Simvastatin inhibits sphingosylphosphorylcholine-induced differentiation of human mesenchymal stem cells into smooth muscle cells

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Abbreviations: FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; hASCs, human adipose tissue-derived mesenchymal stem cells; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MSCs, mesenchymal stem cells; SMCs, smooth muscle cells; SPC, sphingosylphosphorylcholine; statins, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors

Abstract

Sphingosylphosphorylcholine (SPC) induces differentiation of human adipose tissue-derived mesenchymal stem cells (hASCs) into smooth muscle-like cells expressing α -smooth muscle actin (α -SMA) via transforming growth factor- β 1/Smad2- and RhoA/Rho kinase-dependent mechanisms. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) have been known to have beneficial effects in the treatment of cardiovascular diseases. In the present study, we examined the effects of simvastatin on the SPC-induced α -SMA expression and Smad2 phosphorylation in hASCs. Simvastatin inhibited the SPC-induced

α -SMA expression and sustained phosphorylation of Smad2 in hASCs. SPC treatment caused RhoA activation via a simvastatin-sensitive mechanism. The SPC-induced α -SMA expression and Smad2 phosphorylation were abrogated by pretreatment of the cells with the Rho kinase inhibitor Y27632 or overexpression of a dominant negative RhoA mutant. Furthermore, SPC induced secretion of TGF- β 1 and pretreatment with either Y27632 or simvastatin inhibited the SPC-induced TGF- β 1 secretion. These results suggest that simvastatin inhibits SPC-induced differentiation of hASCs into smooth muscle cells by attenuating the RhoA/Rho kinase-dependent activation of autocrine TGF- β 1/Smad2 signaling pathway.

Keywords: cell differentiation; mesenchymal stem cells; myocytes, smooth muscle; rhoA GTP-binding protein; simvastatin; sphingosine phosphorylcholine; transforming growth factor β 1

Introduction

Smooth muscle cells (SMCs) play an important role in angiogenesis, vessel maintenance, and regulation of blood pressure. SMCs exhibit a contractile phenotype characterized by high expression of specific contractile proteins, including α -SMA, calponin-1, SM22 α , smoothelin, h-caldesmon, and smooth muscle myosin heavy chain (Shanahan *et al.*, 1993; Owens *et al.*, 2004). The phenotypic expression of SMCs is 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).

Mesenchymal stem cells (MSCs) have a self-renewal capacity, long-term viability, and potential to differentiate into cells of diverse lineages, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages (Pittenger and Martin, 2004; Caplan, 2007; Chamberlain *et al.*, 2007; Prockop *et al.*, 2010). 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 *in vitro* (Ball *et al.*, 2004). Moreover, injected bone marrow-derived MSCs have been reported to have differentiated into SMCs and to have contributed to the remodeling of vasculature *in vivo* (Davani *et al.*, 2003; Gojo *et al.*, 2003; Yoon *et al.*, 2005). In a previous study, we showed that sphingosylphosphorylcholine (SPC) increased the expression levels of α -SMA and other smooth muscle-specific proteins in human adipose tissue-derived mesenchymal stem cells (hASCs) *via* an autocrine TGF- β /Smad2-dependent mechanism (Jeon *et al.*, 2006). In addition, we have previously reported that SPC stimulated the small GTPase RhoA and that the RhoA-Rho kinase pathway played a key role in SPC-induced differentiation of hASCs to SMCs. RhoA-Rho kinase pathway plays a key role in SMC differentiation by regulating the integrity of the actin cytoskeleton and MRTF-dependent gene transcription (Cen *et al.*, 2004; Miano *et al.*, 2007). Therefore, SPC-induced SMC differentiation of MSCs would be an ideal model for the study of vascular diseases-associated SMC differentiation.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) reportedly exert beneficial effects in patients with cardiovascular diseases *via* pleiotropic functions, including reduction of plaque inflammation and platelet ag-

