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Adrenomedullin and adrenotensin regulate collagen synthesis and proliferation in pulmonary arterial smooth muscle cells

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Abstract

To understand the pathophysiological mechanisms of pulmonary arterial smooth muscle cell (PASMC) proliferation and extracellular-matrix accumulation in the development of pulmonary hypertension and remodeling, this study determined the effects of different doses of adrenomedullin (ADM) and adrenotensin (ADT) on PASMC proliferation and collagen synthesis. The objective was to investigate whether extracellular signal-regulated kinase (ERK1/2) signaling was involved in ADM- and ADT-stimulated proliferation of PASMCs in 4-week-old male Wistar rats (body weight: 100-150 g, n = 10). The proliferation of PASMCs was examined by 5-bromo-2-deoxyuridine incorporation. A cell growth curve was generated by the Cell Counting Kit-8 method. Expression of collagen I, collagen III, and phosphorylated ERK1/2 (p-ERK1/2) was evaluated by immunofluorescence. The effects of different concentrations of ADM and ADT on collagen I, collagen III, and p-ERK1/2 protein expression were determined by immunoblotting. We also investigated the effect of PD98059 inhibition on the expression of p-ERK1/2 protein by immunoblotting. ADM dose-dependently decreased cell proliferation, whereas ADT dose-dependently increased it; and ADM and ADT inhibited each other with respect to their effects on the proliferation of PASMCs. Consistent with these results, the expression of collagen I, collagen III, and p-ERK1/2 in rat PASMCs decreased after exposure to ADM but was upregulated after exposure to ADT. PD98059 significantly inhibited the downregulation by ADM and the upregulation by ADT of p-ERK1/2 expression. We conclude that ADM inhibited, and ADT stimulated, ERK1/2 signaling in rat PASMCs to regulate cell proliferation and collagen expression.

Key words: Adrenomedullin; Adrenotensin; Pulmonary arterial smooth muscle cell; Extracellular signal-regulated kinase-1; Collagen

Introduction

Increased pulmonary blood vessel thickness and stenosis are important pathophysiological components of pulmonary vascular remodeling (1-3). Pulmonary artery smooth muscle cell (PASMC) hypertrophy and hyperplasia, along with the accumulation of collagen and other extracellular-matrix components in the vascular wall, are major contributors to vascular remodeling.

Adrenomedullin (ADM) is a vasoactive polypeptide. Proadrenomedullin (proADM), the precursor to ADM, is cleaved by an endogenous peptidase, peptidylhydroxyglycine α-amidating lyase, into four different active peptides, each of which has a distinct distribution and function (4,5). Among them, ADM and adrenotensin (ADT) are distributed throughout the vascular smooth

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muscle cell layer, whereas proADM N-terminal 20 peptide (PAMP) is found within the tunica adventitia. ADM stimulates blood vessel dilation and inhibits smooth muscle cell proliferation and migration (6,7). ADM activates the potassium channel in smooth muscle cells to enable cellular hyperpolarization (8). Through binding to the CGRP1 receptor, ADM further elevates cAMP levels in vascular smooth muscle cells and expands blood vessel diameter.

ADT is a vasoconstrictive agent that elevates blood pressure and increases the proliferation and migration of vascular smooth muscle cells (9). The mitogen-activated protein kinase (MAPK) intracellular signaling pathway modulates cell proliferation and is induced by a diverse collection of stimuli (10-12).

We have previously explored the distribution of SAPK/JNK, P38, and ERK1/2 in the lungs in a pulmonary hypertension rat model. We found that ERK1/2 pathway activation was the most robustly stimulated. In addition, vascular smooth muscle cell proliferation closely correlated with MAPK signaling and ERK1/2 in particular (13). For these reasons, we focused on ERK1/2 signaling in this study.

In the present study, we evaluated the effects of ADM and ADT on PASMC proliferation, as well as collagen I, collagen III, and phosphorylated (p)-ERK1/2 expression, to determine if the ERK1/2 signal transduction pathway was activated during the response.

Material and Methods

Animals and cell culture

Four-week-old male Wistar rats (body weight: 100-150 g, n = 10) were purchased from the Laboratory Animal Center of Medical School, Shandong University. The Shandong University Institutional Animal Care and Use Committee approved animal care and procedures. Animal use was in accordance with National Institutes of Health and institutional guidelines. Animals were killed using intravenous anesthesia. This study was carried out from June 2011 to February 2012.

