Blood Lipid Mediator Sphingosine 1-Phosphate Potently Stimulates Platelet-derived Growth Factor-A and -B Chain Expression through S1P₁-Gi-Ras-MAPK-dependent Induction of Krüppel-like Factor 5*

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Platelet-derived growth factors (PDGFs), potent mitogens and chemoattractants for mesenchymal cell types, play essential roles in development of several organs including blood vessels, kidney, and lung, and are also implicated in the pathogenesis of atherosclerosis and malignancies. Blood lipid mediator sphingosine 1-phosphate (S1P) regulates migration, proliferation, and apoptosis in a variety of cell types through multiple G protein-coupled receptors of the Edg family, and is necessary for vascular formation at the developmental stage. We found in the present study that S1P induced severalfold increases in the mRNA and protein levels of PDGF-A and -B chains in vascular smooth muscle cells and neointimal cells. S1P stimulation of PDGF mRNA and protein expression was abolished by the small interfering RNA duplexes targeting S1P/Edg1 receptor subtype. S1P stimulated the small GTPase Ras in a G₁-dependent manner, and activated ERK and p38 MAPK in G₁- and Ras-dependent manners. Pertussin toxin pretreatment, adenosine-activated A1nRas expression, the MEK inhibitor PD98059, or the p38 MAPK inhibitor SB203580 markedly suppressed PDGF mRNA and protein up-regulation, indicating the involvement of G₁-Ras-ERK/p38 MAPK in S1P stimulation of PDGF expression. S1P stimulated expression of the transcription factor KLF5 in manners dependent on G₁, Ras, and ERK/p38 MAPK. Down-regulation of KLF5 by small interfering RNA duplexes abolished S1P-induced PDGF-A and -B chain expression. On the other hand, overexpression of KLF5 stimulated basal and S1P-induced PDGF expression. Either S1P stimulation or KLF5 overexpression increased the PDGF-B promoter activity in a cis-element-dependent manner. These results reveal the S1P₁-triggered, G₁-Ras-ERK/p38 MAPK-KLF5-dependent, stimulatory regulation of PDGF gene transcription in vascular smooth muscle cells.

Sphingosine 1-phosphate (S1P)1 is a pleiotropic lysophospholipid mediator present in plasma and is released in large amounts from activated platelets (1–4). S1P exerts diverse activities on various cell types, which include stimulation of gene expression and cell proliferation, suppression of apoptosis, and regulation of cell motility and cytoskeletal reorganization. It is now accepted that many of the S1P actions are mediated through the endothelial differentiation gene (Edg) family G protein-coupled S1P receptor isoforms, which comprise S1P₁/Edg1, S1P₂/Edg5/AGR16, S1P₃/Edg3, S₁P₄/Edg6, and S₁P₅/Edg8 (1–4). A number of studies demonstrated that vascular smooth muscle cells (VSMCs) are targets of S1P; S1P is mitogenic for VSMCs (5), constricts blood vessels (6), and exerts cell type-specific distinct regulation of cell migration (7–9). Intriguingly, a recent study (10) revealed a developmental role of S1P in the vascular maturation; in mice with disruption of the S1P₁ receptor gene, perivascular accumulation of VSMCs and pericytes at the fetal developmental stage was defective. Platelet-derived growth factor (PDGF)-A and -B chains induce a diverse array of cellular responses including cell proliferation, migration, and cell survival in a variety of mesenchymal cell types (11). Especially, PDGF-B chain has been implicated in the formation of various types of vascular proliferative lesions including atherosclerosis, intimal smooth muscle cell accumulation after angioplasty and in grafted vessels, and accelerated atherosclerosis in transplanted organs (12, 13). Besides the pathogenetic roles of PDGFs and PDGF receptors, developmental roles of PDGFs during fetal morphogenesis were demonstrated; it was shown that mice with ablation of either PDGF-B chain gene or PDGF-B chain-specific receptor PDGF-β gene exhibited defects in recruitment and differentiation of vascular mural cells, i.e. vascular remodeling and maturation, after the initial endothelial tube formation (14). It was also shown that the homozygous null mutation of PDGF-A gene led to impaired development of alveolar myofibroblasts (15). Despite the potential importance of PDGFs in vascular occlusive diseases and a variety of physiological processes, only a little is known about the regulator of PDGF expression until recently. We and others have previously shown that angiotensin II, which was implicated in neointima formation after vascular injury, induced expression of PDGF-A and -B in VSMCs (16, 17). However, it is largely un-