gregation, enhanced plaque stability and endothelial function, and inhibition of SMC proliferation and increased apoptosis (Calabro and Yeh, 2005; Liao, 2005). Accumulating evidence suggests that statins attenuate neointimal formation and vascular remodeling by blocking the activation of the Rho family of small G proteins (Rolfe *et al.*, 2005). Statins inhibit the activity of HMG-CoA reductase which catalyses the conversion of HMG-CoA into mevalonate during cholesterol biosynthesis. Mevalonate can be converted into farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), 2 isoprenoid residues that can be anchored onto several intracellular proteins through farnesylation or geranylgeranylation (Wong *et al.*, 2002; Graaf *et al.*, 2004). Simvastatin has been reported to inhibit the relocalization of RhoA to cell membranes and the resulting activation of RhoA by blocking geranylgeranylation (Laufs *et al.*, 1999). However, whether statins can affect the SPC-induced differentiation of MSCs to SMCs has not been studied. In the present study, we show for the first time that simvastatin inhibits the differentiation of hASCs into SMCs by blocking RhoA-Rho kinase-dependent activation of autocrine TGF- β /Smad2 signaling pathway.

Results

Simvastatin inhibits SPC-induced differentiation of hASCs to SMCs

To explore whether statin can affect SPC-induced differentiation of hASCs to SMCs, we examined the effect of simvastatin on the SPC-induced expression of smooth muscle-specific markers, including α -SMA and calponin. As shown in Figure 1, SPC treatment increased the expression of α -SMA and calponin in hASCs, and simvastatin dose-dependently attenuated SPC-induced expression of α -SMA and calponin with a complete inhibition at a 1 μ M concentration, suggesting simvastatin has an inhibitory effect on the SPC-induced differentiation of hASCs to SMCs.

To confirm these results, we determined the effects of simvastatin on α -SMA expression and actin filament formation using immunocytochemistry. As shown in Figure 1B, treatment of hASCs with 2 μ M SPC for 4 days increased α -SMA expression levels, and pretreatment of the cells with simvastatin completely abrogated SPC-induced expression of α -SMA in hASCs.

Figure 1. Effect of simvastatin on SPC-induced expression of smooth muscle markers in hASCs. (A) hASCs were treated with serum-free medium containing 2 μ M SPC or vehicles (0.1% DMSO, w/o) in the presence of indicated concentrations of simvastatin for 4 days. Expression levels of α -SMA, calponin, and GAPDH were determined by Western blotting. (B) Inhibitory effects of simvastatin on SPC-induced α -SMA expression in hASCs were further determined by immunostaining with anti- α -SMA antibody. Scale bar = 50 μ m. Representative data from three independent experiments are shown.

Figure 2. Effects of simvastatin on SPC-induced phosphorylation of Smad2 and ERK. (A) hASCs were treated with serum-free medium containing 2 μ M SPC or vehicles (0.1% DMSO) in the absence or presence of 1 μ M simvastatin for 4 days. Phosphorylation levels of Smad2 and expression levels of Smad2 and GAPDH were determined by Western blotting. (B) The densities of p-Smad2 were quantified and normalized to those of Smad2. Data are expressed as mean $\pm$ SD ($n = 4$; $*P < 0.05$). (C) hASCs were pretreated with serum-free medium containing 1 μ M simvastatin or vehicles (0.1% DMSO) for 1 day and then treated with 2 μ M SPC or vehicles for 10 min. Phosphorylation levels of ERK and expression levels of ERK and GAPDH were determined by Western blotting. Representative data from three independent experiments are shown.

Simvastatin inhibits SPC-induced sustained phosphorylation of Smad2

We previously reported that SPC treatment elicited phosphorylation of Smad2 on day 1 that was sustained until day 4, and that the sustained phosphorylation of Smad2 was responsible for the increased expression of α -SMA (Jeon *et al.*, 2006). Therefore, we sought to determine the effect of simvastatin on SPC-induced Smad2 phosphorylation on day 4. As shown in Figures 2A and 2B, treatment of hASCs with SPC for 4 days induced phosphorylation of Smad2 and pretreatment of the cells with simvastatin markedly attenuated Smad2 phosphorylation.

