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bFGF Induces $S1P_1$ Receptor Expression and Functionality in Human Pulmonary Artery Smooth Muscle Cells

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ABSTRACT

Sphingosine-1-phosphate (S1P), acting through five closely related G-protein coupled receptors termed $S1P_{1-5}$, has recently emerged as a possible regulator of smooth muscle cell (SMC) physiology with the potential to induce contraction, proliferation and stress fiber formation. In the present study, real-time quantitative PCR was used to determine the expression patterns of S1P receptor subtypes in human primary pulmonary artery smooth muscle cells (PASMC). We report here that subconfluent PASMC express predominantly $S1P_2$ and $S1P_3$ receptors and we show that $S1P_1$ receptor mRNA levels are significantly up-regulated following basic fibroblast growth factor (bFGF) treatment. As a consequence, increased responsiveness, as measured by impedance and ERK1/2 phosphorylation, was observed upon stimulation with a specific $S1P_1$ receptor agonist SEW2871. We therefore demonstrate, for the first time, that a growth factor that was previously shown to be involved in physiological and pathological changes of SMC function induced $S1P_1$ receptor expression and we propose that $S1P_1$ receptor up-regulation could contribute to vascular remodeling. J. Cell. Biochem. 105: 1139–1145, 2008.

KEY WORDS: SMOOTH MUSCLE CELLS; SPHINGOSINE-1-PHOSPHATE RECEPTORS; BASIC FIBROBLAST GROWTH FACTOR; PLATELET-DERIVED GROWTH FACTOR

Proliferation and migration of vascular smooth muscle cells (SMC) are important physiological processes during embryonic development and later during vascular repair. Growth factors and cytokines are known to influence SMC physiology [Panettieri, 1998] and recently, sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite, has emerged as a potential regulator of SMC function. A number of biological effects in SMC have been observed in response to S1P, including S1P-stimulated DNA synthesis and proliferation in rat SMC [Kluk and Hla, 2001; Tamama et al., 2001; Lockman et al., 2004], modulation of platelet-derived growth factor (PDGF)-induced migration of rat and mouse SMC [Tamama et al., 2001; Inoue et al., 2007] and contraction of human vascular and airway SMC [Ohmori et al., 2003; Rosenfeldt et al., 2003]. Recent studies also described S1P-induced epidermal growth factor (EGF) receptor expression and tyrosine phosphorylation, resulting in increased rat aortic SMC proliferation [Tanimoto et al., 2004; Hsieh et al., 2008]. In guinea pig airway SMC, the $S1P_1$ receptor and platelet-derived growth factor β (PDGFβ) receptor were shown to form complexes and to promote mitogenic signaling [Waters et al., 2003]. These findings support the idea of a functional cross-talk between S1P and growth factors in SMC physiology.

S1P binds and activates members of the S1P receptor family, a group of five G-protein coupled receptors including the $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ receptors. It is assumed that the biological function of S1P in different cell types depends on the relative S1P receptor expression and the abundance of corresponding intracellular coupling partners. In the case of human SMC, little is known about S1P receptor subtype expression and even less is known about factors that regulate their expression. Non-quantitative RT-PCR or Northern blot experiments were previously used to determine the expression levels of S1P receptor subtypes in SMC derived from blood vessels of various origins. From the five well-known receptors, only $S1P_2$ and $S1P_3$ receptor transcripts were detected in all studies, whereas the presence of the remaining receptors was variable [Kluk and Hla, 2001; Tamama et al., 2001; Ryu et al., 2002; Ohmori et al., 2003]. Regulation of S1P receptor expression was demonstrated in activated T lymphocytes [Jin et al., 2003], during myogenic differentiation [Meacci et al., 2003], in cancer cells [Dolezalova et al., 2003], during endothelial cell differentiation [Hla and Maciag, 1990], and in endothelial cells in response to vascular endothelial growth factor (VEGF) or hydrogen peroxide stimulation [Igarashi et al., 2003, 2007]. Only one study, in rat SMC, has shown changes in S1P receptor expression in response to different cell densities [Kluk and Hla, 2001].