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2 The abbreviations used are: S1P, sphingosine 1-phosphate; Edg, endothelial differentiation gene; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; KLF, Krüppel-like factor; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; RASM, rat aortic smooth muscle cells; siRNA, small interfering RNA; VSMC, vascular smooth muscle cells; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; 8-WS, rat aortic smooth muscle cells from an 8-week Wistar male rat; PTX, pertussis toxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; GST, glutathione S-transferase; RT, reverse transcription; DMEM, Dulbecco’s modified Eagle’s medium.
SIP Stimulates PDGF Expression through KLF5

EXPERIMENTAL PROCEDURES

Materials—SIP and other related lipids, were purchased, aliquoted and stored as described previously (8). Pertussis toxin (PTX) was purchased from List Biological. The following antibodies were employed in the gel electrophoresis and immunoblotting analyses: mouse monoclonal to S1P1 (clone 21, Santa Cruz), mouse monoclonal anti-Rac antibody (Upstate Biotechnology Inc.), mouse monoclonal anti-Ras (clone 18, Transduction Laboratories), mouse monoclonal anti-ERK1 and -2 antibody (clone 13-6200, Zymed Laboratories Inc.), rabbit polyclonal anti-c-Jun N-terminal kinase (JNK) (0, 17), anti-p38 MAPK (C-20) antibodies (Santa Cruz), rabbit polyclonal anti-PDGF-A (N-30) antibody (Santa Cruz), and mouse monoclonal anti-PDGF-B antibody (Mochida). Mouse monoclonal anti-HA antibody and rabbit polyclonal anti-β-galactosidase were purchased from Nacalai Tesque (Kyoto, Japan) and Organon Teknika (West Chester, PA), respectively. Rat monoclonal anti-KLF5 antibody (20) was donated by Dr. Kurabayashi (Gunma University, Maebashi, Japan).

Cell Culture—Rat neointimal VSMCs and newborn male Wistar rat aortic medial VSMCs (RASM) cells, and RASM cells from an 8-week Wistar male rat (8W-RASM cells) were established and maintained as described previously (9, 16). Two days before each experiment, cells were switched to the respective medium supplemented with 0.1% fatty acid-free bovine serum albumin (Sigma). Cells between the 5th and 15th were used in the present study.

Northern Blot Analysis and RT-PCR—Twenty micrograms of total RNA, isolated from VSMCs by the acid-guanidinium isothiocyanate/HCl method were used in the present study. The stimulation of KLF5 by SIP was relieved by Gpi, Ras, and other small G proteins, and their downstream protein kinases extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and Rac, respectively. The transcription factor Krüppel-like factor (KLF)-5/IKLF/BTEB2 belongs to the family of mammalian Krüppel-like transcription factors (18). KLF5 is implicated in phenotypic changes of activated VSMCs, which include alterations in the gene expression, in the site of vascular injury (19). A recent study (20) demonstrated that ablation of KLF5 gene suppressed vascular injury-induced vascular proliferative lesion formation. We observed that KLF5 stimulated PDGF-A and -B chain gene expression in a cis-element-dependent manner and that suppression of SIP-induced KLF5 expression by RNA interference abrogated PDGF mRNA and protein expression. Thus, the present study demonstrates the novel role of SIP-Edg receptor system in the stimulatory regulation of PDGFs, which is exerted at the transcriptional level through mechanisms involving the up-regulation of KLF5.

Determination of Activities of ERK, JNK, and p38 MAPK—The activation of p42 ERK2 was determined by detection of band shift with Western blot analysis of total cell lysate, and the results were quantified as described previously (16). The activation of c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) was determined by the solid-phase kinase assay and immunoprecipitation kinase assay using glutathione S-transferase (GST)-c-Jun and GST-ATF2, respectively, and [-γ-32P]ATP as substrates, as described previously (16, 23). The quantification of activities of MAPK activities was performed by densitometry of the corresponding bands using the Quantity one image analyzing system (PDI, Inc.) for ERK and determination of radioactivity using Fuji BAS 5000 Bio-Image Analyzer for JNK and p38 MAPK, as described previously (16, 23).

Perturbation of SIP expression was assessed as multiples over a value in unstimulated cells, which was expressed as 1.0.

Pull-down Assay of Rho, Rac, and Ras—The amounts of GTP-bound forms of RhoA and Rac were determined as described in detail previously (8, 26). The amount of a GTP-bound form of Ras was determined as described by others (27). Briefly, cell extracts prepared in the same way as for RhoA and Rac were incubated with GST-c-Raf(51–101) bound to glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 45 min. The beads were washed as described for RhoA and Rac assay, and bound to beads was analyzed by Western blotting using monoclonal anti-Ras antibody (Transduction Laboratories). A portion (1/100) of each extract was also analyzed for the amount of total Ras by Western blotting. The quantification of small G proteins was performed by densitometry as described for MAPK activities in the present study.