In this study, we used an adherent tissue explant method for the primary culture of rat PASMCs that is simple and economical. Although this method requires a long culture period, the cells grow well and produce abundant cells after passaging (14). As determined using a mouse monoclonal smooth muscle anti-α-actin antibody (Sigma-Aldrich, USA), the purity of the cultured cells approached 87%. Although primary culture of cells from younger rats was often successful, the target cells were difficult to isolate because the vessels were small and thin. Older rats were not suitable for primary culture because of the low viability of their cells. Therefore, we selected healthy 1-month-old male rats, weighing 100-150 g. Since pulmonary vascular remodeling mainly occurs in distal pulmonary arterioles and manifests as smooth muscle cell hyperplasia, medial hypertrophy, and muscularization of pulmonary arterioles with microthrombosis and luminal stenosis, the PASMCs used in the current study were harvested from the distal pulmonary arterioles.

Lungs harvested from animals that had been anesthetized with 2% pentobarbital were rinsed twice in PBS and then immersed in 75% ethanol for 3 min. The blood vessels from the lungs were opened lengthwise, and the medial smooth muscle was exposed by removing the intima using tip-curved forceps (15,16). The vascular media was cut into 1-mm^3 blocks and transferred to 50-mL culture flasks, so that 25-30-tissue blocks were dispersed uniformly on the bottom of each flask. High-glucose (4500 mg/L) DMEM containing 20% fetal bovine serum was added to the flasks that were placed inverted in an incubator (37°C, 5% CO_2-95% air and controlled humidity) for 3-6 h and then righted and cultured for 4-7 days. At that time, cell growth was observed using an inverted microscope (17,18).

Immunofluorescent characterization of PASMCs

The homogeneity of the cultured cells was confirmed by immunofluorescence using a monoclonal antibody against smooth muscle α-actin (Sigma-Aldrich). Trypsinized cells from passage four were seeded onto 6-well plates (20,000 cells per well) and cultured at 37°C in a 5% CO_2 incubator for 24 h, until confluence. The cells were then incubated with a mouse anti-α-actin smooth muscle monoclonal antibody (1:500) for 1 h at 37°C in a humidified chamber. The blank control was incubated with 10 mM PBS. The cells were then incubated for 1 h at 37°C with an FITC-labeled goat anti-mouse IgG antibody (1:500; Abcam, UK). Finally, the cells were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 1-2 min, sealed with sealing liquid resistant to fluorescence quenching, and then examined under fluorescence microscopy (19).

5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

Cells were exposed to 1 μM, 0.1 μM, or 10 nM ADM and/or ADT for 48 h (9). Unstimulated controls were not exposed to either agent. After 48 h, BrdU was added to a final concentration of 10 μM, and the cells were incubated for a further 3 h at 37°C. Then, the cells were fixed with 4% formalin and preserved overnight at 4°C in a humidified chamber. The cells were subsequently incubated with rabbit anti-BrdU antibody (Abcam) at 4°C overnight (20).

Cell growth analysis

Cells were added to 96-well plates, with 5000 cells per well. Assays were done in quadruplicate. Cell Counting Kit-8 (CCK8; 10 μL per 100-μL reaction system) was added to each well. After 4 h in culture, the cells were incubated for 2 h in 5% CO_2 at 37°C. Cell proliferation

was monitored by determining the absorbance at 450 nm using a GENios Pro fluorescence detector (Tecan, USA). Measurements were recorded every 24 h, beginning at 12 h after treatment, for a total of 156 h.

Immunofluorescence of collagen I, collagen III, and p-ERK1/2

Cells were divided into the following treatment groups (n = 10 wells): 1) 0.1 µM ADM or ADT and rabbit anti-collagen I antibody (1:200; Abcam); 2) 0.1 µM ADM or ADT and rabbit anti-collagen III antibody (1:100; Abcam); 3) 0.1 µM ADM or ADT and rabbit p-ERK antibody (1:200); 4) 0.1 µM ADM or ADT and 10 mM PBS (control). The cells were cultured for 48 h, fixed in 4% paraformaldehyde, and blocked with goat serum. They were then incubated with the above-mentioned primary antibodies and fixed at 4°C in a wet box overnight. Cells incubated with 10 mM PBS served as the control for the antibody staining. Next, the cells were treated with FITC-labeled goat anti-rabbit IgG (1:100), incubated at 37°C in a wet box for 30 min, and stained in DAPI for 1-2 min. Five visual fields in each slide were randomly selected under a fluorescence microscope (400 × magnification) (10,21). The images were processed using the Image-Pro Plus 6.0 (Media Cybernetics, USA) Image Processing Software to obtain the absorbance values under the same time of exposure.

