Sphingosine 1-Phosphate Inhibits Nitric Oxide Production Induced by Interleukin-1β in Rat Vascular Smooth Muscle Cells

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ABSTRACT

Sphingosine 1-phosphate (S1P) is a lipid mediator that exerts potent and diverse biological effects on several cardiovascular cells. We investigated the effect of S1P on interleukin (IL)-1β-induced nitric oxide (NO) production and inducible NO synthase (iNOS) expression in rat vascular smooth muscle cells (VSMCs). S1P inhibited NO production at concentrations higher than 0.1 μM; this was associated with the inhibition of iNOS protein and mRNA expression. S1P also inhibited IL-1β-induced GTP cyclohydrolase I (GTPCH) mRNA expression. Pertussis toxin (PTX) partially attenuated the inhibitory effects of S1P on NO production and iNOS protein induction, whereas it completely blocked the inhibitory effects on iNOS and GTPCH mRNA expression. S1P inhibited iNOS expression in Ca2+-depleted conditions; PTX did not modify this effect. The Rho kinase inhibitor Y 27632 partially but significantly attenuated the inhibitory effect of S1P on iNOS expression in Ca2+-depleted condition but did not affect it in the presence of Ca2+. S1P significantly inhibited IL-1β-induced persistent activation of extracellular signal-regulated kinase (ERK) but had no effect in Ca2+-depleted conditions. Thus, S1P inhibits IL-1β induction of NO production and iNOS expression in rat VSMCs through multiple mechanisms involving both PTX-sensitive and -insensitive G proteins coupled to S1P receptors. Furthermore, Ca2+-dependent ERK inhibition and Ca2+-independent Rho kinase activation might be involved in the inhibitory mechanism of iNOS expression. Through its action on NO production by VSMCs, S1P may play an important role in the progression of local vascular injury associated with thrombosis, atherosclerosis, and hypertension.

Nitric oxide (NO) produced by NO synthase (NOS) in the vascular wall plays important roles in cardiovascular pathophysiology. In addition to its vasodilating effect, NO inhibits platelet aggregation, leukocyte adhesion, proliferation, and migration of vascular smooth muscle cells (VSMCs) and induces VSMC apoptosis. This suggests critical roles for NO in local vascular injury and remodeling (Dusting, 1995). NO can be generated by inducible NOS (iNOS) in VSMCs following stimulation with cytokines such as interleukin (IL)-1β (Busse and Mülsch, 1990). IL-1β is produced by a variety of cells in the vascular wall and participates in the regulation of smooth muscle function in local vascular injury (Clinton and Libby, 1992; Chamberlain et al., 1999). The signaling cascade of iNOS expression initiated by IL-1β in VSMCs is well characterized (Jiang et al., 2001, 2004; Ginnan et al., 2006). The activation of extracellular signal-regulated kinase (ERK) initiated by IL-1β seems to be essential for the activation of nuclear factor-κB and the consequential expression of proinflammatory genes including iNOS (Jiang et al., 2004). Furthermore, it was reported that Rho kinase negatively regulates IL-1β-induced iNOS expression (Wei et al., 2006).

ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; VSMC, vascular smooth muscle cell; iNOS, inducible nitric-oxide synthase; IL, interleukin; ERK, extracellular signal-regulated kinase; S1P, sphingosine 1-phosphate; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; eNOS, endothelial nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; GTPCH, GTP cyclohydrolase I; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BH4, tetrahydrobiopterin; BAPTA-AM, 1,2-bis-(N-methyl-D,L-lysino-2-oxopropyl)-4-(2-hydroxyethyl)-6-cyclodextrin; PKC, protein kinase C; p-ERK, phosphorylated ERK; t-ERK, total ERK.

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The expression of iNOS in VSMCs is negatively modulated by a variety of vasoactive mediators such as platelet-derived growth factor (Scott-Burden et al., 1992), angiotensin II (Nakayama et al., 1994), and 5-hydroxytryptamine (Shimpo et al., 1997). The precise mechanism by which these mediators inhibit iNOS induction remains to be defined.

