ORIGINAL RESEARCH

Endostatin attenuates PDGF-BB- or TGF-β1-induced HSCs activation via suppressing RhoA/ROCK1 signal pathways

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Aim: To testify the hypothesis that endostatin exerts antifibrotic effects in hepatic stellate cells (HSCs) by modulating RhoA (ras homolog gene family, member A)/ROCK 1 (Rho-associated protein kinase 1) signal pathways.

Materials and methods: HSCs-T6 of passages 3–5 were cultured in DMEM and serum starved for 48 hours. HSCs were grouped as follows: control group, TGF-β1 (transforming growth factor β1) group, endostatin+TGF-β1 group, PDGF-BB (platelet-derived growth factor-BB) group, and endostatin+PDGF-BB group. In the PDGF-BB group, HSCs were treated with PDGF-BB (200 ng/mL) for 72 hours; in the TGF-β1 group, they were treated with TGF-β1 (10 ng/mL) for 72 hours. In the Endostatin+TGF-β1 group or Endostatin+PDGF-BB group, HSCs were treated with TGF-β1 (10 ng/mL) or PDGF-BB (200 ng/mL) for 72 hours after pretreatment with endostatin (5 µg/mL) for 1 hour. In the control group, HSCs were only treated with serum-free DMEM for 72 hours. Collagen I was analyzed with ELISA. F-actin was detected with immunofluorescent staining. The mRNAs and proteins of α-smooth muscle actin, RhoA, and ROCK1 were analyzed by using real-time PCR and Western blot, respectively.

Results: TGF-β1 and PDGF-BB promote the proliferation of HSCs significantly at 48 and 72 hours. Endostatin inhibits the proliferation effect induced by TGF-β1 or PDGF-BB significantly ($P<0.01$). The expression of collagen I and F-actin was significantly upregulated in both TGF-β1 and PDGF-BB groups than in the control group ($P<0.01$). Both the collagen I and F-actin expression were downregulated significantly in the endostatin-treated groups ($P<0.05$). Endostatin significantly inhibited the upregulated expression of α-smooth muscle actin, RhoA, and ROCK1 induced by TGF-β1 or PDGF-BB ($P<0.01$).

Conclusion: These results suggested that endostatin inhibited TGF-β1- or PDGF-BB-induced fibrosis in HSCs by modulating RhoA/ROCK signal pathways.

Keywords: endostatin, liver fibrogenesis, hepatic stellate cell, signal pathways, fibrosis

Introduction

Hepatic fibrosis is a worldwide health care burden with excessive synthesis and deposition of extracellular matrix (ECM),[1] which results in high mortality and morbidity. Unfortunately, no ideal therapies are effective in clinical application Therefore, the research for treating liver fibrosis is highly urgent. Hepatic stellate cells (HSCs) are recognized as liver-specific type of pericytes and can be activated by injury or inflammation. The activated HSCs differentiate into myofibroblasts and produce excessive ECM.

Endostatin is a peptide involved in multiple functions of physiological and pathological processes including angiogenesis, fibrosis, sepsis, and acute kidney injury.[2–5]

The antifibrotic activity has emerged as a newly attractive function. For example, endostatin was proved to have protective effects against hepatic fibrosis.[6] However, the precise molecular mechanisms remain unclear.

Multiple signal pathways have relation to liver fibrosis. For example, RhoA (ras homolog gene family, member A)/ ROCK (Rho-associated protein kinase) pathways play important roles in the process of liver fibrosis;[7] therefore, it is hypothesized that endostatin inhibits fibrosis by modulating RhoA/ROCK1 pathways. This study was designed to investigate whether endostatin has an effect on RhoA/ROCK1 pathways in a transforming growth factor (TGF)-β1- or platelet-derived growth factor (PDGF)-induced fibrosis cell model.

Materials and methods
Reagents and antibodies
Primary antibodies against α-smooth muscle actin (α-SMA), RhoA, ROCK1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Affinity Biosciences (Cincinnati, OH, USA). The recombinant endostatin was purchased from the Simcere Pharmaceutical Company (Nanjing, P.R. China). Both PDGF-BB and TGF-β1 were purchased from the ProSpec Bio (Rehovot, Israel). The ELISA kits of human collagen I were purchased from the Abbexa Company (Cambridge, UK).

