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Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via $S1P_2$

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Abstract

Objectives: Sphingosine 1-phosphate (Sph-1-P), a bioactive lipid derived from activated platelets, may play an important role in coronary artery spasm and hence the pathogenesis of ischemic heart diseases, since we reported that a decrease in coronary blood flow was induced by this lysophospholipid in an in vivo canine heart model [Cardiovasc. Res. 46 (2000) 119]. In this study, metabolism related to and cellular responses elicited by Sph-1-P were examined in human coronary artery smooth muscle cells (CASMCs). **Methods and results:** [^{3}H]Sphingosine (Sph), incorporated into CASMCs, was converted to [^{3}H]Sph-1-P intracellularly, but its stimulation-dependent formation and extracellular release were not observed. Furthermore, the cell surface Sph-1-P receptors of S1P family (previously called EDG) were found to be expressed in CASMCs. Accordingly, Sph-1-P seems to act as an extracellular mediator in CASMCs. Consistent with Sph-1-P-elicited coronary vasoconstriction in vivo, Sph-1-P strongly induced CASMC contraction, which was inhibited by JTE-013, a newly-developed specific antagonist of $S1P_2$ (EDG-5). Furthermore, C3 exoenzyme or Y-27632 inhibited the CASMC contraction induced by Sph-1-P, indicating Rho involvement. Finally, exogenously-added [^{3}H]Sph-1-P underwent a rapid degradation. Since lipid phosphate phosphatases, ectoenzymes capable of dephosphorylating Sph-1-P, were expressed in CASMCs, Sph-1-P may be dephosphorylated by the ectophosphatases. **Conclusions:** Sph-1-P, derived from platelets and dephosphorylated on the cell surface, may induce the contraction of coronary artery smooth muscle cells through the $S1P_2$/Rho signaling.

Keywords: Lipid metabolism; Receptor; Signal transduction; Smooth muscle; Vasoconstriction/dilation

1. Introduction

Sphingosine 1-phosphate (Sph-1-P) is a bioactive lysophospholipid capable of inducing a wide spectrum of biological responses, including cell growth, differentiation, survival, and motility [1,2]. Originally, it was reported that Sph-1-P can serve as an intracellular second messenger regulating intracellular Ca^{2+} mobilization and cell growth and survival [3,4]. Furthermore, the dynamic balance between the intracellular levels of ceramide (Cer) and Sph-1-P, with the consequent regulation of opposing signaling pathways, was proposed to be an important factor that determined the cell fate [5]. However, recent evidence has indicated that Sph-1-P also acts as an intercellular mediator, interacting with the S1P (also called endothelial differentiation gene (EDG)) family of G protein-coupled receptors [1,2,6]. $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), $S1P_3$ (EDG-3), $S1P_4$ (EDG-6), and $S1P_5$ (EDG-8) exhibit overlapping, as well as distinct patterns of expression in various tissues as Sph-1-P receptors; the eventual cellular responses to extracellular Sph-1-P depend on the types of S1P receptors expressed [6,7]. Accordingly, Sph-1-P is now considered to be a unique lipid mediator that has dual actions, signaling inside and outside of the cell.

Blood platelets are unique in that they store Sph-1-P abundantly (possibly due to the existence of highly active

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sphingosine (Sph) kinase and to a lack of Sph-1-P lyase) and release this bioactive lipid extracellularly upon stimulation [8,9]. This is consistent with the fact that Sph-1-P is a normal constituent of plasma and serum; the Sph-1-P levels of the latter are higher [10]. In view of the diverse biological effects of Sph-1-P, including those toward vascular cells, Sph-1-P released from activated platelets may be involved in a variety of physiological and pathophysiological processes, in which critical platelet–vascular cell interactions (including thrombosis, hemostasis, angiogenesis, atherosclerosis, and ischemia) occur.

Platelet-derived mediators play an important role in coronary artery constriction and hence the pathogenesis of ischemic heart diseases, and inhibition of the spastic effects of these mediators would be useful therapeutically. We recently reported that in a canine isolated, blood-perfused papillary muscle preparation, which is a well-established in vivo model, a decrease in the coronary blood flow and the resultant negative inotropic effect were induced by Sph-1-P [11]. In this study, metabolism related to and cellular responses elicited by Sph-1-P were examined in human coronary artery smooth muscle cells (CASMCs) to provide an insight into the in vivo responses produced by Sph-1-P.

2. Methods

2.1. Materials

Recombinant *Clostridium botulinum* C3 exoenzyme was prepared as described previously [12], and kindly donated by Dr. S. Narumiya (Department of Pharmacology, Kyoto University Faculty of Medicine). Y-27632, a specific Rho kinase inhibitor [13], was a gift from Welfide (Osaka, Japan).

