Sphingosylphosphorylcholine induces cytosolic Ca^{2+} elevation in endothelial cells in situ and causes endothelium-dependent relaxation through nitric oxide production in bovine coronary artery

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Abstract Sphingosylphosphorylcholine (SPC) increased intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and nitric oxide (NO) production in endothelial cells in situ on bovine aortic valves, and induced endothelium-dependent relaxation of bovine coronary arteries precontracted with U-46619. The SPC-induced vasorelaxation was inhibited by N^{ω}-monomethyl-L-arginine, an inhibitor of both constitutive and inducible NO synthase (NOS), but not by 1-(2-trifluoromethylphenyl) imidazole, an inhibitor of inducible NOS (iNOS). Immunoblotting revealed that endothelial constitutive NOS, but not iNOS, was present in endothelial cells in situ on the bovine aortic valves. We propose that SPC activates $[Ca^{2+}]i$ levels and NO production of endothelial cells in situ, thereby causing an endothelium-dependent vasorelaxation.

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Key words: Sphingolipid; Cytosolic Ca^{2+}; Nitric oxide; Vasorelaxation; 2,3-Diaminonaphthalene; Endothelial cell

1. Introduction

Sphingolipids are novel second messengers mediating a variety of cellular responses, including cell proliferation and apoptosis. One of the sphingolipids, sphingosylphosphorylcholine (SPC), which is generated by sphingomyelin *N*-deacylation, induces elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in various types of cultured cells [1–3]. However, characterization of $[Ca^{2+}]i$ elevation as induced by SPC has remained to be elucidated.

In endothelial cells, elevation of $[Ca^{2+}]i$ is a prerequisite for cellular responses via production of nitric oxide (NO) and prostacyclin, and the activation of other Ca^{2+}-dependent enzymes. A diffusible gas, NO plays a most important role through the activation of guanylyl cyclase in the endothelium-dependent vasorelaxation. The formation of NO is induced by nitric oxide synthase (NOS), which is classified into constitutive NOSs (endothelial NOS and neural NOS) and inducible NOS (iNOS) [4,5]. The constitutive NOSs, but not iNOS, are activated by the elevation of $[Ca^{2+}]i$. In particular, the elevation of $[Ca^{2+}]i$ caused by the influx of extracellular Ca^{2+}, but not the release of intracellular Ca^{2+}, plays an important role in the activation of endothelial NOS (eNOS) [6]. However, it has not been clarified whether or not SPC can stimulate the influx of extracellular Ca^{2+} in endothelial cells. In addition, the physiological effects of sphingolipids on endothelial cells are not well understood.

We now provide the first evidence that SPC regulates vascular tone by activating endothelial NO production, as evidenced by the following: (1) SPC induced a $[Ca^{2+}]i$ increase, as mediated by both the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+} in endothelial cells in situ, (2) SPC increased NO production in endothelial cells in situ, and (3) SPC induced an endothelium-dependent vasorelaxation, which was blocked by a NOS inhibitor.

2. Materials and methods

2.1. Materials

SPC was purchased from Sigma (St. Louis, MO, USA) or Biomol (Plymouth Meeting, PA, USA). Adenosine 5′-triphosphate (ATP), U-46619, and fatty acid-free bovine serum albumin were from Sigma. Fura-2/AM and 2,3-diaminonaphthalene (DAN) were from Dojindo (Kumamoto, Japan). Acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate (DiI-Ac-LDL) was from Biomedical Technologies Inc. (Massachusetts, USA). N^{ω}-Monomethyl-L-arginine (L-NMMA) was from Wako Pure Chemical (Osaka, Japan), and 1-(2-trifluoromethylphenyl) imidazole (TRIM) was from Calbiochem (San Diego, CA, USA). Bradykinin (BK) was from Peptide Institute (Osaka, Japan). All other chemicals were purchased from Katayama Chemical (Osaka, Japan). Positive controls for the immunoblotting of eNOS (human endothelial lysate) and iNOS (mouse macrophage lysate) were from Transduction Laboratories Inc. (Lexington, KY, USA).

2.2. Preparations

Bovine aortic valves and coronary arteries were obtained from a local abattoir immediately after the animals were killed. 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_2PO_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). Endothelial cells in situ of aortic valves were identified by their specific uptake of DiI-Ac-LDL [7,8].

