that the contraction of vascular smooth muscle stimulated by alpha agonist is mediated by the activation of protein kinase C (9, 16), phospholipase D (19) and calcium (18). The possibility that sphingomyelin signaling is involved in regulating vascular tone would have to be considered if the sphingomyelin pathway could be shown to alter any of the contractile mechanisms of the alpha agonist.

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Indeed, previous studies have demonstrated that ceramide inhibits the activities of protein kinase C (7), phospholipase D (2) and calcium channels (21). Thus, the second messenger ceramide could regulate vascular tone through those mechanisms. We hypothesized that the sphingomyelin pathway has an important role in regulating vascular tone. By applying ceramide in phenylephrine-precontracted vessels in a tissue bath, we demonstrated ceramide-induced relaxation in isolated vessels.

Wong et al., have demonstrated that ceramide inhibits the calcium influx stimulated by fMet-Leu-Phe in neutrophils (21). This calcium inhibitory effect of ceramide can be blocked by a protein phosphatase inhibitor okadaic acid (21). We therefore tested the hypothesis that ceramide-induced relaxation in endothelium removed aortic rings is mediated by activation of a protein phosphatase and/or inhibition of protein kinase C. Our results demonstrated that while sphingomyelin signaling has an important role in regulating vascular tone, neither a protein phosphatase nor protein kinase C is likely to be involved in ceramide-induced relaxation of vascular smooth muscle.

Materials and Methods

Male Sprague-Dawley rats weighing approximately 200-250 g were used for the experiments. After anesthetizing with pentobarbital (50 mg/kg) intraperitoneally, the chest was opened and the thoracic aorta was carefully removed and placed in a cold physiological salt solution (PSS), (130 mmole/L NaCl, 4.7 mmole/L KCl, 1.18 mmole/L KH_2PO_4, 1.17 mmole/L $MgSO_4$, 1.6 mmole/L $CaCl_2$-$2H_2O$, 14.9 mmole/L $NaHCO_3$, 5.5 mmole/L dextrose, and 0.03 mmole/L $CaNa_2EDTA$). Each aorta was then cleaned of all loosely adherent tissue and six rings (4 mm long) were cut from each and prepared for the following experiments. In some of the aortic rings, the endothelium was removed by rubbing the aortic segment with a pair of forceps. The complete removal of endothelium was tested by applying acetylcholine (10^{-6} M) in near half-maximal (EC_{50}) phenylephrine-precontracted vessels. The entire experimental procedure conforms to the guidelines set by the American Physiological Society and the University of Michigan Unit for Laboratory Animal Medicine.

Relaxation Response to Ceramide

The endothelium-intact or -removed aortic rings were mounted between a force transducer (Grass FT03, Quincy, MA) and a displacement device in a jacketed organ bath filled with PSS. The PSS was kept at 37°C and gassed with 95% O_2 and 5% CO_2. Isometric tension was recorded throughout the experiment. After preparation, a passive force of 3 g was applied to the rings. After vessel segments were allowed to equilibrate for 40 minutes, a previously determined EC_{50} dosage of phenylephrine was applied to the bath. When the EC_{50} phenylephrine-induced contraction reached a plateau, natural ceramide (10^{-6} and 10^{-5} mole/L) from bovine brain sphingomyelin was applied cumulatively to the bath. The relaxation response to ceramide was normalized to the percent relaxation of the contraction induced by EC_{50} phenylephrine.

In some experiments, endothelium-intact aortic rings were pretreated with a nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 10^{-4} mole/L) or a guanylate cyclase inhibitor methylene blue (10^{-5} mole/L) for 20 minutes. Cumulative doses of ceramide were administrated to the bathes which contained rings precontracted with near EC_{50} phenylephrine. These experiments were performed to determine if endothelium-derived nitric oxide is involved in ceramide-induced relaxation. After washout the effects of ceramide on endothelium-removed aortic rings, the contractile responses to phenylephrine (10^{-10} mole/L to 10^{-5} mole/L) were repeated to demonstrate the viability of the vessels (data not shown).

