Sphingosylphosphorylcholine induces differentiation of human mesenchymal stem cells into smooth-muscle-like cells through a TGF-β-dependent mechanism

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Summary

Mesenchymal stem cells (MSCs) can differentiate into diverse cell types including adipogenic, osteogenic, chondrogenic and myogenic lineages. In the present study, we demonstrated for the first time that sphingosylphosphorylcholine (SPC) induces differentiation of human adipose-tissue-derived mesenchymal stem cells (hATSCs) to smooth-muscle-like cell types. SPC increased the expression levels of several smooth-muscle-specific genes, such as those for α-smooth-muscle actin (α-SMA), h_1-calponin and SM22α, as effectively as transforming growth factor β (TGF-β1) and TGF-β3. SPC elicited delayed phosphorylation of Smad2 after 24 hours exposure, in contrast to rapid phosphorylation of Smad2 induced by TGF-β treatment for 10 minutes. Pretreatment of the cells with pertussis toxin or U0126, an MEK inhibitor, markedly attenuated the SPC-induced expression of α-SMA and delayed phosphorylation of Smad2, suggesting that the $G_{i/o}$-ERK pathway is involved in the increased expression of α-SMA through induction of delayed Smad2 activation. In addition, SPC increased secretion of TGF-β1 through an ERK-dependent pathway, and the SPC-induced expression of α-SMA and delayed phosphorylation of Smad2 were blocked by SB-431542, a TGF-β type I receptor kinase inhibitor, or anti-TGF-β1 neutralizing antibody. Silencing of Smad2 expression with small interfering RNA (siRNA) abrogated the SPC-induced expression of α-SMA. These results suggest that SPC-stimulated secretion of TGF-β1 plays a crucial role in SPC-induced smooth muscle cell (SMC) differentiation through a Smad2-dependent pathway. Both SPC and TGF-β increased the expression levels of serum-response factor (SRF) and myocardin, transcription factors involved in smooth muscle differentiation. siRNA-mediated depletion of SRF or myocardin abolished the α-SMA expression induced by SPC or TGF-β. These results suggest that SPC induces differentiation of hATSCs to smooth-muscle-like cell types through $G_{i/o}$-ERK-dependent autocrine secretion of TGF-β, which activates a Smad2-SRF/myocardin-dependent pathway.

Key words: Sphingosylphosphorylcholine, Mesenchymal stem cells, Differentiation, α-smooth-muscle actin, Smooth muscle cells, Myocardin

Introduction

Mesenchymal stem cells (MSCs) have a self-renewal capacity, long-term viability, and the potential to differentiate into diverse cell types, such as adipogenic, osteogenic, chondrogenic and myogenic lineages (Barry and Murphy, 2004; Prockop, 1997; Pittenger et al., 1999; Short et al., 2003). Local environment and resident cellular populations are likely to be crucial factors in determining their differentiation fates (Minguell et al., 2001; Caplan, 1991). In vitro, bone marrow-derived MSCs have been shown to differentiate to smooth muscle cells (SMCs) in response to transforming growth factor β (TGF-β) (Kinner et al., 2002; Wang et al., 2004), mechanical stress (Kobayashi et al., 2004) and direct contact with vascular endothelial cells (Ball et al., 2004). In vivo, injected bone marrow-derived MSCs are able to differentiate into SMCs and contribute to the remodeling of vasculature (Davani et al., 2003; Yoon et al., 2005; Gojo et al., 2003). The phenotype of SMCs is characterized by expression of contractile proteins, such as α-smooth-muscle actin (α-SMA), h_1-calponin and SM22α (Owens et al., 2004), and the expression of SMCs has been implicated in vascular development as well as in a variety of cardiovascular diseases, including hypertension and atherosclerosis (Liu et al., 2004; Owens et al., 2004). However, the molecular identity of extracellular factors and the underlying molecular mechanisms involved in the differentiation of MSCs into SMCs are largely unknown.

Lysophospholipids, such as sphingosine-1-phosphate (S1P), lysophosphatidylcholine (LPC) and sphingosylphosphorylcholine (SPC), play a role in cardiovascular physiology and pathophysiology (Dart and Chin-Dusting, 1999; Anliker and

Chun, 2004; Meyer zu Heringdorf et al., 2002; Spiegel and Milstien, 2003). They are also implicated in cardiovascular development (Osborne and Stainier, 2003). In particular, S1P has been reported to play a crucial role in vascular maturation and expression of smooth-muscle-specific genes in SMCs (Liu et al., 2000; Lockman et al., 2004). However, involvement of lysophospholipids in smooth muscle differentiation has not yet been clearly elucidated. SPC is a sphingolipid generated by *N*-deacylation of sphingomyelin, one of the most abundant lipids in the cell membrane. SPC has been shown to be involved in a variety of physiological responses, such as proliferation, growth inhibition, migration and wound healing (Xu, 2002; Meyer zu Heringdorf et al., 2002). In vasculature, SPC has been reported to induce migration and morphogenesis of endothelial cells and contraction of SMCs (Boguslawski et al., 2000; Kim et al., 2005), suggesting that SPC may play a role in the maintenance of vascular structure, biochemical stability and functionality of vessels. The SPC-induced cellular responses are sensitive to pertussis toxin (PTX), indicating an involvement of $G_{i/o}$-coupled receptors (Xu, 2002; Chin and Chueh, 1998; Seufferlein and Rozengurt, 1995). In a previous study, we demonstrated that SPC stereo-specifically stimulates or inhibits the proliferation of human adipose-tissue-derived mesenchymal stem cells (hATSCs), depending on the concentration of SPC used (Jeon et al., 2005; Jeon et al., 2006). However, it has not yet been ascertained whether lysophospholipids, including SPC, can modulate vascular maturation by affecting the differentiation of hATSCs to SMCs.

Serum-response factor (SRF) is a widely expressed transcription factor involved in orchestrating disparate programs of gene expression linked to muscle differentiation and cellular growth. SRF has been shown to be required for expression of most SMC-specific markers by binding to highly conserved CArG cis elements ($CC(A/T)_6GG$) found in the promoters of most SMC-specific genes. Myocardin, a member of the SAP domain family of nuclear proteins (Aravind and Koonin, 2000), functions as an SRF cofactor and plays a central role in the SRF-dependent expression of SMC-specific genes (Du et al., 2003; Li et al., 2003; Yoshida et al., 2003; Wang et al., 2003). TGF-β signaling is thought to direct, in part, the differentiation of mesenchymal lineage cell types to SMCs (Bertolino et al., 2005). TGF-β binds to two different types of serine/threonine kinase receptors, termed type I and type II (Massague, 2000; Moustakas et al., 2001). The type I receptor is activated by the type II receptor upon ligand binding and transduces signals into the cytoplasm through phosphorylation of Smad2 and Smad3. Accumulating evidence has demonstrated that the SRF/myocardin-dependent pathway plays a crucial role in the TGF-β-induced SMC differentiation through a Smad-dependent pathway (Hu et al., 2003; Qiu et al., 2005; Sinha et al., 2004). However, whether the SRF/myocardin-dependent pathway is involved in the lysophospholipid-induced SMC differentiation is largely unknown.

In the present study, we demonstrated for the first time that SPC induces the differentiation of hATSCs toward smooth-muscle-like cell types by stimulating autocrine release of TGF-β1 through $G_{i/o}$-dependent activation of extracellular signal-regulated kinase (ERK). We also demonstrated that Smad2 and SRF/myocardin play a pivotal role in the SPC-induced SMC differentiation.