To analyze the expression of NOS, homogenates of endothelial cells on bovine aortic valves were subjected to 7.5% SDS-polyacrylamide gel electrophoresis. After blocking, the blots were incubated with anti-

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Abbreviations: SPC, sphingosylphosphorylcholine; NO, nitric oxide; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; ATP, adenosine 5′-triphosphate; DAN, 2,3-diaminonaphthalene; DiI-Ac-LDL, acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate; L-NMMA, N^{ω}-monomethyl-L-arginine; TRIM, 1-(2-trifluoromethylphenyl) imidazole; BK, bradykinin; PSS, physiological salt solution; IP3, inositol 1,4,5-trisphosphate

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eNOS or anti-iNOS antibody (Transduction Laboratories Inc.) and the antigens were visualized, as described [9].

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

The endothelial cells in situ of bovine aortic valves were loaded with 10 μM fura-2/AM (the acetoxymethyl ester form of fura-2), as described [7,8,10]. Using double staining with DiI-Ac-LDL and fura-2, we confirmed that fura-2 fluorescence was exclusively emitted from the endothelial cells on the surface of the aortic valves, as described [7,8]. Changes in [Ca^{2+}]i were continuously monitored with fluorescence intensities (510 nm) at alternating 340 nm (F_{340}) and 380 nm (F_{380}) excitation and their ratio ($R = F_{340}/F_{380}$) in a spectrofluorometer (Hitachi F2000), all operated using our own program. Before the start of the experimental protocol, strips of aortic valves were stimulated with 10 μM ATP for 1 min to confirm a reference response. The fluorescence ratio values were normalized by assigning the values in normal PSS and at the peak response to 10 μM ATP to be 0% and 100%, respectively.

2.4. Measurements of NO production of the endothelial cells in situ

To assess NO production in endothelial cells in situ, we measured the concentration of nitrite in PSS using a fluorometry of DAN according to the method of Misko et al. [11], except that 5 mM EDTA was first added to sample solutions to avoid the possible interference with a fluorescence measurement of DAN by divalent cations [12]. Before application of 20 μM SPC, strips of aortic valves were stimulated for 3 min by a well-known activator of eNOS, 10 μM ATP [13], as a reference response. ATP, SPC or vehicle was applied to the aortic valve (5×8 mm) pinned in the Sylgard chamber by exchanging the solutions (600 μl). At the end of the treatment (3 min), the solution was aspirated from the chamber and used for nitrite measurements. To determine the basal NO production in endothelial cells in situ at rest, fresh PSS (600 μl) was applied to the aortic valve for 3 min and was collected before each experiment.

The intensity of the fluorescent signal generated by 1-(H)-naphthotriazole, the product of the reaction between nitrite and DAN, was measured with excitation at 365 nm and emission at 405 nm, using a spectrofluorometer (Hitachi F2000). The limit of detection of nitrite concentration in PSS was 10 nM. Standard sodium nitrite solutions (10 nM–1 μM) were freshly prepared to obtain a standard curve regarding the relationship between fluorescence intensity and nitrite concentration in PSS, for each measurement. The level of NO production was expressed as a percentage, assigning value at the 'zero' level (= background fluorescence of solution only, without the aortic valves) and the value obtained from the aortic valves at rest (treated with fresh PSS for 3 min) to be 0% and 100%, respectively.

We also used a three-electrode potentiostatic EMS-100 system (BIO-LOGIC, France) to measure NO production directly [14]. In each experiment NO biosensor calibration was performed by cumulative applications of various concentrations of NO standard solutions. The NO stock solution (1.4 mM) was made by saturating distilled water with NO gas for 30 min after substitution of all the dissolved gases with Ar gas, as described [15]. The NO biosensor is specific for NO and did not respond to nitrite (up to 10 μM). The NO biosensor was positioned about 10 μm above the aortic valve (5×8 mm), which was pinned in the chamber filled with 600 μl PSS. Six μl of 2 mM SPC (final concentration 20 μM) was added to the chamber by pipetting.