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator that is formed from sphingosine by the action of sphingosine kinase. S1P is abundantly stored in platelets and released into the circulation upon stimulation (Yatomi et al., 2001). Several studies have demonstrated that S1P exerts potent and diverse biological effects on a variety of cells types including vascular endothelial cells and VSMCs. Five S1P receptor subtypes (S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅) have been identified in a wide variety of cells. These receptors are coupled to multiple pertussis toxin (PTX)-sensitive and -insensitive G proteins (Pyne and Pyne, 2000; Kluk and Hla, 2002). Rat VSMCs express high levels of S1P₂ and S1P₃ mRNAs and, under certain conditions, a low level of S1P₁ mRNA (Tamama et al., 2001; Kluk and Hla, 2001; Nodai et al., 2007). S1P₂ and S1P₃ receptors have common signaling characteristics and couple to Gₛ, G₁₀, and G₁₂/13 proteins (Pyne and Pyne, 2000; Kluk and Hla, 2002). Furthermore, S1P activates mitogen-activated protein kinase (MAPK), including ERK and p38 (Usui et al., 2004; Nodai et al., 2007), as well as G₁₂/₁₃-activated Rho kinase (Takawa, 2002). Through these cascades, S1P stimulates proliferation of VSMCs, inhibits or stimulates their migration (Levade et al., 2001), and elicits proinflammatory cascades, S1P stimulates proliferation of VSMCs, inhibits or stimulates their migration (Levade et al., 2001), and elicits proinflammatory and anti-apoptotic effects in VSMCs (Stone et al., 2004; Sanada et al., 2005). However, the role of S1P in the regulation of iNOS expression and subsequent NO production in rat renal mesangial cells (Xin et al., 2004), the effect of S1P on NO production by VSMCs is unclear.

The aim of the present study was to investigate the effect of S1P on NO production and iNOS expression in rat VSMCs and to provide mechanistic insight into these effects. Based on our results, we propose that S1P inhibits IL-1β-induced iNOS expression via Ca²⁺-dependent ERK inhibition and Ca²⁺-independent Rho kinase activation.

Materials and Methods

Cell Culture. VSMCs were enzymatically isolated from aortic media of 6- to 7-week-old Wistar rats using collagenase and elastase and cultured until confluence in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, with medium changes every 2 to 3 days (Hirafuji et al., 2002). Primary VSMCs were used throughout the experiments. Cells were incubated with 3 ng/ml IL-1β and S1P for 24 h in medium containing 0.1% bovine serum albumin. S1P was dissolved in physiological saline containing 10 mM NaOH and further diluted with the serum-free culture medium containing 0.1% bovine serum albumin. In some experiments, VSMCs were treated with PTX (400 ng/ml) for 24 h before IL-1β stimulation, and subsequent experiments were carried out identically in a paired fashion. The present study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Animal Research Committee of Health Sciences University of Hokkaido.

Determination of Nitrite/Nitrate. NO in the culture medium was measured as nitrite/nitrate, stable oxidation products of NO. The culture medium was applied to a copperized cadmium reduction column to reduce nitrate to nitrite and then reacted with Griess reagent, using a NOx AutoAnalyzer (ENO-10; Eicom, Kyoto, Japan) (Hirafuji et al., 2002). Detected NO was normalized to protein concentration (nanomoles per milligram of protein). Protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. Results were expressed as a percentage, taking samples treated with IL-1β alone as 100%.

Western Blot Analysis. S1P protein expression and ERK activation were evaluated by Western blot analysis as described previously (Hirafuji et al., 2002; Machida et al., 2005). The blot was incubated for 2 h with anti-iNOS, anti-endothelial NOS (eNOS), anti-neuronal NOS (nNOS) antibody, and antibodies against phosphorylated and total ERK. The immunoblot was incubated with horseradish peroxidase-conjugated secondary antibody and visualized by an enhanced chemiluminescence kit. iNOS (130 kDa), eNOS (140 kDa), and nNOS (155 kDa) bands corresponding to appropriate positive controls were analyzed by densitometry using NIH Image (version 1.61).