Immunoblotting analysis

Immunoblotting was performed as described previously (22). To identify the intracellular signaling pathways used by ADM and ADT for regulation of proliferation and collagen synthesis of cultured PASMCs, 10 µM PD98059, a cell-permeable ERK/MAPK inhibitor, was added to the cultured PASMCs 30 min prior to ADM or ADT treatment. All the procedures were performed on ice or at 4°C, and all the solutions were precooled. At the regulated time points, cells were harvested (adherent cells were trypsinized), rinsed twice in PBS buffer, transferred to microfuge tubes, and centrifuged at 1000 *g* for 5 min. After the supernatant was removed, the cell pellet was resuspended in cell lysate buffer, incubated in an ice bath for 40 min, and centrifuged at 18,000 *g* for 10 min. The collected supernatant contained the total cell protein.

We separated 20 µg total protein by 12% SDS-PAGE, and then transferred the proteins to a nitrocellulose membrane (Hybond™, ECLTM, Amersham Pharmacia, UK). After blocking for 2 h in Tris buffer supplemented with 0.05% Tween-20 (TBS-T) and 5% nonfat milk, the membrane was incubated with the corresponding primary antibody (Abcam) at 4°C overnight, rinsed in TBS-T three times for 10 min, and then incubated with the secondary antibody (Abcam) at room temperature for 2 h. The membrane was rinsed again in TBS-T three times for 10 min, and visualized using an ECL Chemiluminescence system (Bio-Rad, USA).

Statistical analysis

Data are reported as means ± SD. Comparison between groups was analyzed using ANOVA, and the Bonferroni test was used when $P<0.05$. Data were processed using the SPSS 15.0 software (SPSS, USA), and $P<0.05$ was considered to be statistically significant.

Results

Pulmonary artery smooth muscle cell characterization

PASMCs were identified as smooth muscle cells by positive staining with mouse anti-α-actin antibody and FITC-labeled goat anti-mouse antibody (Figure 1). The cell cytoplasm was stained with FITC, shown in green (Figure 1 A1), and cell nuclei were stained with DAPI, shown in blue (Figure 1 A2). The image in Figure 1 A3 is Figure 1 A1 merged with Figure 1 A2. The mean homogeneity of the cells approached 87%.

Effect of ADM and ADT on the proliferation of cultured PASMCs

Both the BrdU incorporation test (Figure 2) and the CCK8 test (Figure 3) showed that ADM, when used at concentrations ranging from 1 µM to 10 nM, inhibited the proliferation of treated PASMCs, which showed a significant

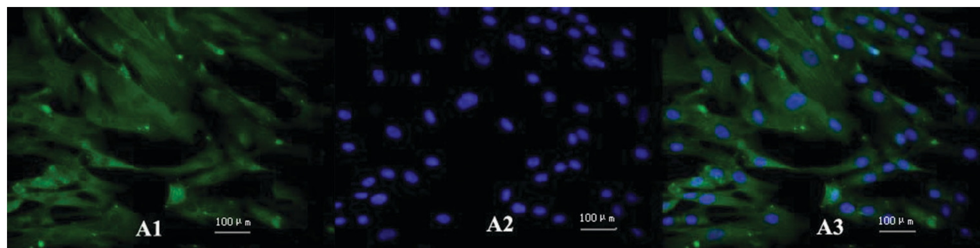

Figure 1. Identification of rat pulmonary arterial smooth muscle cells using a mouse anti-human smooth muscle α-actin monoclonal antibody. The images were acquired using an inverted phase contrast microscope (200 × magnification). *A1*, The cell cytoplasm was stained with FITC (green). *A2*, The cell nuclei were stained with DAPI (blue). *A3*, A1 merged with A2.