Plasmids, Adenoviruses and Gene Transfer—Replication-deficient adenoviruses containing each of the genes of AsnRas, AsnRac, Myc-tagged AsnRhoA, and LacZ were described and amplified as described previously (8, 16, 25). Adenoviruses containing either Myc-tagged human Ala19ThrPhe112–115–11K1 gene (16, 24) or rat KLF5 (19), driven by the CAG promoter that consists of the cytomegalovirus 1E enhancer and chicken β-actin promoter, were generated by use of homologous recombination, as described in detail previously (28). The cells were infected with adenoviruses at a multiplicity of infection of ~100, and allowed to recover in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) with 10% fetal calf serum for 3 h, and then switched to serum-free DMEM, and 24 h later the cells were used for transfection, fetal calf serum-containing DMEM was added to make a final serum concentration of 10% and incubated for 18 h. The cells were then switched to serum-free DMEM, and 24 h later the cells were harvested for experiments.
PDGF-B Promotor Assay—Newborn RASM cells were co-transfected with Sis-Luc and either of the expression vectors encoding KLF5 or an empty vector (pME18S), using LipofectAMINE as described previously (16). Cells were allowed to recover after transfection for 3 h in DMEM containing 10% fetal calf serum and then serum-deprived for 48 h. Cell lysates were prepared, and luciferase activity was measured with a Lumat LB95001 luminometer (Berthold) using the luciferase assay system (Promega), as described previously (16, 24). Luciferase activity was normalized for **/H9252-galactosidase activity measured in parallel cultures co-transfected with the **/H9252-galactosidase expression plasmid pSV/**/H9252gal and either of the expression vectors or empty vector.

PDGF Protein Analysis—Proteins in the conditioned media collected from newborn RASM cells were precipitated by trichloroacetic acid and analyzed by Western analysis using anti-PDGF-A chain and -B chain-specific antibodies, as described previously (16).

**Statistics**—The data are presented as the means ± S.E. of three or more determinations. The statistical significance of differences between the two groups was determined by Student’s t test, whereas multiple comparisons were analyzed by Scheffe’s test.

**RESULTS**

**S1P Up-regulates PDGF mRNA Expression in Newborn RASM and Neointimal VSMCs, but Not in 8W-RASM Cells**—Newborn RASM expressed a low level of PDGF-B chain mRNA at quiescent states. Stimulation with S1P (100 nM) induced a marked increase in PDGF-B mRNA level, which peaked after 4 h and gradually declined thereafter, but remained elevated for at least 8 h (Fig. 1A, upper). S1P stimulation did not alter GAPDH gene expression. The PDGF-B gene mRNA level normalized for GAPDH mRNA level showed a maximal 5-fold increase above the unstimulated level (Fig. 1A, bottom). Neointimal VSMCs derived from the neointima of injured artery (16) also responded to S1P with induction of PDGF-B mRNA, with a similar time course and to comparable extents as newborn RASM. In RASM cells from an 8-week-old rat (8W-RASM), by contrast, S1P failed to induce any increase in PDGF-B gene expression over the basal unstimulated level. PDGF-A chain mRNA level was in a detectable level in quiescent newborn RASM cells (Fig. 1A). S1P stimulated PDGF-A chain mRNA with a maximal 5-fold increase in newborn RASM cells.

As shown in Fig. 1B, the stimulatory effect of S1P on PDGF-B expression in newborn RASM increased dose-dependently with half-maximal and maximal effects obtained at 100 nM and 1 μM, respectively.

Besides S1P, dihydro-S1P and, to a lesser extent, sphingosylphosphorylcholine, both of which are weak agonists for S1P receptors (8, 29, 31), induced dose-dependent increases in PDGF-B chain mRNA level, with expected reduced potencies (Fig. 1C). Other sphingolipids, including sphingosine and sphingomyelin, which are not S1P receptor agonists, or lyso- phosphatidic acid (LPA), which is an agonist for LPA receptor
subfamily of the Edg G protein-coupled receptor family (3), did not induce PDGF-B mRNA expression under our experimental condition (up to 1 μM).

Northern analyses of total cellular RNA revealed that newborn and 8W-RASM as well as neointimal VSMCs expressed comparable levels of S1P2 and S1P3 mRNAs (Fig. 1D). However, the expression levels of S1P1 mRNA were quite different among the three cell types; it was abundant in newborn RASM and neointimal VSMCs, whereas it was barely detectable, if any, in 8W-RASM. The results in neointimal cells are consistent with a previous report (5). The mRNAs of S1P4 or S1P5 were not detectable in either cell type.