Inhibition of Protein Kinase C and Phosphatase on Ceramide-Induced Relaxation

To determine if inhibition of protein kinase C or activation of a protein phosphatase are involved in ceramide-induced relaxation in endothelium-removed aortic rings, ceramide-induced relaxation of these vessels was studied in the presence of protein kinase C inhibitor chelerythrine (3 μmolole/L), or a protein phosphatase inhibitor okadaic acid (10 nmole/L). Chelerythrine has been previously identified as a potent protein kinase C inhibitor with an IC_{50} of 0.66 μM (6).

Response to Different Sphingomyelin-Related Compounds

To study the effectiveness of different fatty acyl chains of ceramide on ceramide-induced responses, N-acetylsphingosine (C_2-ceramide), N-hexanoyl-sphingosine (C_6-ceramide) or N-palmitoyl-sphingosine (C_{16}-ceramide) were applied cumulatively to endothelium-intact aortic rings. The breakdown product of ceramide, D-sphingosine, was also tested on endothelium-intact vessels.

Pharmacological Agents

The following agents were purchased from

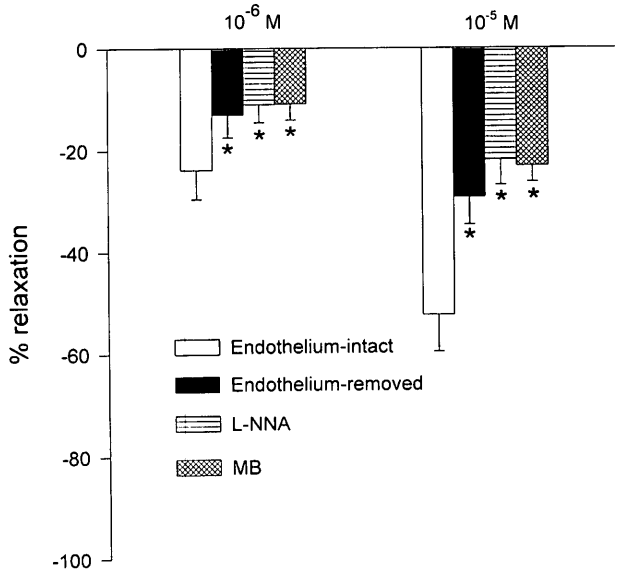

Fig. 1. Relaxation effect of natural ceramide in endothelium-intact aortic rings (open bars, n = 8) pretreated with endothelium removal (closed bars, n = 8), N^{ω}-nitro-L-arginine (L-NNA, 10^{-4} mole/L, horizontal hatched bars, n = 8) or methylene blue (MB, 10^{-5} mole/L, n = 8, cross-hatched bars). Values are mean ± S.E.M. Asterisks indicate a significant difference when compared to the response of endothelium-intact aortic rings ($p<0.05$).

Sigma Chemical Company (St. Louis, MO): natural ceramide (type III) from bovine brain sphingomyelin, D-sphingosine from bovine brain sphingomyelin, L-phenylephrine HCl, N^{ω}-nitro-L-arginine, methylene blue and acetylcholine hydrochloride. N-acetyl-sphingosine (C_2-ceramide), N-hexanoylsphingosine (C_6-ceramide), N-palmitoyl-sphingosine (C_{16}-ceramide) were purchased from Calbiochem (La Jolla, CA). Chelerythrine chloride and okadaic acid were purchased from Research Biochem. International (Natick, MA). The natural ceramide, C_2-ceramide, C_6-ceramide, C_{16}-ceramide and sphingosine were prepared as 2×10^{-2} M stock solutions in 95% ethyl alcohol. The concentration of ethanol in the tissue bathes did not exceed 0.1%. With this concentration of ethanol, previous study from our laboratory showed no signicant change of vessel tone.

Statistics

Results are expressed as the mean±standard error of the means (S.E.M.) for all observations. Statistical analysis of the data was performed by Student's paired and un-paired tests. Multiple comparison of means was accomplished by ANOVA followed by the Newman-Keuls test when appropriate. A P-value less than 0.05 was considered to be statistically significant. When necessary, Bonferroni correction was applied to adjust for multiple comparisons.