2.5. Force recording of arterial rings with or without endothelium

The bovine coronary arterial rings were mounted vertically at the organ bath filled with PSS, gassed with 5% CO_2 and 95% O_2, and maintained at 37°C. A force transducer (TB-611T, Nihon Koden, Japan) was used to measure the isometric force [8]. In an equilibration period of 1 h, a passive force of about 2 g was applied to the rings, and high-K^+ PSS (118 mM K^+) was applied repeatedly to reach a steady amplitude. After recording a reference response to 118 mM K^+ depolarization, the effects of 20 μM SPC on the force were examined at the plateau phase of the precontraction induced by 100 nM U-46619 (9,11-dideoxy-9α,11α-epoxymethanoprostaglandin F2α), and thereafter NOS inhibitors (100 μM TRIM and 3 mM L-NMMA) were added. The remaining endothelium was examined by application of 1 μM BK. In some strips, the endothelium was removed by rubbing with a cotton web.

3. Results and discussion

3.1. SPC-induced changes in [Ca^{2+}]i in endothelial cells in situ

SPC dose-dependently (1–30 μM) induced [Ca^{2+}]i elevation, which consisted of an initial transient and a subsequently sustained plateau phase (Figs. 1A and 2). The observed SPC-induced increase in [Ca^{2+}]i is not simply due to a non-specific, lipid-induced hyperpermeabilization of the cell membrane, since after washing SPC, the [Ca^{2+}]i reverted to the prestimulation level and the [Ca^{2+}]i elevation induced by

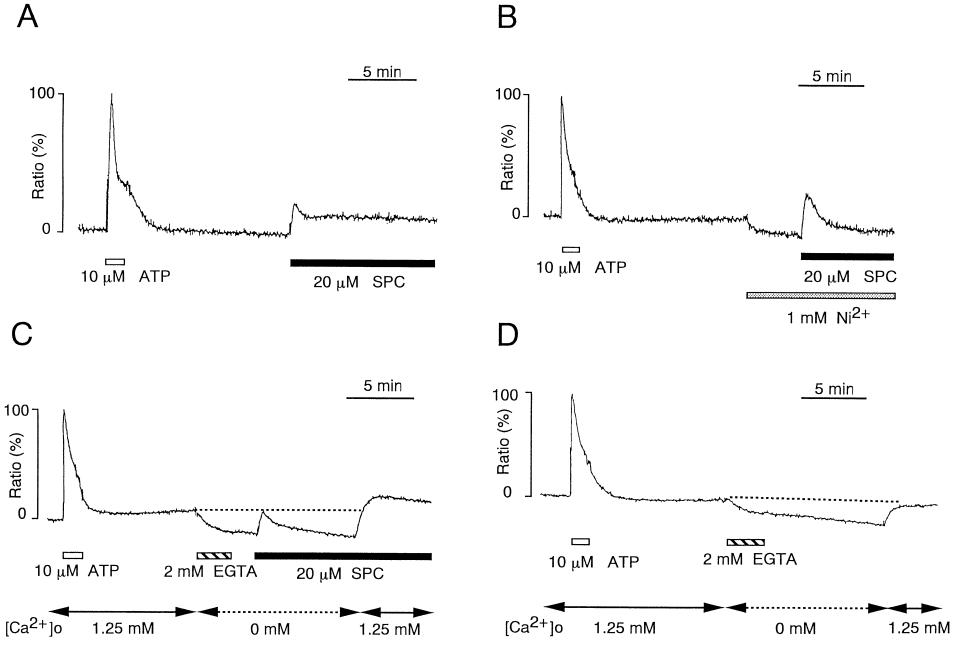

Fig. 1. Representative recordings showing changes in [Ca^{2+}]i induced by 20 μM SPC, under various conditions. SPC (20 μM) was applied 15 min after the stimulation with 10 μM ATP in PSS (1.25 mM Ca^{2+}; A), under blockade of Ca^{2+} influx with Ni^{2+} (B), and in the absence of extracellular Ca^{2+} (C). Extracellular Ca^{2+} was restored in the absence (D) and presence (C) of SPC.