Newborn RASM cells were stimulated with S1P or left unstimulated for 4 h, and then actinomycin D, a transcription inhibitor, was added (0 time points) (Fig. 1E). The PDGF-B mRNA level was then monitored over the next 4 h. The PDGF-B mRNA levels in both cell groups decayed with time at comparable rates, with the half-life being 83 and 76 min in the absence and presence of S1P, respectively. The results suggest that S1P up-regulates PDGF-B mRNA level mainly by stimulating transcription, rather than by inhibiting degradation.

S1P1 Mediates S1P Stimulation of PDGF mRNA Expression

8W-RASM cells barely express S1P1 mRNA, different from newborn RASM and neointimal VSMCs (Fig. 1D). One likely reason why 8W-RASM cells fail to respond to S1P with stimulation of PDGF-B chain mRNA might be simply a lack or a very low expression of S1P1 receptor. To examine this possibility, we established clones of 8W-RASM that overexpresses either S1P1 (8W-RASM-S1P1) or S1P2 (8W-RASM-S1P2). As shown in Fig. 2A, the overexpression of S1P1 and S1P2 hardly affected mRNA expression of other S1P receptor isoforms. In 8W-RASM-S1P1 cells, we found that S1P did induce an 8-fold increase in the expression level of PDGF-B mRNA, with a peak response obtained at 4 h (Fig. 2B). The other two clones that overexpressed S1P1 gave similar results. In contrast, 8W-RASM-S1P2 cells and vector control 8W-RASM cells showed
only a marginal response, if any, like naive 8W-RASM cells. These results suggest that S1P₁ may be the principal receptor isoform that mediates S1P up-regulation of PDGF-B chain gene expression, but are inconsistent with the notion that 8W-RASM cells lack the intracellular machinery for induction of the growth factor.

To confirm that S1P₁ mediates S1P stimulation of PDGF expression, we down-regulated the expression of S1P₁ mRNA by RNA interference. Treatment of the newborn RASM cells with the siRNA targeting S1P₁, effectively reduced S1P₁ mRNA level (Fig. 2C, upper). We also observed that the siRNA targeting S1P₁, specifically reduced HA-tagged S1P₁, protein expression level in the VSMCs transfected with HA-tagged S1P₁, but not β-galactosidase (LacZ) expression in the cells transfected with LacZ (Fig. 2C, lower). The siRNA targeting S1P₁, but not scrambled RNA duplex, markedly inhibited S1P-induced expression of PDGF-A and -B chains, compared with scrambled RNA duplex. These observations indicate that S1P₁ mediates S1P up-regulation of PDGF-A and -B chains.

The G_{i}-Ras/Rac Pathway and Pertussis Toxin (PTX)-insensitive G-Rho Pathway Are Involved in S1P Stimulation of PDGF-A and -B in Newborn RASM—In both newborn RASM (Fig. 2E) and 8W-RASM-S1P₁ cells (Fig 2F), PTX pretreatment (100 ng/ml for 24 h) inhibited S1P stimulation of PDGF-B chain mRNA expression by −70%. In neointimal cells as well, S1P-induced PDGF-B expression was sensitive to PTX (data not shown). We also observed that S1P-induced PDGF-A mRNA expression was sensitive to PTX. These observations indicate that the G₁ signaling pathway plays a major role in mediating S1P up-regulation of PDGF-B chain mRNA. It is of note, however, that the rest (−30%) of the S1P action was mediated via a PTX-insensitive signaling pathway (see below).

We determined how S1P regulates the activities of Ras, Rac, and Rho small GTPases downstream of the S1P receptor in newborn RASM. Shown in Fig. 3A are the results of the determination of cellular amounts of GTP-bound, active forms of these small GTPases. S1P rapidly stimulated GTP loading of Ras and Rho, and Rac, although to a lesser extent, with peak stimulations of ◆◆ ◆- and 2-fold, respectively. S1P did not affect the activity of Cdc42. PTX pretreatment reduced S1P stimulation of Ras by ◆ ◆0% (Fig. 3B), which indicated that Gᵢ plays an important role in mediating S1P stimulation of Ras. Unlike Ras, S1P activation of Rho was insensitive to PTX pretreatment (Fig. 3B).

We examined roles for these small GTPases in S1P stimulation of PDGF-B chain mRNA expression, by using adenovirus-mediated expression of the dominant negative mutants of these G proteins (8, 16, 25). The expression of Asn^{17}Ras and Asn^{17}Rac, but not Asn^{19}RhoA or LacZ, only slightly (5 and 3%, respectively) reduced the numbers of viable cells 2 days after transfection, as counted by trypan blue exclusion. The expression of Asn^{17}Ras inhibited S1P stimulation of PDGF-B chain mRNA expression by −90% compared with the LacZ control.