The following materials were obtained from the indicated suppliers: anti-RhoA monoclonal antibody (MoAb) (Santa Cruz Biotech, Santa Cruz, CA, USA); glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Buckinghamshire, UK); tetramethyl rhodamine isothiocyanate-phalloidin, suramin, serotonin, endothelin-1, and D-*erythro*--Sph (Sigma, St. Louis, MO, USA); Sph-1-P (Biomol, Plymouth Meeting, PA, USA); pertussis toxin (Kaken Pharmaceutical, Tokyo, Japan); recombinant human platelet-derived growth factor-BB (PDGF) (GenzymeTECHNE, Cambridge, MA, USA); thrombin (Mochida Pharmaceutical, Tokyo, Japan); angiotensin II (Bachem California, Torrance, CA, USA); [Arg^8]-vasopressin (Seikagaku, Tokyo, Japan); isoproterenol (Wako Pure Chemical Industries, Tokyo, Japan); U46619 (Calbiochem-Novabiochem, CA, USA); D-*erythro*-[3-^{3}H]Sph and [3-^{3}H]Sph-1-P (DuPont NEN, Boston, MA, USA).

2.2. Characterization of the $S1P_2$ antagonist JTE-013

The pyrazolopyridine derivative JTE-013 was a gift from the Central Pharmaceutical Research Institute, Japan Tobacco Incorporation, Osaka, Japan. JTE-013 is a specific $S1P_2$ antagonist (PCT (WO) Patent: publication number, WO 01/98301; publication date, December 27, 2001). It was confirmed that JTE-013 inhibited a specific binding of radio-labeled Sph-1-P to the cell membranes of Chinese hamster ovary (CHO) cells stably transfected with human $S1P_2$ and rat $S1P_2$, with IC_{50} values of 17 ± 6 nmol/l and 22 ± 9 nmol/l, respectively. In contrast, this compound at concentrations up to 10 μmol/l did not affect the Sph-1-P binding to the cell membranes of CHO cells stably transfected with human $S1P_1$. Furthermore, only 4.2% inhibition was observed with 10 μmol/l JTE-013 when the effect of this compound was examined on the Sph-1-P binding to the CHO cell membranes expressing $S1P_3$.

2.3. Cell culture

Human CASMCs were purchased from the Applied Cell Biology Research Institute (Kirkland, WA, USA), and maintained in Dulbecco's modified Eagle's medium with 20% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), 10 ng/l of recombinant human basic fibroblast growth factor (bFGF) (Becton Dickinson Labware, Lincoln Park, NJ, USA), penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37 °C under an atmosphere of 5% CO_2 and 95% room air. The cells were not used after the seventh passage.

2.4. Metabolism of [^{3}H]Sph or [^{3}H]Sph-1-P

CASMCs were incubated with 1 μM (0.2 μCi) [3-^{3}H]Sph or 100 nM (0.2 μCi) [3-^{3}H]Sph-1-P. Lipids were extracted from the cells and medium separately, and then analyzed for [^{3}H]sphingolipid metabolism as described previously [14]. Finally, portions of the extracted lipids were applied to silica gel high-performance thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany), and the plates were then developed in butanol/acetic acid/water (3:1:1), followed by autoradiography.

2.5. RNA isolation, Northern blot analysis, and RT-PCR

Total RNA was prepared from CASMCs with a total RNA isolation system (Isogen, Wako Pure Chemical Industries, Osaka, Japan), and the isolation of mRNA was performed with Oligotex™-dT30⟨Super⟩ (Takara Biomedicals, Tokyo, Japan), according to the manufacturer's instructions.

For Northern analysis, total RNAs were separated on a 1% agarose gel and transferred to a Hybond N^+™ nylon filter (Amersham Pharmacia Biotech). The membranes were probed with PCR products from cDNA of *$S1P_{1-4}$*, or *β-actin*, which had been labeled with digoxigenin using a PCR DIG Probe Synthesis Kit™ (Boehringer Mannheim, Mannheim, Germany). The probe binding was detected using alkaline phosphatase-conjugated anti-digoxigenin

antibody and visualized with CSPD™ according to the manufacturer's recommendations (Boehringer Mannheim). Quantitative analysis was performed using a PDI400oe Scanner and Quantity One 2.5a software for Macintosh.

For RT-PCR, the isolated mRNA was reverse transcribed using a SuperScript™ Preamplification System (Gibco BRL, Life Technologies, Rockville, MD, USA). Reverse transcribed cDNA was amplified in a Perkin-Elmer 9600R thermal cycler (Perkin-Elmer, Norwalk, CT, USA) using Takara Taq™ (rTaq DNA polymerase) (Takara Biomedicals).