Reverse Transcription-Polymerase Chain Reaction. mRNA expression level for iNOS and GTP cyclohydrolase I (GTPCH) was determined by semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR) with total RNA (Hirafuji et al., 2002). RT-PCR for iNOS and GTPCH mRNA was carried out using primers specific for rat iNOS (Hirafuji et al., 2002) and GTPCH (Hattori and Gross, 1993), respectively. Concurrent RT-PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message was performed as an internal control (Hirafuji et al., 2002). Total RNA was isolated from VSMCs using RNaseasy total RNA kit (QIAGEN, Hilden, Germany). RT and PCR reactions were carried out using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and the Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN), respectively. After an initial denaturation at 95°C for 3 min, PCR amplification was performed for either 30 cycles (for iNOS and GAPDH) or 35 cycles (for GTPCH) using a three-step protocol: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Samples were incubated for an additional 5 min at 72°C before completion. iNOS, GTPCH, and GAPDH primers were predicted to amplify products of 250, 372, and 306 bp, respectively. PCR products were separated on an ethidium bromide-stained 1.0% agarose gel and scanned using a Light-Capture (ATTO, Tokyo, Japan). Quantities of each product were analyzed by densitometry using NIH Image and calculated relative to GAPDH.

NOS Activity Assay. NOS activity assay was performed according to Kim and Lancaster (1993). The reaction mixture consisted of 1 mM NADPH, 20 μM FAD, 20 μM FMN, 0.5 mM tetrahydrobiopterin (BH₄), 4 mM L-arginine, and 5 mM glutathione, in a final volume of 200 μl in 40 mM Tris-HCl, pH 7.7. The reaction was initiated by addition of cell extract (30 μl) at 37°C and terminated by addition of 400 μl of 0.5 M NaOH and 600 μl of methanol after 2 h. After centrifugation, nitrite/nitrate was measured in the supernatant.

Materials. Fetal calf serum, penicillin, streptomycin, and the medium were obtained from Invitrogen. IL-1β was from Collaborative Biomedical Products (Bedford, MA). S1P and Cₛ-cameride were from Cayman Chemicals (Ann Arbor, MI). Sphingomyelin and sphingosine were from BIOMOL (Plymouth Meeting, PA). PTX was from HyClone (Logan, UT). PD 98059, SB 203580, GF 109203X, and Y 27632 were obtained from Calbiochem (San Diego, CA). Phosphorylated and total ERK. The immunoblot was incubated with Horseradish peroxidase-conjugated secondary antibody and visualized by an enhanced chemiluminescence kit. iNOS (130 kDa), eNOS (140 kDa), and nNOS (155 kDa) bands corresponding to appropriate positive controls were analyzed by densitometry using NIH Image (version 1.61).

Statistical Analysis. Statistical analysis was performed using Mann-Whitney's U test for unpaired data and analysis of variance.
followed by Dunnett’s test for multiple comparisons. $p$ Values $< 0.05$ were considered significant.

**Results**

**Effect of S1P on NO Production.** To investigate the effect of S1P on NO production by VSMCs, VSMCs were stimulated with IL-1β for 24 h in the absence or presence of S1P. NO was determined in the medium as nitrite/nitrate. IL-1β-induced NO production in VSMCs was 204.0 ± 4.81 nmol/mg protein (mean ± S.E.M., $n = 4$). In the 0.1 to 10 μM range, S1P inhibited the production of NO induced by IL-1β in a concentration-dependent manner. At 10 μM, S1P caused an almost complete inhibition (95.8 ± 2.8%). In contrast, at 0.01 μM, S1P tended to stimulate IL-1β-induced NO production (Fig. 1A, open columns). In the absence of IL-1β, S1P alone had no effect on NO production (data not shown). Sphingosine, C6-ceramide, and sphingomyelin at concentrations up to 10 μM had no significant effects on the IL-1β-induced NO production (data not shown). The inhibitory effect of S1P was attenuated by pretreatment with PTX (Fig. 1A, hatched columns). In PTX-pretreated VSMCs, S1P slightly stimulated the NO production at 0.01 μM. As shown in Fig. 1B, 1400W, a specific iNOS inhibitor (Garvey et al., 1997), inhibited IL-1β-induced NO production in a concentration-dependent manner (Fig. 1B). The complete inhibition of NO production by 100 μM 1400W suggests that NO production induced by IL-1β is almost iNOS-derived.

