Sphingomyelinase causes endothelial-dependent vasorelaxation through endothelial nitric oxide production without cytosolic Ca^{2+} elevation

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Abstract Neutral sphingomyelinase (N-SMase) elevated nitric oxide (NO) production without affecting intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in endothelial cells in situ on aortic valves, and induced prominent endothelium-dependent relaxation of coronary arteries, which was blocked by N’-monomethyl-L-arginine, a NO synthase (NOS) inhibitor. N-SMase induced translocation of endothelial NOS (eNOS) from plasma membrane caveolae to intracellular region, eNOS phosphorylation on serine 1179, and an increase of ceramide level in endothelial cells. Membrane-permeable ceramide (C_{8}-ceramide) mimicked the responses to N-SMase. We propose the involvement of N-SMase and ceramide in Ca^{2+}-independent eNOS activation and NO production in endothelial cells in situ, linking to endothelium-dependent vasorelaxation.

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1. Introduction

Sphingomyelinase (SMase), a key enzyme of sphingolipid signaling pathway [1], catalyzes hydrolysis of sphingomyelin to produce intracellular ceramide, which is a lipid second messenger for various cellular actions including cell differentiation and proliferation [2]. Various SMases have been found in mammalian cells, tissues and biological fluids including serum [1].

Nitric oxide (NO) generated by endothelial NO synthase (eNOS) plays an important role in regulating vascular tone. An elevation of intracellular Ca^{2+} concentrations ([Ca^{2+}]_i) has been believed to be necessary to activate eNOS [3]. However, recently accumulating reports show that eNOS activity is also regulated by phosphorylation of the enzyme, cellular localization, and protein–protein interaction [4]. Although it is reported that SMase induces endothelium-dependent relaxation [5], the mechanism of SMase-induced vasorelaxation has never been elucidated. The aim of this study is to clarify how SMase induces endothelium-dependent relaxation.

In the present study, we found for the first time that in endothelial cells in situ neutral SMase (N-SMase) (E.C. 3.1.4.12) produces ceramide and induces Ca^{2+}-independent eNOS activation, probably mediated by translocation of eNOS from the plasma membrane caveolae to intracellular region and by phosphorylation on serine 1179 of eNOS.

2. Materials and methods

2.1. Materials

SMase from Bacillus cereus, ATP, and U46619 were purchased from Sigma (USA). Fura-2/AM was from DOJINDO (Japan). 4,5-Diamidinofluorescein diacetate (DAF-2/DA) was from Daiichi Chemical (Japan). Bradykinin (BK) was from Peptide Institute (Japan). N’-Monomethyl-L-arginine (LNMMA) was from Wako Pure Chemical (Japan). All other chemicals were from Katayama Chemical (Japan).

2.2. Preparations of the tissue specimens

Bovine aortic valves and coronary arteries (left anterior descending arteries) were obtained from a local abattoir. The tissue specimens were placed in ice-cold physiological salt solution (PSS (mM): 123 NaCl, 4.7 KCl, 15.5 NaHCO_{3}, 1.2 KH_{2}PO_{4}, 1.2 MgCl_{2}, 1.25 CaCl_{2}, and 11.5 D-glucose) and transported to our laboratory. All solutions were gassed with a mixture of 5% CO_{2} and 95% O_{2} (pH adjusted to 7.4 at 37 °C).

2.3. Force recording of arterial rings with or without endothelium

Tension study was done, as previously described [6,7]. The presence of endothelium was examined by application of 1 μM BK. Effects of N-SMase on the force were then examined at the plateau phase of the sustained contraction induced by 100 nM U46619. In some rings, the endothelium was removed by rubbing with a cotton web.

2.4. Measurement of [Ca^{2+}]_i of the endothelial cells in situ

Monitoring of [Ca^{2+}]_i in the endothelial cells in situ was done, as previously described [6,7]. Briefly, the fluorescence intensities at alternating 340 nm (F340) and 380 nm (F380) excitation and their ratio (R = F340/F380) were monitored at 510 nm emission. The fluorescence ratio values were normalized by the peak response to 10 μM ATP (100%).