The oligonucleotide primer pairs used for *lipid phosphate phosphatase* (*LPP*)-1, 2, and 3 were:

5′-GTACGTGGCCCTCGATGT-3′ (sense) and

5′-TGGTGATTGCTCGGATAGTG-3′ (antisense)

for *LPP*-1 (GenBank #AB000888);

5′-CTCGACGTGCTGTGCTTACT-3′ (sense)

and 5′-GTGCGGGTATCCATAGTGGT-3′ (antisense)

for *LPP*-2 (GenBank #AF056083);

5′-GCAAAACTACAAGTACGACAAAGC-3′ (sense)

and 5′-TGTCCACAGGTGAAAGGATTT-3′

(antisense) for *LPP*-3 (GenBank #AB000889).

Amplification was conducted with 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. The absence of contaminating DNA was confirmed by control reactions with RNA that had not been reverse transcribed. The PCR products (5 μl) were resolved by electrophoresis on a 2% agarose gel in TBE buffer (90 mM Tris–borate, 2 mM EDTA, pH 8.3) and stained with ethidium bromide. The PCR products were cut from the gels, solubilized, and sequenced with Dye Terminator Cycle Sequencing FS Ready Reaction Kits (Perkin-Elmer) and analyzed using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) according to the supplied protocols.

2.6. Cell contraction assay

In order to visualize agonist-induced contractility, suspensions of CASMCs were seeded onto dishes coated with 0.2% gelatin, and the cells were allowed to adhere to and spread on the substrate for 24 h. The cells were serum-starved for 3 h, and then stimulated with the indicated agonist for 30 min. The cells were fixed with 3% paraformaldehyde for 40 min, and then permeabilized with 0.2% Triton X-100 for 10 min. Actin filaments were detected with 0.1 μg/ml of tetramethyl rhodamine isothiocyanate-phalloidin. Cell morphology was observed with a confocal microscope, and the images were digitalized with a digital scanner (Canoscan D2400U, Canon, Tokyo, Japan). For quantitative evaluation, the maximal cord length of the cells was measured and was defined as the cell length. To preclude the possibility that the reduced viability of the cells accounted for the observed effects, we performed the following control experiments. For every batch, the cells challenged with the contractile agonist were subsequently exposed to 10 μmol/l isoproterenol. Only those batches of CASMCs that reduced their maximum cell length in response to the agonist and relaxed after the β-adrenergic receptor stimulation were used for the analysis.

2.7. Rho activity assay

Rho activity was assayed by the detection of cellular GTP-Rho. The coding sequence for the Rho-binding domain of Rho kinase (amino acids 759–1097) was amplified by PCR, and then cloned into a pGEX-2T vector (Amersham Pharmacia Biotech). The construct was transformed into *E. coli*, and the GST fusion protein was purified according to the manufacturer's recommendations. Affinity-precipitation of cellular GTP-Rho was performed with the GST fusion protein as described previously [15]. When incubated with lysates from COS cells expressing a HA-tagged mutant of Rho, this GST fusion protein was confirmed to precipitate recombinant V14Rho (GTP bound) but not N19 Rho (GDP-bound) (data not shown).

2.8. Statistics

When indicated, statistical analysis was performed by Student's *t*-test, and $P<0.05$ was considered significant.

3. Results

3.1. Sph metabolism and S1P expression in CASMCs

Sph-1-P is a bioactive sphingolipid, acting as an intracellular second messenger in some cells and as an extracellular mediator in others. In this context, we first checked the intracellular Sph-1-P formation (from Sph) and *S1P* expression in CASMCs.

[^{3}H]Sph incorporated into CASMCs was phosphorylated by Sph kinase to [^{3}H]Sph-1-P (Fig. 1A). The [^{3}H]Sph-1-P formation was transient, possibly due to degradation by Sph-1-P lyase, and was not affected by the established SMC agonists such as angiotensin II, endothelin-1, PDGF, vasopressin, and thrombin (Fig. 1B). Accordingly, it is unlikely that Sph-1-P acts as an intracellular second messenger in CASMCs. Furthermore, [^{3}H]Sph-1-P was not detected in the medium under these conditions (Fig. 1B), indicating that stimulation-dependent extracellular Sph-1-P release does not occur in these cells.

We then checked the expression of the cell surface Sph-1-P receptors. Northern blot analysis of CASMC RNA showed that $S1P_3$ was abundantly expressed, while $S1P_1$ and $S1P_2$ transcripts were expressed at lower levels in these cells; $S1P_4$ was not expressed (Fig. 2). Accordingly, it was confirmed that CASMCs express cell surface Sph-1-P receptors. The facts that stimulation-dependent Sph-1-P formation was not observed and that the cell surface