**Effect of S1P on NOS Expression.** The effect of S1P on IL-1β-induced NOS protein expression was investigated by Western blot analysis. The expression of iNOS protein induced by IL-1β stimulation for 24 h was markedly inhibited by S1P (1 and 10 μM) (Fig. 2A, open columns). This inhibitory effect was reduced by PTX (Fig. 2A, hatched columns). In contrast to iNOS expression, both IL-1β and S1P had no effect on eNOS expression (Fig. 2B). IL-1β also had no effect on nNOS expression (Fig. 2C). S1P at 10 μM slightly inhibited nNOS expression, although it was statistically not significant ($p < 0.1$ versus IL-1β alone; Fig. 2C). Because the IL-1β-induced NO production was almost attributable to iNOS induction, we next investigated the effect of S1P on IL-1β-induced iNOS mRNA expression by semiquantitative RT-PCR. S1P inhibited IL-1β-induced iNOS mRNA expression in a concentration-dependent manner (Fig. 3A), and 10 μM S1P caused a moderate but significant inhibition (Fig. 3B, open columns). This inhibitory effect was prevented by PTX (Fig. 3, A and B, hatched columns). GTPCH, the first and rate-limiting enzyme in the de novo BH4 synthetic pathway, is coinduced with iNOS in VSMCs (Gross and Levi, 1992). S1P at 10 μM also caused a significant inhibition of IL-1β-induced GTPCH mRNA expression; this effect was prevented by PTX (Fig. 3, C and D).

**Effect of S1P on NOS Activity.** The effect of S1P on NOS activity was then investigated. NOS activity was significantly induced by IL-1β and was markedly inhibited by S1P in a concentration-dependent manner (Fig. 4).

**Involvement of Ca2+ in the Inhibition of iNOS Expression by S1P.** S1P induces an increase of intracellular Ca2+ concentration in many cell types including VSMCs (Vekshina, 2001; Xu et al., 2006). Thus, we investigated the involvement of Ca2+ in the inhibitory effect of S1P on iNOS expression. Ca2+ depletion with the extracellular Ca2+ chelator EGTA (1 mM) and the intracellular Ca2+ chelator BAPTA-AM (10 μM) significantly increased IL-1β-induced iNOS expression (Fig. 5A). S1P inhibited IL-1β-induced iNOS expression in Ca2+-depleted conditions (Fig. 5B, open columns); this inhibition was not affected by PTX pretreatment (Fig. 5B, hatched columns).

**Involvement of Rho Kinase in the Ca2+-Independent Effect of S1P.** Rho kinase activated by G$_{12/13}$ protein is another important kinase in S1P signaling in VSMCs (Takuya, 2002). Thus, we next investigated whether Rho kinase is involved in the mechanism of iNOS inhibition. As shown in Fig. 6A, Y 27632 (10 μM), a Rho kinase inhibitor, had no significant effect on the induction of iNOS by IL-1β and the iNOS inhibition by S1P in the presence of Ca2+. However, in Ca2+-depleted conditions, Y 27632 partially but significantly antagonized the iNOS inhibition by S1P (Fig. 6B).

**Role of MAPK in the Inhibition of iNOS Expression by S1P.** PD 98059 (10 μM), a selective inhibitor of ERK activation, significantly inhibited IL-1β-induced iNOS expression. In contrast, SB 203580 (10 μM), a selective inhibitor of p38 MAPK, did not affect the induction of iNOS expression by IL-1β. Neither PD 98059 nor SB 203580 affected the inhibition of iNOS expression by S1P (Fig. 7).

**Effect of S1P on ERK Activation.** Shown in Fig. 8 is a representative blot image of ERK activation by IL-1β, S1P alone, and in combination. IL-1β caused biphasic ERK activation, i.e., a transient phase reaching a peak at 20 to 30 min.
Western blot. iNOS (A), eNOS (B), and nNOS (C) protein expression was analyzed by Western blot. iNOS (A), eNOS (B), and nNOS (C) protein expression was analyzed by Western blot. 

Fig. 2. Effect of S1P on IL-1β-induced NOS protein expression in VSMCs. iNOS (A), eNOS (B), and nNOS (C) protein expression was analyzed by Western blot. β-Actin protein expression was also evaluated as a loading control. A, same protocol as in Fig. 1. Top panels, representative blotting image. Bottom panel, summary of densitometric analysis expressed as a percentage, taking samples treated with IL-1β alone as 100%. Bars, mean ± S.E.M. (n = 6). **, p < 0.01 versus IL-1β; †, p < 0.05 versus IL-1β alone as 100%.