2.5. Measurement of NO production of the endothelial cells in situ

Nitric oxide production in endothelial cells in situ was measured with fluorescent indicator DAF-2 [8,9]. Bovine aortic valvar strips (4 x 3 mm) were loaded with 10 μM DAF-2/DA for 30 min at 37 °C in PSS. Changes in NO production were monitored using diaminorotiazolofluorescein produced from DAF-2 in spectrofluorometer.
(Hitachi F2000). The fluorescence intensities at 495 nm excitation were monitored at 515 nm emission. Before starting the experimental protocol, all strips were stimulated by 1 μM BK for 5 min in order to obtain a reference response. The fluorescence values were normalized by the peak response to 1 μM BK (=100%).

2.6. Immunostaining of endothelial cells in situ

Confocal images of immunocytochemistry of strips of bovine aortic valves (2 × 3 mm) were observed under a confocal laser scan microscope (LSM510, Zeiss), as described [7]. The fixed and permeabilized cells were incubated with monoclonal mouse anti-eNOS (Transduction Laboratories) and rabbit anti-caveolin-1 (Santa Cruz Biotechnology) antibodies, then incubated with an Alexa546-conjugated anti-mouse IgG (Molecular Probe) and an Alexa488-conjugated anti-rabbit IgG (Molecular Probe).

2.7. Measurement of eNOS phosphorylation on serine 1179

To assess eNOS phosphorylation, we performed immunoblot analyses in lysates of endothelial cells of bovine aortic valves, as described [10]. The strips of valves were immediately frozen with liquid nitrogen, crushed by using SK mill (Tokken, Japan), and lysed in lysis buffer of 50 mM Tris, pH 7.4, 1 mM EDTA, 1% vol/vol NP-40, 150 mM NaCl, 2 mM Na3VO4, 0.25% sodium deoxycholate, 1 mM NaF, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.008% bromophenol blue, and 1% protease inhibitor cocktail. After centrifugation (14 000 g, 20 min), the solubilized preparations were subjected to 10% SDS–PAGE and electroblotted onto PVDF membrane. After blocking, membranes were incubated with anti-eNOS antibody (Transduction Laboratories) and electroblotted onto PVDF membrane. After blocking, membranes were incubated with anti-eNOS antibody (Transduction Laboratories) and rabbit anti-caveolin-1 (Santa Cruz Biotechnology) antibodies, then incubated with an Alexa546-conjugated anti-mouse IgG (Molecular Probe) and an Alexa488-conjugated anti-rabbit IgG (Molecular Probe).

2.8. Measurement of ceramide concentration

Bovine aortic valves were preincubated with 50 μM PDMP, a glycosyltransferase inhibitor, and 30 μM oleylthanolamine, a ceramidase inhibitor, at 37 °C for 75 min. After stimulation, endothelial cells of bovine aortic valves were removed with a razor blade in ice-cold buffer (50 mM Tris–HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin and 10 μg/ml aprotinin) and collected by centrifugation (15 000 g, 5 min at 4 °C). Lipids were extracted from cell homogenates according to the Folch method. The chloroform phase was dried, dissolved by 100 μl chloroform, and spotted on thin layer chromatography (TLC) plates (Silica gel 60, Merck, Germany). Developmental conditions and immunostaining method were described previously [11]. TLC plates were developed in a multiple development system. After blocking, the TLC plates were incubated with an anti-ceramide antibody (Azwell Inc., Japan) at 1:100 dilution, then treated with alkaline phosphatase-conjugated anti-mouse IgM (Sigma), and stained using a BCIP-NBT Kit (Bio-Rad, USA). After immunostaining, TLC plates were sprayed with 48% H2SO4, heated at 170 °C for 30 min, then the proportion of ceramide to total phospholipids was determined on a NIH image.