Results

Sphingosylphosphorylcholine increases the expression levels of α-SMA in human adipose tissue-derived mesenchymal stem cells

To explore whether lysophospholipids can induce differentiation of hATSCs to SMCs, hATSCs were treated with 0.5, 2.0 and 5.0 μM D-*erythro*-SPC, and then the expression levels of α-SMA were determined by western blotting with an anti-α-SMA antibody. As shown in Fig. 1A, the expression level of α-SMA in hATSCs was drastically increased in response to treatment with 0.5 μM SPC, and maximally increased by treatment with 2 μM SPC without significant

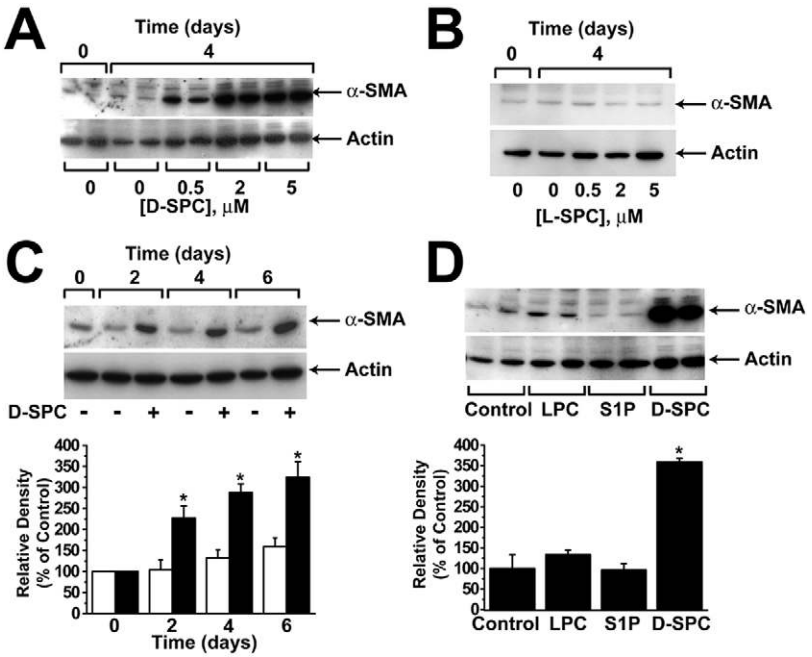

Fig. 1. Dose- and time-dependent effects of SPC on the expression levels of α-SMA. Serum-starved hATSCs were treated with the indicated concentrations of D-*erythro*-SPC (A) or L-*threo*-SPC (B) for 4 days. (C) Serum-starved hATSCs were treated with vehicle or 2 μM D-*erythro*-SPC for the indicated time periods. (D) Serum-starved hATSCs were treated with 0.1% DMSO (control), 5 μM LPC, 1 μM S1P or 5 μM D-*erythro*-SPC for 4 days. The expression levels of α-SMA and actin were determined by western blotting with anti-α-SMA and anti-actin antibodies, respectively (upper panels). The densities of α-SMA and actin were quantified from three independent experiments, and the expression levels of α-SMA were normalized to total actin levels in the samples (lower panels). The data are presented as a percentage of control. *Significantly different from control value ($P<0.05$).

change in the expression level of total actin. It has been reported that D-*erythro*-SPC, but not L-*threo*-SPC, exists naturally and stereo-specifically activates its cognate receptors (Meyer zu Heringdorf et al., 1998). Furthermore, we have reported that D-*erythro*-SPC stereo-specifically stimulates proliferation of hATSCs (Jeon et al., 2006). Therefore, in order to explore whether the SPC-induced differentiation of hATSCs is stereo-specific, we examined the effects of L-*threo*-SPC on the expression of α-SMA. As shown in Fig. 1B, the expression levels of α-SMA were not increased by treatment with concentrations of L-*threo*-SPC up to 5 μM, suggesting that the SPC-induced differentiation of hATSCs to SMCs is stereo-specific, and that only D-*erythro* stereoisomer of SPC is active. Exposure of hATSCs to 2 μM SPC for 2 days increased the expression levels of α-SMA, and the amounts of α-SMA were increased time-dependently up to 6 days (Fig. 1C). In contrast to the SPC-induced increase in the expression levels of α-SMA, the exposure of hATSCs to structurally related lysophospholipids, such as LPC and S1P, had no impact on the expression levels of α-SMA (Fig. 1D). These results suggest that D-*erythro*-SPC specifically induces differentiation of hATSCs to SMCs by activating plasmalemmal receptors.

Stimulatory effects of SPC and TGF-β on the expression levels of α-SMA

It has been reported that the TGF-β family of cytokines increases the expression levels of α-SMA in a variety of cell types, including bone marrow-derived MSCs (Kinner et al., 2002; Wang et al., 2004). To explore whether TGF-β isoforms increase the expression of α-SMA in hATSCs, we examined the effects of TGF-β1 and -β3 on the expression levels of α-SMA. Both TGF-β1 and -β3 increased the protein levels of α-SMA with a maximal increase at 2 ng/ml concentration. The stimulatory effect of TGF-β3 on α-SMA expression was slightly more potent than that of TGF-β1 (Fig. 2A,B).

To compare the effects of SPC and TGF-β3 on the expression levels of α-SMA, we treated hATSCs with 2 μM D-*erythro*-SPC and/or 2 ng/ml TGF-β3 for 4 days. The stimulatory effect of D-*erythro*-SPC on the expression of α-SMA was as potent as that of TGF-β3 (Fig. 2C,D). Furthermore, treatment of hATSCs with D-*erythro*-SPC in the presence of TGF-β3 further increased the expression levels of α-SMA and h_1-calponin, another smooth-muscle-specific marker. These results indicate that both SPC and TGF-β3 stimulate differentiation of hATSCs to SMCs.

SPC increases the mRNA levels of α-SMA and other smooth-muscle-specific genes

To explore whether SPC stimulates the expression of the SMC-specific markers, we examined mRNA levels of several smooth muscle-specific markers by real time or semi-quantitative RT-PCR analyses. hATSCs were treated with 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for the indicated time, and the level of α-SMA mRNA was determined by real-time RT-PCR analysis.

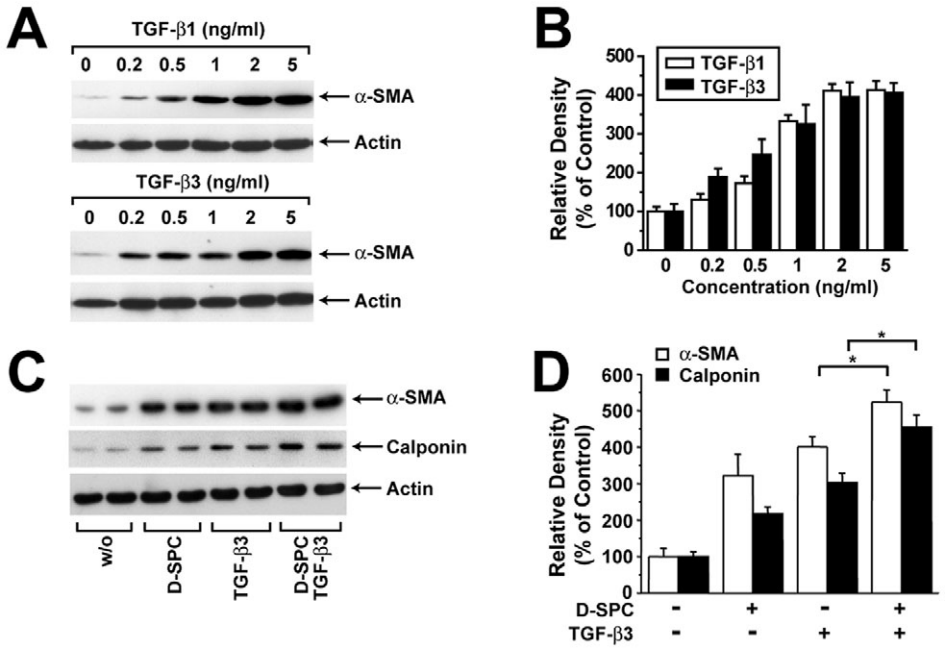

Fig. 2. Effects of SPC and TGF-β on the differentiation of hATSCs to SMCs. (A) Serum-starved hATSCs were treated with indicated concentrations of TGF-β1 or TGF-β3 for 4 days, and the expression levels of α-SMA and actin were determined by western blotting with anti-α-SMA and anti-actin antibodies, respectively. Representative data from three independent experiments are shown. (B) The quantities of α-SMA and actin were determined from the three experiments in A, and the expression levels of α-SMA were normalized to total actin levels in the samples. (C) Serum-starved hATSCs were challenged with 2 μM D-*erythro*-SPC and/or 2 ng/ml TGF-β3 for 4 days. The expression levels of α-SMA, h_1-calponin, and actin were probed with anti-α-SMA, anti-h_1-calponin and anti-actin antibodies, respectively. Representative data from three independent experiments are shown. (D) The quantities of α-SMA, h_1-calponin and actin were determined from the three experiments in C, and the expression levels of α-SMA and h_1-calponin were normalized to total actin levels in the samples. The data are presented as a percentage of control (mock-treated cells). These data were analysed using Student's *t*-test and considered to be significantly different from the control when $P<0.05$ (*).