In the present study quantitative real-time PCR was employed to determine the expression patterns of S1P receptor subtypes in primary human SMC derived from various blood vessels with

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particular emphasis on the analysis of S1P receptor expression and functional consequences in primary human pulmonary artery smooth muscle cells (PASMC). We report here that subconfluent PASMC express predominantly $S1P_2$ and $S1P_3$ receptors and we show that the $S1P_1$ receptor mRNA levels are significantly lower when compared to $S1P_2$ and $S1P_3$ receptor transcript levels. However, $S1P_1$ receptor expression was significantly up-regulated following basic fibroblast growth factor (bFGF) treatment and consequently increased receptor signaling was observed upon stimulation with a specific $S1P_1$ receptor agonist. We demonstrate, for the first time, that bFGF, which was previously shown to be involved in physiological and pathological changes of SMC function, induces $S1P_1$ receptor expression and we propose that $S1P_1$ receptor up-regulation could contribute to changes in SMC function.

MATERIALS AND METHODS

CELLS AND REAGENTS

Human AoSMC, CASMC, and PASMC (Cambrex, Walkersville, MD) were cultured in a humidified atmosphere (5% CO_2/95% air) at 37°C in SmGM-2 BulletKit medium (Cambrex). Cell passages 7–9 were used for all experiments.

SU5402, AG1478, and SEW2871 were purchased from Calbiochem (San Diego, CA). S1P was purchased from Biomol (Plymouth Meeting, PA), $VEGF_{165}$ was purchased from Biosource International (Nivelles, Belgium) and PDGF-BB was purchased from Oncogene (San Diego, CA).

QUANTIFICATION OF S1P RECEPTORS mRNA BY REAL-TIME PCR (Q-PCR)

Total RNA was isolated, reverse-transcribed and quantified in real-time using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described [Birker-Robaczewska et al., 2003]. The specific primer sets and probes for the $S1P_2$ receptor were as follows: forward primer: 5′-TGGCCGCCTCCGATCT-3′, TaqMan probe: 5′-CTGGCAGGCGTGGC-3′ and reverse primer: 5′-GAGAGCAAGGTATTGGCTACGAA-3′. Otherwise, TaqMan Assays-on-Demand (Applied Biosystems) were used: $S1P_1$ AoD Hs00173499_m1, $S1P_3$ AoD Hs00245464_s1; $S1P_4$ AoD Hs00269446_s1; $S1P_5$ AoD Hs00258220_s1. The amplification of 18s rRNA or/and GAPDH, using the pre-developed TaqMan Assay Reagent (Applied Biosystems) was examined as an internal control. Expression values were calculated as cycle threshold (C_T) − 18s or C_T − GAPDH and expressed in relative expression values (1 = no detectable expression, defined as $C_T \geq 38$ cycles). All amplifications were performed in duplicate.

ERK1/2 DETECTION

PASMC were seeded in 12-well plates at a density 10^5 cells/well and grown for 48 h in PASMC-conditioned medium supplemented or not with 8 ng/ml bFGF. Next, the cells were starved for 4 h in basal SmBM medium (Cambrex) containing 0.1% fatty acid-free BSA. Cells were subsequently stimulated for 10 min with tested compounds and lysed immediately thereafter. Cell lysates were analyzed for phosphorylated pERK1/2 and total ERK1/2 by ELISA according to manufacturer's protocol (Biosource International, Nivelles, Belgium). pERK1/2 levels were normalized against total ERK1/2.

IMPEDANCE MEASUREMENTS

The assay was performed using the 6×96 well real-time cell electronic sensing (RT-CES™) device (ACEA Inc., San Diego, CA), placed in a 37°C/5% CO_2 incubator. PASMC were seeded in 96-well electrode plates at 2×10^4 cells/well density and grown for 48 h in PASMC-conditioned medium supplemented or not with 8 ng/ml bFGF. Next, the cells were starved for 4 h in basal SmBM medium (Cambrex) containing 0.1% fatty acid-free BSA. Cells were subsequently stimulated with tested compounds and the induced impedance changes were monitored for several hours. Analysis of the impedance traces was performed using ACEA software.