Discussion

In the present study, we demonstrated that S1P strongly inhibited IL-1β-induced NO production and iNOS expression in rat VSMCs. At a concentration of 10 μM, S1P almost completely inhibited IL-1β-induced NO production. S1P-related compounds, such as sphingosine, C6-ceramide, and sphingomyelin, had no effect on the IL-1β-induced NO production. The IL-1β-induced NO production was almost iNOS-derived because 1400W, a specific iNOS inhibitor (Garvey et al., 1997), strongly inhibited NO production. In fact, S1P inhibited IL-1β-induced NOS activity and iNOS protein and mRNA expressions in a concentration-dependent manner. In agreement with a previous report (Seasholtz and Brown, 2004), rat VSMCs expressed both eNOS and nNOS. However, neither IL-1β nor S1P had any significant effect on both eNOS and nNOS expression. Therefore, the inhibition of NO production by S1P can be mainly attributed to the inhibition of iNOS induction.

The inhibitory effect of S1P on iNOS protein induction (~40.7% inhibition) was smaller than that on NO production (~95.8% inhibition). We observed that S1P inhibited IL-1β-induced GTPCH mRNA expression, the first and rate-limiting enzyme of BH4 synthesis, a cofactor of NOS, in the de novo pathway in VSMCs (Gross and Levi, 1992). Because NO production is critically regulated by the availability of BH4, as well as by the amount of iNOS protein (Gunnett et al., 2005), the reduction of BH4 availability by inhibiting GTPCH plus S1P (10 μM) B and C, cells were stimulated with IL-1β (3 ng/ml) for 24 h in the absence or presence of various concentrations of S1P. Top panels, representative blotting image. Bottom panel, summary of densitometric analysis expressed as a percentage, taking samples treated with IL-1β alone as 100%. Bars, mean ± S.E.M. (n = 3).
expression could be one reason for the potent inhibition of NO production by S1P. However, this does not seem to be the main reason in the present study because S1P almost completely inhibited NOS activity, even in the presence of excess amount of substrate and all cofactors including BH4. Although at present we have no clear explanation for this discrepancy between NO production and iNOS expression, slight inhibition of nNOS expression by S1P may also account for potent inhibition of NO production. A limitation of the current study is that we did not measure the effect of S1P and IL-1β on each NOS isozyme activity. Indeed, an effect on protein expression does not necessarily reflect a change in enzyme activity. Thus, future studies with isozyme-specific NOS inhibitors will be required to confirm the contribution of each NOS isozyme to NO inhibition by S1P.

A previous study from our laboratory has shown that rat VSMCs express high mRNA levels of S1P2 and S1P3 receptors (Nodai et al., 2007). S1P2 and S1P3 receptors have common signaling characteristics and couple to Gq, Gi, and G12/13 proteins (Pyne and Pyne, 2000; Kluk and Hla, 2002). We showed that PTX almost completely abolished the inhibitory effects of S1P on iNOS mRNA expression, suggesting that these effects are mediated via S1P receptors coupled to the PTX-sensitive Gi protein. In contrast, PTX only partially attenuated the inhibitory effects of S1P on iNOS induction at higher concentrations, suggesting the involvement of PTX-insensitive Gi proteins, possibly Gq and/or G12/13 proteins, in the S1P-induced inhibition of iNOS expression. From these results, S1P also seems to cause a post-transcriptional regulation of iNOS protein expression via PTX-insensitive G proteins.

The present study further demonstrates that S1P caused a PTX-insensitive inhibition of IL-1β-induced iNOS expression under Ca2+-depleted conditions, suggesting the involvement of a Ca2+-independent pathway via a PTX-insensitive G protein-coupled receptor. Rho kinase, which is activated by the PTX-insensitive G12/13 protein, is one likely step in the main S1P signaling cascade in VSMCs (Takuwa, 2002). We showed that Y 27632, a Rho kinase inhibitor, blocked the S1P-induced inhibition of iNOS in Ca2+-depleted conditions but had no effect in the presence of Ca2+. In fact, Rho kinase was reported to negatively regulate iNOS induction by IL-1β (Wei...
iNOS includes a G12/13 protein-Rho kinase step. Moreover, a Ca²⁺/Rho kinase-dependent pathway involved in the S1P-induced inhibition of iNOS expression was analyzed by Western blot. A, effect of Ca²⁺ depletion on IL-1β-induced iNOS expression. Cells were either untreated or pretreated with EGTA (1 mM) and BAPTA-AM (10 μM) for 1 h to deplete them of Ca²⁺. Cells were then treated with IL-1β (3 ng/ml) for 24 h. Top panel, representative blotting image. Bottom panel, summary of densitometric analysis expressed as a percentage, taking the samples treated with IL-1β alone as 100%. Bars, mean ± S.E.M. (n = 4). **, p < 0.01 versus IL-1β.