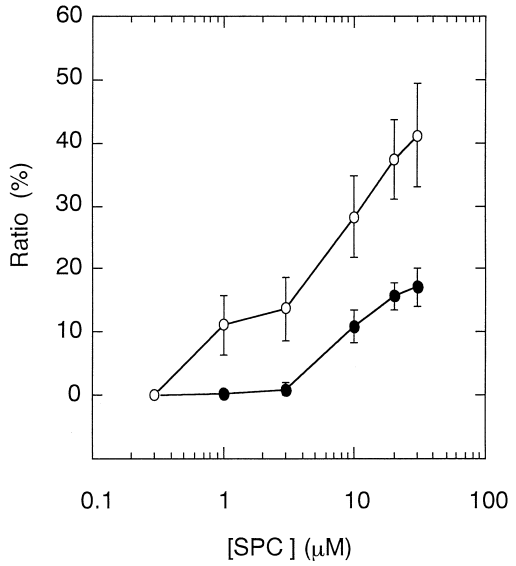

Fig. 2. Concentration-response curves for the SPC-induced $[Ca^{2+}]i$ elevations. Abscissa, concentration of SPC; ordinate, the levels of the $[Ca^{2+}]i$ elevations (% of the 10 μM ATP-induced response). Open and closed symbols indicate the levels of an initial transient and a subsequently sustained elevation of $[Ca^{2+}]i$, respectively. The data were obtained from five separate experiments (as carried out according to the protocol shown in Fig. 1A) for each dose and are expressed as mean ± S.E.M.

10 μM ATP was reproduced. In addition, neither sphingosine nor phosphorylcholine (up to 30 μM) induced $[Ca^{2+}]i$ elevation (data not shown), thus supporting the notion of a specific effect of SPC on the $[Ca^{2+}]i$ in the endothelial cells in situ. To estimate the relative contribution of extracellular Ca^{2+} to the SPC-induced $[Ca^{2+}]i$ changes, SPC was applied either in the presence of 1 mM Ni^{2+} or in the absence of extracellular Ca^{2+} (buffered with 2 mM EGTA). Blockade of the Ca^{2+} influx with Ni^{2+} abolished the sustained elevation of $[Ca^{2+}]i$ induced by SPC, whereas the initial transient elevation of $[Ca^{2+}]i$ remained (Fig. 1B). An identical result was obtained when SPC was applied in the absence of extracellular Ca^{2+} (Fig. 1C). Reapplication of Ca^{2+} restored the sustained elevation of $[Ca^{2+}]i$ induced by SPC (Fig. 1C), whereas this procedure in the absence of SPC did not generate a $[Ca^{2+}]i$ elevation above the resting level (Fig. 1D). These findings suggest that the $[Ca^{2+}]i$ elevation induced by SPC may be mediated by both the influx of extracellular Ca^{2+} and the release of intracellular Ca^{2+} in endothelial cells in situ.

We found that the SPC-induced transient elevation of $[Ca^{2+}]i$ is mediated by the release of intracellular Ca^{2+} (Fig. 1), which may involve activation of the recently reported novel Ca^{2+} channels at store sites [16]. In the present study, we demonstrated for the first time that SPC stimulates the influx of extracellular Ca^{2+}, thereby inducing the sustained elevation of $[Ca^{2+}]i$ in endothelial cells in situ (Fig. 1). Cellular mechanisms of the SPC-induced influx of extracellular Ca^{2+} are unknown. SPC is reported to activate the K^+ channels, thereby inducing hyperpolarization in guinea pig atrial myocytes [17]. Therefore, SPC may increase Ca^{2+} influx by activating K^+ channels in the endothelial cells, since Ca^{2+} influx in endothelial cells is enhanced by the hyperpolarization of membrane potential [18]. Recently it was reported that sphingosine 1-phosphate (SPP), one of the sphingolipids, is a ligand for the G protein-coupled receptor EDG1 [19] and activates phospholipase C, which in turn produces inositol 1,4,5-trisphosphate (IP_3), a trigger of Ca^{2+} release [20]. SPC and SPP increased the $[Ca^{2+}]i$ in endothelial cells in situ, with a similar potency (EC_{50} = 5 μM for SPC and 1 μM for SPP) in the present study (unpublished data). In contrast, SPC is much less potent than SPP for activating the reported sphingolipid receptors (EDG1, EDG3, and H218): the EC_{50} values of SPC and SPP are 100 nM and 1 nM (for EDG1), 100 nM and 10 nM (for EDG3), and 50 nM and 5 nM (for H218), respectively [20,21]. Therefore, it is unlikely that the sphingolipid receptors have an important role in the SPC-induced $[Ca^{2+}]i$ elevations, at least in endothelial cells in situ.