STATISTICAL ANALYSIS

Results were analyzed with a one-sided Wilcoxon rank sum test as implemented in R 2.7.0 (www.r-project.org). In all cases, statistical significance was defined as $P < 0.05$.

RESULTS

EXPRESSION OF S1P RECEPTOR SUBTYPES

To compare the S1P receptor subtype expression patterns in different human vascular smooth muscle cell types, the expression levels of the different S1P receptors were analyzed in cultured primary aortic smooth muscle cells (AoSMC), coronary artery smooth muscle cells (CASMC) and pulmonary artery smooth muscle cells (PASMC). Total RNA was isolated from low passage number cells that were cultured until they reached subconfluency and the amount of mRNA corresponding to each S1P receptor subtype ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$) was measured by quantitative real-time PCR (Q-PCR) using receptor-specific TaqMan probes. The observed expression pattern in all examined SMC types was very similar irrespective of SMC tissue origin. $S1P_2$ and $S1P_3$ receptor transcripts were generally highly abundant, whereas lower and more variable $S1P_1$ receptor expression levels were found. The expression levels of the $S1P_5$ receptor were consistently low, while those of the $S1P_4$ receptor were below the detection limits in all three SMC types (Fig. 1).

INDUCIBLE $S1P_1$ RECEPTOR TRANSCRIPTION IN PASMC

Increased $S1P_1$ receptor expression was previously reported in rat pup primary vascular SMC cultures at high cell density and minor changes in $S1P_3$ and $S1P_2$ receptor mRNA levels were observed at different stages of cell monolayer confluency [Kluk and Hla, 2001]. To investigate if similar regulation could be observed in adult human SMC, PASMC were chosen as example. The cells were seeded at different densities and total RNA was isolated after 24 h. Q-PCR analysis revealed a pronounced decrease in $S1P_1$ receptor mRNA with increasing cell density, moderate decrease in $S1P_2$ receptor

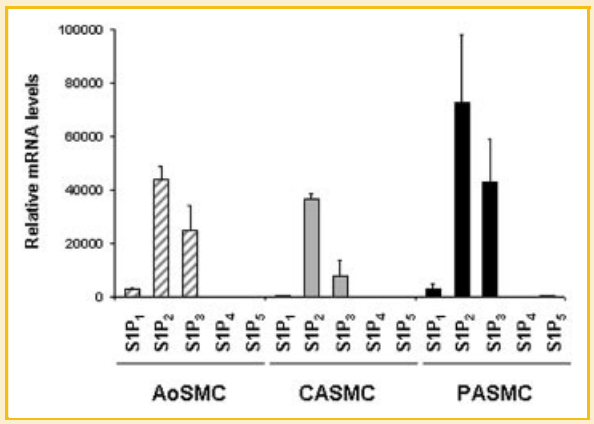

Fig. 1. S1P receptor subtype expression in primary human SMC. Total mRNA was prepared from subconfluent AoSMC (hatched bar), CASMC (striped bar) and PASMC (filled bar) and expression levels of $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ receptor mRNA were determined by quantitative PCR. Data are means ± SD of three independent experiments. AoSMC, aortic smooth muscle cells; CASMC, coronary artery smooth muscle cells; PASMC, pulmonary artery smooth muscle cells; SMC, smooth muscle cells.

mRNA, whereas no significant changes were observed in $S1P_3$ receptor expression levels (Fig. 2A). Furthermore, $S1P_1$ receptor mRNA levels decreased further after a prolonged incubation time of 72 h, whereas $S1P_2$ receptor expression remained constant and $S1P_3$ receptor expression appeared to increase (Fig. 2A). This suggested that cell density, possibly through cell contact inhibition, influenced $S1P_1$ receptor expression. However, a gradual depletion of unknown stimulating factor(s) during cell culture could also contribute to the observed decrease in $S1P_1$ receptor transcription.

To further elucidate this hypothesis, $S1P_1$ receptor expression levels were measured in PASMC that were seeded in regular medium or in medium that was pre-conditioned with PASMC for 72 h and subsequently supplemented with different components such as 5% fresh fetal bovine serum (FBS) with or without additional growth factor cocktail (GF), containing epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin. Q-PCR analysis was then performed 24 h after seeding.