As shown in Fig. 3A, exposure of the cells to D-*erythro*-SPC or TGF-β3 significantly increased the level of α-SMA mRNA at 12 hours, and it was maximally increased at 24 hours (Fig. 3A). Semi-quantitative RT-PCR analysis demonstrated that treatment with SPC, as well as TGF-β3, increased the mRNA levels of other SMC-specific markers, such as h_1-calponin and SM22α, in hATSCs (Fig. 3B). The SPC- or TGF-β3-induced increase of h1-calponin and SM22α mRNA levels was confirmed by real-time RT-PCR analysis (Fig. 3C). These results indicate that SPC induces differentiation of hATSCs to smooth-muscle-like cell types by stimulating the expression of these genes at the level of transcription.

SPC induces α-SMA-mediated formation of stress fiber in hATSCs

Since SPC and TGF-β3 increased the expression levels of α-SMA and other smooth-muscle-specific markers, we next examined actin organization by double staining for α-SMA and actin filaments. As shown in Fig. 4, treatment of hATSCs with 2 ng/ml TGF-β3 or 2 μM D-*erythro*-SPC for 4 days increased the expression levels of α-SMA, which is localized in actin filaments. The increased assembly of actin filaments and thick fiber in response to SPC or TGF-β3 was highly correlated with the increased expression of α-SMA. These results suggest that not only TGF-β but also SPC induces the formation of intracellular actin filaments in human mesenchymal cell types.

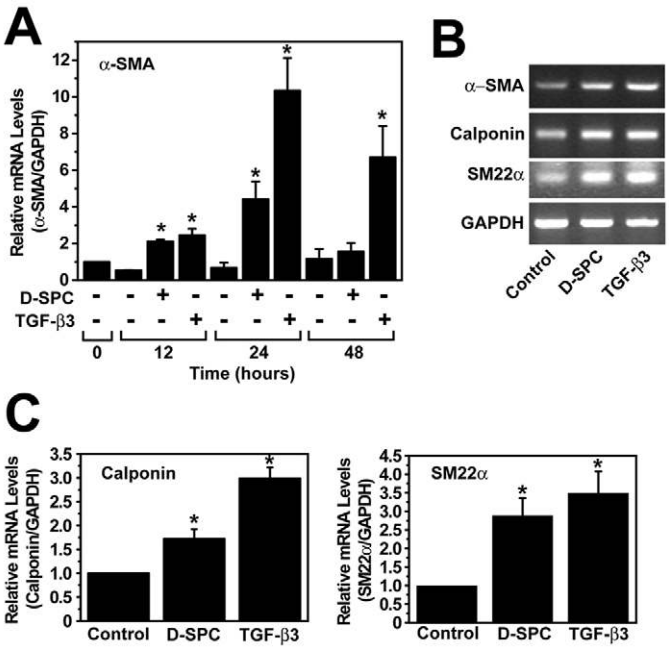

Fig. 3. Effects of SPC and TGF-β3 on the transcriptional levels of smooth-muscle-specific markers. (A) Serum-starved hATSCs were treated with vehicles, 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for the indicated time, and α-SMA mRNA was quantified by real time RT-PCR analysis. Data represent average values ± s.e.m. of triplicate determinants. *Significantly different from control value (P<0.05). (B) Serum-starved hATSCs were exposed to 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 24 hours, and the levels of α-SMA, h_1-calponin, SM22α and GAPDH mRNA were determined by semi-quantitative RT-PCR using gene-specific primers. Representative data from three independent experiments are shown. (C) hATSCs were treated with vehicles, 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 24 hours, and the expression levels of h_1-calponin and SM22α were quantified by real time RT-PCR. Data represent average values ± s.e.m. of triplicate determinants. *Significantly different from control value (P<0.05).

SPC induces differentiation of hATSCs to SMCs through a $G_{i/o}$-dependent pathway

SPC treatment increased the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in hATSCs, and pretreatment of the cells with PTX completely blocked the SPC-induced elevation of $[Ca^{2+}]_i$ (Jeon et al., 2006). By contrast, TGF-β3 did not increase $[Ca^{2+}]_i$ in hATSCs (data not shown), suggesting that SPC specifically transduces its signal through activation of $G_{i/o}$-coupled receptors, whereas TGF-β induces α-SMA expression through oligomerization of type I and type II TGF-β receptor kinases and subsequent phosphorylation of cytoplasmic signaling molecules, Smads (Hu et al., 2003; Qiu et al., 2005; Sinha et al., 2004). To assess whether SPC and TGF-β3 can induce phosphorylation of Smads, we examined the effects of SPC and TGF-β3 on the phosphorylation of Smad2. TGF-β3 treatment rapidly induced phosphorylation of Smad2 within 10 minutes and the TGF-β3-induced Smad2 phosphorylation was sustained through day 1 (Fig. 5A,B). However, SPC treatment elicited phosphorylation of Smad2 on day 1, and then the SPC-induced phosphorylation of Smad2 was sustained by day 4. Both TGF-β3 and SPC significantly increased the phosphorylation level of Smad2 on day 4 (Fig. 5A,B), indicating late phosphorylation of Smad2.

To examine whether the SPC-induced delayed phosphorylation of Smad2 is dependent on $G_{i/o}$, we next determined the effect of PTX on the SPC-induced phosphorylation of Smad2. As shown in Fig. 5C, the delayed phosphorylation of Smad2 induced by SPC treatment for 4 days was abolished by pretreatment of hATSCs with PTX, suggesting that a $G_{i/o}$-dependent pathway is involved in the delayed activation of Smad2 by SPC treatment. However, the TGF-β3-induced phosphorylation of Smad2 on day 4 was not affected by pretreatment of the cells with PTX. In addition, the acute phosphorylation of Smad2 by TGF-β3 treatment for 10 minutes was not sensitive to PTX. These results suggest that the $G_{i/o}$-dependent pathway is specifically involved in the late activation of Smad2 in response to SPC in hATSCs.

To elucidate the involvement of the $G_{i/o}$-dependent pathway in the SPC-induced SMC differentiation, we examined the effects of PTX on the SPC-induced expression of α-SMA. The D-*erythro*-SPC-stimulated expression of α-SMA was abolished by pretreatment of the cells with PTX (Fig. 5D,E). However, the TGF-β3-induced expression of α-SMA was not affected by pretreatment of the cells with PTX. These results suggest that SPC induces differentiation of hATSCs to SMCs through $G_{i/o}$-dependent delayed activation of Smad2.

ERK is involved in the SPC-induced SMC differentiation by mediating the delayed phosphorylation of Smad2 in response to SPC

In a previous study, we demonstrated that SPC induced activation of ERK in hATSCs (Jeon et al., 2005). Therefore, we next examined the effects of SPC and TGF-β3 on the phosphorylation levels of ERK. Both SPC and TGF-β3 were

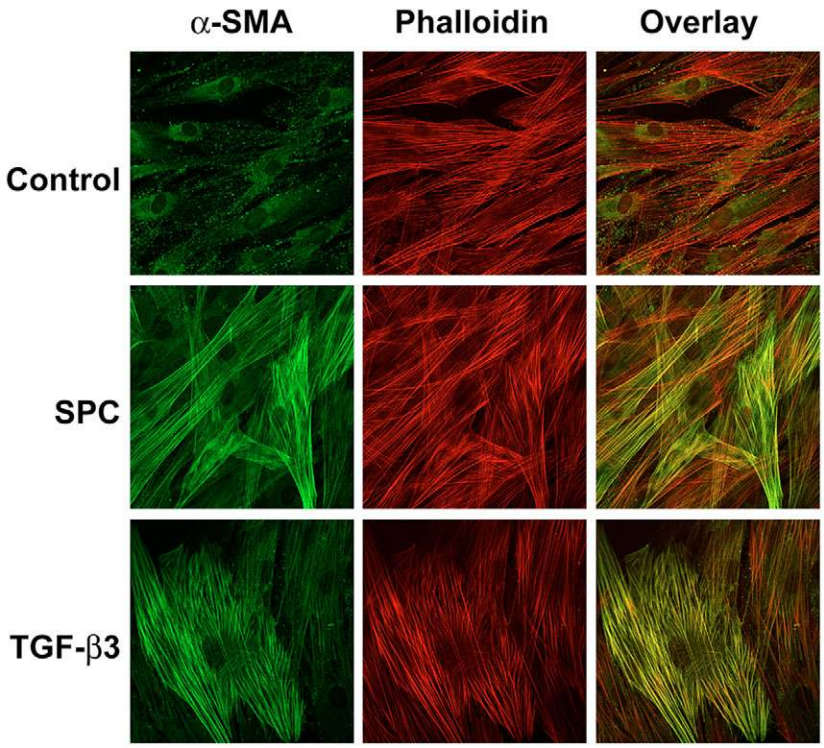

Fig. 4. Effects of SPC and TGF-β3 on the formation of actin stress fibers and the localization of α-SMA in hATSCs. Serum-starved hATSCs were treated with vehicles, 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 4 days, and immunostaining performed. α-SMA and F-actin were double-stained with anti-α-SMA and Alexa Fluor 568 phalloidin, and analysed using a Leica TCS-SP2 laser scanning confocal microscope (Leica Microsystems, Germany) with a magnification of 400×. The overlaid images of the double staining are shown. Representatives of three independent experiments are shown.

able to induce phosphorylation of ERK as early as 10 minutes and then declining to the basal level by 3 hours (Fig. 6A). To explore whether the SPC-induced phosphorylation of ERK is dependent on $G_{i/o}$, we examined the effect of PTX on the phosphorylation levels of ERK in response to SPC. The phosphorylation of ERK induced by SPC was abolished by pretreatment of the cells with PTX (Fig. 6B). These results suggest that SPC induces activation of ERK by a signaling pathway involving a $G_{i/o}$-dependent pathway.

