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The PDE1A-PKCα Signaling Pathway Is Involved in the Upregulation of α-Smooth Muscle Actin by TGF-β_1 in Adventitial Fibroblasts

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Key Words
Adventitial fibroblasts · Cyclic nucleotide phosphodiesterases · PDE1A · Protein kinase Cα · α-Smooth muscle actin · Transforming growth factor-β_1

Abstract
Background: Increasing evidence has suggested that differentiation of adventitial fibroblasts (AFs) to myofibroblasts plays an important role in arterial remodeling. The molecular mechanisms by which myofibroblast formation is regulated still remain largely unknown. This study aimed to evaluate the role of cyclic nucleotide phosphodiesterase 1A (PDE1A) in the formation of adventitial myofibroblasts induced by transforming growth factor (TGF)-β_1. ***Methods and Results:*** AFs were cultured by the explant method. Western blot and immunocytochemistry were applied for α-smooth muscle actin (SMA) or protein kinase C (PKC) α protein analysis. Results showed that TGF-β_1 upregulated PDE1A protein expression in rat aortic AFs and pharmacological inhibition of PDE1A blocked TGF-β_1-induced α-SMA expression, a marker of myofibroblast formation, suggesting that the upregulation of PDE1A may mediate TGF-β_1-induced AF transformation. Moreover, calphostin C (a PKC inhibitor) inhibited TGF-β_1-induced α-SMA expression, whereas phorbol-12-myristate-13-acetate (a PKC activator) induced it. Finally, the upregulation of PKCα expression by TGF-β_1 was also inhibited by PDE1A inhibition. ***Conclusions:*** Taken together, our data suggest that TGFβ_1 induces α-SMA expression and myofibroblast formation via a PDE1A-PKCα-dependent mechanism. Our study thus unveils a novel signaling mechanism underlying TGF-β_1-induced adventitial myofibroblast formation.

Introduction

Adventitial fibroblasts (AFs) are the most abundant cell type in the adventitia. Following biological or mechanical injury, AFs differentiate into myofibroblasts, which become capable of proliferating, migrating and synthesizing extracellular matrices. Differentiation of AFs into myofibroblasts has long been thought to play an important role in vascular remodeling [1–3]. Among a variety of regulatory factors, transforming growth factor (TGF)-β_1 is known to induce myofibroblast formation, which is mainly characterized by upregulation of α-

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smooth muscle actin (SMA) expression [4–6]. We have previously shown that TGF-β_1 upregulated α-SMA expression in cultured AFs likely via the protein kinase C (PKC) α pathway [7]. However, the molecular mechanisms by which adventitial myofibroblast formation is regulated still remain largely unknown.

Cyclic nucleotide phosphodiesterase (PDE) 1A is a Ca^{2+}/calmodulin-stimulating PDE family member and preferentially hydrolyzes cyclic guanosine monophosphate (cGMP). Increasing evidence indicates that cGMP and TGF-β_1 signaling pathways play distinct roles in regulating a variety of cellular responses, including myofibroblast formation [8–13]. In vascular smooth muscle cells (VSMCs), PDE1A is involved in phenotypic modulation, proliferation and survival [14]. Previously, the number of PDE1A-positive cells was markedly increased in the adventitial layer of rat carotid arteries with balloon injury as well as mouse carotid arteries with ligation-induced remodeling [14], leading to the hypothesis that TGF-β_1 may induce myofibroblast formation by upregulating PDE1A expression. In this study, TGF-β_1 upregulated PDE1A protein levels in rat AFs. PDE1A inhibition attenuated TGF-β_1-induced expression of α-SMA. In addition, PKCα was involved in PDE1A-mediated myofibroblast formation. Our study thus elucidates our understanding of the signaling mechanisms underlying TGF-β_1-induced AF transformation.