These experiments demonstrated a fourfold increase of $S1P_1$ receptor transcripts in growth factor (GF)-supplemented/pre-conditioned medium (Fig. 2B), whereas a more moderate response was observed in fresh FBS-supplemented/pre-conditioned medium. In addition, a cumulative effect of combined GF supplement/fresh FBS was seen. Summarizing, these results suggest that gradual growth factor depletion, rather than contact inhibition, is responsible for $S1P_1$ receptor mRNA down-regulation.

bFGF-INDUCED $S1P_1$ RECEPTOR EXPRESSION IN PASMC

In order to identify the specific growth factor(s) that were able to induce $S1P_1$ receptor expression, PASMC were seeded in pre-conditioned medium and treated for 24 h with increasing concentrations of EGF, bFGF or insulin. $S1P_1$ receptor mRNA levels increased fivefold in presence of increasing concentrations of bFGF and the observed accumulation of $S1P_1$ receptor mRNA was completely suppressed in presence of the FGF receptor kinase inhibitor SU5402, but not the EGF receptor kinase inhibitor AG1478 (Fig. 3). $S1P_2$ receptor and $S1P_3$ receptor mRNA levels remained largely unaffected under all experimental conditions (data not shown). A moderate but dose-independent increase in $S1P_1$ receptor expression was also observed in presence of EGF (Fig. 3). This effect was not inhibited by AG1478 or SU5402, indicating a non-specific effect of the peptide. Insulin was not able to induce any change in $S1P_1$ receptor expression (data not shown). Furthermore, $VEGF_{165}$ and PDGF-BB, two other growth factors involved in smooth muscle cell physiology, were not able to influence $S1P_1$ receptor mRNA levels (data not shown).

bFGF-INDUCED INCREASED $S1P_1$ RECEPTOR FUNCTIONALITY IN PASMC

To address the functional consequence of the bFGF-induced $S1P_1$ receptor up-regulation, the specific $S1P_1$ receptor agonist SEW2871 was used and PASMC stimulation was measured using impedance technology. This technology allows real-time monitoring of cell shape changes upon receptor activation [Xi et al., 2008]. To this end, PASMC were seeded in pre-conditioned medium in presence or absence of bFGF and SEW2871-induced dose-dependent responses were measured. In bFGF pre-treated PASMC the response was of much higher magnitude and more sustained than in untreated PASMC, which is consistent with the higher levels of $S1P_1$ receptor upon bFGF pre-incubation (Fig. 4A). Since signal integration from $S1P_1$ and PDGFβ receptors was demonstrated in SMC [Waters et al., 2003], the response of PASMC to PDGF-BB stimulation was also examined under the same experimental settings. Interestingly, the magnitude of response to PDGF-BB was also much stronger in bFGF pre-treated PASMC compared to the untreated cells (Fig. 4B) and an additive response was observed when both SEW2871 and PDGF-BB were used for stimulation (Fig. 4C). The response to bFGF was very weak and unchanged by PASMC pre-treatment (Fig. 4D).

Since $S1P_1$ receptors couple exclusively to $G\alpha_i$ proteins, the functionality of the induced $S1P_1$ receptors was further addressed in an ERK1/2 phosphorylation assay. As in the impedance assay, ERK1/2 activation was measured upon stimulation with the specific $S1P_1$ receptor agonist SEW2871 in bFGF-treated or untreated PASMC. In PASMC that were cultured in pre-conditioned medium lacking additional additives, stimulation with SEW2871 was unable to induce ERK1/2 phosphorylation, which is consistent with the very low levels of $S1P_1$ receptor in these cells (Fig. 5A). However, when PASMC were cultured for 48 h in pre-conditioned medium in the presence of bFGF, the specific $S1P_1$ receptor agonist induced a twofold increase in ERK1/2 phosphorylation compared to the response in untreated cells (Fig. 5B). PDGF signaling was also strongly enhanced by bFGF treatment. PDGF-BB induced a fivefold increase in ERK1/2 phosphorylation in bFGF pre-treated PASMC compared to a twofold increase in untreated cells (Fig. 5A,B). ERK1/2 activation after co-stimulation with SEW2871 and PDGF-BB did not exceed the magnitude of activation induced by PDGF-BB alone (Fig. 5A,B).