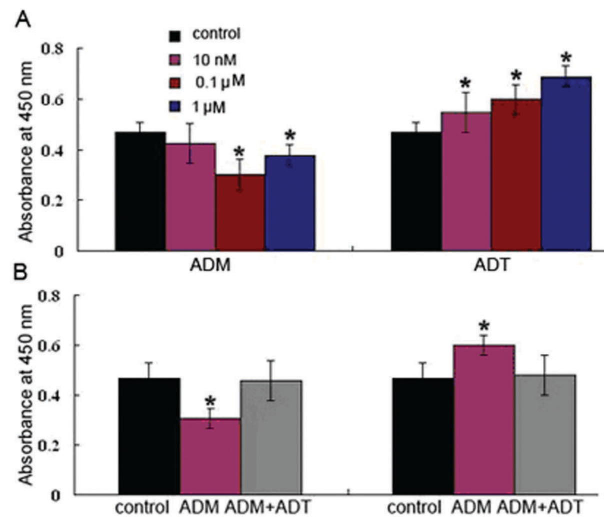

Figure 2. *A*, Effect of increasing concentrations of ADM or ADT on cell proliferation in cultured PASMCs. Cell proliferation was assayed with BrdU incorporation. Data are reported as mean ± SE absorbance for n = 6 rats per group. *$P < 0.05$ *vs* control (one-way ANOVA). *B*, Effect of 0.1 μM ADM, ADT, or ADM + ADT on the cell proliferation of cultured rat PASMCs. Cell proliferation was assayed with BrdU incorporation. PASMCs: pulmonary arterial smooth muscle cells; ADM: adrenomedullin; ADT: adrenotensin. Data are reported as mean ± SE absorbance for n = 6 rats per group. *$P < 0.05$ *vs* control (one-way ANOVA).

decrease in proliferation compared to the unstimulated control (BrdU, $P < 0.05$; CCK8, $P < 0.01$). In contrast, ADT upregulated proliferation in treated PASMCs at concentrations of 1 μM, 0.1 μM, and 10 nM (BrdU, $P < 0.01$; CCK8, $P < 0.05$). Simultaneous addition of 0.1 μM ADM and 0.1 μM ADT did not affect cell proliferation (no statistical difference compared with the control; $P > 0.05$), indicating that ADM and ADT exhibit reciprocal inhibition effects on the proliferation of cultured PASMCs.

Effect of 0.1 μM ADM and 0.1 μM ADT on collagen I expression in cultured PASMCs

The cells in Figure 4(a) A1, treated with ADM and stained for collagen I, show low fluorescence, indicating low collagen I expression; whereas those in Figure 4(a) B1, treated with ADT and also stained for collagen I, show strong fluorescence, indicating high collagen I expression. The cell nuclei for the two groups of cells (Figure 4(a) A2 and 4(a) B2, respectively) are shown with blue fluorescence. The findings indicate that ADM inhibited, whereas ADT upregulated, collagen I expression in cultured PASMCs.

Effect of 0.1 μM ADM and 0.1 μM ADT on the expression of collagen III in cultured PASMCs

The cells in Figure 4(b) A1, treated with ADM and stained for collagen III, show low fluorescence, indicating low collagen III expression, whereas those in Figure 4(b) B1, treated with ADT and stained for collagen III, show high fluorescence, indicating high collagen III expression. The cell nuclei in both groups [Figure 4(b) A2 and 4(b) B2] were stained with blue fluorescence. Figure 4(b) A3 and Figure 4(b) B3 are Figure 4(b) A1 merged with Figure 4(b) A2 and Figure 4(b) B1 merged with Figure 4(b) B2, respectively. These findings indicate that ADM inhibited the expression of collagen III in PASMCs, whereas ADT enhanced it.

Effect of 0.1 μM ADM and 0.1 μM ADT on the expression of p-ERK1/2 in cultured PASMCs

The cells in Figure 4(c) A1, treated with ADM and stained for p-ERK1/2, show low fluorescence, indicating low p-ERK1/2 expression, whereas those in Figure 4(c) B1, treated with ADT and stained for p-ERK1/2, show high fluorescence, indicating high p-ERK1/2 expression. Figure 4(c) A2 and Figure 4(c) B2 show the nuclei with blue fluorescence. Figure 4(c) A3 and Figure 4(c) B3 are