To determine the role of ERK in SPC-induced SMC differentiation, we examined the effects of U0126 on the expression level of α-SMA in response to SPC or TGF-β3 treatment. As shown in Fig. 6C, the SPC-induced expression of α-SMA was abrogated by pretreatment of hATSCs with U0126. Furthermore, the TGF-β3-stimulated expression of α-SMA was attenuated by pretreatment of the cells with U0126. These results indicate that both SPC and TGF-β3 induce the differentiation of hATSCs to SMC through an ERK-dependent pathway. Since a $G_{i/o}$-dependent pathway is involved in the delayed phosphorylation of Smad2 induced by SPC, we next examined the role of ERK in the phosphorylation levels of Smad2 in response to SPC or TGF-β3. As shown in Fig. 6C, the late phosphorylation of Smad2 by treatment of hATSCs with SPC or TGF-β3 for 4 days was completely abolished by pretreatment of the cells with U0126. However, the acute phosphorylation of Smad2 by TGF-β3 treatment for 10 minutes was not affected by U0126 pretreatment (Fig. 6D). These results suggest that the TGF-β3-induced acute phosphorylation of Smad2 does not require ERK activation, whereas the sustained phosphorylation of Smad2 in response to SPC or TGF-β3 is mediated by the ERK-dependent pathway.

SPC induces differentiation of hATSCs to SMCs by stimulating secretion of TGF-β1

Recently, a selective inhibitor of TGF-β type I receptor kinase activity, SB-431542, has been developed and shown to suppress TGF-β-mediated gene transcription (Laping et al., 2002; Inman et al., 2002). Therefore, we examined the effect of SB-431542 on the expression levels of α-SMA in hATSCs treated with SPC or TGF-β3 for 4 days. As shown in Fig. 7A, the TGF-β3-induced expression of α-SMA was completely inhibited by pretreatment of the cells with SB-431542. Moreover, the SPC-induced expression of α-SMA was blocked by pretreatment with SB-431542, suggesting that TGF-β type I receptor kinase plays a key role in the SPC-induced differentiation of hATSCs to SMCs. Since the sustained phosphorylation of Smad2 is required for the SPC-induced SMC differentiation, we next examined the involvement of a TGF-β type I receptor kinase in the SPC-induced phosphorylation of Smad2. The phosphorylation of Smad2 by SPC or TGF-β3 on day 4 was completely blocked by pretreatment of the cells with SB-431542 (Fig. 7A). These results suggest that both SPC and TGF-β3 induce differentiation of hATSCs to SMCs through TGF-β type I receptor kinase-dependent delayed activation of Smad2.

To explore whether the SPC-induced late activation of Smad2 is mediated by increased release of TGF-β isoforms, we determined the expression levels of TGF-β1, -β2 and -β3 by ELISA. As shown in Fig. 7B, hATSCs expressed TGF-β1, and SPC stimulated the levels of TGF-β1 secreted. The increased secretion of TGF-β1 as a result of SPC treatment was abrogated by pretreatment of the cells with U0126, implying that ERK is involved in the SPC-induced expression of TGF-β1. However, TGF-β2 and -β3 were barely detected by

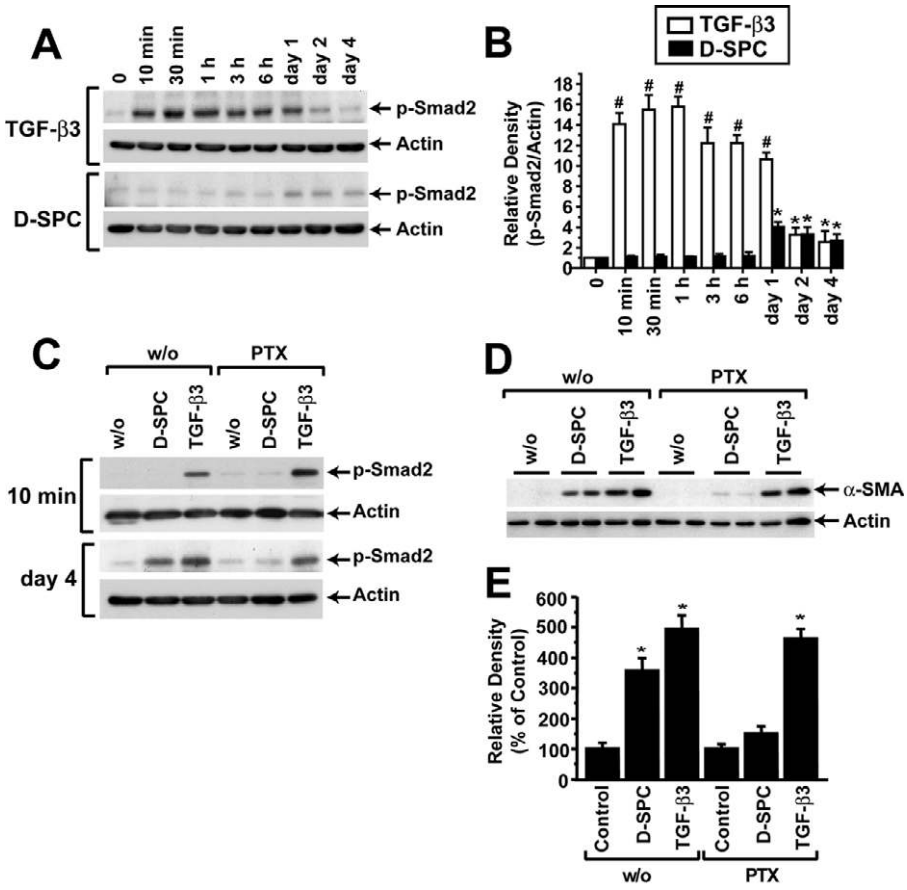

Fig. 5. The role of PTX-sensitive G proteins in the SPC-induced differentiation of hATSCs to SMCs. (A) Serum-starved hATSCs were treated with 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for the indicated time periods, and the phosphorylation level of Smad2 was determined by western blotting with anti-phospho-Smad2 antibody. Anti-actin antibody was used to confirm equal loading. (B) The densities of p-Smad2 and actin were quantified in duplicate, and the phosphorylation levels of Smad2 were normalized to total actin levels in the samples. Relative densities of p-Smad2 are shown as mean ± s.e.m. Asterisks indicate significant difference from control values (**P*<0.05, #*P*<0.01). (C) hATSCs were pretreated with 100 ng/ml PTX for 24 hours. The cells were then exposed to 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 10 minutes or 4 days, and the phosphorylation level of Smad2 was determined by western blotting with an anti-phospho-Smad2 antibody. Anti-actin antibody was used to confirm equal loading. Representative data from three independent experiments are shown. (D) Serum-starved hATSCs were pretreated with 100 ng/ml PTX for 24 hours. The cells were then exposed to 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 4 days, and the expression levels of α-SMA and actin were determined by western blot analysis. Representative data from three independent experiments are shown. (E) The densities of α-SMA and actin were quantified from the duplicate determinations, and the expression levels of α-SMA were normalized to total actin levels in the samples. The data are presented as a percentage of control (mock-treated cells). *Significantly different from control value (*P*<0.05).