2.9. Statistics

The values are expressed as means ± S.E. Student’s t test was used to determine statistical significance. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of N-SMase on contraction in bovine coronary artery

We examined if N-SMase may induce endothelium-dependent vasorelaxation. N-SMase (0.05 U/ml) caused a remarkable relaxation (61.6 ± 4.96%, n = 19) of bovine coronary arteries with endothelium precontracted by 100 nM U46619 (Fig. 1A), and a response to 100 nM U46619 was maintained after washing (data not shown). Although N-SMase (0.1 U/ml) caused a larger endothelium-dependent relaxation, the response to 100 nM U46619 reduced after washing (data not shown). Therefore, we use 0.05 U/ml of N-SMase in our experiments. The relaxations reached the maximum at about 15 min after application of N-SMase (0.05 U/ml). The N-SMase-induced relaxation was blocked by a NOS inhibitor (3 mM LNMMA) (Fig. 1A) and the removal of endothelium (Fig. 1B), suggesting that N-SMase induces endothelium-dependent relaxation presumably by the activation of eNOS. C8-ceramide (10 μM) induced similar effects (Fig. 1C and D), although the response was smaller than the N-SMase response.

3.2. Effects of N-SMase on [Ca2+]i and NO production in endothelial cells in situ

Because most of eNOS activations are accompanied with [Ca2+]i elevation, we examined the in situ effects of N-SMase (0.05 U/ml) on the [Ca2+]i levels in endothelial cells on bovine aortic valves. N-SMase induced no apparent changes in
[Ca^{2+}], although 10 μM ATP did cause a [Ca^{2+}] increase (n = 5, Fig. 2A).

We then determined if N-SMase may increase NO production in endothelial cells in situ. A fluorometrical measurement of NO production using DAF-2DA showed that N-SMase (0.05 U/ml) gradually increased NO production (n = 3), the extent of which was comparable to that of the BK-induced one (Fig. 2B).

The [Ca^{2+}]-independent NO production by N-SMase may be due to either Ca^{2+}-independent activation of eNOS or simple activation of iNOS (a Ca^{2+}-independent NOS), which is sometimes induced in the cultured endothelial cells. However, in endothelial cells in situ we studied, only eNOS, but not iNOS, was expressed (data not shown). Taken together, these findings suggest that N-SMase activates eNOS (but not iNOS) without a [Ca^{2+}] elevation and thereby stimulates NO production in endothelial cells in situ.

3.3. Effects of N-SMase on localization of eNOS and caveolin-1 in endothelial cells in situ

The interaction of eNOS with caveolin in plasma membrane caveolae in endothelial cells is important for the regulation of its activity [12,13]. Therefore, we next examined if N-SMase may induce translocation of eNOS in endothelial cells in situ. At rest, both eNOS and caveolin-1 are colocalized in the plasma membrane (Fig. 3). After treatment of N-SMase (0.05 U/ml, 15 min), eNOS was translocated from the plasma membrane to intracellular region (especially the perinuclear region), whereas caveolin-1 remained at the plasma membrane even after the stimulation (Fig. 3). These results suggest that N-SMase induces translocation of eNOS from the caveolae to the intracellular region without a [Ca^{2+}] elevation in endothelial cells.

3.4. Effects of N-SMase on eNOS phosphorylation on serine 1179 in endothelial cells in situ

Phosphorylation of eNOS on serine 1179 is a major mechanism for Ca^{2+}-independent activation of eNOS [14,15]. Therefore, we examined its involvement in eNOS activation by N-SMase in endothelial cells in situ. N-SMase (0.05 U/ml, 15 min) significantly (P < 0.05) increased phosphorylation of eNOS on serine 1179 to 179.9 ± 30.4% (n = 5) (Fig. 4A and B).

3.5. Effects of N-SMase on ceramide level in endothelial cells of bovine aortic valves

We determined if N-SMase increases ceramide in endothelial cells in situ of bovine aortic valves. N-SMase (0.05 U/ml, for 15 min) increased remarkably ceramide concentrations.
(260.5 ± 21.6% of the control level) significantly (Fig. 5). This finding strongly suggests that elevation of NO production by N-SMase may be mediated by an increase of intrinsic ceramide concentration in endothelial cells in situ.