**Fig. 3.** S1P stimulation of PDGF-B chain mRNA expression is dependent on Ras, Rac, and Rho. A, S1P stimulates Ras, Rac, and Rho. The newborn RASM cells were stimulated with 100 nM S1P for indicated time periods, and the cellular amounts of GTP-bound Ras, Rac, and RhoA (GTP-Ras, Rac, and -Rho, respectively) were determined by pull-down assays (8, 9). A portion (1/100) of the cell lysates was also subjected to Western blot analysis for evaluating the amounts of total Ras, Rac, and RhoA in each sample. B, S1P-induced activation of Ras, but not Rho, are PTX-sensitive. Newborn RASM cells were pretreated with 100 ng/ml PTX or not pretreated for 24 h and stimulated with 100 nM S1P for 1 min. C, S1P stimulation of PDGF-B mRNA expression is dependent on Ras, Rac, and Rho. Newborn RASM cells were infected with adenoviruses carrying each gene of Asn^{17}Ras, Asn^{17}Rac, and Asn^{19}RhoA or a control adenovirus encoding LacZ (9, 16). 48 h later, the cells were stimulated with 100 nM S1P for 4 h. In each panel, experiments were repeated with similar results at least three times.
The expression of Asn 17Rac also markedly suppressed PDGF-B chain mRNA expression, despite only marginal stimulation of Rac by S1P. The expression of Asn19Rho inhibited S1P stimulation of PDGF-B chain mRNA expression by \(-40\%\) (Fig. 3C).

**ERK, p38 MAPK, and Rho Kinase Mediate S1P Stimulation of PDGF-A and -B Chain Expression**—We next explored whether MAPKs and Rho kinase were involved at the sites downstream of Ras, Rac, and Rho in S1P stimulation of PDGF-B gene expression. In newborn RASM cells S1P rapidly activated ERK, with peak activation at 5 min, as evaluated by band shift of p42 ERK2 (Fig. 4A). S1P also induced activation of c-Jun N-terminal kinase (JNK) and p38 MAPK with peak activation at 10 and 5 min, respectively, as evaluated by the solid phase and the immunoprecipitation kinase assays, respectively (Fig. 4, B and C). PTX pretreatment inhibited S1P activation of these MAPKs by \(-70\%\) (Fig. 4, D-F). Adenovirus-mediated expression of Asn17Ras strongly inhibited S1P activation of ERK and p38 MAPK (Fig. 4, D and F), compared with the expression of LacZ, whereas Asn14Ras rather potentiated JNK activation (Fig. 4E). The expression of Asn17Rac, by contrast, strongly attenuated activation of JNK and also ERK (Fig. 4, D and E), whereas it was without effect on p38 MAPK activation (Fig. 4F). The expression of Asn19Rho modestly inhibited S1P activation of ERK (Fig. 4D), but not JNK or p38 MAPK (Fig. 4, E and F).

The MEK inhibitor PD98059 (30 \(\mu\)M) and the p38 kinase inhibitor SB203580 (10 \(\mu\)M) each inhibited S1P stimulation of PDGF-B expression by 90 and 60\%, respectively (Fig. 5A). The combination of the two inhibitors nearly totally abrogated S1P stimulation (Fig. 5A). Adenovirus-mediated expression of the dominant negative mutant of JNK (16, 24), which completely abolished S1P-induced activation of endogenous JNK (data not shown), did not at all inhibit S1P induction of PDGF-B expression (Fig. 5B). The Rho kinase inhibitor Y27632 (10 \(\mu\)M) reduced S1P stimulation of PDGF-B mRNA expression (Fig. 5C) by 30\%, suggesting that Rho kinase mediated the stimulatory effect of Rho (Fig. 3C).

S1P also stimulated PDGF-A chain mRNA in Ras- and ERK/p38 MAPK-dependent manners (Fig. 6, A and B). Consistent with the stimulatory actions of S1P on PDGF-A and -B chain mRNA expression, S1P stimulated secretion of PDGF-A and -B chain proteins (Fig. 7A). PTX greatly reduced secretion into the media of both A and B chains of PDGF (Fig. 7A), and the expression of Asn17Ras abolished secretion of PDGF proteins (Fig. 7B). PD98059 (30 \(\mu\)M) and SB203580 (10 \(\mu\)M) each inhibited S1P stimulation of PDGF-A and -B proteins substantially (Fig. 7A). These observations indicate that S1P stimulates secretion of PDGF proteins in a manner dependent on the \(G_i\)-Ras-ERK/p38 MAPK pathways.