Materials and Methods

Materials

All the experimental procedures were performed according to the guidelines for the care and use of laboratory animals established by our institute. Phorbol-12-myristate-13-acetate (PMA), calphostin C, TGF-β_1, β-actin and α-SMA antibodies were obtained from Sigma-Aldrich (St. Louis, Mo., USA), and PKCα antibody was from Santa Cruz. PDE1 inhibitor IC86340 and PDE1A antibody were provided by Dr. Yan (University of Rochester). Hoechst 33342 was from Invitrogen. PE rabbit-anti-mouse secondary antibody was obtained from Dako Cytomation (Denmark).

Cell Culture

AFs were isolated from thoracic aortas of 200-gram male Sprague-Dawley rats using the explant method and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (NCS), 100 U/ml penicillin and 100 μg/ml streptomycin, as described previously [15]. In brief, thoracic aortas of 3 or 4 rats were aseptically removed and cleaned of fat tissue and blood cells. The adventitia was cut into small pieces and placed in tissue dishes in DMEM containing 20% fetal bovine serum. Fibroblasts grew from these explants within 3–5 days. They were then subcultured in 10% NCS. Passage 3–5 cells were used during all the experiments. AFs were α-SMA negative, desmin positive and von Willebrand factor negative [16].

Western Blot Analysis

Following treatment, cells were washed twice with ice-cold PBS and then lysed in buffer containing protease inhibitors (10 mM Tris-HCl, pH 7.5, 150 mM NaCl; 0.1% SDS; 1.0% Triton X-100; 2 μg/ml aprotonin; 2 ng/ml leupeptin; 1 mM PMSF) on ice for 15 min and sonicated. Cell lysates were then centrifuged (13,000 rpm, 20 min at 4°C) and supernatant was preserved at 20°C for Western blot analysis. Protein concentrations were determined using the Bradford method. Aliquots of protein (20 μg) were subjected to SDS-PAGE as described. Proteins were then transferred to PVDF membranes and blocked with 5% skimmed milk/TBST for 2 h at room temperature. Membranes were incubated with primary antibodies directed against PDE1A (1:500), PKCα (1:1,000), α-smooth muscle actin (SMA; 1:5,000) and β-actin (1:5,000) at 4°C overnight. After washing membranes with TBST three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using ECL (Roche, Mannheim, Germany) and quantified by scanning densitometry.

Immunocytochemistry

AFs were growth arrested with medium containing 0.1% BSA for 24 h. Quiescent cells were pretreated with IC86340 or IBMX for 30 min, and then treated without (control) or with TGF-β_1 (10 ng/ml) for 24 h. After treatment, cells were fixed in ice-cold ethanol, permeabilized with 0.1% PBS-Triton for 15 min, blocked with 3% BSA and incubated with an anti-α-SMA antibody (1:200). Immunofluorescent staining of α-SMA was completed by incubating the cells with a PE rabbit anti-mouse IgG. Cellular nuclei were stained with Hoechst 33342. The specificity of primary antibody was confirmed either with the pre-immune serum with/without incubation in primary antibody. A Zeiss fluorescence microscope was used to visualize the stained cells, and photographs were taken by SDS software.

Statistics

Results are expressed as means ± SEM. Statistical evaluation of the data was performed by analysis of variance. Values were considered to be significantly different when $p < 0.05$.

Results

TGF-β_1 Upregulated PDE1A Protein in AFs

We first determined if TGF-β_1 upregulates PDE1A protein expression. Growth-arrested AFs by serum starvation for 24 h were treated with TGF-β_1 at various time points and subjected to Western blot analysis. As shown in figure 1a, PDE1A protein level was increased after 4 h of TGF-β_1 (10 ng/ml) stimulation, peaked at 24 h and declined thereafter. Subsequently, AFs were treated with various doses of TGF-β_1 for 24 h. As shown in figure 1b, TGF-β_1 upregulated PDE1A protein level in a dose-dependent manner.