ELISA, and SPC had no significant impact on the expression levels of TGF-β2 and -β3 (data not shown). To confirm that SPC induces expression of TGF-β1 through an ERK-dependent pathway, we determined the mRNA level of TGF-β1 by RT-PCR. As shown in Fig. 7C, exposure of the cells to D-*erythro*-SPC significantly increased the level of TGF-β1 mRNA at 6 hours, which then declined to basal level by day 4. These results suggest that the SPC-induced expression of TGF-β1 follows the SPC-stimulated ERK phosphorylation, which was maximal after exposure of the cells for 10 minutes (Fig. 6A). Moreover, the SPC-induced expression of TGF-β1 preceded the SPC-induced late phosphorylation of Smad2, which was initiated on day 1 (Fig. 5A). Consistent with the result that the SPC-induced secretion of TGF-β1 is dependent on ERK activation (Fig. 7B), pretreatment of the cells with U0126 completely abrogated the SPC-stimulated gene transcription of TGF-β1 (Fig. 7D,E). These results suggest that SPC induces TGF-β1 secretion through an ERK-dependent pathway in hATSCs.

To assess whether autocrine release of TGF-β1 is involved in the SPC-induced SMC differentiation, we determined the effects of anti-TGF-β1 neutralizing antibody on the SPC-induced increase of α-SMA. As shown in Fig. 7F, the expression of α-SMA induced by SPC and TGF-β1, but not TGF-β3, was blocked by pre-incubation of the cells with the anti-TGF-β1 neutralizing antibody, suggesting that SPC increases α-SMA expression through induction of TGF-β1 release. Furthermore, pretreatment of the cells with TGF-β1 neutralizing antibody abrogated the late activation of Smad2 induced by SPC or TGF-β1, but not TGF-β3. Together with

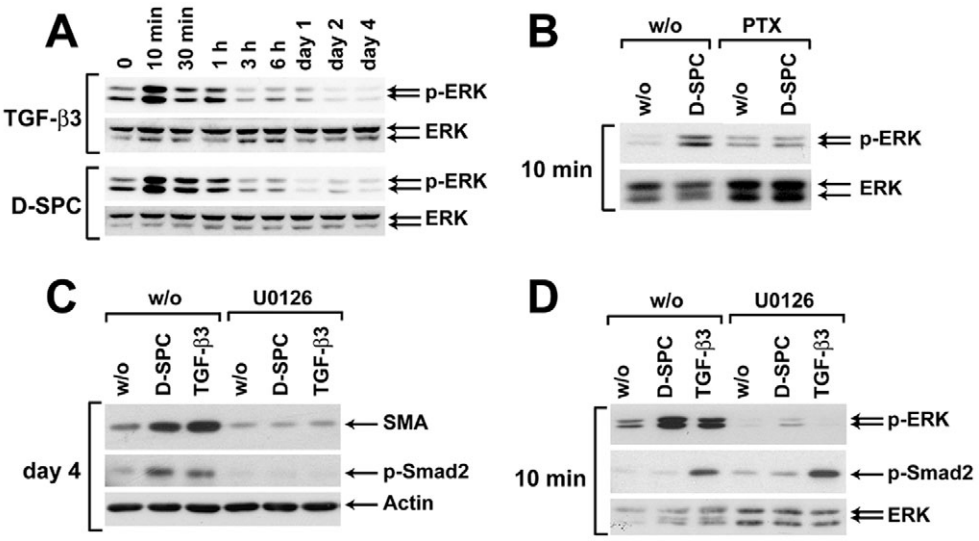

Fig. 6. The role of ERK in SPC-induced expression of α-SMA and delayed phosphorylation of Smad2. (A) Serum-starved hATSCs were treated with 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for the indicated time periods. (B) hATSCs were pretreated with 100 ng/ml PTX for 24 hours, and then exposed to 2 μM D-*erythro*-SPC for 10 minutes. (C) hATSCs were pretreated with 10 μM U0126 for 15 minutes, and then exposed to 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 4 days. (D) hATSCs were pretreated with 10 μM U0126 for 15 minutes, and then exposed to 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 10 minutes. The expression levels of α-SMA, actin, and ERK were determined by western blotting with anti-α-SMA, anti-actin and anti-ERK antibodies, respectively. The phosphorylation levels of ERK and Smad2 were determined by western blotting with anti-p-ERK and anti-p-Smad2 antibodies, respectively. Representative data from three independent experiments are shown.

the result that pharmacological inhibition of TGF-β type I receptor blocked the SPC-induced expression of α-SMA and delayed phosphorylation of Smad2, these results clearly suggest that SPC induces differentiation of hATSCs to SMC through TGF-β1-Smad2 pathway.

Smad2 is involved in the SPC-induced SMC differentiation

Smad2 has been reported to play a crucial role in the TGF-β1-induced expression of α-SMA in proximal tubule epithelial cells (Phanish et al., 2006). In order to clarify whether Smad2 plays a crucial role in the SMC differentiation induced by SPC or TGF-β3, we examined the effect of small interference RNA (siRNA)-mediated knockdown of Smad2 on the SPC-stimulated expression of α-SMA. Transfection of hATSCs with Smad2 siRNA down-regulated the expression level of Smad2 and it completely abrogated the late phosphorylation of Smad2 induced by SPC or TGF-β3 (Fig. 8). We next examined the effect of siRNA-mediated depletion of Smad2 on the SMC differentiation induced by SPC or TGF-β3. As shown in Fig. 8, Smad2 siRNA inhibited the SPC- or TGF-β3-induced expression of α-SMA and h1-calponin, suggesting that the SPC-induced SMC differentiation is dependent on Smad2.

SPC-induced SMC differentiation is dependent on SRF and myocardin

It has recently been shown that myocardin, an SRF co-factor, is absolutely required for the expression of SMC-specific proteins (Du et al., 2003; Yoshida et al., 2003; Wang et al., 2001; Wang et al., 2003). Therefore, we next determined the effects of SPC or TGF-β3 on the mRNA levels of these myocardin family proteins by semi-quantitative RT-PCR. As shown in Fig. 9A, myocardin mRNA was increased in response to treatment with D-*erythro*-SPC or TGF-β3. In addition to myocardin, the expression levels of SRF were also increased in hATSCs, when treated with D-*erythro*-SPC or TGF-β3. To confirm the stimulatory effects of SPC and TGF-β3 on the expression of myocardin and SRF, the mRNA levels were quantified by real-time RT-PCR analysis. As shown in Fig. 9B, the expression levels of myocardin and SRF were increased in response to exposure of the cells to SPC or TGF-β3, suggesting that the SPC-induced increase of myocardin and SRF may play a crucial role in the SPC-induced SMC differentiation.

To further assess whether SRF and myocardin are involved in the SPC-induced SMC differentiation, we examined the effects of siRNA-mediated knockdown of SRF and myocardin expression on the SPC-stimulated expression of α-SMA. As shown in Fig. 9C, the transfection of SRF and myocardin siRNAs significantly attenuated the levels of SRF and myocardin mRNA, respectively, whereas the level of GAPDH mRNA was not affected by the knockdown of SRF and myocardin genes.

We next examined the effects of downregulated expression of SRF and myocardin on the SMC differentiation induced by SPC or TGF-β3. The TGF-β3-stimulated expression of α-SMA was attenuated by the siRNA-based knockdown of SRF and myocardin (Fig. 9D,E), suggesting that the TGF-β3-induced expression of α-SMA is dependent on SRF and myocardin. Moreover, the SPC-induced expression of α-SMA was also abrogated

by the knockdown of SRF and myocardin. These results indicate that SRF and its co-factor, myocardin, play a crucial role in SPC-induced SMC differentiation.