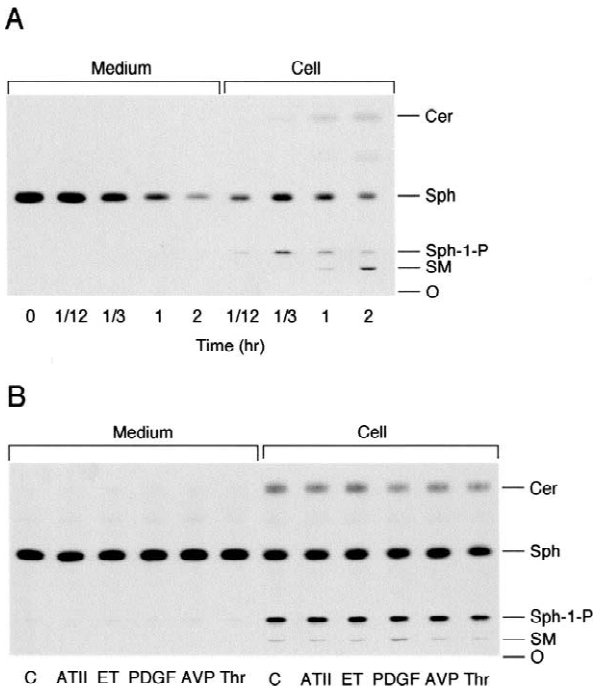

Fig. 1. Metabolism of [^{3}H]Sph in human CASMCs. (A) CASMCs were incubated with [^{3}H]Sph for the indicated durations. Lipids were then extracted from the cells and medium, and analyzed for [^{3}H]sphingolipids. Locations of standard lipids are indicated on the right. SM, sphingomyelin; O, origin. (B) CASMCs were incubated with [^{3}H]Sph for 1 min and then challenged without (C) or with 1 μmol/l angiotensin II (ATII), 3 nmol/l endothelin-1 (ET), 5 nmol/l PDGF, 1 μmol/l arginine vasopressin (AVP), or 1 U/ml of thrombin (Thr) for 20 min. The results shown are representative of three independent experiments.

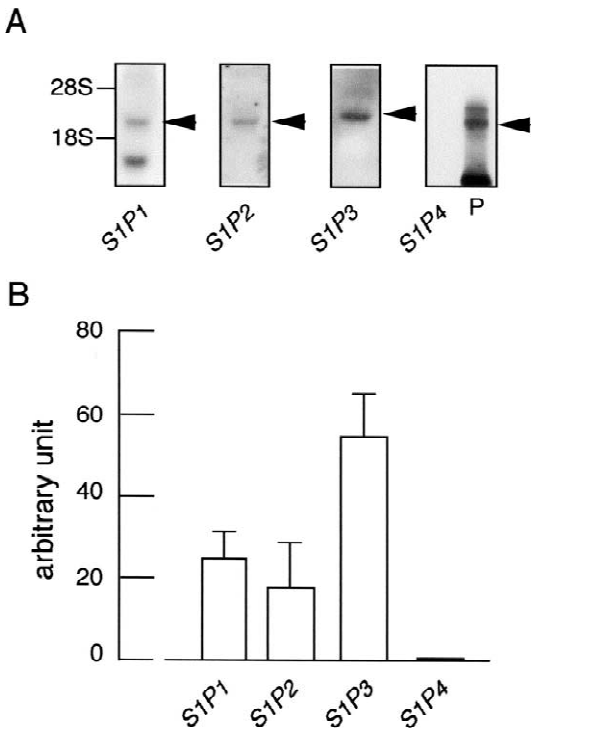

Fig. 2. Expression of *S1P* Sph-1-P receptor transcripts in CASMCs. (A) Total RNA from CASMCs (10 μg/lane) and PCR products for the positive control for $S1P_4$ (P) (when indicated) were hybridized with each fragment of $S1P_{1-4}$ PCR products, as described in the Methods. The locations of the 28S and 18S rRNA bands are indicated on the left. (B) The levels of *S1P* expression were adjusted by that of *β-actin* expression, and their relative quantities were calculated as an arbitrary unit. Columns and error bars represent the mean±S.D. ($n=3$).

Sph-1-P receptors are expressed on CASMCs suggest Sph-1-P acting as an extracellular mediator in these cells.

3.2. Sph-1-P-induced CASMC contraction via $S1P_2$

We next examined the actions of Sph-1-P in CASMCs to provide an insight into the mechanism by which Sph-1-P exerted the response in an in vivo heart model (see Introduction). We evaluated CASMC contraction by examining the cell shape change. As shown in Figs. 3A and 4A, Sph-1-P strongly induced CASMC contraction.

Of the S1P receptors expressed in CASMCs, $S1P_1$ is known to activate only members of the G_i family, while $S1P_2$ and $S1P_3$ have a broader coupling profile than $S1P_1$ and can communicate with several G proteins, including $G_{12/13}$ [1,2,6]. We first examined the effect of JTE-013, a specific and potent $S1P_2$ antagonist, to check $S1P_2$ involvement in the Sph-1-P-induced CASMC contraction. This pyrazolopyridine derivative strongly and concentration-dependently inhibited Sph-1-P-induced CASMC contraction (Fig. 3A and B). On the other hand, this $S1P_2$ antagonist failed to affect CASMC contraction induced by serotonin or U46619 (a stable thromboxane A2 analogue) (Fig. 3C). Although $S1P_3$ is abundantly expressed in CASMCs (see Fig. 2), the $S1P_3$ antagonist suramin [16] did not inhibit Sph-1-P-induced CASMC contraction (data not shown); the data, however, should be interpreted cautiously because of its low specificity. Furthermore, pertussis toxin, which inactivates G_i, had no effect (Fig. 4B), excluding $S1P_1$ involvement.