Altogether, these results demonstrate that bFGF specifically induced expression of $S1P_1$ receptors in PASMC, which is associated with increased $S1P_1$ receptor signaling. In addition, enhanced

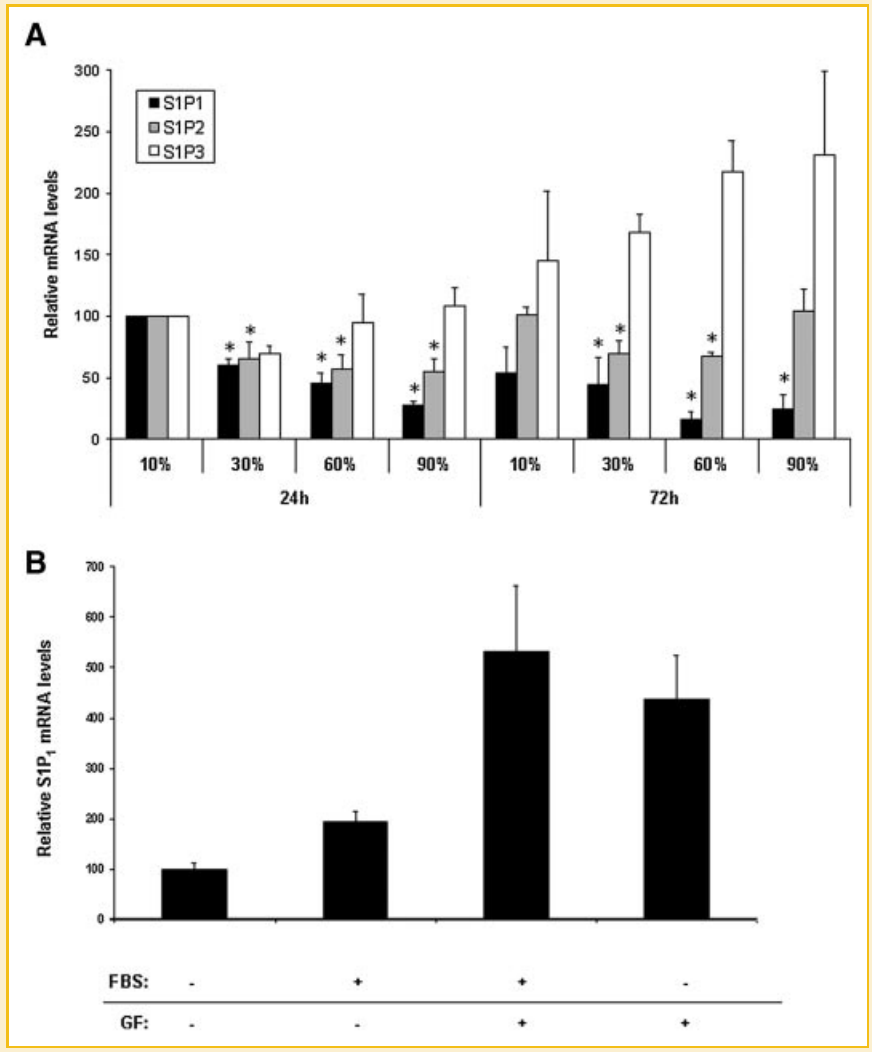

Fig. 2. $S1P_1$ receptor mRNA levels are regulated in PASMC. A: Different PASMC densities (10%, 30%, 60%, 90%) were seeded in full medium and cultured for either 24 h (left panel) or 72 h (right panel) without medium change and $S1P_1$ mRNA levels were measured by Q-PCR. Data are means ± SEM of three independent experiments performed in duplicate. $^*P < 0.05$ compared to cells seeded at 10% density at 24 h. B: 10^5 PASMC were seeded in a 12-well plate in PASMC-conditioned medium in presence or absence of FBS and/or growth factors (EGF, bFGF, and insulin) supplements. After 24 h cells were lysed and $S1P_1$ receptor mRNA levels quantified by Q-PCR. Results are expressed as relative increase compared to cells seeded in conditioned medium (FBS 1x, GF 1x) (set as 100%), and represent the mean ± SD of one representative experiment done in duplicate. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; FBS, fetal bovine serum; GF, growth factors.

signaling of PDGF receptor(s) paralleled increased $S1P_1$ receptor signaling.