We have shown that SPC elicited acute ERK phosphorylation in 10 min and ERK phosphorylation played a key role in SPC-induced sustained phosphorylation of Smad2 (Jeon *et al.*, 2006). Therefore, we examined the effect of simvastatin in SPC-induced ERK phosphorylation. As shown in Figure 2C, treatment of hASCs with SPC for 10 min induced phosphorylation of ERK, whereas SPC-induced ERK phosphorylation was not attenuated by pretreatment with simvastatin. This suggests that ERK is not a target of simvastatin-induced inhibition of Smad2 phosphorylation and α -SMA expression.

Figure 3. Effect of simvastatin on SPC-induced RhoA activation. (A) hASCs were treated with 2 μ M SPC for the indicated time periods. (C) hASCs were pretreated with serum-free medium containing 1 μ M simvastatin or vehicles (0.1% DMSO) for 1 day and then treated with 2 μ M SPC or vehicles for the indicated time periods. The amounts of RhoA in total lysates and membrane fractions were determined by Western blotting. (B, D) The densities of RhoA in membrane fractions were quantified and normalized to those of RhoA in total lysates. Data are expressed as mean $\pm$ SD ($n = 4$; $*P < 0.05$). (E) hASCs were pretreated with 1 μ M simvastatin (0.1% DMSO) or vehicles for 1 day, followed by treatment with 2 μ M SPC for 5 min. The amounts of RhoA in the whole cell lysates (Total RhoA) or the GTP-bound RhoA precipitated from the lysates were revealed by Western blotting. Representative data from three independent experiments are shown.

Simvastatin inhibits SPC-induced RhoA activation

Increasing body of evidence suggests that RhoA-mediated rearrangement of the actin cytoskeleton plays a pivotal role in SMC differentiation (Cen *et al.*, 2004). It has been reported that activated RhoA translocated from the cytosol to the plasma membrane through geranylgeranylation, and that simvastatin inhibited this translocation (Laufs *et al.*, 1999).

To explore whether SPC can induce RhoA activation, hASCs were treated with SPC for the indicated periods, and the amounts of membrane-associated RhoA were determined. SPC treatment increased the amounts of RhoA associated with cell membrane in a time-dependent manner, with a maximal increase at 5 min (Figures 3A and 3B). The SPC-induced translocation of

Figure 4. Role of RhoA-Rho kinase pathway in the SPC-induced phosphorylation of Smad2. (A) hASCs were transfected with the dominant-negative mutant of RhoA (DN-RhoA) or control vector and then treated with serum-free medium containing 2 μ M SPC or vehicles for 4 days. (C) hASCs were treated with serum-free medium containing 2 μ M SPC or vehicles in the absence or presence of 10 μ M Y27632 for 4 days. Phosphorylation levels of Smad2 and expression levels of Smad2, α -SMA, and GAPDH were determined by Western blotting. (B, D) The densities of α -SMA were quantified and normalized to those of GAPDH. Data are expressed as mean $\pm$ SD ($n = 4$; * $P < 0.05$).

RhoA was completely abrogated by pretreatment of the cells with simvastatin (Figures 3C and D). To confirm these results, we determined the amounts of GTP-loaded RhoA after treatment with SPC and/or simvastatin. As shown in Figure 3E, SPC treatment increased GTP-RhoA levels and pretreatment with simvastatin abrogated SPC-induced increase of GTP-RhoA.

RhoA-Rho kinase is responsible for the SPC-induced α -SMA expression and Smad2 phosphorylation

In order to delineate whether RhoA is involved in the SPC-induced α -SMA expression and Smad2 phosphorylation, we examined the effect of a dominant-negative mutant of RhoA (RhoA^{N19}) on SPC-induced α -SMA expression and Smad2 phosphorylation. As shown in Figures 4A and 4B, overexpression of RhoA^{N19} attenuated the SPC-induced α -SMA expression and Smad2 phosphorylation, suggesting that RhoA plays a key role in SPC-induced α -SMA expression through a Smad2-dependent mechanism.