These results indicate that Sph-1-P-induced CASMC contraction is mediated specifically by $S1P_2$.

3.3. Involvement of the Rho/Rho kinase pathway in Sph-1-P-induced CASMC contraction

A guanine nucleotide exchange factor for Rho, p115RhoGEF, has been identified as a target for the α subunit of the $G_{12/13}$ family, with which $S1P_2$ communicate [1,2,6]. Furthermore, the small GTPase Rho and its downstream targets Rho kinase (and myosin light chain phosphatase) play an important role in phosphorylation of myosin light chain and thereby induce actomyosin contractile force [17,18]. Accordingly, the involvement of this signaling pathway was examined. As expected, pretreatment of CASMCs with the specific Rho inactivator C3 exoenzyme [12] or the Rho kinase inhibitor Y-27632 [13] reduced the CASMC contraction induced by Sph-1-P (Fig.

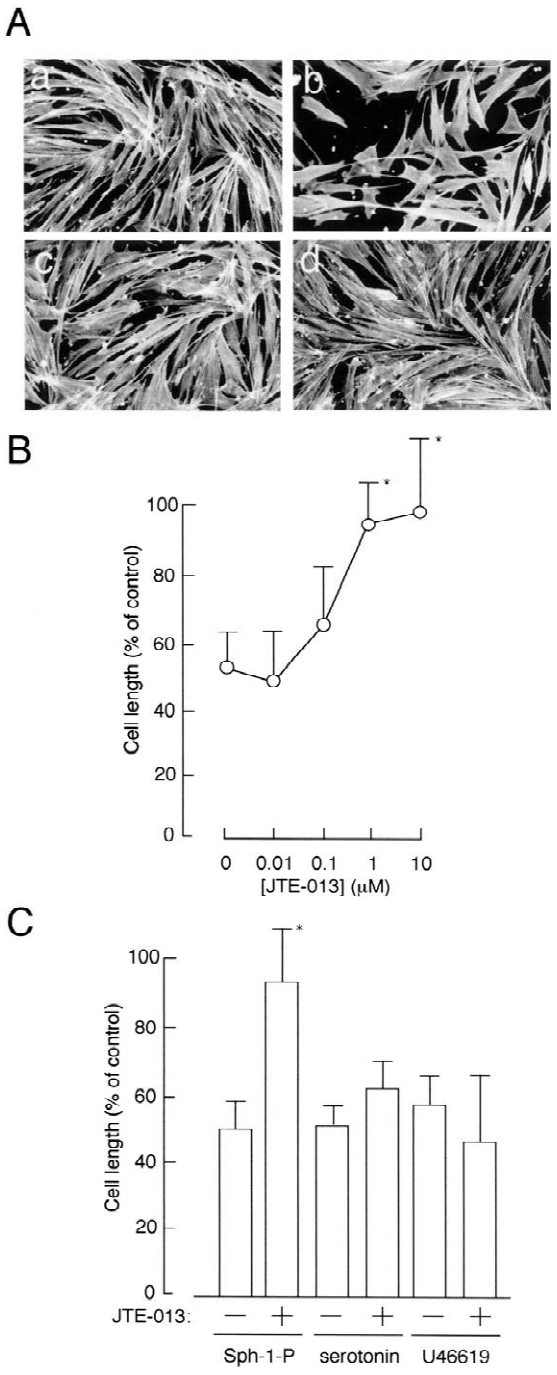

Fig. 3. Sph-1-P-induced CASMC contraction and its inhibition by JTE-013, a specific $S1P_2$ antagonist. (A) CASMCs pretreated without (a, b) or with 1 μmol/l (c) or 10 μmol/l (d) JTE-013 for 10 min were challenged without (a) or with 100 nmol/l Sph-1-P (b–d). The cells were fixed, permeabilized, and then stained with tetramethyl rhodamine isothiocyanate-phalloidin for evaluation of their contraction. The results are representative of three independent experiments. (B) CASMCs pretreated with various concentrations of JTE-013 for 10 min were stimulated with 100 nmol/l Sph-1-P. The average cell length from 100 cells for each condition is shown as a percentage of the control cells (without treatment). Data represent the mean±S.D. ($n=3$). (C) CASMCs were pretreated without (−) or with 1 μmol/l JTE-013 for 10 min (+), and then challenged with 100 nmol/l Sph-1-P, 1 μmol/l serotonin, or 1 μmol/l U46619. Columns and error bars represent the mean±S.D. ($n=3$). *Statistically significant compared with control (without JTE-013 pretreatment).