DISCUSSION

In the present study, we analyzed and compared the expression profiles of known S1P receptor subtypes in primary human smooth muscle cells isolated from various blood vessels. A similar expression pattern was detected in all three investigated SMC types, with a predominant expression of the $S1P_2$ and $S1P_3$ receptor, which is in agreement with previous reports [Kluk and Hla, 2001; Tamama et al., 2001; Coussin et al., 2002; Ryu et al., 2002]. This highly consistent expression pattern suggests that $S1P_2$ and $S1P_3$ receptors play a role in general SMC physiology. $S1P_2$ receptor activation has been implicated in functions related to SMC migration and proliferation [Tamama et al., 2001; Ryu et al., 2002; Inoue et al., 2007], whereas $S1P_3$ receptor activation has been shown to be involved in SMC contraction, at least in cerebral and basilar arteries [Salomone et al., 2003].

Regulation of S1P receptor expression was previously demonstrated in rat vascular SMC [Kluk and Hla, 2001]. There, $S1P_1$, $S1P_2$, and $S1P_3$ receptor mRNA levels increased at higher cell densities. Using quantitative RT-PCR, we now provide evidence that $S1P_1$ receptor expression is also highly regulated in human PASMC. However, in contrast to rat vascular SMC, we observed that $S1P_1$, but not $S1P_2$ or $S1P_3$, receptor transcript levels decreased with increasing cell densities or cell culture duration. It remains to be clarified if the observed differences between the different studies are due to the species differences or SMC developmental stage.

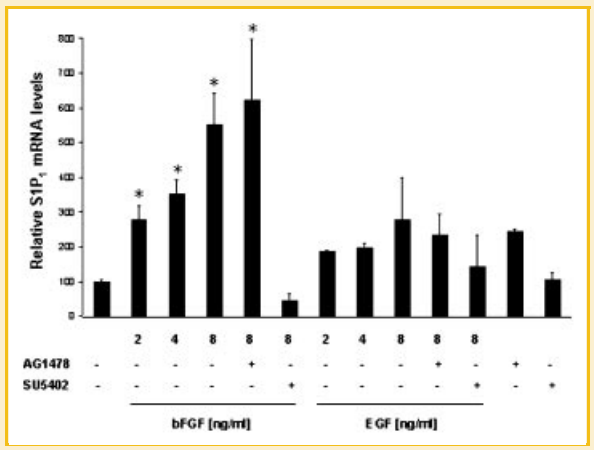

Fig. 3. bFGF-mediated $S1P_1$ receptor mRNA induction in PASMC. 10^5 PASMC were seeded in a 12-well plate in PASMC-conditioned medium supplemented with increasing concentrations of bFGF and EGF and cultured for 24 h. When indicated, cells were incubated with AG14787 (5 μM) or SU5402 (20 μM) for 45 min before addition of bFGF or EGF. Q-PCR results are expressed as relative increase compared to untreated cells (set as 100%), and represent the mean ± SD of two experiments done in duplicate. $^*P < 0.05$ compared to untreated cells.

In the present study we further demonstrate that bFGF specifically promoted a concentration-dependent up-regulation of $S1P_1$ receptors. Induction of $S1P_1$ receptor expression by bFGF was seen within the physiologically relevant concentration range (2–5 ng/ml) [Vesely et al., 2004], whereas a variety of other growth factors that were tested, including insulin, EGF, $VEGF_{165}$ and PDGF-BB, had no marked effect on $S1P_1$ receptor expression under these experimental conditions. The expression levels of other S1P receptor subtypes were not significantly changed by bFGF treatment. In a recent study by Igarashi et al. [2003] VEGF was shown to induce $S1P_1$ receptor mRNA and $S1P_1$ receptor protein levels in endothelial cells, thereby leading to enhanced S1P signaling responses. In our hands $VEGF_{165}$ had no effect on $S1P_1$ expression in PASMC, suggesting that multiple growth factors are able to regulate $S1P_1$ receptor mRNA expression in a cell type-specific manner.