**S1P Stimulates Expression of the Transcription Factor KLF5 in Manners Dependent upon \(G_i\), Ras, and ERK/p38 MAPK**—KLF5 is a transcription factor implicated in the transcriptional activation of the non-muscle myosin heavy chain-B (NMHC-B).
and PDGF-A chain genes in activated VSMCs (19, 20). We first examined whether S1P was capable of inducing the expression of KLF5. In newborn RASM cells S1P (100 nM) markedly stimulated the expression of KLF5 mRNA (Fig. 8A). KLF5 mRNA level peaked at 4 h with a maximal 4-fold elevation, and then gradually declined to the basal level by 10 h. KLF5 protein level reached a peak at 6 h and was sustained till 10 h in S1P-stimulated cells, whereas α-tubulin protein level did not change (Fig. 8B). S1P up-regulation of KLF5 mRNA was strongly inhibited by PTX pretreatment (Fig. 8C), by the ex-
expression of Asn17Ras (Fig. 8D), and by either PD98059 or SB203580 (Fig. 8E). KLF5 protein level was also inhibited by PTX, Asn17Ras expression, PD98059, or SB203580 (Fig. 8E and G). These results suggest that S1P stimulates KLF5 expression through mechanisms involving Gi, Ras, and ERK/p38 MAPK.

Inhibition of KLF5 Expression by siRNA Suppresses S1P-induced PDGF Expression—The transfection of newborn RASM cells with siRNA targeting KLF5 gene, but not scrambled RNA duplex, successfully abolished S1P stimulation of KLF5 protein expression (Fig. 9A). α-Tubulin protein expression was not affected by transfection with siRNA duplexes. The siRNA targeting KLF5 gene, but not scrambled RNA, strongly suppressed S1P-induced expression of both A and B chain mRNAs (Fig. 9B) and proteins (Fig. 9C) of PDGF, indicating the requirement of KLF5 for S1P stimulation of PDGF expression.

We overexpressed KLF5 protein in newborn RASM cells by using an adenovirus carrying KLF5 gene and examined

Fig. 7. S1P stimulates secretion of PDGF-A and -B chain proteins. A, inhibition of PDGF secretion by PTX (100 ng/ml), the MEK inhibitor PD98059 (30 μM) and p38 MAPK inhibitor SB203580 (10 μM). Newborn RASM cells were stimulated with 100 nM S1P for 36 h or left unstimulated in the presence or absence of indicated inhibitors, and analyzed as in Fig. 2D. B, S1P stimulation of PDGF secretion is dependent on Ras. The cells were infected with adenoviruses as in Fig. 3C, and stimulated with S1P as in A. The representative results from three independent experiments are shown. In each panel, experiments were repeated with similar results at least twice.

Fig. 8. S1P stimulates KLF5 expression in newborn RASM in Gi-, Ras-, and ERK/p38 MAPK-dependent manners. A, S1P (100 nM) stimulates KLF5 mRNA expression in a time-dependent manner. The cell lysate was analyzed by Western blotting using anti-KLF5 antibody. α-Tubulin protein is also shown to confirm equal loading of proteins (bottom). C, PTX inhibits S1P stimulation KLF5 mRNA expression. The cells were pretreated with PTX as in Fig. 2E, and then stimulated with 100 nM S1P for 4 h. D, expression of Asn17Ras inhibits S1P stimulation of KLF5 mRNA expression. The cells were infected with adenoviruses as in Fig. 3C, and then stimulated with S1P as in C. E, the MEK inhibitor PD98059 and the p38 MAPK inhibitor SB203580 inhibit S1P stimulation of KLF5 mRNA expression. The cells were pretreated with PD98059 and SB203580 as in Fig. 5A, and then stimulated with 100 nM S1P as in C. F, PTX, the MEK inhibitor PD98059, and the p38 MAPK inhibitor SB203580 inhibit S1P stimulation of KLF5 protein expression. The cells were pretreated with PTX, PD98059, and SB203580 as in C and E, and then stimulated with 100 nM S1P for 6 h. G, expression of Asn17Ras inhibits S1P stimulation of KLF5 protein expression. The cells were infected with adenoviruses as in Fig. 3C, and then stimulated with S1P as in F. In each panel, experiments were repeated with similar results at least twice.
PDGF-A and -B chain mRNA and protein expression. The cells infected with the adenovirus carrying KLF5 gene, but not control adenovirus carrying LacZ gene, displayed overexpression of KLF5 protein (data not shown). The cells overexpressing KLF5 showed augmentation of both basal and S1P-stimulated levels of PDGF-A and -B chain mRNAs (Fig. 10 A), compared with LacZ-transfected cells. Consistent with these, KLF5 overexpression stimulated both basal and S1P-stimulated secretion of PDGF-A and -B chain proteins (data not shown).