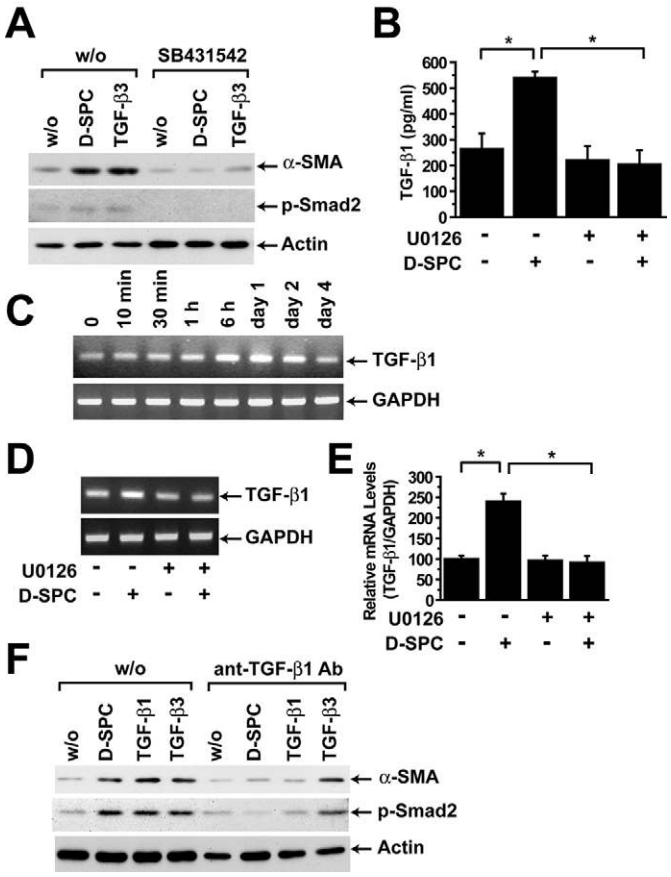

Fig. 7. The role of the TGF-β-dependent pathway in SPC-induced SMC differentiation and the late activation of Smad2. (A) Serum-starved hATSCs were pretreated with 10 μM SB-431542 or vehicle, and then treated with 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 4 days. The expression levels of α-SMA and actin, and the phosphorylation levels of ERK and Smad2 were determined by western blotting. Representative data from three independent experiments are shown. (B) hATSCs were exposed to 2 μM D-*erythro*-SPC for 2 days in the absence or presence of 10 μM U0126, and then the conditioned media were subjected to ELISA for quantification of TGF-β1 protein. Data are shown as mean ± s.e.m. (n=4) and representatives of three independent experiments. *P<0.05. (C) Serum-starved hATSCs were exposed to 2 μM D-*erythro*-SPC for the indicated time periods, and TGF-β1 and GAPDH mRNAs were quantified by semi-quantitative RT-PCR. Representative data from three independent experiments are shown. (D) Serum-starved hATSCs were treated with 2 μM D-*erythro*-SPC in the absence or presence of 10 μM U0126 for 6 hours. The mRNA levels of TGF-β1 and GAPDH were determined by semi-quantitative RT-PCR. (E) The relative level of TGF-β1 was normalized to those of GAPDH. The data are shown as mean ± s.e.m. of triplicate determinations. *P<0.01. (F) Serum-starved hATSCs were treated with 2 μM D-*erythro*-SPC, 2 ng/ml TGF-β1, or 2 ng/ml TGF-β3 together with anti-TGF-β1 neutralizing antibody (0.2 μg/ml) for 4 days. The expression levels of α-SMA and actin, and the phosphorylation level of p-Smad2 were determined by western blotting. Representative data from three independent experiments are

Discussion

In this study, we demonstrated that SPC induced differentiation of hATSCs to SMCs, as evidenced by increased expression of SM-specific proteins, such as α-SMA, SM22α and h_1-calponin, and enhanced formation of actin stress fibers in the cells. The SPC-stimulated expression of α-SMA was stereo-specific for D-*erythro*-SPC and was prevented by pretreatment of hATSCs with PTX, indicating involvement of $G_{i/o}$-coupled receptors in the SPC-induced smooth muscle differentiation. Furthermore, SPC induced delayed phosphorylation of Smad2, and pharmacological inhibition of the $G_{i/o}$-ERK pathways abrogated the late activation of Smad2 and SMC differentiation induced by SPC (Figs 5, 6). Therefore, these results indicate, for the first time, that activation of $G_{i/o}$-coupled receptors by SPC induces SMC differentiation by trans-activating the TGF-β-dependent Smad2 pathway (Fig. 10).

The SPC-induced activation of TGF-β-Smad2 signaling cross-talk pathways provides a new signaling mechanism for the SPC-induced cellular responses. In the present study, we demonstrated that SPC induced the secretion of TGF-β1 through an ERK-dependent pathway in hATSCs. Pharmacological inhibition of TGF-β type I receptor kinase or preincubation of the cells with anti-TGF-β1 neutralizing antibody prevented SMC differentiation and the late activation of Smad2 induced by SPC (Fig. 7). Furthermore, siRNA-mediated depletion of Smad2 expression completely abrogated the SPC-induced expression of α-SMA and h_1-calponin (Fig. 8), suggesting a pivotal role of the TGF-β1-Smad2 signaling pathway in SPC-induced SMC differentiation. Accumulating evidence demonstrates that expression of TGF-β1 can be induced by various cytokines, including IL-1β, IL-6, and IL-10 and angiotensin II (Aoki et al., 2006b; Aoki et al., 2006a; Sinuani et al., 2006; Wang et al., 2006), and that the IL-1β-

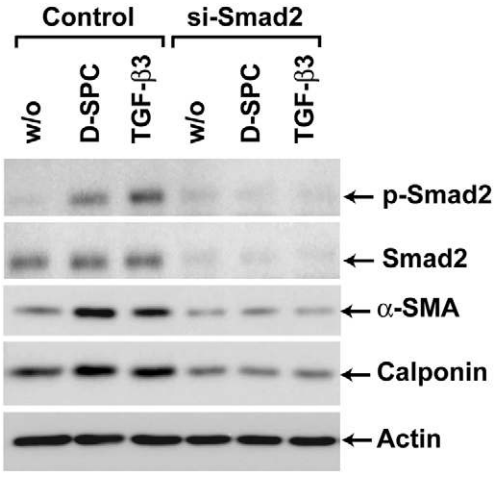

Fig. 8. The role of Smad2 in the SPC-induced differentiation of hATSCs to SMCs. Serum-starved hATSCs were transfected with control siRNA (Control) or Smad2 siRNA (si-Smad2), and then treated with 2 μM D-*erythro*-SPC or 2 ng/ml TGF-β3 for 4 days. The phosphorylation level of Smad2 and the expression level of Smad2 were determined by western blotting with anti-p-Smad2 and anti-Smad2 antibodies, respectively. The expression levels of α-SMA, h_1-calponin and actin were determined by western blotting. Representative data from three independent experiments are shown.

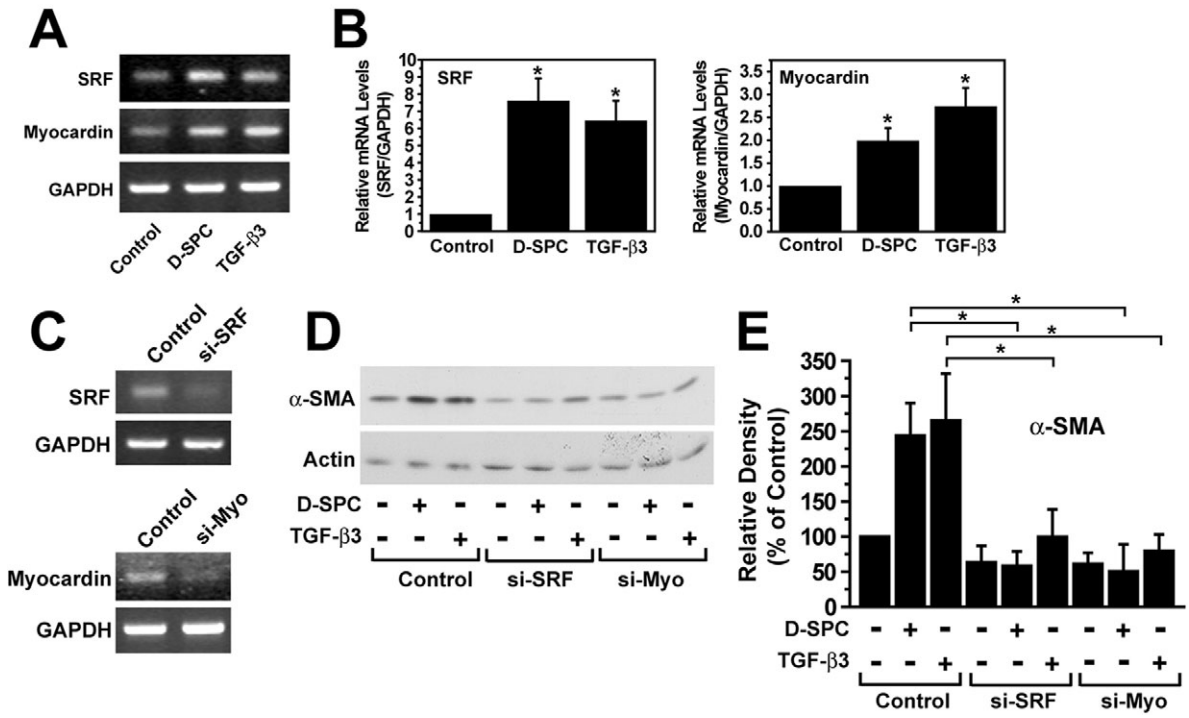

Fig. 9. The role of SRF and myocardin in the SPC-induced differentiation of hATSCs to SMCs. Serum-starved hATSCs were treated with vehicles, 2 μM D-*erythro*-SPC, or 2 ng/ml TGF-β3 for 24 hours, and SRF and myocardin mRNA levels were determined by semi-quantitative RT-PCR (A) or real time RT-PCR (B). Representative data from three independent experiments are shown in A. The data in B are shown as mean ± s.e.m. (n=3). (C) hATSCs were transfected with control siRNA or siRNAs specific for SRF or myocardin, respectively. The levels of SRF and myocardin mRNAs were determined by semi-quantitative RT-PCR. Representative data from three independent experiments are shown. (D) The siRNA-transfected hATSCs were treated with vehicle, 2 μM D-*erythro*-SPC, or 2 ng/ml TGF-β3 for 4 days, and the expression levels of α-SMA and actin were determined by western blot analysis. (E) The quantity of α-SMA and actin were determined in triplicate, and the expression levels of α-SMA were normalized to total actin levels in the samples. The data are presented as a percentage of mock-treated control. *Significantly different from control value (P<0.05).