Thus, our results suggest that the Ca²⁺-independent pathway involved in the S1P-induced inhibition of iNOS includes a G12/13 protein-Rho kinase step. Moreover, because Rho kinase was reported to decrease nNOS expression in VSMCs (Seasholtz and Brown, 2004), it is possible that modulation of Rho kinase by S1P was responsible for the slight inhibition of nNOS expression. Several studies have reported that ERK and p38 MAPK are implicated in the IL-1β-induced regulation of iNOS expression in rat VSMCs (Jiang et al., 2001; Hirafuji et al., 2002; Jiang et al., 2004; Ginnan et al., 2006). In agreement with a previous report (Jiang et al., 2001), IL-1β caused a biphasic ERK activation, characterized by a transient phase peaking at 20 to 30 min, and by a long-lasting phase peaking within 3 h that persisted at a high level for up to 24 h. The persistent activation of ERK is considered to be essential for iNOS induction by IL-1β, although the signaling cascade leading to the ERK activation is poorly understood (Jiang et al., 2001). An ERK-dependent regulation of iNOS expression by IL-1β may be negatively regulated by p38 MAPK (Guikema et al., 2005); however, this was not clear in the present study. Similar to IL-1β, S1P strongly activates ERK and p38 MAPK in rat VSMCs (Usui et al., 2004; Nodai et al., 2007). Activated p38 MAPK plays a major role in the angiotensin II-induced inhi-

**Fig. 5.** Effect of Ca²⁺ depletion on S1P-induced iNOS inhibition in VSMCs. iNOS protein expression was analyzed by Western blot. A, effect of Ca²⁺ depletion on IL-1β-induced iNOS expression. Cells were either untreated or pretreated with EGTA (1 mM) and BAPTA-AM (10 μM) for 1 h to deplete them of Ca²⁺. Cells were then treated with IL-1β (3 ng/ml) for 24 h. Top panel, representative blotting image. Bottom panel, summary of densitometric analysis expressed as a percentage, taking the samples treated with IL-1β alone as 100%. Bars, mean ± S.E.M. (n = 4). **, p < 0.01 versus IL-1β.

**Fig. 6.** Effect of Y 27632 on S1P-induced the iNOS inhibition in VSMCs. iNOS protein expression was analyzed by Western blot. Cells were pretreated with Y 27632 (10 μM) in the absence (A) or presence (B) of EGTA (1 mM) and BAPTA-AM (10 μM) for 1 h to deplete them of Ca²⁺. Cells were then treated with IL-1β (3 ng/ml) in the absence or presence of S1P (10 μM) for 24 h. Top panel, representative blotting image. Bottom panel, summary of densitometric analysis expressed as a percentage, taking samples treated with IL-1β alone as 100%. Bars, mean ± S.E.M. (n = 4). **, p < 0.01 versus IL-1β; ††, p < 0.01 versus IL-1β plus S1P.
S1P inhibits the induction of iNOS expression by IL-1β (Jiang et al., 2004). However, this may not be the case for the inhibitory effect of S1P because inhibition of p38 MAPK with SB 203580 did not restore it.

S1P induces an increase of intracellular Ca^{2+} concentration via both PTX-sensitive and -insensitive G proteins in rat VSMCs (Tamama et al., 2001). In the present study, S1P significantly inhibited ERK activation both in the absence and the presence of IL-1β. However, the stimulatory effect of IL-1β and the inhibitory effect of S1P were both abolished in Ca^{2+}-depleted conditions. Thus, the persistent activation of ERK by IL-1β and its inhibition by S1P probably involve a Ca^{2+}-dependent pathway. This inhibition of IL-1β-induced ERK activation by S1P was also observed in PTX-pretreated cells, suggesting that PTX-insensitive G protein-coupled S1P receptors might be involved in the inhibitory effect by S1P on ERK activation.

Ca^{2+} depletion significantly enhanced the IL-1β-induced iNOS expression without an increase in ERK activity. Although the mechanism of this phenomenon is unclear, another Ca^{2+}-dependent pathway, which is augmented by S1P stimulation, may negatively modulate iNOS induction downstream of ERK.