Based on two previous reports, it appears possible that bFGF induced an increase in nuclear factor-κB (NF-κB) expression, leading to $S1P_1$ receptor up-regulation in PASMC. A first report showed that bFGF rapidly enhanced transcriptional activity of NF-κB in rat vascular SMC [Hoshi et al., 2000] and a second report described three potential NF-κB binding sites in the $S1P_1$ receptor promoter region [Liu and Hla, 1997]. Further experiments are required to establish the exact mechanism of bFGF-induced $S1P_1$ receptor up-regulation in human PASMC.

bFGF is produced by a number of cells types, including SMC, and it was shown to have diverse activities in SMC physiology, such as protection from apoptosis [Miyamoto et al., 1998], induction of other growth factors and growth factor receptors [Bonner et al., 1996; Belgore et al., 2003], promotion of proliferation [Padro et al., 2002] and migration [Pickering et al., 1997]. It was also reported that vascular structures resulting from the exposure to both bFGF and

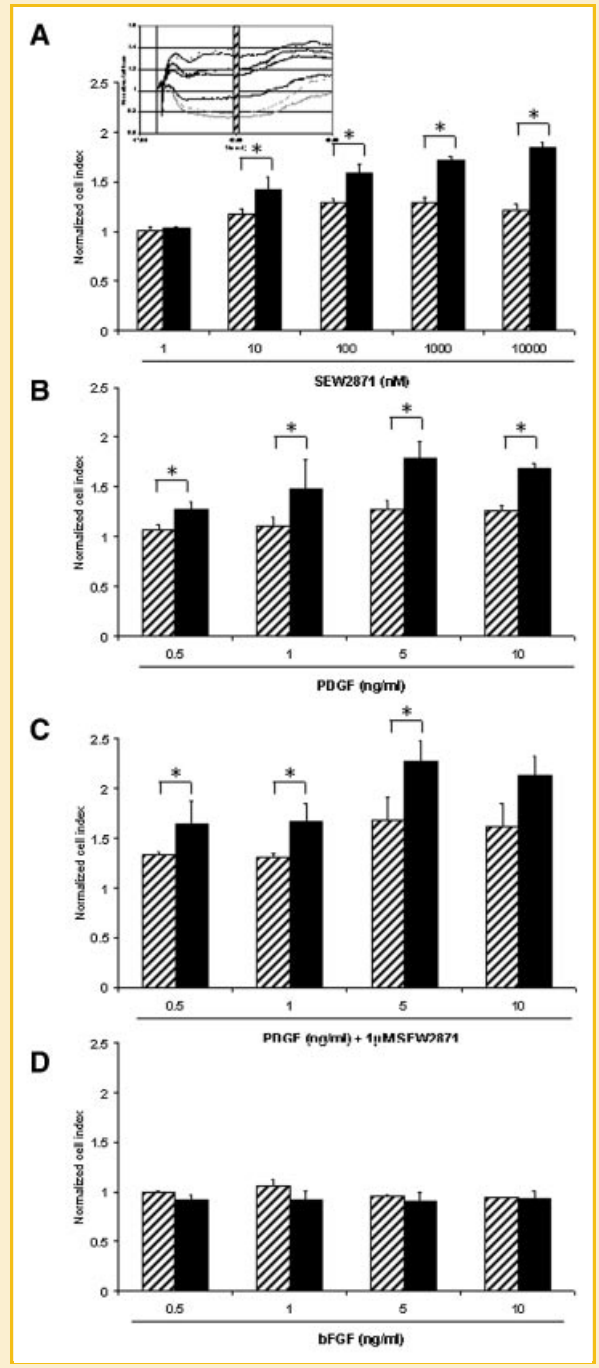

Fig. 4. Increased response of bFGF pre-treated PASMC to $S1P_1$ receptor agonist and PDGF-BB. Untreated (hatched bars) or pre-treated with 8 ng/ml bFGF for 48 h (filled bars) PASMC were serum starved for 4 h then stimulated with SEW2871 (A), PDGF-BB (B), PDGF-BB + 1 μM SEW2871 (C) or bFGF (D). Impedance changes in response to compound addition were monitored for several hours (inset A shows SEW2871 response in bFGF pre-treated PASMC). Traces were normalized to the time point of compound addition and the results expressed as fold induction of cell index compared to vehicle stimulation 1 h later (indicated by hatched bar on inset A). Data are means ± SD of three independent experiments. Impedance changes between bFGF pre-treated and untreated PASMC were statistically significant ($^*P < 0.05$). PDGF-BB, platelet-derived growth factor BB.

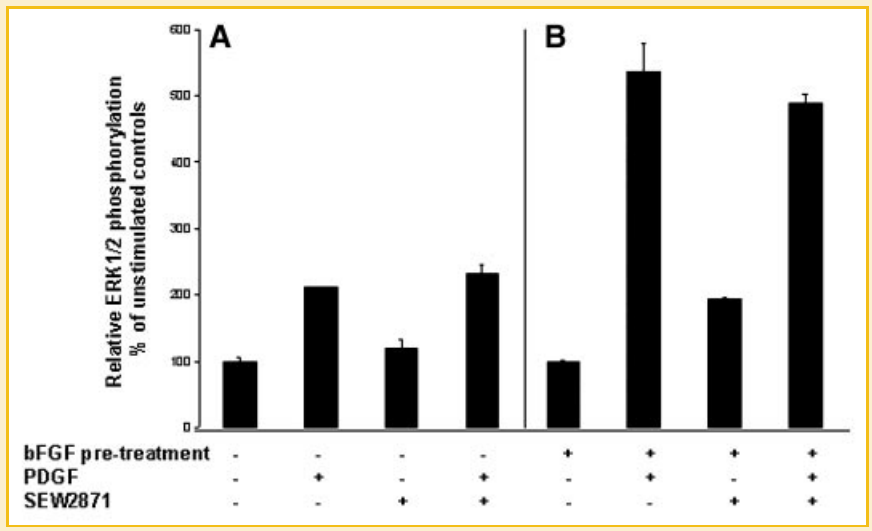