3.2. SPC-induced NO production

It has been reported that influx of extracellular Ca^{2+} is essential for the activation of eNOS and the resultant stimu-

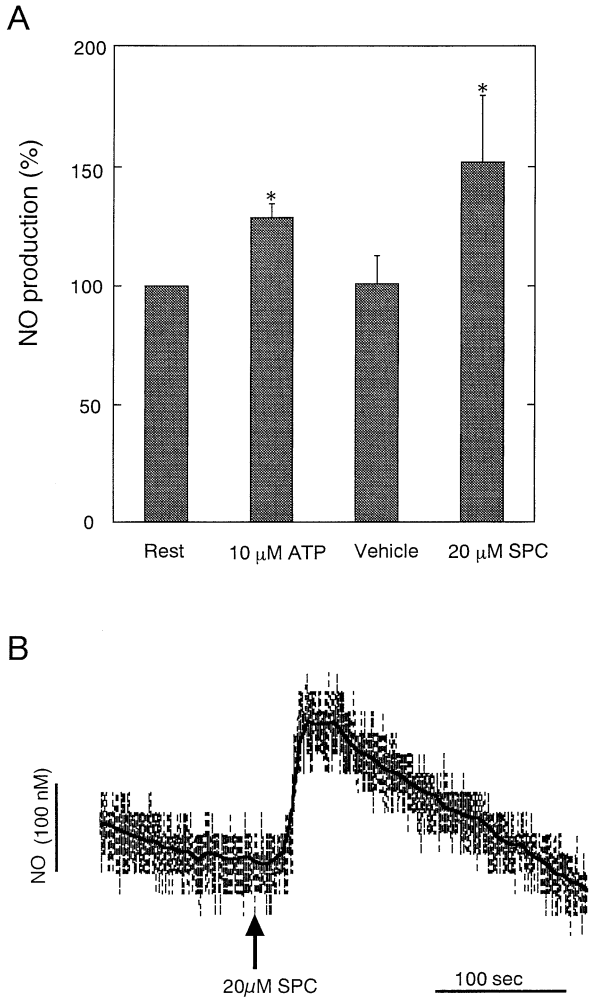

Fig. 3. A: Effects of 10 μM ATP and 20 μM SPC on NO production in endothelial cells in situ. NO production (3 min) was expressed as a percentage, assigning the value obtained at rest (= basal NO production by endothelial cells in situ) to be 100%. Vehicle was that of 20 μM SPC and consisted of 0.2% ethanol, 0.02 mg/ml bovine serum albumin, and 1% PBS. The data were obtained from four separate experiments and are expressed as means ± S.E.M. *$P < 0.05$ by Student's t-test was considered to be significant. B: A representative differential pulse amperogram obtained with NO biosensor after application of 20 μM SPC.

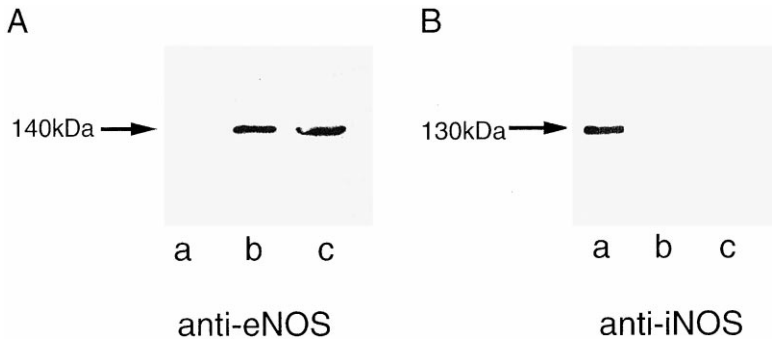

Fig. 4. Western blot analysis of endothelial cells (10 μg of protein) of bovine aortic valve. Lanes a and b are positive controls for iNOS (mouse macrophage lysate) and for eNOS (human endothelial lysate), respectively. Lane c is homogenates of endothelial cells in situ of bovine aortic valve.

lation of NO production in endothelial cells [6]. Therefore, in order to investigate the functional role of the SPC-induced $[Ca^{2+}]i$ elevation, NO production in endothelial cells in situ was assessed not only by a fluorometric measurement of the production of nitrite using DAN but also by a direct measurement of NO using a NO electrode.