To explore whether Rho kinase, a major downstream target of RhoA, is involved in SPC-induced α -SMA expression and Smad2 phosphorylation in

Figure 5. Effect of simvastatin on SPC-induced secretion of TGF- β 1. hASCs were treated with serum-free medium containing 2 μ M SPC or vehicles (w/o) in the absence or presence of 10 μ M Y27632 or 1 μ M simvastatin for 2 days. The conditioned medium was subjected to ELISA for determination of TGF- β 1 protein levels. Data represent mean $\pm$ SD ($n = 4$). *indicates $P < 0.05$.

hASCs, we examined the effect of Y27632, a Rho kinase-specific inhibitor, on α -SMA expression and Smad2 phosphorylation induced by SPC. As shown in Figures 4C and 4D, SPC-induced α -SMA expression and Smad2 phosphorylation were abrogated by pretreatment of the cells with Y27632, suggesting that Rho kinase is involved in SPC-induced α -SMA expression and Smad2 phosphorylation. Moreover, basal expression levels of α -SMA in the absence of SPC treatment were slightly inhibited by either DN-RhoA overexpression or pretreatment with Y27632, suggesting a potential role of RhoA in basal expression of α -SMA as well as in the SPC-induced α -SMA expression.

Simvastatin inhibits SPC-induced secretion of TGF- β 1 through Rho kinase-dependent mechanism

We previously reported that autocrine secretion of TGF- β 1 is responsible for SPC-induced Smad2 phosphorylation and α -SMA expression (Jeon *et al.*, 2006). To explore whether the inhibitory effects of simvastatin on SPC-induced Smad2 phosphorylation and α -SMA expression are due to the decreased secretion of TGF- β 1, we measured the effect of simvastatin on SPC-induced TGF- β 1 secretion. As shown in Figure 5, SPC stimulated secretion of TGF- β 1 from hASCs, and pretreatment of the cells with simvastatin abrogated SPC-induced secretion of TGF- β 1. Furthermore, pretreatment of the cells with Y27632 inhibited SPC-induced TGF- β 1 secretion. These results suggest that simvastatin inhibits the SPC-induced autocrine TGF- β 1/Smad2 signaling pathway and α -SMA expression by inhibiting Rho kinase-dependent TGF- β 1 secretion.

Discussion

The RhoA-Rho kinase signaling pathway plays a key role in the SPC-induced differentiation of hASCs to SMCs. Inhibition of the RhoA-Rho kinase pathway by over-expression of DN-RhoA or pre-treatment with Y27632 completely abrogated SPC-induced α -SMA expression (Jeon *et al.*, 2008). It has been documented that the RhoA/Rho kinase-dependent pathway regulates transcription of SMC-specific genes (Mack *et al.*, 2001; Wamhoff *et al.*, 2004). Sphingosine-1-phosphate increased RhoA activity and expression level of α -SMA in smooth muscle cells, and that inhibition of Rho kinase by Y27632 attenuated the sphingosine-1-phosphate-stimulated α -SMA expression (Lockman *et al.*, 2004). In the present study, we demonstrated that simvastatin treatment blocked SPC-induced translocation of RhoA to membrane fractions and abrogated SPC-induced α -SMA expression in hASCs. Inactive GDP-bound RhoA becomes activated by membrane translocation and subsequent exchange of GDP and GTP in the presence of guanine nucleotide exchange factor (Bokoch *et al.*, 1994). Statins lead to the deprivation of mevalonate and downstream metabolites such as mevalonate, GGPP and FPP by blocking HMG-CoA reductase (Nakagami *et al.*, 2003). GGPP and FPP are transferred to proteins such as small GTPases by GGPP and FPP, respectively (Wong *et al.*, 2002; Graaf *et al.*, 2004). Statins has been reported to inhibit membrane localization and GTP binding activity of RhoA (Kusama *et al.*, 2001; Yoshida *et al.*, 2001). Taken together, these results support the notion that ablation of RhoA-Rho kinase signaling cascade is responsible for simvastatin-induced inhibition of SPC-induced differentiation of hASCs to SMCs.