Culture of rat HSCs
The rat HSCs line, HSC-T6, was obtained from ATCC (Manassas, VA, USA). Cell passages 3–5 were cultured in DMEM with 10% fetal bovine serum (Wisent, Canada), and seeded in six-well plates at a density of 5×10^5 cells per well for 24 hours, then serum-starved for 48 hours before treatment.

Treatments and groups
HSCs were divided into five groups: control group, TGF-β1 group, endostatin+TGF-β1 group, PDGF-BB group, and endostatin+PDGF-BB group. In the TGF-β1 group, HSCs were treated with 10 ng/mL TGF-β1; in the PDGF-BB group, the HSCs were treated with 200 ng/mL PDGF-BB for 72 hours. In the endostatin+TGF-β1 group or endostatin+PDGF-BB group, HSCs were pretreated with endostatin (5 µg/mL) for 1 hour and then treated with TGF-β1 (10 ng/mL) or PDGF-BB (200 ng/mL) for 72 hours. Endostatin was removed before treatment with TGF-β1 or PDGF-BB. In the control group, HSCs were treated only with serum-free DMEM for 72 hours. All cells were cultured in serum-free DMEM.

Cell viability assay
Based on our previous studies of concentration determination, the aim of the cell viability assay was to choose the best working time of treatments. The cell viability was measured by CCK-8 assay. HSC-T6 cells were cultured and seeded in the 96-well microplates at a density of 1×10^4 per 100 mL, then treated according to the manufacturer's guidance. The cell viability was measured at 0, 24, 48, and 72 hours. The optical density was measured by using the microplate reader at 450 nm. An additional endostatin-alone treatment group was used to analyze the potential effect of endostatin on the viability of HSCs. HSCs were incubated with endostatin (5 µg/mL) for 1 hour, and then cultured in serum-free DMEM for 72 hours. All experiments were repeated three times.

ELISA assay
HSC-T6 cells were seeded in six-well plates at a density of 5×10^5/mL, and treated as described above in section "Treatments and groups." The concentrations of collagen I in the supernatants were detected using ELISA kits according to the manufacturer's guidance. Absorbance value was measured at 450 nm with the microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The concentration of type I collagen was measured consistent with the corresponding standard curves. The OD value was measured at 450 nm on a microplate reader (Bio-Rad). The concentrations of collagen I were quantified according to the standard curves. The supernatant fluid was harvested and measured three times.

Immunofluorescent staining
HSCs were treated with 4% (0.04 g/mL) paraformaldehyde solution followed by 0.2% (v/v) Triton X-100. Immunofluorescent staining with the antibody against F-actin was performed according to the manufacturer's instruction as described previously.[8] DAPI reagent (4′,6-diamidino-2-phenylindole, MP Biomedicals, 1:7,500) was used to stain the nucleus.

Real-time PCR analysis (RT-qPCR)
The total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The expression of target genes was assayed by using the SYBR Green Real-Time PCR system (Thermo Fisher Scientific). The target gene primer sequences are displayed in Table 1. All experiments were repeated three times.

Table 1 Primer probe sequences used for real-time PCR

mRNA	Sequence (5′–3′)	Amplicon size (bp)
α-SMA	F: CATCACCAACTGGGACGACA	96
	R: TCCGTTAGCAAGGTCGGATG	
RhoA	F: GCCAAAATGAAGCAGGAGCC	92
	R: TACCCAAAAGCGCCAATCCT	
ROCK1	F: AATCTTCCAGTTGGTTCTGCCT	94
	R: CTCTATTTGGTACAGAAAGCCAACC	
GADPH	F: TCTCTGCTCCTCCCTGTTCT	95
	R: ATCCGTTCACACCGACCTTC	

Abbreviations: α-SMA, α-smooth muscle actin; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R, reverse; RhoA, ras homolog gene family, member A; ROCK1, Rho-associated protein kinase 1.

Western blot analysis

The procedure was performed according to our previous study.[3] The proteins were transferred to a polyvinylidene fluoride membrane and then incubated overnight at 4°C with the following primary antibodies: α-SMA (1:10,000 dilution), RhoA (1:1,000 dilution), and ROCK1 (1:1,000 dilution). GAPDH (1:2,000 dilution) was used as an internal control. Secondary antibodies (goat anti-rabbit IgG, 1:2,000 dilution) were then added. The quantification of target protein bands was analyzed with ImageJ software (v.1.60) and normalized to GAPDH. All experiments were repeated three times.