*S1P Stimulates PDGF-B Chain Promoter Activity in a Manner Dependent on KLF5-binding cis-Element*—We evaluated the effect of S1P on PDGF-B chain gene transcription by measuring PDGF-B chain promoter activity by adopting the PDGF-B promoter-luciferase reporter vector (Sis-Luc) (16). S1P induced modest but consistent increases in the PDGF-B promoter activity in newborn RASM cells (∼1.7- and 2.3-fold increases at 0.1 and 1 μM, respectively) (Fig. 10B). The co-transfection of KLF5 expression vector together with Sis-Luc dose-dependently stimulated the promoter activity in a dose-dependent manner with a maximal 4-fold stimulation (Fig. 10C).

The inspection of the 5′-upstream region of the transcription start site of PDGF-B chain gene revealed the existence of the two sequences at −257 to −252 (site 1) and −56 to −51 (site 2) (Fig. 10D), which are compatible to the reported core nucleotide sequences of the KLF5-binding cis-regulatory element found in the 5′-upstream region of the NMHC-B gene (19). We generated the three mutants of Sis-Luc that were mutated at either site 1 or site 2, or both, so as to lose KLF5 binding activity (19) (Sis-Luc-mut1, Sis-Luc-mut2, and Sis-Luc-mut1+2, respectively) (Fig. 10D). The co-transfection of either wild type Sis-Luc or Sis-Luc-mut2 together with KLF5 expression vector conferred 3-fold stimulation over the value obtained with empty vector (Fig. 10E). However, the co-transfection of Sis-Luc-mut1 or Sis-Luc-mut1+2 together with KLF5 expression vector did not show any stimulation, suggesting that the site 1 acts as the cis-regulatory element to mediate KLF5-induced enhancement of PDGF-B gene transcription. Consistent with this notion, S1P stimulation of luciferase activity in the cells transfected with Sis-Luc-mut1+2 was greatly reduced compared with wild type Sis-Luc (Fig. 10F). All these data suggest that KLF5 link the Ras-ERK/p38 MAPK pathways to stimulation of PDGF-B chain gene transcription.

**FIG. 9. The suppression of KLF5 expression by siRNA abolishes S1P-induced PDGF mRNA and protein expression in newborn RASM cells. A**, RNA interference inhibits S1P stimulation of KLF5 protein expression. The cells were transfected with either siRNA targeting KLF5 or scrambled RNA duplex, and 48 h later stimulated with S1P (100 nM) for 6 h. **B**, the siRNA targeting KLF5 abolishes S1P stimulation of PDGF-A and -B chain mRNA expression. The cells were transfected with the duplex RNA as in A, and 48 h later stimulated with S1P (100 nM) for 4 h. **C**, the siRNA targeting KLF5 abolishes S1P stimulation of PDGF-A and -B chain protein expression. The cells were transfected with the duplex RNAs as in A and B, and stimulated with S1P and analyzed for the contents of PDGF proteins as in Fig. 2D. In each panel, experiments were repeated with similar results at least twice.

**DISCUSSION**

The present study demonstrated for the first time that S1P stimulates PDGF-A and -B chain mRNA and protein expression via S1P1 receptor in VSMCs. PDGFs are potent mitogens and chemoattractants for VSMCs. It was shown in the occlusive vascular lesions that the intimal VSMCs, endothelial cells, and infiltrating cells of the non-vascular origin express PDGFs, which was suggested to stimulate migration and proliferation of VSMCs in the lesions (11–13). Available evidence (1–4) suggests that S1P is not only a blood-borne lipid mediator present largely in forms bound to albumin and lipoproteins, but also a locally produced autocrine/paracrine mediator. In addition to plasma lipoproteins, LDL that is accumulated in the atherosclerotic lesions may release S1P and contribute to local S1P action (32). LDL was also shown to possess the capability to stimulate S1P production in VSMCs by activating sphingosine kinase activity (34). In injured vessels, activated platelets may also release a large amount of S1P (35). Thus, in the atherosclerotic lesions, local concentration of S1P might be presumably elevated. It was observed that expression of S1P1 among S1P receptors is elevated in the neointima (5). Therefore, neointimal VSMCs are an interesting candidate cell type to produce PDGFs in response to S1P stimulation. A recent study (36) demonstrated that PDGF-B chain acted on VSMCs to stimulate S1P release, resulting in stimulation of cell migration via activation of S1P receptors on the VSMCs in an autocrine/paracrine fashion. This report and our present results suggest the existence of mutually stimulatory interactions between PDGF and S1P. It is unknown presently whether the interactions have any physiological and pathological role in the vasculature. We previously showed that angiotensin II, which stimulates PDGF-B chain gene expression in vitro in neoangiogenesis.
mal and newborn VSMCs (16), is involved in PDGF-β receptor stimulation and neointima formation in vivo in balloon-injured vessels (37). It is worth trying to see the effect of a S1P receptor blocker on PDGF receptor activation in vascular injury models in the future studies.