Results

Role of Endothelium-Derived nitric Oxide on Ceramide-Induced Relaxation

Removal of endothelium significantly inhibited the relaxation response to ceramide when compared to those of endothelium-intact aortic rings (Fig. 1). At concentrations of 10^{-6} mole/L and 10^{-5} mole/L, ceramide-induced relaxations were 13 ± 5% and 29 ± 5% for endothelium-removed aortic rings and were 24 ± 6% and 52 ± 7% for endothelium-intact aortic rings, respectively. This indicates that an endothelium-derived relaxation factor contributes to ceramide-induced relaxation. Whether or not endothelium-derived nitric oxide is involved in the ceramide-induced relaxation in endothelium-intact aortic rings was studied by pretreating the vessels with a nitric oxide synthase inhibitor L-NNA (10^{-4} mole/L, Fig. 1) or with a guanylate cyclase inhibitor methylene blue (10^{-5} mole/L, Fig. 1). Both L-NNA and methylene blue pretreatment significantly inhibited the 10^{-6} and 10^{-5} mole/L ceramide-induced relaxations (11 ± 4% and 22 ± 5% for L-NNA pretreated; and 11 ± 3% and 23 ± 3% for methylene blue pretreated, respectively).

Inhibition of Protein Kinase C and Phosphatase on Ceramide-Induced Relaxation

In endothelium-removed aortic rings, ceramide-induced relaxation was not inhibited by a protein kinase C inhibitor chelerythrine or a protein phosphatase inhibitor okadaic acid (data not shown).

Relaxation Responses to Different Sphingomyelin Compounds

The relaxation responses to C_2-ceramide, C_6-ceramide, C_{16}-ceramide and D-sphingosine are presented in Figure 2. All of the above compounds showed dose-dependent relaxation responses in endothelium-intact aortic rings. At lower dose (10^{-7} mole/L), there was no significant difference in the degree of relaxation induced by these compounds. At higher dose (10^{-5} mole/L), the relaxation response to C_6-ceramide was significantly larger than that of C_2-ceramide.

Viability of The Vessels After Ceramide Treatment

After washout the effects of natural ceramide (10^{-5} mole/L), the concentration response curve to

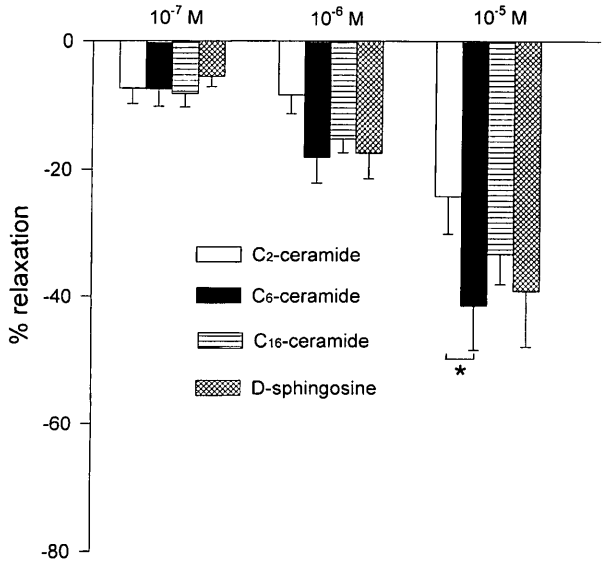

Fig. 2. Relaxation response to C_2-ceramide (open bars, n = 8), C_6-ceramide (closed bars, n = 8), C_{16}-ceramide (horizontal hatched bars, n = 8) and D-sphingosine (cross-hatched bars, n = 8) in EC_{50} phenylephrine precontracted endothelium-intact aortic rings. Asterisk indicates statistical significance of differences between groups ($p < 0.05$). Values are mean ± S.E.M.

phenylephrine was not significantly altered (data not shown).

Discussion

The second messenger of sphingomyelin pathway, ceramide, has been shown to regulate biological activity at the cellular level. The experiments presented here provide evidence that sphingomyelin signaling has an important role in regulating vascular tone by both endothelium-dependent and -independent mechanisms. Endothelium-derived nitric oxide is clearly involved in ceramide-induced relaxation in vascular smooth muscle.