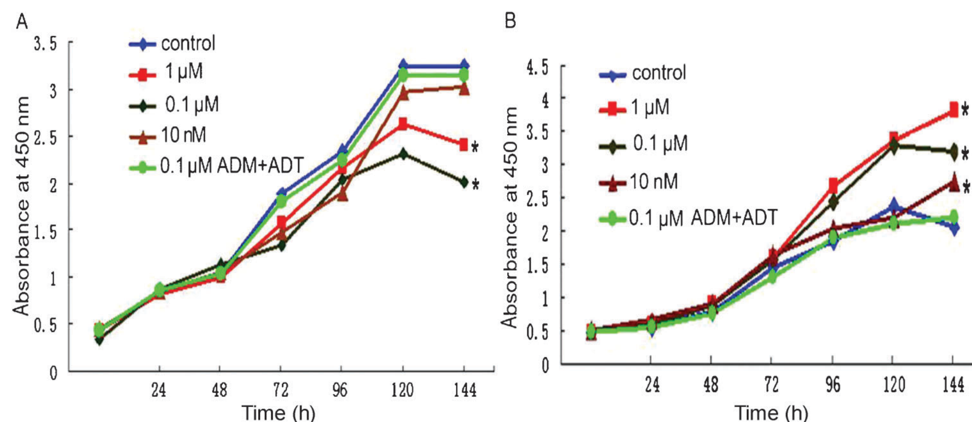

Figure 3. Proliferation curve of cultured PASMCs by the CCK8 test. *A*, Increasing concentrations of ADM or 0.1 μM ADM + ADT. *B*, Increasing concentrations of ADT or 0.1 μM ADM + ADT. PASMCs: pulmonary arterial smooth muscle cells; ADM: adrenomedullin; ADT: adrenotensin. Data are reported as mean ± SE absorbance for n = 6 rats per group. *$P < 0.05$ *vs* control (one-way ANOVA).