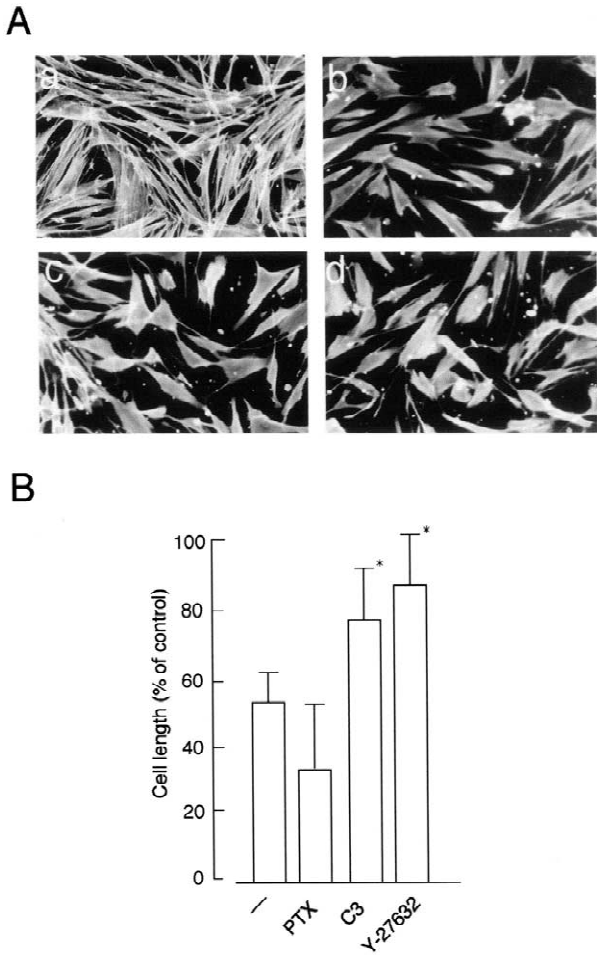

Fig. 4. Rho-dependent CASMC contraction induced by Sph-1-P. (A) CASMCs were stimulated without (a) or with 100 nmol/l (b), 1 μmol/l (c), or 10 μmol/l (d) Sph-1-P for 30 min, and the cell contraction was determined as described in the legend to Fig. 3. The results are representative of three independent experiments. (B) CASMCs were pretreated without (−) or with 500 ng/ml of pertussis toxin for 2 h (PTX), 10 μg/ml of C3 exoenzyme for 30 h (C3), or 20 μmol/l Y-27632 for 30 min. The cells were then stimulated with 100 nmol/l Sph-1-P, and then stained for evaluation of their contraction. Columns and error bars represent the mean±S.D. of at least three independent experiments. *Statistically significant compared with control (without pretreatment).

4B), indicating the importance of the Rho/Rho kinase pathway in Sph-1-P-induced CASMC contraction.

We next checked Rho activation in CASMCs stimulated with Sph-1-P. The Rho activity increased as early as 1 min after the Sph-1-P stimulation, and was sustained for at least 30 min, when it was assayed by an affinity-precipitation of cellular GTP-Rho (Fig. 5A). As expected, the Rho activation induced by Sph-1-P was abolished by pretreatment with C3 exoenzyme (Fig. 5B). Pertussis toxin did not inhibit the Rho activation induced by Sph-1-P; rather, it enhanced the Sph-1-P effect (Fig. 5B) for unknown reasons. These data further strengthened the idea that the Sph-1-P-elicited CASMC response observed is mediated via Rho.

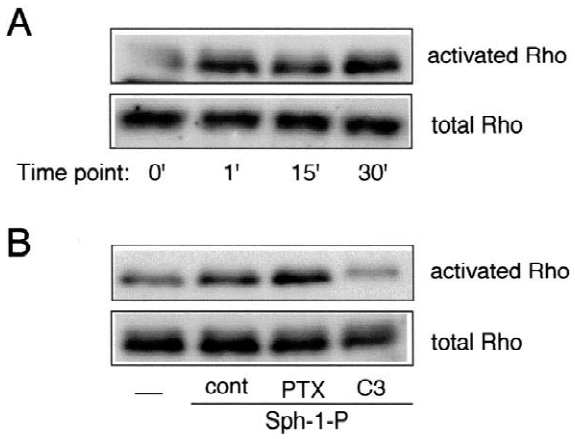

Fig. 5. Sph-1-P activates the Rho GTPase. (A) CASMCs were stimulated with 1 μmol/l Sph-1-P for the indicated durations. (B) CASMCs pretreated without (− or control) or with 500 ng/ml of pertussis toxin (PTX) for 2 h or 10 μg/ml of C3 exoenzyme (C3) for 30 h were stimulated without (−) or with 1 μmol/l Sph-1-P for 15 min. The cell lysates were incubated with GST fusion protein containing the Rho-binding domain of Rho kinase. The bound proteins were resolved on SDS–PAGE, and then immunoblotted with anti-RhoA MoAb (upper panel). Whole cell lysates prepared before the precipitation were also analyzed (lower panel). The results are representative of three independent experiments.