In addition to the pathogenic roles, PDGFs are also shown to possess the developmental role in the vasculature. Investigations of mice with ablation of PDGF-B chain and PDGF-β receptor genes suggested that the PDGF-B chain is involved in the recruitment of perivascular smooth muscle cells and pericytes after the initial formation of endothelial tubes, by stimulating proliferation and migration of PDGF-β receptor-expressing progenitor cells (14, 38). The knockout mice displayed thinning of the arterial media and aneurysma-like distension of small vessels, as a result of a defect in the vascular remodeling. A more recent study (39) using the in vitro culture system of ES cells demonstrated that PDGF-B chain also promotes differentiation of the progenitor cells into the lineage of VSMC. The recent study of targeted deletion of S1P1 gene revealed that VSMCs in the S1P1 knockout mice failed to migrate to uniformly surround primitive capillaries (10). Thus, there is some similarity in the phenotypes between the S1P1- and PDGF-B chain- or PDGF-β-disrupted mice. One intriguing possibility is that S1P acts on vascular cells via S1P1 to stimulate PDGF-B chain gene expression, thus contributing to vascular remodeling in the developmental stage.

Both newborn RASM and neointimal cells express S1P1 and respond to S1P with stimulation of PDGF-B chain mRNA expression (Fig. 1A). In contrast, naive 8W-RASM cells that do not express S1P1 fail to stimulate PDGF-B expression in response to S1P, and the overexpression of S1P1, but not S1P2, confers responsiveness to S1P (Fig. 2 B and F). Finally, down-regulation of S1P1 expression by RNA interference inhibited S1P stimulation of PDGF-A and -B chain expression (Figs. 3 C, 6 A, and 7 B). Consistent with the role of S1P1, S1P stimulation of PDGF expression was strongly dependent on Gαi (Figs. 2 E and F) and 7A).

The present study fully characterized the signaling cascade of S1P stimulation of PDGF gene expression (Fig. 11). S1P stimulated the small G protein Ras largely in a PTX-sensitive manner (Fig. 3, A and B). S1P activated ERK and p38 MAPK, and JNK, in Ras-dependent and -independent manners, respectively (Fig. 4). S1P-induced PDGF expression was strongly dependent on Ras (Figs. 3C, 6A, and 7B), and on ERK and p38...
MAPK, but not JNK (Figs. 5 (A and B), 6B, and 7A). Thus, the major signal to stimulate PDGF-A and -B chain gene expression involves S1P1-Gi-Ras-ERK/p38 MAPK. In addition to this pathway, we also observed the involvement of Rac and Rho in PDGF gene expression (Fig. 3C) downstream of PTX-sensitive G, and PTX-insensitive G protein, respectively (Fig. 3B). The latter was most likely G12/13, because we previously demonstrated in the heterologous expression experiments of each Edg receptor isoform that S1P1 and S1P3, but not S1P2, mediate Rho stimulation via the G12/13 class (8, 25). Our results suggested that Rac contributed to stimulation of PDGF gene expression at least in part via ERK activation (Fig. 4D), whereas Rho seemed to act through Rho kinase (Fig. 5C).

The Krüppel-like transcription factors play diverse roles in regulation of growth, development, differentiation, and apoptosis (18). KLF5, which was originally identified as a transcription factor to stimulate NMHC-B gene expression (19), has recently been shown to mediate expression of de-differentiated phenotypes, the so-called synthetic phenotype, in VSMCs (20). KLF5 was recently shown to positively regulate cell proliferation and to induce a transformed phenotype in fibroblasts (40). KLF5 is abundantly expressed in developing blood vessels, but not in adult vessels, and in activated VSMCs in vascular lesions (20). The study of KLF5 gene-disrupted mice (20) demonstrated that KLF5 is essential for normal development of the vascular media and neointima formation after vascular injury. The same group showed that angiotensin II-induced up-regulation of PDGF-A chain expression in vascular endothelial cells. It remains to be determined how Egr-1 and KLF5 precisely interact in stimulation of PDGF-B chain gene transcription.

In conclusion, these data demonstrate that S1P can stimulate expression of PDGF-A and -B chains in VSMCs through mechanisms involving KLF5 up-regulation, which is mediated by G12/13

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