and IL-6-induced expression of TGF-β1 is mediated by an ERK-dependent pathway (Aoki et al., 2006b; Aoki et al., 2006a). Moreover, angiotensin II induces the late activation of Smad2/3 by an ERK-dependent pathway, and angiotensin II-induced secretion of TGF-β1 is responsible for the activation of Smad2/3 in vascular SMCs (Wang et al., 2006). These reports support the present findings that ERK plays a pivotal role in the SPC-induced secretion of TGF-β1. Nevertheless, the molecular mechanisms by which the SPC-induced activation of ERK stimulated secretion of TGF-β1 should further be explored.

Several GPCR agonists, such as thrombin, angiotensin II, vasopressin, and S1P, have been reported to increase the expression levels of smooth muscle markers in cultured cell systems (Dulin et al., 2001; Hautmann et al., 1997; Lockman et al., 2004; di Gioia et al., 2000; Garat et al., 2000; Owens et al., 2004). However, in the present study, LPC and S1P, lysophospholipids structurally related to SPC, showed no significant impact on the expression of α-SMA in hATSCs. Therefore, SPC appears to be a new extracellular factor regulating SMC differentiation through GPCR activation in hATSCs. Several GPCRs, such as OGR1, GPR4, G2A and GPR12, have been shown to be high affinity receptors for SPC (Xu, 2002; Meyer zu Heringdorf et al., 2002). However, the molecular identity of SPC receptors involved in the SPC-induced differentiation still remains unclear, since some of the SPC receptors have recently been reported to be activated by protons, but not by extracellular SPC (Bektas et al., 2003; Ludwig et al., 2003; Murakami et al., 2004). Therefore, the identity of SPC receptors involved in the SMC differentiation needs to be clarified in future.

Our present study demonstrated that SPC increased the expressions of myocardin and SRF at the transcriptional level, and that siRNA-mediated knockdown of these transcription factors attenuated the expression of α-SMA in response to SPC or TGF-β3 in hATSCs. It is well established that most SMC-specific differentiation marker genes are dependent on the SRF/myocardin-dependent pathway (Miano, 2003; Wang and Olson, 2004), and that the onset of SRF expression correlates closely with SMC marker expression during development (Browning et al., 1998; Croissant et al., 1996). TGF-β induces the expression of SRF, which in turn activates CArG-dependent SMC differentiation (Hirschi et al., 2002; Kawai-Kowase et al., 2004). Furthermore, mouse embryos lacking myocardin die during the early stage of smooth muscle development and fail to express multiple smooth muscle marker genes in embryonic dorsal aorta and other vascular structures (Li et al., 2003). Forced expression of myocardin is sufficient to induce the expression of smooth-muscle-specific genes in human mesenchymal stem cells (Ventura, 2005; van Tuyn et al., 2005),

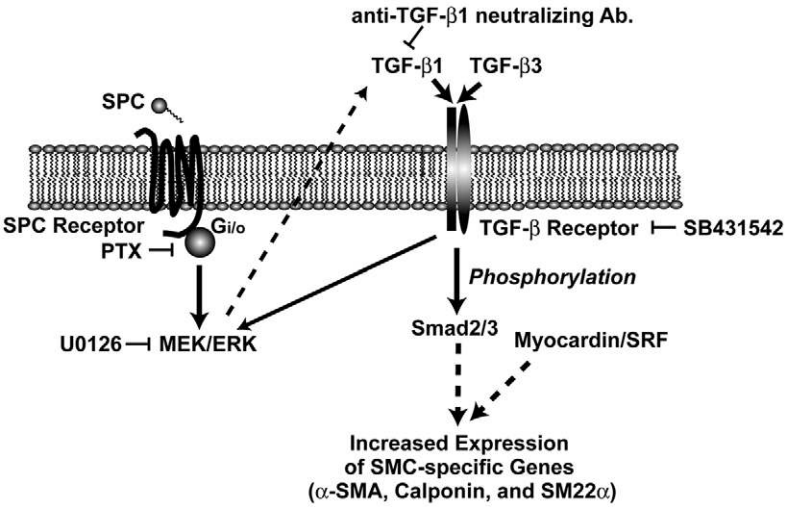

Fig. 10. Schematic illustration of the molecular mechanisms involved in the SPC-induced differentiation of hATSCs. SPC increases the expression of smooth-muscle-specific genes, including α-SMA, through a $G_{i/o}$-ERK-dependent pathway. SPC-induced activation of ERK stimulates the expression and secretion of TGF-β isoforms, which elicit late activation of Smad2 through TGF-β type I receptor kinase. Activated Smad2 cooperates with SRF and myocardin to induce expression of smooth-muscle-specific genes.

and dominant negative myocardin mutants and myocardin-specific siRNAs inhibit SMC differentiation marker gene expression in a variety of smooth muscle cell types, including primary rat aortic SMC, A7r5, Pac1 and A10 SMC lines (Du et al., 2003; Yoshida et al., 2003; Wang et al., 2003). Therefore, these earlier results appear to support our present study that SRF and myocardin are required for SMC differentiation of hATSCs in response to SPC or TGF-β3. The molecular mechanism by which myocardin/SRF and Smad2 stimulate SMC differentiation has not yet been clearly elucidated. A recent report demonstrated that Smad3 is involved in the expression of SM22α by direct association with both SRF and myocardin (Qiu et al., 2005). Together with the present study, indicating that Smad2 is required for the SPC-induced expression of α-SMA and calponin (Fig. 8), these results suggest that activation of Smad2/3 plays a key role in the SPC-induced SMC differentiation by interaction with SRF/myocardin (Fig. 10).

Possible involvement of SPC in angiogenesis and vasculogenesis has been suggested by previous studies indicating that SPC can promote migration and morphogenesis of endothelial cells (Boguslawski et al., 2000; Kim et al., 2005) and migration of vascular SMCs (Boguslawski et al., 2002). The present study provides clues that SPC may play a role in angiogenesis and vasculogenesis by promoting the differentiation of hATSCs to SMCs. However, because of lack of precise information on the distribution and phenotypic characteristics of hATSCs within adipose tissues, physiological or pathological significance of SPC-induced differentiation of hATSCs is still unclear. Several studies suggest that vascular pericytes or solitary SMCs, which are progenitors of SMCs involved in blood vessel formation, have phenotypic and physiological characteristics similar to MSCs (Short et al., 2003). Similar to mesenchymal stem cells, pericytes possess adipogenic, osteogenic, and chondrogenic differentiation potential (Farrington-Rock et al., 2004; Schor et al., 1995). The present study, together with these earlier studies, suggest that hATSCs may be progenitors for SMCs within adipose tissue, and that SPC may play a pivotal role in angiogenesis and vasculogenesis by inducing differentiation of MSCs to SMCs.

Both SPC and TGF-β have been shown to stimulate dermal wound healing (Sun et al., 1996; O'Kane and Ferguson, 1997). TGF-β induces differentiation of fibroblasts to myofibroblasts, which exhibit elevated levels of α-SMA and other smooth-muscle-specific genes. Myofibroblasts in turn have an ability to enhance contraction of extracellular matrix, therefore, being essential for wound closure (Gabbiani, 2003). Although it is not certain whether SPC increases the expression of α-SMA in fibroblasts, the present study suggests that SPC mimics the TGF-β-induced physiological responses, including SMC differentiation and wound healing, by stimulating autocrine release of TGF-β. Because of phenotypic similarity of SMCs and myofibroblasts, it is tempting to speculate that the stimulatory effect of SPC on wound healing could be due to differentiation of mesenchymal cells to myofibroblasts or smooth-muscle-like cells in the subcutaneous region of injured dermal tissues. Obviously, further studies are needed to clarify whether SPC-induced differentiation of MSCs to SMCs or myofibroblasts is involved in wound healing.