We have previously showed that SPC-induced activation of the autocrine TGF- β 1 signaling loop is responsible for the sustained phosphorylation of Smad2 on day 4 (Jeon *et al.*, 2006). In the present study, we demonstrated for the first time that inhibition of Rho kinase by treatment with Y27632 or simvastatin blocked SPC-induced secretion of TGF- β 1 and sustained Smad2 phosphorylation. Although the molecular mechanisms by which activation of Rho kinase regulates expression of TGF- β 1 are largely elusive, a possible role of Rho kinase in the regulation of TGF- β 1 is supported by a report that mechanical stretch of human airway SMCs augmented TGF- β 1 expression through a RhoA-Rho kinase-dependent mechanism (Mohamed and Boriek, 2010). Inhibition of Rho kinase by treatment with Y27632 has been reported to abrogate renal fibrosis-associated increase of α -SMA expression and TGF- β 1-induced myofibroblastic dif-

ferentiation of gingival fibroblasts (Nagatoya *et al.*, 2002; Smith *et al.*, 2006). Consistently, inhibition of RhoA by C3 exoenzyme or DN-RhoA and pharmacological inhibition of Rho kinase by pretreatment with Y27632 blocked TGF- β 1-induced expression of α -SMA in Monc-1 neural crest stem cells (Chen *et al.*, 2006). Taken together with these results, the present study suggests that Rho kinase is involved in the SPC-induced autocrine secretion of TGF- β 1 and subsequent activation of TGF- β receptor and Smad2 phosphorylation.

Differentiation and phenotypic modulation of vascular smooth muscle cells (VSMCs) are implicated in the development of vascular diseases including atherosclerosis (Rzucidlo *et al.*, 2007). Accumulating evidence suggests that MSCs from various tissues reside in a perivascular location and can be identified as pericytes that play a key role in development of microvessels (Covas *et al.*, 2008; Feng *et al.*, 2010). It has been reported that hASCs also originate from perivascular cells (Cai *et al.*, 2011). Moreover, several studies suggest that pericytes surrounding capillaries and microvessels but also adventitial cells located around larger arteries and veins natively express MSC markers and multipotent differentiation potentials similar to MSCs (Chen *et al.*, 2009; Corselli *et al.*, 2010). Therefore, these results suggest that MSCs may play a role in the vascular development and vascular diseases. SPC has been reported to exist in high-density lipoprotein (HDL) particles (Tolle *et al.*, 2008). Increasing body of evidence demonstrate that HDL exerts anti-atherogenic and anti-inflammatory effects (Nofer *et al.*, 2004; Scanu and Edelstein, 2008). HDL induced expression of TGF- β 2, an anti-inflammatory cytokine, in human umbilical vein endothelial cells and SPC mimicked the effect of HDL (Norata *et al.*, 2005). These results suggest that SPC included in HDL may promote differentiation of tissue-resident MSCs or pericytes to SMCs, and that statins can interfere the differentiation of MSCs to SMCs. Similar to the results of the present study, it has been reported that TGF- β 1-induced α -SMA expression in human tenon fibroblasts was attenuated by treatment with lovastatin, a member of the statin family (Meyer-Ter-Vehn *et al.*, 2008). However, lovastatin treatment has been shown to stimulate α -SMA expression in VSMCs and prevent phenotypic dedifferentiation of VSMCs (Wada *et al.*, 2008; Wagner *et al.*, 2010). These results raise a possibility that statins differentially affect expression of SMC markers in different cell types. To clarify functional role of statins on MSC differentiation, it is necessary to determine further whether statins can affect differentiation of MSCs to SMCs using *in vivo* vascular disease animal

models such as atherosclerosis and vascular injury that are associated with phenotypic modulation of SMCs.

Methods

Materials

Trypsin, α -minimum essential medium, fetal bovine serum, and Lipofectamine 2000 reagent were purchased from Invitrogen (Carlsbad, CA). Y27632 and simvastatin were purchased from Calbiochem (La Jolla, CA). D-erythro-SPC was purchased from Matreya (Pleasant Gap, PA). Anti- α -SMA antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-phospho-Smad2 (Ser465/467) and anti-Smad2 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-RhoA antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Millipore (Temecula, CA). Peroxidase-labeled secondary antibodies were obtained from Amersham Biosciences (Franklin Lakes, NJ).