Removal of the endothelium significantly inhibited the relaxation response to ceramide suggesting an endothelium-derived relaxation factor involved in the mechanism of ceramide-induced relaxation. Pretreatment of endothelium-intact aortic rings with an exogenous nitric oxide synthase inhibitor L-NNA also significantly inhibited the relaxation responses to ceramide in endothelium-intact aortic rings. These indicate that endothelium-derived nitric oxide mediates ceramide-induced relaxation. A similar inhibitory effect to ceramide-induced relaxation was noted in endothelium-intact vessels incubated with the guanylate cyclase inhibitor methylene blue. Thus, synthesis of nitric oxide and its subsequent stimulation of guanylate cyclase contribute to ceramide-induced relaxation in vascular smooth muscle.

Although the exact mechanism involved in ceramide-induced relaxation in endothelium-denuded aortic rings is not made clear by this study, previous studies have demonstrated that ceramide potently and reversibly inhibits protein kinase C (7) and that it activates a protein phosphatase in different types of cultured cell (1, 13, 20). Activation of protein kinase C has been reported to induce the contractile response in vascular smooth muscle (14, 16, 19). Therefore, the in-activation of protein kinase C by ceramide could contribute to ceramide-induced relaxation in rings precontracted with phenylephrine since phenylephrine-induced contraction is mediated via protein kinase C (9, 16, 19). However, in endothelium-removed aortic rings incubated with a protein kinase C inhibitor chelerythrine, ceramide-induced relaxation was not inhibited. One previous study has demonstrated that the protein phosphatase inhibitor okadaic acid, augments myosin light chain phosphorylation (3); this phosphorylation of the myosin light chain could contribute to an increase in vascular tone (14). Ceramide-induced relaxation could theoretically be mediated by the activation of a protein phosphatase which subsequently de-phosphorylates the myosin light chain. However, a contrary finding in this study was that ceramide-induced relaxation was not inhibited by the protein phosphatase inhibitor okadaic acid.

Wong, et al. have shown that ceramide decreases the intracellular free calcium in neutrophils (21). Thus, ceramide could act to inhibit calcium influx from the extracellular space or from intracellular stores. Ceramide may also activate the calcium extrusion pump to remove intracellular calcium. These possible effects of ceramide could contribute to the current observations that ceramide induces relaxation in endothelium-intact and -removed aortic rings.

Different fatty acyl chains of ceramide were used to determine which compound is biological more active in inducing smooth muscle relaxation. At the dosage 10^{-5} mole/L, C_6-ceramide induced a larger relaxation response than C_2-ceramide in endothelium-intact vessels. Although C_2-and C_6-ceramide are both able to activate protein phosphatase (1), the larger relaxation response induced by C_6-ceramide may indicate the difference in membrane permeability of ceramides.

D-sphingosine is a metabolic product of the breakdown of ceramide by the enzyme, ceramidase. Our study demonstrated that D-sphingosine also induced a dose-dependent relaxation in endothelium-intact vessels. Previous studies have shown that D-sphingosine has an effect similar to ceramide in the stimulation of protein phosphatase, and the

inactivation of protein kinase C and calcium channels (4, 5, 15). This opens the possibility that ceramide needs to be metabolized to D-sphingosine in order to induce smooth muscle relaxation. Another possible mechanism involved in our observations is that ceramide-induced relaxation may be mediated by a down-stream messenger of the sphingomyelin pathway. For example, ceramide is converted to glucosylceramide by the enzyme, glucosylceramide synthase (17). A previous study showed that the specific glucosylceramide synthase inhibitor threo-1-phenyl1-2-decanoylamino-3-morpholino-1-propanol (PDMP), inhibits the cell cycle arrest induced by ceramide in NIH 3T3 cells (17). The effect of glucosylceramide was not investigated in this study, but the possibility that glucosylceramide contributes to the current observations cannot be ruled out.

In summary, our results indicate that ceramide is biologically active in regulating vascular tone. Both endothelium-dependent and independent mechanisms mediate the relaxation response to ceramide in vascular smooth muscles.

Acknowledgments

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