Fluorometry of DAN revealed that SPC (20 μM) and ATP (10 μM) significantly increased NO production at 3 min ($P < 0.05$), although a vehicle for SPC had no stimulatory effect on NO production (Fig. 3A).

The differential pulse amperogram obtained with the NO biosensor is shown in Fig. 3B. SPC (20 μM) induced a rapid elevation of NO concentration, with a maximum elevation of 186 nM, whereas no change was induced by a simple addition of PSS. SPC (up to 20 μM) solution itself, without aortic valves, had no effect on the amperogram of the NO biosensor (data not shown). Using these two different (biochemical and electrophysiological) techniques for the NO measurements, we obtained evidence that SPC stimulates NO production in endothelial cells in situ.

eNOS is constitutively expressed in endothelial cells, whereas iNOS is induced by various stimuli, such as interferon, lipopolysaccharide, and trauma [4]. We examined the expression of the NOS isoforms in endothelial cells in situ on bovine aortic valves, using immunoblotting with antibodies against eNOS and iNOS. Anti-eNOS antibody recognized a protein with an approximate molecular mass of 140 kDa both in homogenates of endothelial cells in situ on bovine aortic valves and in human endothelial lysate used as positive control for eNOS (Fig. 4A). The band at 140 kDa was undetectable in mouse macrophage lysate used as positive control for iNOS (Fig. 4A). The band at 140 kDa was prevented by preadsorption of anti-eNOS antibody with purified bovine eNOS protein, and the staining was not observed without

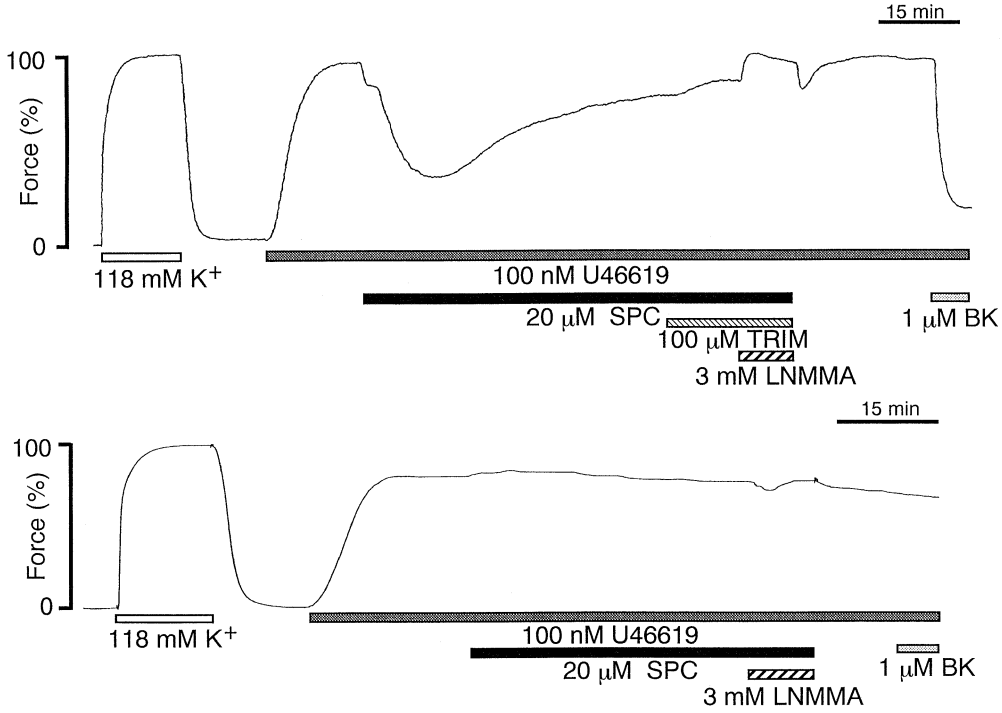

Fig. 5. Representative recordings showing the effects of 20 μM SPC on the contraction of the bovine coronary arteries, with (A) and without (B) endothelium, precontracted by 100 nM U-46619. The effects of the selective inhibitor for iNOS (100 μM TRIM) and of non-selective NOS inhibitor (3 mM L-NMMA) on the SPC-induced vasorelaxation were also observed. The presence (A) and absence (B) of endothelium was assessed by the presence and absence of the relaxing response to 1 μM BK, respectively.