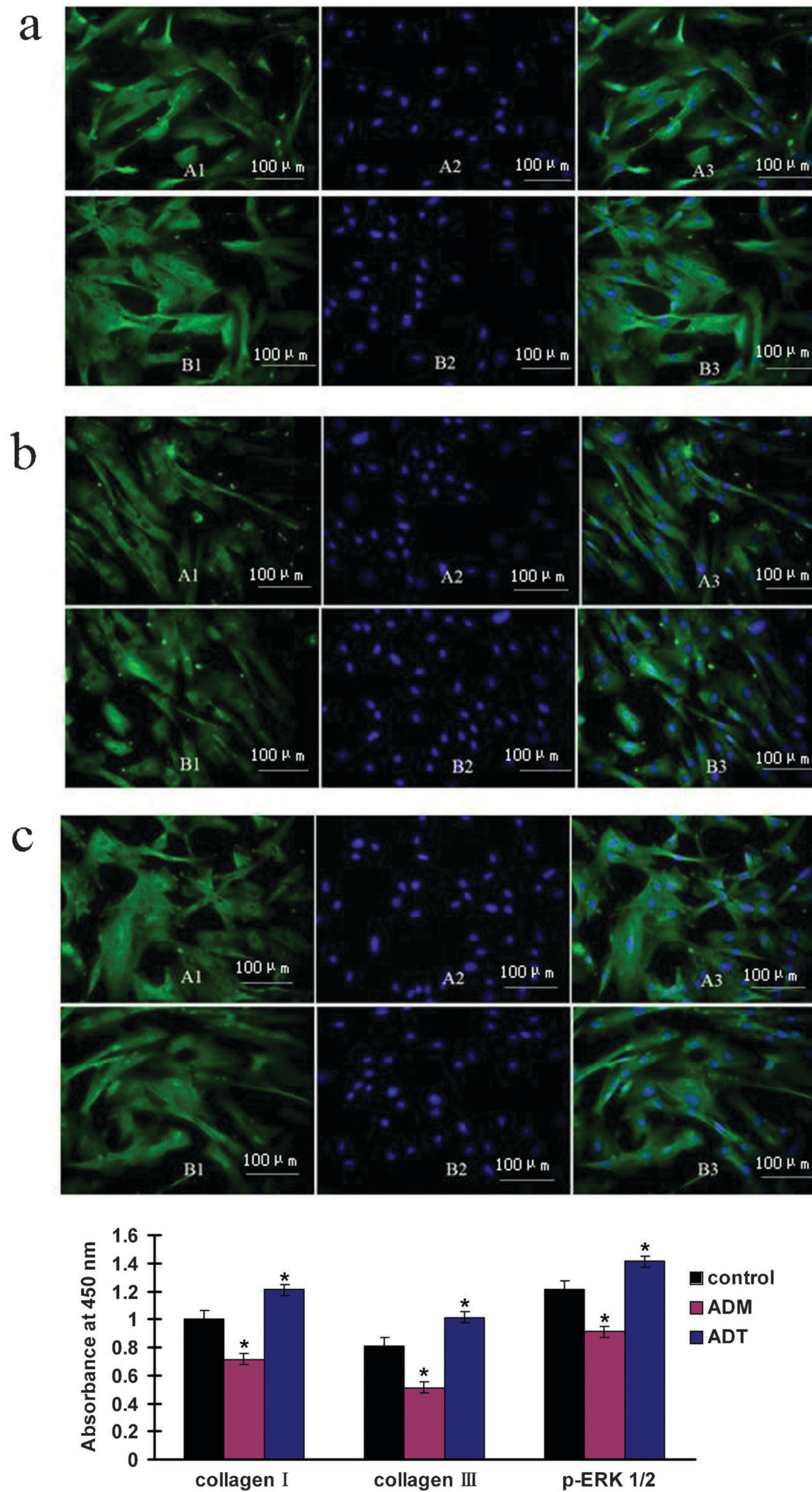

Figure 4. Protein expression of collagen I, collagen III and p-ERK1/2 in cultured PASMCs. *Panels a, b, and c*, Influence of 0.1 μM ADM (A1-A3) and ADT (B1-B3) on the expression of (*a*) collagen type I (*b*) collagen type III and (*c*) p-ERK1/2 in cultured PASMCs (400 × magnification). The cell cytoplasm was stained with FITC (green; A1 and B1). The cell nuclei were stained with DAPI (blue; A2 and B2). A3 and B3 represent the overlapped images of A1 merged with A2 and B1 merged with B2, respectively. *Bottom panel*, Summary data showed the influence of 0.1 μM ADM and ADT on the expression of collagen I, collagen III, and p-ERK1/2 in cultured PASMCs by immunofluorescence. PASMCs: pulmonary arterial smooth muscle cells; ADM: adrenomedullin; ADT: adrenotensin. Data are reported as mean ± SE absorbance for n = 10 rats per group. *$P < 0.05$ *vs* control (one-way ANOVA).

Figure 4(c) A1 merged with Figure 4(c) A2 and Figure 4(c) B1 merged with Figure 4(c) B2, respectively. These images indicate that ADM inhibited the expression of p-ERK1/2 in PASMCs, whereas ADT enhanced it. The primary antibody control showed no staining, indicating that the primary antibody was specific.

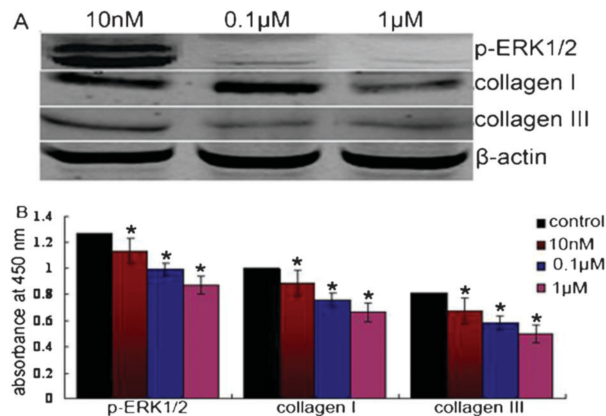

Figure 5. *A*, Effect of different concentrations of ADM on the protein expression of collagen I, collagen III, and p-ERK1/2 in cultured PASMCs by immunoblotting. β-actin was used as an internal control. *B*, Effect of stimulation of cultured PASMCs with 10 nM, 0.1 μM, 1 μM ADM on the expression of collagen I, collagen III, and p-ERK1/2. PASMCs: pulmonary arterial smooth muscle cells; ADM: adrenomedullin. Data are reported as mean ± SE absorbance for n = 6 rats per group. *P<0.05 *vs* control (one-way ANOVA).

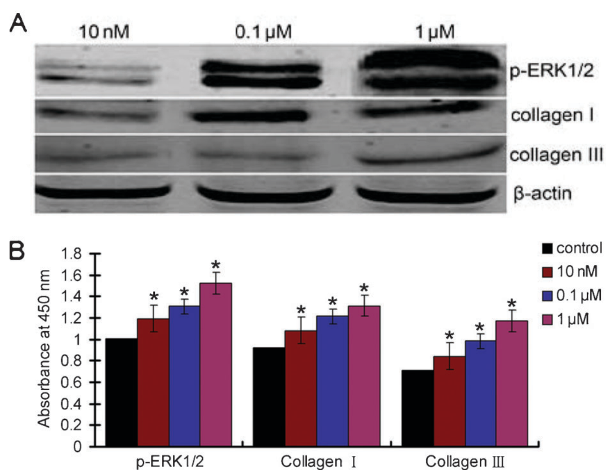

Figure 6. *A*, Effect of different concentrations of ADT on the protein expression of collagen I, collagen III, and p-ERK1/2 in cultured PASMCs by immunoblotting. β-actin served as an internal control. *B*, Summary data of the influence of 10 nM, 0.1 μM, and 1 μM ADT on the expression of collagen I, collagen III, and p-ERK1/2 in cultured PASMCs. PASMCs: pulmonary arterial smooth muscle cells; ADT: adrenotensin. Data are reported as mean ± SE absorbance for n = 6 rats per group. *P<0.05 *vs* control (one-way ANOVA).