4. Discussion

The present study demonstrates for the first time that N-SMase induces activation of eNOS without elevation of [Ca\(^{2+}\)], in endothelial cells in situ, and the mechanisms of eNOS activation by N-SMase involve translocation of eNOS from the caveolae to the perinuclear region, an increase of eNOS phosphorylation on serine 1179, and an increase of ceramide level in endothelial cells in situ. In addition, the Ca\(^{2+}\)-independent eNOS activation induced by N-SMase causes prominent endothelium-dependent vasorelaxation.

Two major mechanisms for activation of eNOS in endothelial cells have been proposed: (1) dissociation of eNOS from caveolin-1, a caveola-localizing protein that inactivates eNOS; (2) eNOS phosphorylation on serine 1179. Firstly, proper subcellular localization of eNOS is critical for activation of eNOS by agonists [16]. In a resting state of endothelial cells, caveola-bound eNOS is inactivated by interaction with caveolin-1, whereas upon receptor activation the resultant [Ca\(^{2+}\)] elevation stimulates the dissociation of eNOS from caveolin-1, thereby activating eNOS [17]. This process has been considered to be a major mechanism for the Ca\(^{2+}\)-dependent activation of eNOS in endothelial cells [18]. However, we previously reported that eicosapentanoic acid induces Ca\(^{2+}\)-independent activation of eNOS in endothelial cells, which is accompanied with the translocation of membrane eNOS to the cytosol [7]. In addition, Igarashi et al. previously demonstrated that C\(_2\)-ceramide, a membrane permeable ceramide, stimulates NO production without [Ca\(^{2+}\)], elevation in cultured endothelial cells, which is accompanied with the translocation of eNOS from caveolin-bound caveolae [19]. Therefore SMase, which produces intrinsic ceramide, may also activate eNOS by the same mechanism. Secondly, eNOS can be activated [Ca\(^{2+}\)]-independently by the phosphorylation on serine 1179, through the activation of Akt [14,15,20]. Although eNOS activation by VEGF, which increases eNOS phosphorylation on serine 1179, is not accompanied with translocation of eNOS [21], our study shows that the N-SMase-induced eNOS activation involves both of translocation of eNOS from the plasma membrane to the perinuclear region (Fig. 3) and increase of phosphorylated eNOS on serine 1179 (Fig. 4). TNF-\(\alpha\), which activates N-SMase, increases ceramide level and activates eNOS, which is accompanied with phosphorylation of Akt on serine 473 and of eNOS on serine 1179 in eNOS-Tet off cells [22,23]. In the present study, N-SMase increased ceramide level in the endothelial cells in situ (Fig. 5). Taken together, these findings raise the possibility that ceramide may activate eNOS not only by stimulating its dissociation from caveolin-1, but also by increasing eNOS phosphorylation on serine 1179 probably by Akt, without elevation of [Ca\(^{2+}\)], in the endothelial cells in situ. Indeed, C\(_8\)-ceramide showed similar effects as N-SMase in the experiment of tension study (Fig. 1C and D).
In the present study, Ca\(^{2+}\)-independent NO production by N-SMase was accompanied with a marked and sustained vaso-relaxation (Fig. 1). Zn\(^{2+}\)-stimulated SMase has been found in fetal bovine serum [24]. Although the SMase shows optimal activity at pH 5.5, its activity at pH 7.4 is about 0.001 U/ml at optimal condition. If the SMase has the same effects of N-SMase, the SMase might induce endothelium-dependent vaso-relaxation. As considering the elevation of ceramide level by a physiological agonist, BK [19], N-SMase and ceramide may play a physiological role in the regulation of vascular tone in an endothelial Ca\(^{2+}\)-independent manner. Further studies are required for the determination of this notion.

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