treatment of anti-eNOS antibody (data not shown). On the other hand, anti-iNOS antibody recognized a protein with an approximate molecular mass of 130 kDa in mouse macrophage lysate, but not in homogenate of endothelial cells and human endothelial lysate ($n = 10$) (Fig. 4B). These observations indicate that eNOS, but not iNOS, is mainly expressed in the endothelial cells in situ on the bovine aortic valves used in the present study. Taken together, the findings of the present study suggest that SPC activates eNOS through $[Ca^{2+}]i$ elevation and thereby stimulates NO production in endothelial cells in situ.

SPC stimulated the NO production more strongly than ATP did, while the SPC-induced $[Ca^{2+}]i$ elevation was smaller than that induced by ATP. These results are compatible with the idea that SPC potentiates the Ca^{2+} sensitivity of NO production, supporting its regulatory role for the activation of eNOS. Direct interaction of eNOS with caveolin, a scaffolding protein in caveolae, inhibits the eNOS activity and promotes sequestration of inactive eNOS within caveolar microdomains, by acylation in the N-terminal region of eNOS [22–25]. Ca^{2+}-calmodulin promotes the dissociation of caveolin from eNOS and therefore activates eNOS [26]. Since SPC has an alkyl chain with a hydroxy moiety as well as an acyl chain in the molecular structure, SPC may inhibit competitively the binding of eNOS to caveolin, thereby activating eNOS.

3.3. SPC-induced endothelium-dependent vasorelaxation

We further examined the effects of SPC on coronary arterial tone with or without endothelium. SPC (20 μM) caused a relaxation of coronary arteries with endothelium precontracted by 100 nM U-46619. The SPC-induced relaxation was blocked by an inhibitor for all isoforms of NOS (3 mM L-NMMA) [27], but not by a specific inhibitor for iNOS (100 μM TRIM) [28] (Fig. 5A). The presence of endothelium was confirmed by the addition of 1 μM BK at the end of each experiment. SPC had no relaxing effect on the bovine coronary arteries without endothelium precontracted by U-46619 (Fig. 5B). The endothelium-dependent relaxation was not observed in case of treatment with phosphocholine and sphingosine, both metabolites of SPC [1] (data not shown). This evidence shows that SPC directly causes endothelium-dependent vasorelaxation, as mediated by NO, which may be produced by the activation of eNOS through a $[Ca^{2+}]i$ increase in endothelial cells.

3.4. Physiological implications

SPC is produced by *N*-deacylation of sphingomyelin, one of the most abundant membrane lipids, although the enzyme regulating this step has yet to be identified. On the other hand, sphingomyelin, a precursor of SPC, is also converted to ceramide by the activation of sphingomyelinase in response to physiological stimuli, such as Fas and tumor necrosis factor-α [29–31]. Therefore, activation of sphingomyelinase may also inhibit the conversion from sphingomyelin to SPC by consumption of sphingomyelin, resulting in a decrease in cellular SPC. Tissue accumulation of SPC was observed in patients with Niemann-Pick disease, in which sphingomyelinase is deficient [31,32], thus sphingomyelinase may regulate the production of SPC. In addition, since oxidized LDL activates sphingomyelinase in human macrophages [33], oxidized LDL may inhibit SPC production by activating sphingomyelinase and thereby suppress the endothelium-dependent vasorelaxation mediated by SPC, as demonstrated in the present study. This notion is supported by findings that endothelium-dependent relaxation is impaired in the isolated arteries exposed to oxidized LDL [34]. The impairment of endothelium-dependent vasorelaxation plays an important role in vascular hypercontractility observed in cardiovascular diseases, such as hypertension, vasospasm, and ischemic heart disease. Our experiments are ongoing in an attempt to clarify the pathophysiological role of the sphingomyelin-SPC pathway in vascular disease.

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