Summary analysis of the effects of 0.1 μM ADM and 0.1 μM ADT on the expression of collagen I, collagen III, and p-ERK1/2 in cultured rat PASMCs

The absorbance ratio (Figure 4, bottom panel) showed that ADM inhibited the expression of collagens I and III and p-ERK1/2 in cultured rat PASMCs (P<0.01), whereas ADT enhanced them (P<0.05).

Effect of ADM and ADT on the expression of collagen I, collagen III, and p-ERK1/2 protein in cultured PASMCs by immunoblotting

Absorbance results showed that ADM inhibited the expression of collagen I and collagen III protein and

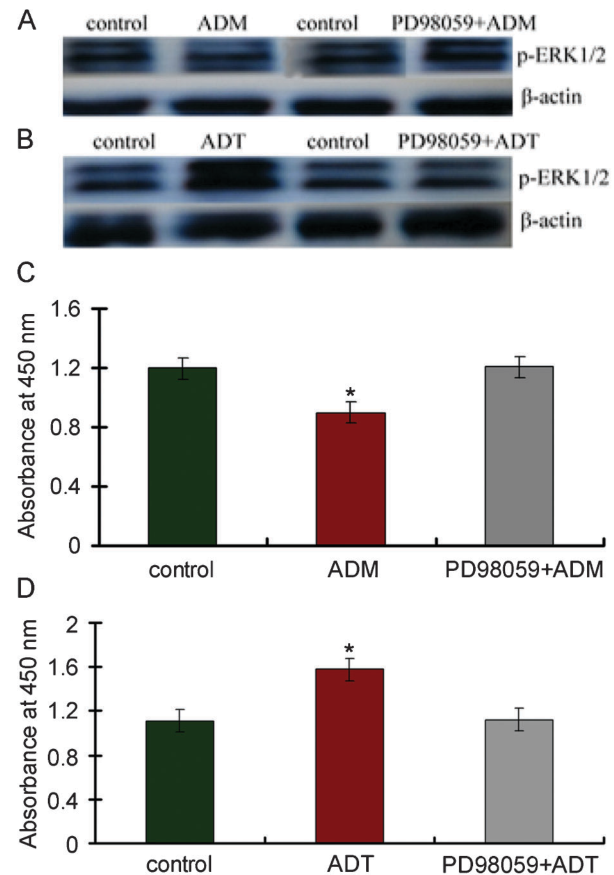

Figure 7. Effect of PD98059 on p-ERK1/2 protein expression of cultured PASMCs stimulated by (*A*) 0.1 μM ADM or (*B*) 0.1 μM ADT, shown by immunoblotting. β-actin served as an internal control. Summary data of the influence of PD98059 on the expression of p-ERK1/2 in cultured PASMCs stimulated by (*C*) 0.1 μM ADM or (*D*) 0.1 μM ADT. PASMCs: pulmonary arterial smooth muscle cells; ADM: adrenomedullin; ADT: adrenotensin. Data are reported as mean ± SE absorbance for n = 6 rats per group. *P<0.05 *vs* control (one-way ANOVA).

decreased p-ERK1/2 protein in a dose-dependent manner ($P<0.05$, $P<0.05$, and $P<0.01$, respectively; Figure 5). In contrast, ADT increased protein expression for collagen I, collagen III, and p-ERK1/2 in a dose-dependent manner ($P<0.05$, $P<0.05$, and $P<0.01$, respectively; Figure 6). These results indicate that the ERK1/2 signaling pathway was activated, concomitant with the increase in proliferation, by ADT stimulation, and that, in contrast, ERK1/2 signaling was inhibited and cell proliferation was decreased by ADM stimulation.

Cellular mechanisms of ADM and ADT on proliferation and collagen synthesis in cultured PASMCs

Absorbance showed that the cell-permeable ERK/MAPK inhibitor PD98059 (10 μM) significantly inhibited the downregulating effect of ADM and the upregulating effect of ADT on p-ERK1/2 protein expression ($P<0.05$; Figure 7), indicating that ADM and ADT may regulate the proliferation and collagen synthesis of PASMCs through the ERK1/2 signaling pathway.