Statistics

The data were analyzed with Prism 6.0 software by using the one-way analysis of variance. The differences with $P<0.05$ were considered significant.

Results

Endostatin suppressed PDGF-BB- or TGF-β1-induced HSC proliferation

There is no significant difference among these groups at 0 and 24 hours. TGF-β1, 10 ng/mL, or PDGF-BB, 200 ng/mL, significantly promoted HSC-T6 proliferation at 48 and 72 hours ($P<0.01$, Figure 1). Endostatin significantly suppressed the proliferation effect at 48 and 72 hours in both endostatin-pretreated and endostatin-alone groups ($P<0.01$). Therefore, we selected 72 hours as the working time in the subsequent experiments.

Effect of endostatin on the expression of collagen I

As shown in Figure 2, PDGF-BB and TGF-β1 induced a significant increase in collagen I protein level. The upregulated expression of collagen I induced by PDGF-BB and TGF-β1 was significantly inhibited by endostatin ($P<0.01$, Figure 2).
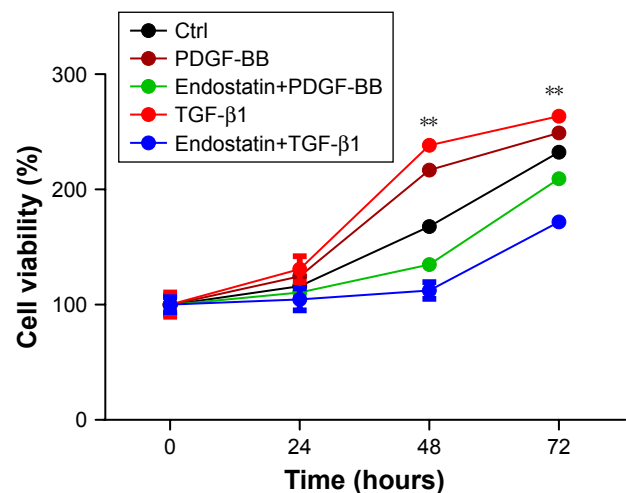

Figure 1 Endostatin inhibits HSC-T6 cells proliferation.
Notes: The cell proliferation was measured by CCK-8 assay. There is no significant difference in cell viability among these groups at 0 and 24 hours. TGF-β1 and PDGF-BB significantly promote proliferation of HSCs at 48 and 72 hours. Endostatin significantly inhibited TGF-β1- and PDGF-BB-induced proliferation at 48 and 72 hours in both endostatin-pretreated and endostatin-alone groups. Results were expressed as mean ± SD. **$P<0.01$.
Abbreviations: CCK-8, Cell Counting Kit-8; HSCs, hepatic stellate cells; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

Endostatin inhibited the expression of F-actin

The expression of F-actin in TGF-β1- or PDGF-BB-induced HSC-T6 cells was significantly upregulated than that in the
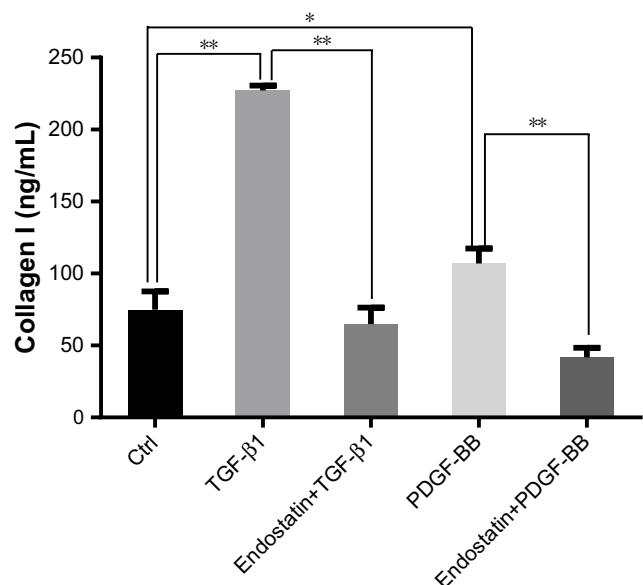
Figure 2 ELISA of collagen I.
Notes: The expression of collagen I in both PDGF-BB and TGF-β1 groups was significantly increased than that in the control group. Endostatin suppressed the upregulated collagen I expression induced by PDGF-BB or TGF-β1 significantly. Results are expressed as mean ± SD. *$P<0.05$, **$P<0.01$.
Abbreviations: PDGF, platelet-derived growth factor; TGF, transforming growth factor.