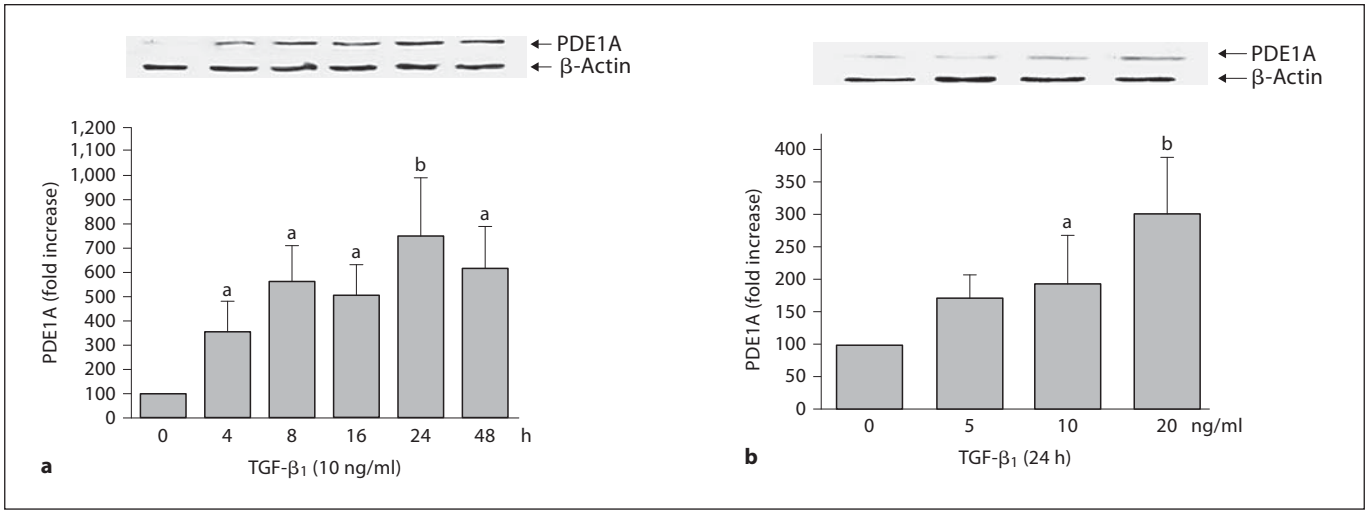

Fig. 1. TGF-β_1 upregulated PDE1A expression in rat aortic AFs. Growth-arrested rat aortic AFs were stimulated with 10 ng/ml TGF-β_1 for the indicated time points (**a**) or various doses of TGF-β_1 for 24 h (**b**). Protein levels of PDE1A were measured by Western blot analysis. β-Actin was used as loading control. Values are expressed as means ± SEM of triplicates from a representative experiment. Mean values for the percent change in PDE1A were calculated by comparing TGF-β_1-stimulated samples versus samples collected at time 0, or without TGF-β_1, which was arbitrarily set at 100% for each experiment. [a] $p < 0.05$, [b] $p < 0.01$, vs. samples at time 0 or without TGF-β_1. Similar results were obtained from at least three independent experiments.

PDE1A Is Involved in α-SMA Induction by TGF-β_1

To determine the role of PDE1A in AF transformation, we next assessed the effect of IC86340, a specific PDE1 inhibitor, on TGF-β_1-induced α-SMA protein expression. As shown in figure 2a, TGF-β_1-induced α-SMA expression was inhibited by IC86340 in a dose-dependent manner. The PDE1 family consists of three gene products, PDE1A, PDE1B and PDE1C. PDE1A is the main PDE1 isozyme expressed in AFs, whereas PDE1B and PDE1C are either not expressed or expressed at very low levels (data not shown). Therefore, the effect of IC86340 on α-SMA expression is likely due to its inhibitory effect on PDE1A. Furthermore, a general PDE inhibitor, IBMX, also inhibited TGF-β_1-induced α-SMA expression in a dose-dependent manner (fig. 2b). Similar results were also confirmed by performing immunocytochemical analysis in the same cell type (fig. 2c). To further understand whether the PDE1A location is associated with the phenotype of fibroblasts, we determined the subcellular localization of PDE1A induced by TGF-β_1 in AFs. PDE1A was primarily expressed in the cytoplasm of non-stimulated cells and translocated into the nucleus upon TGF-β_1 stimulation for 30 min (fig. 2d). Together, our data suggest that TGF-β_1 upregulates PDE1A protein expression, which may be involved in TGF-β_1 upregulation of α-SMA and adventitial myofibroblast formation.