ERK activation by S1P was significantly enhanced by the nonselective PKC inhibitor GF 109203X, but not by the PKCδ inhibitor rottlerin. This suggests that S1P-induced ERK activation is negatively regulated by a conventional PKC isozyme that requires Ca^{2+} and by the lipid mediators, diacylglycerol and phosphatidylycerine (Newton, 1997). ERK activation by IL-1β was also slightly enhanced by GF 109203X. Therefore, it is possible that S1P inhibited the IL-1β-induced persistent ERK activation through a mechanism involving a PKC isozyme. In contrast to the persistent activation, transient ERK activation by IL-1β is Ca^{2+}-independent and is mediated by PKCδ, one of the novel isozymes that require only diacylglycerol and phosphatidylycerine for their activation (Ginnan et al., 2006). Thus, the roles of PKCs in IL-1β signaling leading to ERK activation and S1P signaling leading to ERK inhibition are somewhat complicated, possibly due to multiple PKC isozymes that are exerting similar and/or opposite effects depending on the duration of the stimulus. Further studies are required to clarify their precise roles.

Xin et al. (2004) reported that S1P inhibits IL-1β-induced iNOS expression in rat renal mesangial cells. In their study, S1P transactivates the transforming growth factor-β receptor and triggers activation of the Smads, signal transducers for the members of transforming growth factor-β, signaling cascade followed by inhibition of iNOS expression. Xin et al. (2004) also suggest that S1P activates ERK, and this inhibits Smads activation, which may also positively modulate iNOS induction. In fact, Xin et al. demonstrate that inhibition of ERK activation strongly potentiates the inhibitory effect of S1P on iNOS induction. Therefore, the S1P signaling mechanism that induces iNOS inhibition in VSMCs seems to be different from that operating in renal mesangial cells.

S1P levels in human plasma and serum have been found to be approximately 0.19 and 0.48 μM, respectively, by a chemical method (Yatomi et al., 2001) and over 2-fold higher by a high-performance liquid chromatographic method (Caligan et al., 2000). S1P concentration could possibly attain even higher levels at sites of vascular injury, where platelet acti-
vation or thrombus formation occurs, although S1P tightly binds to plasma components such as lipoproteins (Murata et al., 2000; Aoki et al., 2005). As was the case in VSMCs, a low concentration of S1P simulates NO production in vascular endothelial cells by activating endothelial NO synthase via the S1P1 receptor (Igarashi et al., 2001; Kwon et al., 2001). The iNOS induction in VSMCs is considered to function primarily as a defensive and compensatory mechanism for lack of endothelial function at the site of a local vascular injury. Therefore, the S1P inhibition of NO production that we demonstrated in this study may be of important relevance in local pathophysiological conditions. Through its action on NO production by VSMCs as well as endothelial cells, S1P may play important roles in the progression of local vascular injury associated with thrombosis, atherosclerosis, and hypertension. Furthermore, in addition to their vasodilatory effects (Masumoto et al., 2001), Rho kinase inhibitors may be helpful therapeutically, in view of their antagonism of NOS inhibition by S1P.

In conclusion, our study suggests that S1P inhibits IL-1β induction of NO production and iNOS expression in rat VSMCs. ERK activation was detected as p-ERK expression by Western blot. Each p-ERK was normalized to t-ERK. A, effect of IL-1β on ERK activation. Cells were either untreated or pretreated with EGTA (1 mM) and BAPTA-AM (10 μM) for 1 h to deplete them of Ca2+. Cells were then either not treated or treated with IL-1β (3 ng/ml) or S1P (10 μM) for 24 h. B and C, effect of S1P on ERK activation. Cells were either untreated or pretreated with EGTA (1 mM) and BAPTA-AM (10 μM) for 1 h to deplete them of Ca2+. Cells were then untreated (B) or treated with IL-1β (3 ng/ml; C) in the absence or presence of S1P (10 μM) for 24 h. Top panels, representative blotting images of p-ERK (top) and t-ERK (bottom). Bottom panels, summary of densitometric analysis expressed as a percentage, taking IL-1β alone as 100%. Bars, mean ± S.E.M. (n = 4), *p < 0.05 versus untreated control.
in the inhibition of iNOS expression. Further studies are required to clarify the precise mechanisms of NO inhibition by S1P.

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