3.4. Degradation of exogenously-added Sph-1-P in the presence of CASMCs

We finally evaluated the fate of Sph-1-P in the presence of CASMCs by pursuing the metabolic changes of [3-^{3}H]Sph-1-P, which should retain the radioactivity after its dephosphorylation. When [^{3}H]Sph-1-P was extracellularly added to CASMCs, [^{3}H]Sph-1-P associated with CASMCs underwent a marked metabolism; instead, [^{3}H]Sph, [^{3}H]Cer, and [^{3}H]sphingomyelin were formed (Fig. 6A). These results can be best explained by the idea that non-polar [3-^{3}H]Sph, formed from polar [3-^{3}H]Sph-1-P by ectophosphatase activity [19], is incorporated into CASMCs and then converted to [3-^{3}H]Cer (and then to [3-^{3}H]sphingomyelin) intracellularly; Sph (but not Sph-1-P) is hydrophobic and easily passes through the lipid bilayer. In fact, [^{3}H]Sph, added exogenously, was incorporated into CASMCs and converted to [^{3}H]Cer and [^{3}H]sphingomyelin (see Fig. 1A).

Recently, several isoenzymes of mammalian lipid phosphate phosphatase (LPP) (type 2 phosphatidic acid phosphatase) have been cloned [20], and they are believed to act at the outer leaflet of the cell surface bilayer, accounting for the ecto-lipid phosphate phosphatase activities (toward Sph-1-P, lysophosphatidic acid, or phosphatidic acid) previously described [21]. Accordingly, we finally checked the expression of LPPs by RT-PCR. RNA from CASMCs was prepared and reverse transcribed, followed by PCR amplification of specific transcripts. As demonstrated in Fig. 6B, CASMCs were found to express mRNA

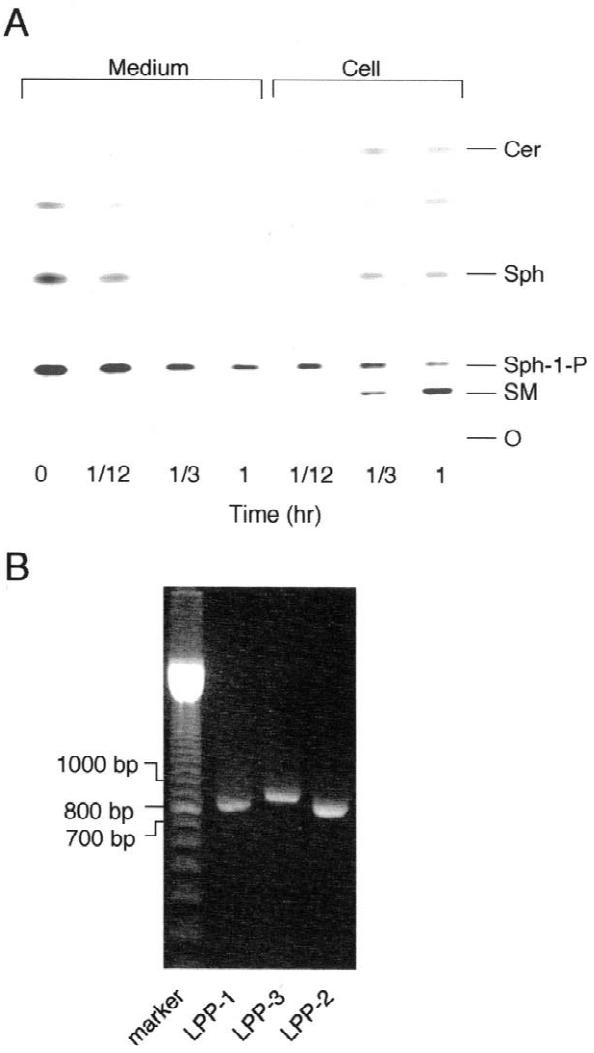

Fig. 6. Metabolism of exogenous [^{3}H]Sph-1-P and detection of mRNA expression for LPPs in CASMCs. (A) CASMCs were incubated with [3-^{3}H]Sph-1-P for the indicated durations. Lipids were then extracted from the cells and medium, and analyzed for [^{3}H]sphingolipids. There are two faint radioactive bands other than Sph-1-P in time 0; these may have been formed by Sph-1-P degradation during its storage. (B) Amplified products for LPP-1, 2, and 3 were resolved on 2% agarose gels. The marker lane contained the size standards with the positions (base pairs (bp)) indicated. The specific amplified products for LPP-1, 2, and 3 were 825, 828, and 899 bp, respectively. The results are representative of three independent experiments.

transcripts for LPP-1, 2, and 3; the sequence of this PCR product was confirmed (data not shown).