The PDE1A-PKC Pathway Mediates α-SMA Induction by TGF-β_1

Due to the important role for PKCα in TGF-β_1-induced cellular responses [7], we next investigated if PKCα is involved in PDE1A-mediated α-SMA induction by TGF-β_1. We found that TGF-β_1 upregulated PKCα protein expression (fig. 3a). PMA, a PKC activator, induced α-SMA protein expression in a dose-dependent manner (fig. 3b). Calphostin C, a PKC inhibitor, inhibited TGF-β_1-induced α-SMA expression (fig. 3d). Treatment of the cells with either IBMX or IC86340 inhibited PKCα upregulation by TGF-β_1 (fig. 3c). Notably, cotreatment of the cells with both the PKC inhibitor calphostin C and the PDE1A inhibitor IC86340 did not further attenuate α-SMA induction compared to cells treated with calphostin C alone (fig. 3d), suggesting that PDE1A and PKC may act in the same pathway. These findings suggest that PDE1A may act upstream of PKCα in mediating α-SMA induction by TGF-β_1.

Discussion

Myofibroblasts, characterized by the appearance of the cytoskeletal protein α-SMA, contribute to vascular remodeling, in particular to the establishment of tension

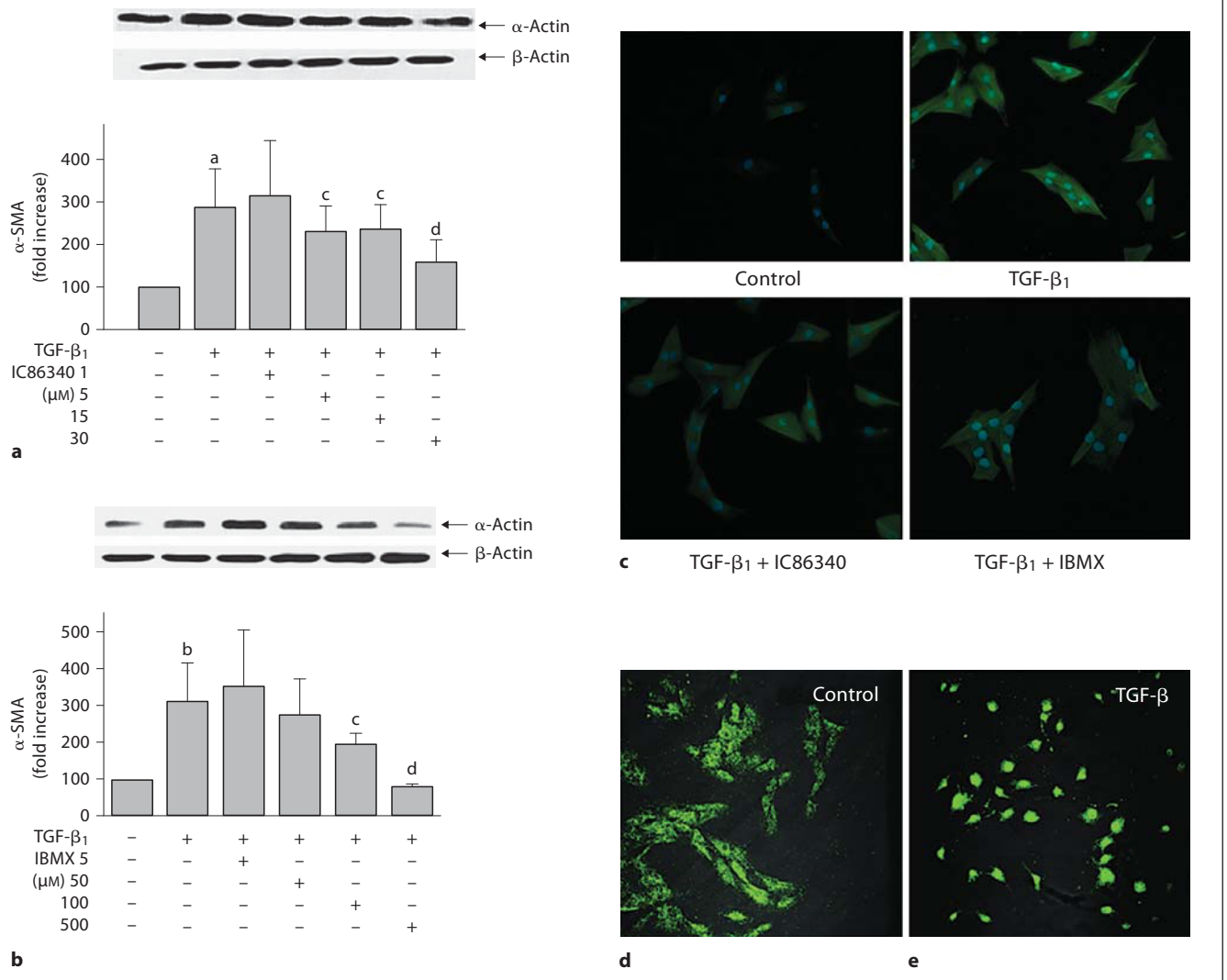

Fig. 2. IBMX and IC86340 inhibited α-SMA expression induced by TGF-β_1. Growth-arrested rat aortic AFs were pretreated with IC86340 (**a**) or IBMX (**b**) for 30 min in the presence or absence of 10 ng/ml TGF-β_1 for 24 