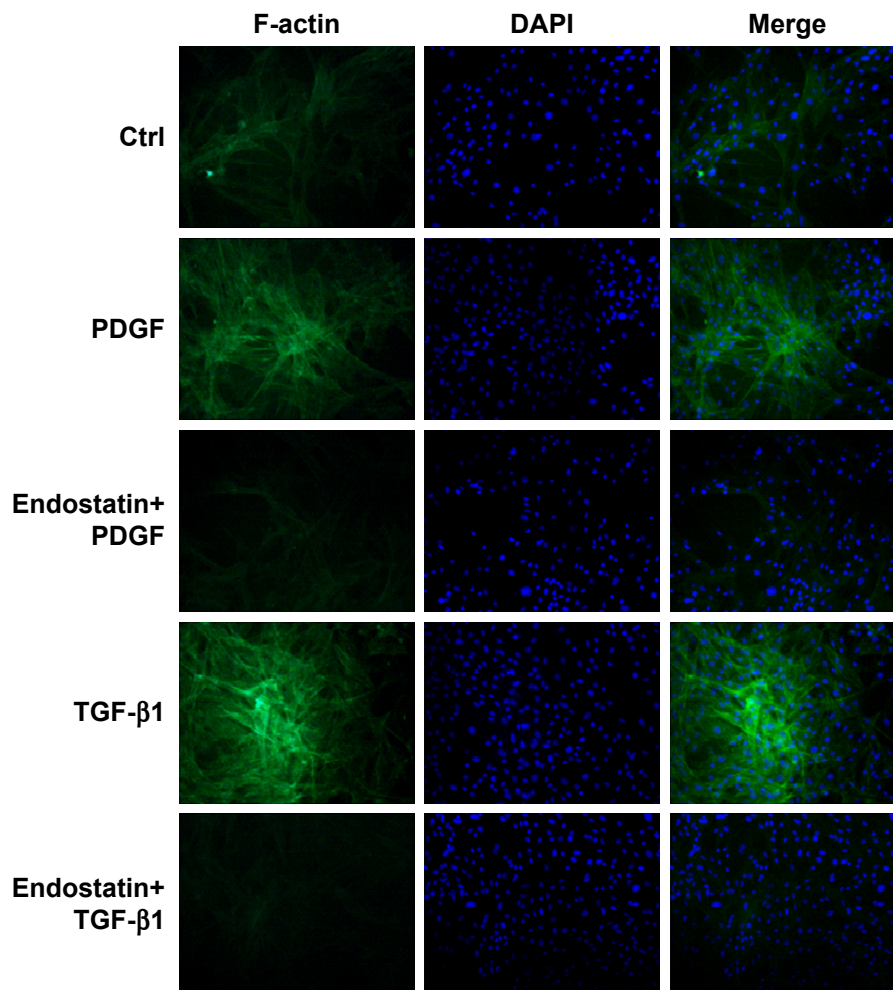| F-actin	DAPI	Merge

Figure 3 Effects of endostatin on the expression of F-actin in HSCs induced by PDGF-BB or TGF-β1.
Notes: Immunofluorescence using antibodies against F-actin (green) was performed. DAPI was used to stain the nucleus (blue) in HSCs. The F-actin-positive ratios in the PDGF-BB- and TGF-β1-induced groups are significantly higher than those in the blank control group. Endostatin markedly inhibited the expression of F-actin induced by PDGF-BB or TGF-β1. Magnification ×200.
Abbreviations: HSCs, hepatic stellate cells; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

control group. Endostatin reduced the TGF-β1- or PDGF-BB-induced expression of F-actin significantly (Figure 3, $P < 0.01$).

Endostatin inhibited the expressions of α-SMA, RhoA, and ROCK1 at the mRNA level

The expression of α-SMA, RhoA, and ROCK1 at mRNA level in the PDGF-BB- or TGF-β1-induced groups was significantly higher than that in the control group. Endostatin significantly inhibited the expression of α-SMA, RhoA, and ROCK1 at the mRNA level ($P < 0.01$, Figure 4A–C).

Endostatin inhibited the expressions of α-SMA, RhoA, and ROCK1 at the protein level

The expressions of α-SMA, RhoA, and ROCK1 at protein level were significantly upregulated in the PDGF-BB- and

TGF-β1-induced groups. Endostatin significantly inhibited the expressions of α-SMA, RhoA, and ROCK1 at the protein level ($P < 0.01$, Figure 5A–D).