Materials and Methods

Materials

α-Minimum essential medium, phosphate-buffered saline (PBS), trypsin, fetal bovine serum, and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA). PTX was from BIOMOL (Plymouth Meeting, PA). S1P and LPC were from Avanti Polar Lipids (Alabaster, AL), and D-*erythro*- and L-*threo*-SPC were from Matreya (Pleasant Gap, PA). The anti-actin antibody (clone C4) was purchased from MP Biomedicals (Irvine, CA). Human recombinant TGF-β1 and TGF-β3, anti-TGF-β1 neutralizing antibody (catalog number, AF-101-NA), and enzyme-linked immunosorbent assay (ELISA) kits (catalog number: DY240 for TGF-β1; DY302 for TGF-β2; DY243 for TGF-β3) were purchased from R&D systems, Inc. (Minneapolis, MN). Anti-α-SMA, anti-h_1-calponin antibodies and SB-431542 were from Sigma-Aldrich (St Louis, MO, USA). Anti-phospho-Smad2 (Ser465/467) and anti-Smad2 antibodies were from Cell Signaling Technology (Beverly, MA). Peroxidase-labeled secondary antibodies were from Amersham Biosciences. Alexa Fluor 488 goat anti-mouse antibody and Alexa Fluor 568 phalloidin were from Molecular Probes, Inc. (Eugene, OR). LightCycler FastStart DNA Master Sybr Green I was purchased from Roche Applied Science (Mannheim, Germany).

Cell culture

Subcutaneous adipose tissue was obtained from elective surgeries with the patient's consent as approved by the Institution Review Board, and hATSCs were isolated as previously described (Lee et al., 2004). Briefly, adipose tissues were washed at least three times with sterile PBS and treated with an equal volume of collagenase type I suspension (1 g/l of HBSS buffer with 1% bovine serum albumin) for 60 minutes at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugation at 300 ***g*** for 5 minutes. The cell pellet

was resuspended in α-minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and cells were plated in tissue culture dishes at 3500 cells/cm^2. The primary hATSCs were cultured for 4-5 days until they reached confluence and were defined as passage '0'. The passage number of hATSCs used in these experiments was 3-10. The hATSCs were positive for CD29, CD44, CD90, and CD105, all of which have been reported to be marker proteins of mesenchymal stem cells (Lee et al., 2004). However, c-kit, CD34 and CD14, which are known as hematopoietic markers, were not expressed in hATSCs.

Western blotting

Confluent, serum-starved hATSCs after treatment were washed with ice-cold PBS, and then lysed in lysis buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, 1% Triton X-100, pH 7.4). Lysates were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and then stained with 0.1% Ponceau S solution (Sigma-Aldrich). After blocking with 5% nonfat milk, the membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence western blotting system (ECL; Amersham Biosciences).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted by the TRIzol method (Invitrogen). For RT-PCR analysis, 2 μg aliquots of RNA were subjected to cDNA synthesis with 200 U of M-MLV reverse transcriptase (Invitrogen) and 0.5 μg of oligo(dT) 15 primer (Promega, Madison, WI, USA). The cDNA in 2 μl of the reaction mixture was amplified with 0.5 U of GoTaq DNA polymerase (Promega) and 10 pmol each of sense and antisense primers as follows: GAPDH 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′; α-SMA 5′-CCTGACTGAGCGTGGCTATT-3′, 5′-GATGAAGGATGGCTGGAACA-3′; SM22α 5′-GCCTTCTTTCCCCAGA-CA-3′, 5′-CCTCCAGCTCCTCGTCAT-3′; h_1-calponin 5′-GCCCAGAAGTAT-GACCACCA-3′, 5′-TGATGAAGTTGCCGATGTTC-3′; myocardin 5′-TCCAA-CGGCTTCTACCACTT-3′, 5′-CACCTTCTGCTTCTCCACCA-3′; SRF 5′-AGC-CTGAGCGAGATGGAGAT-3′, 5′-TTGCTGAAGGTCGTGTAGC-3′; TGF-β1 5′-TACTACGCCAAGGAGGTCAC-3′, and 5′-GCTGAGGTATCGCCAGGAAT-3′. The thermal cycle profile was as follows: denaturation for 30 seconds at 95°C, annealing for 45 seconds at 52-58°C depending on the primers used, and extension for 45 seconds at 72°C. For semi-quantitative assessment of expression levels, each PCR reaction was carried out for 30 cycles. PCR products were size fractionated on 1.2% ethidium bromide/agarose gel and quantified under UV transillumination.

Real-time RT-PCR

Quantitative PCR was performed and analysed on a capillary real-time thermocycler (Light-Cycler, Roche Diagnostics, Mannheim, Germany). Amplification was done in the presence of SYBR Green I as follows: in 20 μl of final volume, cDNA was mixed with LightCycler-Fastart DNA Master SYBR Green (Roche Diagnostics), 10 pmol each of forward primer and reverse primer, and 4 mM $MgCl_2$. Glass capillaries were placed into the Light-Cycler rotor, and the following run protocol was used: a pre-denaturing step at 95°C for 10 minutes, an amplification and quantification program repeated for 50 cycles at 95°C for 10 seconds, annealing for 10 seconds at 52-58°C, depending on the primers used, and extension for 30 seconds at 72°C. At the end of elongation at each cycle, SYBR Green I fluorescence was measured. Data was analysed using the 'Fit Point Method' in the LightCycler software 3.3 (Roche Diagnostics). Relative quantification was made against serial dilution of GAPDH cDNA used as a housekeeping gene.

Immunocytochemistry and microscopy

The phase-contrast images of hATSCs were collected using a Leica inverted microscopy (DM IRB) with 10× objective and a digital CCD camera (Leica, DFC300FX). Immunostaining and confocal microscopy were used to determine the subcellular distribution and organization of proteins. Cells were fixed in 4% paraformaldehyde in PBS for 15 minutes. For immunostaining, specimens were incubated with anti-α-SMA antibody for 2 hours and Alexa Fluor 488-conjugated anti-mouse secondary antibody (Molecular Probes) for 1 hour. To stain F-actin filaments, the specimen was incubated with Alexa Fluor 543-conjugated phalloidin for 30 minutes, followed by confocal microscopy. The images of the specimen were collected with a Leica TCL SP2 confocal microscope system.

Enzyme-linked immunosorbent assay

Commercially available sandwich ELISA kits (R&D Systems, MN), which are specific for human TGF-β isoforms, were used to evaluate the secretion of TGF-β isoforms. Conditioned culture media were collected and centrifuged at 13,000 rpm for 30 minutes to remove particulate. ELISA was carried out according to the manufacturer's instructions. The absorbance (450 nm) for each sample was analysed using an ELISA reader and was interpolated with a standard curve.

Transfection with small interference RNA (siRNA)

The SRF siRNA (catalog number M-009800-01), myocardin siRNA (catalog number M-015905-00), and nonspecific control siRNA (D-001206-13-05) were purchased from Dharmacon, Inc. (Lafayette, CO). The Smad2 siRNA duplex was synthesized, desalted, and purified by Dharmacon, Inc. Smad2 Sequences were as follows: 5′-GUCCCAUGAAAAGACUUAAdTdT-3′ (sense) and 5′-UUAAGUC-UUUUCAUGGGACdTdT-3′ (antisense). For siRNA experiments, hATSCs were plated on 60-mm dishes at 70% confluence, and they were then transfected with SRF siRNA, myocardin siRNA, Smad2 siRNA, or control siRNA using the Lipofectamine™ 2000 reagent according to manufacturer's instructions. Briefly, Lipofectamine™ 2000 reagent was incubated with serum-free medium for 10 minutes, and then respective siRNAs were added to the mixtures. After incubation for 15 minutes at room temperature, the mixtures were diluted with serum-free medium and added to each well. The final concentration of SRF siRNA, myocardin siRNA, Smad2 siRNA, or control siRNA in each well was 100 nM. After incubation for 6 hours, the cells were cultured in growth medium for 24 hours, and the expression levels of SRF, myocardin, and Smad2 were determined by RT-PCR analysis.

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