Fig. 5. Effect of bFGF pre-treatment on $S1P_1$ receptor mediated ERK1/2 phosphorylation. Untreated (A) or pre-treated with 8 ng/ml bFGF for 48 h (B) PASMC were serum starved for 4 h then stimulated for 10 min with 2 ng/ml PDGF-BB, 1 μM SEW2871 or both. The results show the mean relative ERK1/2 phosphorylation ± SD normalized against total ERK1/2 of one representative experiment done in duplicate. ERK1/2, extracellular signal-regulated kinase 1/2.

S1P had highly developed adherens junction complexes that were not seen in similar structures arising from exposure to bFGF alone [Lee et al., 1999]. Our results further support the idea of cross-talk between $S1P_1$ and bFGF receptors.

We show that bFGF induced the expression of functional $S1P_1$ receptors in PASMC, which are then able to activate ERK1/2 phosphorylation upon agonist stimulation. These results provide new insight into the mechanism of mitogenic bFGF signaling. It was previously shown [Xu et al., 2002] that sphingosine kinase and $G\alpha_i$ proteins participate in the mitogenic effects of bFGF in SMC. Here, we show that $S1P_1$ receptors are up-regulated by bFGF and we suggest the S1P acts as an additive component that could potentiate the bFGF-induced mitogenic response.

In summary, we provide evidence that $S1P_1$ receptor expression in human PASMC is highly up-regulated by bFGF, resulting in a potentiation of S1P-induced intracellular signaling. To our knowledge this is the first report showing induction of S1P receptor subtype expression by bFGF in SMC and we propose a coordinated effect of bFGF and $S1P_1$ receptor-mediated signaling in vascular remodeling. Our results provide important information on possible integration of bFGF and S1P signals in SMC biology and may therefore contribute to the better understanding of SMC disease states, where increased levels of these two agents are found.

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