h. Protein levels of α-SMA were measured by Western blot analysis (**a**, **b**) and by immunofluorescent staining using an anti-α-SMA antibody (**c**). Nuclei were stained with Hoechst 33342. ×200. **a**, **b** Mean values for the percent change in α-SMA were calculated in comparison to samples without TGF-β_1, which was arbitrarily set at 100% for each experiment. [a] $p < 0.05$, [b] $p < 0.01$, vs. TGF-β_1 at zero; [c] $p < 0.05$, [d] $p < 0.01$ vs. TGF-β_1 alone. **d** Representative microscopic images showing PDE1A localization in cells treated with vehicle (**d**) or 10 ng/ml TGF-β (**e**). PDE1A is localized in cytoplasmic regions in non-stimulated cells (**d**). PDE1A is primarily localized in the nucleus after TGF-β stimulation for 30 min (**e**).

and enhanced extracellular matrix [17]. Although a number of hormones and growth factors are known to induce formation of myofibroblast, little is known regarding effective approaches to attenuate this transformation process [4–6]. In our study, inhibition of PDE1A attenuated myofibroblast formation in rat AF and PKCα was an important signaling transducer in PDE1A-mediated AF transformation.

Of particular interest, TGF-β_1 upregulated PDE1A protein level in a time- and dose-dependent manner and PDE1A inhibition decreased α-SMA expression induced by TGF-β_1 in rat AF, possibly explaining, at least in part,