4. Discussion

As expected from our previous in vivo study showing that Sph-1-P induces coronary artery vasoconstriction [11], this lysophospholipid was found to induce contraction of CASMCs. The facts that stimulation-dependent Sph-1-P formation from Sph was not affected upon activation and

that the cell surface Sph-1-P receptors (*EDG*-1, 3, and 5) were expressed suggest Sph-1-P acting as an extracellular mediator in CASMCs. Furthermore, CASMCs themselves failed to release Sph-1-P extracellularly and hence are not a likely source for extracellular Sph-1-P. It can be speculated that these cells may receive this lysophospholipid mediator from the outside, probably from platelets; Sph-1-P is abundantly stored in platelets and released extracellularly upon stimulation [8,9]. In vivo, Sph-1-P derived from platelets has to pass through vascular endothelial cells to interact with CASMCs, as is the case with thromboxane A_2. Although further studies are needed to solve this problem, not only endothelial cells but also smooth muscle cells are likely to be exposed to significant levels of Sph-1-P under the conditions in which the integrity of vascular endothelial cells is disturbed.

As for the signaling pathway involved in this Sph-1-P-induced response, the Rho/Rho kinase pathway seems to be important, based on the inhibitory effects by C3 exoenzyme and Y-27632. In fact, Sph-1-P was confirmed to activate Rho, a member of the small GTPase protein. Rho is converted to an active form by Rho-GEF through the activation of $G_{12/13}$ [17,18], and plays important roles in a variety of cellular functions, including cytoskeletal reorganization, integrin activation, and gene expression [17,18]. Rho, through its target Rho kinase (and the myosin binding subunit of myosin phosphatase) [17,18], regulates myosin light chain phosphorylation, and hence induces smooth muscle contraction in a Ca^{2+}-independent manner [22]. Recently, hydroxyfasudil, an inhibitor of Rho kinase, was shown to inhibit serotonin-induced coronary spasm in a porcine model both in vivo and in vitro [23,24], and RT-PCR analysis revealed that the expression of Rho kinase mRNA was significantly increased in the spastic segments of coronary arteries [23]. These results indicate that Rho kinase is a key molecule in coronary artery hypercontraction induced by vasospastic agents (including Sph-1-P).

Platelets release a variety of vasospastic agents upon their activation, including serotonin and thromboxane A_2, and hence are involved in coronary artery vasoconstriction. Sph-1-P should be added to the list of such vasospastic substances released from activated platelets. In CASMCs, the Sph-1-P receptors *S1P*$_1$, *S1P*$_2$, and *S1P*$_3$ were confirmed to be expressed. Of these, $S1P_2$, which has been shown to preferentially activate $G_{12/13}$ and hence Rho, is the most probable candidate receptor for transducing the Sph-1-P effect on CASMC contraction because of the specific inhibition of the Sph-1-P-induced response by JTE-013, an $S1P_2$ antagonist. This is compatible with the facts that Sph-1-P-induced CASMC contractile response is not inhibited when pertussis toxin inactivates G_i, only with which $S1P_1$ couples [16,25] and that the $S1P_3$ antagonist suramin fails to affect the response.

Finally, Sph-1-P degradation in the presence of CASMCs may be important when the in vivo effects of Sph-1-P are evaluated. Although the possibility of Sph-1-P degradation occurring after its intracellular uptake by S1P-mediated and ligand-dependent recycling [26] cannot be ruled out, it is most likely that Sph-1-P is metabolized at the cell surface by LPPs, which were confirmed to be expressed in CASMCs. Based on the previous results, K_ds for S1P receptors are much lower than the concentrations of Sph-1-P in the plasma [2,6,10]. Probably, due to Sph-1-P dephosphorylation at the cell surface, the concentrations of Sph-1-P interacting with S1P receptors may be lower than the plasma Sph-1-P levels. This is also consistent with a recent in vitro transfection study showing that LPPs may limit the bioactivity of Sph-1-P by regulating its concentration (interacting with S1P receptors) [27]. The Sph-1-P-induced effects on CASMCs in vivo may depend on various factors, including Sph-1-P release from platelets, S1P expression on CASMCs, and LPP activity on CASMCs.

Sph-1-P is attracting much attention as a bioactive sphingolipid released from platelets. Considering the great variety of responses induced by Sph-1-P, the development of its receptor agonists/antagonists of the S1P family may lead to a new therapeutic approach to regulate various diseases. Very recently, it was shown that lymphocyte trafficking is altered by Sph-1-P and by its related synthetic compounds and that the inhibition of lymphocyte recirculation by the Sph-1-P receptor agonists may result in therapeutically useful immunosuppression [28]. In the present study, we have shown for the first time that Sph-1-P induces CASMC contraction through $S1P_2$; antagonists of this Sph-1-P receptor may have potential as drugs to control vascular diseases.

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