Cell culture

Subcutaneous adipose tissue was obtained from elective surgeries with the patient's consent as approved by the Institution Review Board of Pusan National University Hospital. To isolate hASCs, adipose tissues were washed at least three times with sterile PBS and treated with an equal volume of collagenase type I suspension [1 g/liter of Hank's balanced salt solution (HBSS) buffer with 1% bovine serum albumin] for 60 min at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugation at $300 \times g$ for 5 min. The cell pellet was resuspended in α -minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and cells were plated in tissue culture dishes at 3500 cells/cm². Primary hASCs were cultured for 4-5 days until they reached confluence and were defined as passage "0." The passage number of hASCs used in these experiments was 3-10. Adipose tissues were collected from six different patients, and six different hASC clones exhibited similar differentiation potentials toward osteogenic and adipogenic lineages as well as SMCs. Multipotent differentiation and proliferation capacities of hASCs remained consistent up until 10th passage. These six hASC clones exhibited SPC-induced α -SMA expression which could be inhibited by simvastatin treatment. They were positive for CD29, CD44, CD73, CD90, and CD105, whereas CD31 and CD45 were not expressed in hASCs (Supplemental Data Figure S1).

Western blotting

Confluent, serum-starved hASCs were treated with the appropriate conditions, 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 Na₃VO₄, 30 mM sodium pyrophosphate, 25 mM

β -glycerol phosphate, and 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). Images were obtained using a LAS3000 image capture system (FUJIFILM, Japan). The immunoblots were quantified by measuring the intensity of the protein bands with LAS3000 software. Immunoblot images were obtained at different exposure times, and images whose band intensities were within the linear range between the intensity increase and exposure time were selected for quantification of images.

Measurement of membrane translocation of RhoA

To determine the amounts of membrane-associated RhoA, membrane fractions were isolated as previously described (Negre-Aminou *et al.*, 2001). Cells were made quiescent and stimulated as described in the figure legends. They were then washed in ice-cold PBS, lysed by incubation in hypotonic buffer (5 mM Tris-HCl, 5 mM NaCl, 1 mM CaCl₂, 2 mM EGTA, 1 mM MgCl₂, and 2 mM dithiothreitol, pH 7.0) containing a mixture of protease inhibitors. Membrane fractions were separated from cytosolic fractions by centrifugation ($100,000 \times g$, 1 h at 4°C) of the lysates. Membrane fractions were prepared by resuspending the pellets in the hypotonic buffer. The lysates and membrane fractions were diluted in SDS-PAGE buffer and the protein levels of RhoA were determined by Western blotting.

RhoA activation assay

A commercial pull-down assay (Rho activation assay kit, Upstate) was used to measure the effect of SPC on RhoA activity in hASCs. The cells were treated washed twice with α -MEM and incubated for 24 h in fresh modified α -MEM without serum, SPC was added, and the cell suspension was centrifuged at the indicated time after SPC addition. After lysis of the cell pellet (lysis buffer, Upstate) and the activated RhoA was precipitated using a fusion protein consisting of GST and the Rho binding domain of Rhotekin, according to the manufacturer's protocol. Protein concentrations were equalized between treatment groups prior to pull-down assay.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in PBS for 15 min. For immunostaining, specimens were incubated with anti- α -SMA antibody for 2 h and Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h. The specimens were finally washed and mounted in Vectashield medium (Vector Laboratories) with 4',6-diamidino-2-phenylindole (DAPI) for visualization of nuclei. Fluorescence images were collected using a Leica TCL-SP2 confocal microscope system (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

ELISA

ELISA was carried out according to the manufacturer's instructions. In brief, cells were seeded onto 24-well culture plates at a density of 1×10^4 cells/well and cultured in growth medium for 48 h to confluence. After treatment of the confluent cells under appropriate conditions, conditioned media were collected and centrifuged at $15000 \times g$ for 30 min to remove particulates. A commercially available sandwich ELISA kit (R&D Systems Inc., Minneapolis, MN) was used to evaluate the secretion of TGF- β 1 in the conditioned medium. The absorbance (450 nm) for each sample was analyzed by an ELISA reader and was interpolated with a standard curve.

Supplemental data

Supplemental data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-2-11.pdf.

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