Discussion

In the present study, we provide new evidence that ADM and ADT exhibit reciprocal effects on the proliferation of cultured rat PASMCs. ADM exerted an inhibitory effect, and ADT, a stimulatory effect, on the proliferation of cultured rat PASMCs, particularly at a concentration of 0.1 μM. ADM is a vasoactive polypeptide that functions to dilate blood vessels and inhibit vascular smooth muscle cell proliferation and migration by interacting with specific receptors (23).

As previously reported, ADM and ADT participate in hypoxia-induced pulmonary vascular remodeling (24,25). A recent animal study revealed that ADM could delay and reverse pulmonary vascular remodeling, which suggested an encouraging therapeutic potential (2). It was also demonstrated that inhaled or intravenously administered ADM could decrease the pulmonary blood pressure of patients with pulmonary hypertension and reverse pulmonary vascular hypertrophy (26). These effects indicated the clinical significance of ADM.

In contrast to ADM, ADT induces blood vessel constriction and promotes the proliferation of vascular smooth muscle cells. ADT also stimulates synthesis and secretion of extracellular matrix in cultured rat mesangial cells by interacting with a receptor that has not yet been identified (9). The present study builds on these past reports to extend the functions of ADM and ADT to PASMCs.

The BrdU incorporation test and CCK8 assay both showed that ADM, especially at a concentration of 0.1 μM, inhibited PASMC proliferation, possibly by inhibiting DNA synthesis and mitosis. It was also shown that ADT stimulated PASMC proliferation in a dose-dependent manner, possibly also through the promotion of DNA synthesis and mitosis. Hypertrophy of the pulmonary vascular wall and luminal stenosis caused by the proliferation of PASMCs is an important influence in development of pulmonary hypertension and pulmonary vascular remodeling (27).

Collagen I and III expression in rat PASMCs decreased after exposure to ADM, but increased after exposure to ADT. ADM may indirectly influence collagen expression by reducing the number of PASMCs by inhibiting cell proliferation. ADT, however, may enhance the mRNA expression of procollagen I and III in PASMCs at the transcription level, which would serve to upregulate the synthesis of collagen I and III proteins (28). Additionally, ADT may impair collagen degradation, thereby increasing the total content of collagen I and III proteins in rat PASMCs. Collagen is a major component of the extracellular matrix; collagen I and III are present in vascular walls. Collagen helps to determine vascular tension resistance and elasticity (29). The accumulation of collagen protein in the vessel wall can increase vessel thickness and stiffness, playing an important role in hypoxia-induced pulmonary vascular remodeling (8). Collagen is located outside the cell, thus the collagen expression seen inside the cell in this study may be newly synthesized collagen that has not yet been excreted.

As previously reported, the addition of ADM to cultured smooth muscle cells could inhibit cell migration, excessive aggregation of microtubules, and excessive stabilization of the cytoskeleton, thus delaying or suppressing hypertrophy, hyperplasia, and migration of smooth muscle cells (30). In addition, ADM can induce smooth muscle cells into hyperpolarization by activating cation channels, causing a decrease in intracellular calcium. ADM can also activate phosphatidylinositol-3 kinase and protein kinase B/Akt, enhancing the activity of endothelial nitric oxide synthase via phosphorylation (31). ADM has also been shown to modify endothelial progenitor cells within the pulmonary tissue of rats with pulmonary hypertension, which could decrease the pulmonary blood pressure markedly and inhibit smooth muscle cell remodeling (32,33).

The present study showed that the expression of p-ERK1/2 in PASMCs changed after exposure to ADM and ADT. The cell-permeable ERK/MAPK inhibitor PD98059 blocked the downregulatory effect of ADM and the upregulatory effect of ADT on the expression of p-ERK1/2, indicating that ADM and ADT regulate the proliferation and collagen synthesis of cultured PASMCs through the ERK1/2 signaling pathway. Activated ERK1/2 can initiate and upregulate the proliferation genes for PASMCs via nuclear translocation, leading to the proliferation of the cells. ADM can also directly inhibit the ERK/MAPK signaling pathway, decreasing production of downstream kinases related to proliferation and secretion

of extracellular matrix, thus inhibiting cell differentiation and proliferation by suppressing the functionality of transcription factors (32). In contrast, ADT may regulate the ERK/MAPK signaling pathway through a mechanism opposite to that of ADM. Although ADT and ADM are derived from the same precursor, they have opposing biological functions, which are necessary for maintaining stability and equilibrium in the ERK/MAPK signal transduction pathway.

In conclusion, our results indicate that therapeutic agents that enhance ADM activity while inhibiting ADT effects may provide a novel strategy for the control of pulmonary vascular remodeling.

Acknowledgments

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