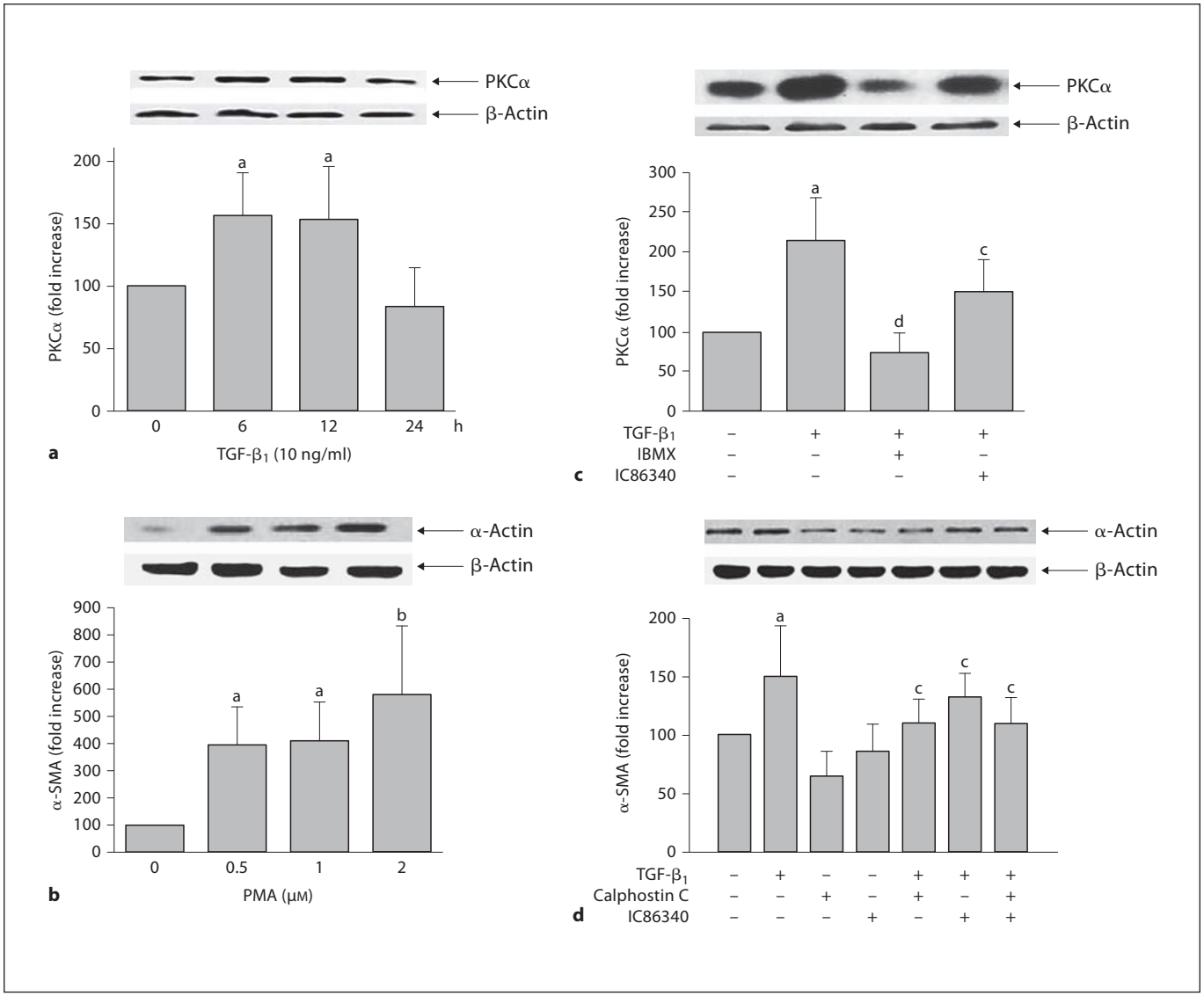

Fig. 3. PKCα was involved PDE1A-mediated myofibroblast formation induced by TGF-β_1. **a** TGF-β_1 upregulated PKCα protein expression in a time-dependent manner in rat aortic AF. Growth-arrested rat aortic AFs were stimulated with 10 ng/ml TGF-β_1 for 0, 6, 12 and 24 h. Protein levels of PKCα were measured by Western blot analysis. β-Actin was used as loading control. Values are expressed as means ± SEM of triplicates from a representative experiment. **b** PMA upregulated α-SMA in AFs. Growth-arrested rat aortic AFs were treated with different doses of PMA for 24 h. Protein levels of α-SMA were measured by Western blot analysis. Data are normalized under serum-free conditions. Values represent means ± SEM of at least three experiments and were compared using one-way ANOVA. **c** IC86340 and IBMX inhibited PKCα expression induced by TGF-β_1. Growth-arrested rat aortic AFs were pretreated with IC86340 or IBMX for 30 min in the presence or absence of 10 ng/ml TGF-β_1 for 12 h. Protein levels of PKCα were measured by Western blot analysis. **d** Effects of the PKC inhibitor calphostin C and the PDE1A inhibitor IC86340 on α-SMA expression. AFs were pretreated with 100 μM calphostin C or 15 μM IC86340 or both of them for 30 min and then treated with 10 ng/ml TGF-β_1 for 24 h. Calphostin C or IC86340 alone inhibited α-SMA protein expression. When used together, no difference was observed on the inhibitory effect compared with calphostin C or IC86340 alone. Mean values for the percent change in PKCα or α-SMA were calculated in comparison to samples without TGF-β_1 or PMA, which was arbitrarily set at 100% for each experiment. [a] $p < 0.05$, [b] $p < 0.01$, vs. TGF-β_1 or PMA at zero; [c] $p < 0.05$, [d] $p < 0.01$, vs. TGF-β_1 alone.

how TGF-β_1 antagonizes cGMP signaling via upregulating PDE1A and in turn downregulating the cGMP level. Previously, Kim et al. [18] reported that angiotensin II increased PDE5A expression in VSMCs, providing a possible explanation regarding how angiotensin II antagonizes cGMP signaling. To our knowledge, this is the first report showing that TGF-β_1 upregulates PDE1A expression, and that inhibition of PDE1A represents a novel mechanism by which cGMP antagonizes TGF-β_1 signaling pathways. Thus, our study elucidates our understanding of signaling mechanisms underlying TGF-β_1-induced adventitial transformation.

PDE1A preferentially hydrolyzes cGMP, which plays an important role in the regulation of a variety of cellular responses, e.g. proliferation, apoptosis, transformation, extracellular matrix expression and vascular tone [19–25]. Previous data showed that two cGMP-elevating agents, the PDE5 inhibitor sildenafil and L-arginine, both reduced α-SMA expression in fibroblasts cultured from patients with Peyronie's disease [8]. Kapoun et al. [12] also reported that in primary human cardiac fibroblasts brain natriuretic peptide opposed TGF-β_1-regulated genes related to myofibroblast conversion including α-SMA via cGMP signaling [10–12]. Consistent with these observations, our data demonstrated that PDE1A inhibition blunted α-SMA expression in AF, suggesting that this effect is possibly due to elevated cGMP levels.

In our previous study, subcellular localization of PDE1A was associated with VSMC phenotype [14]. The cytoplasmic PDE1A is associated with the contractile phenotype whereas the nuclear PDE1A is associated with the synthetic phenotype of VSMC. It is known that synthetic VSMCs are vascular myofibroblast-like cells, which are very similar to adventitial myofibroblasts. Therefore, we examined PDE1A localization in AFs in response to TGF-β_1 stimulation and found that PDE1A is located in both cytoplasmic and nuclear regions in non-stimulated cells. TGF-β_1 stimulation causes a quick accumulation of PDE1A in the nucleus within 30 min after TGF-β_1 treatment, suggesting that TGF-β_1 stimulates PDE1A translocation to the nucleus even before upregulating PDE1A expression, and nuclear PDE1A plays a critical role in regulating α-SMA expression during the phenotypic modulation of AFs.

Another novel finding is that PDE1A signaling was involved in TGF-β_1-induced expression of PKCα protein. As shown in figure 3, inhibiting PDE1A activity by IC86340 attenuated PKCα upregulation by TGF-β_1, thereby suggesting that PDE1A may act upstream of PKCα in mediating α-SMA induction by TGF-β_1. This finding may also improve our understanding of PDE1A-mediated cell signaling mechanisms. Future studies will focus on determining the therapeutic potential of blocking PDE1A signaling in vascular diseases related to adventitial transformation.

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