Discussion

Hepatic fibrosis is actually the result of a wound-healing process. HSCs are located in the Disse space between hepatocytes and sinusoidal endothelial cells. HSCs have been recognized as liver-specific pericytes.[9] HSCs could be activated by stimulations and differentiated into myofibroblasts.[10–12] In our study, an in vitro TGF-β1- or PDGF-BB-induced HSCs fibrosis model was used. TGF-β1 and PDGF-BB promote the expressions of collagen I, α-SMA, and F-actin significantly.

Endostatin is the C-terminal of collagen type XVIII present in the liver sinusoidal and basement membrane, and discovered to ameliorate liver fibrosis.[13] Our previous studies found that endostatin ameliorates scar formation in the rabbit
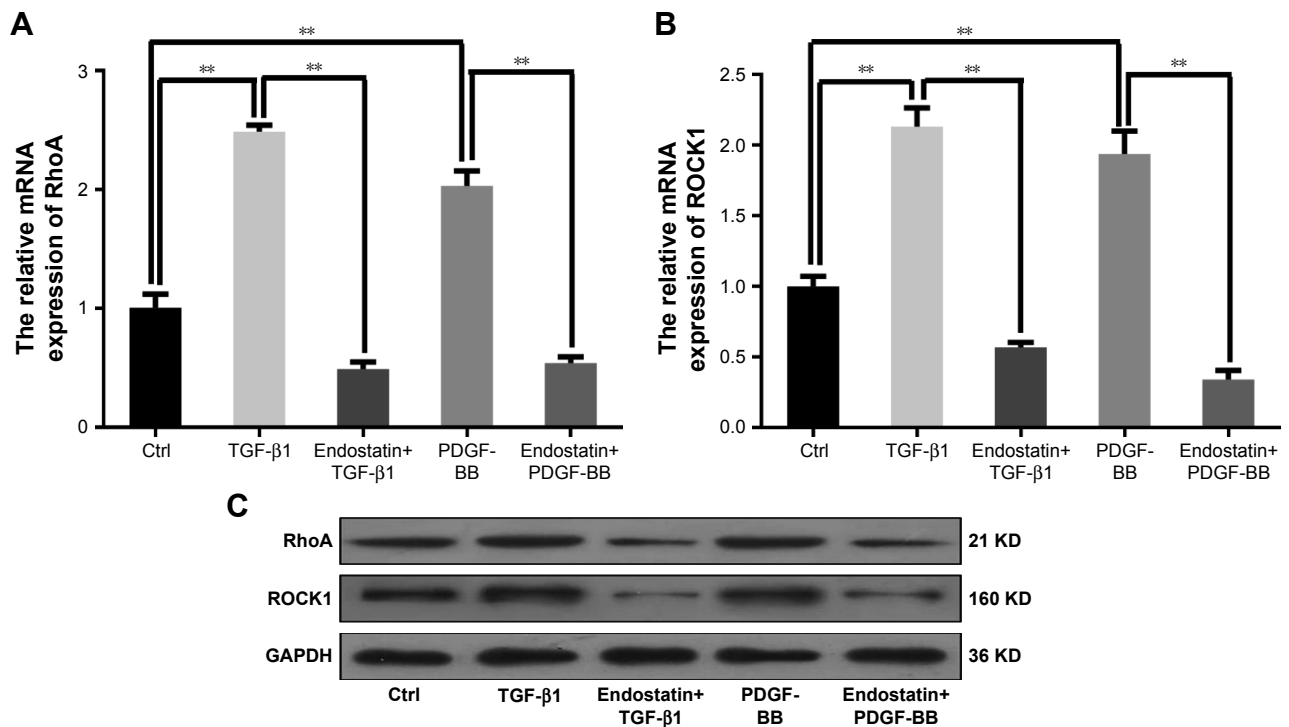
Figure 4 Endostatin inhibits the expression of α-SMA, RhoA, and ROCK1 at mRNA level.
Notes: Transcript levels of α-SMA, RhoA, and ROCK1 were analyzed by RT-PCR (**A–C**). Endostatin significantly suppressed the expressions of α-SMA, RhoA, and ROCK1 at mRNA level in HSC-T6 cells. Data are expressed as mean ± SD. **P<0.01 (n=3 per group).
Abbreviations: α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSC, hepatic stellate cell; RhoA, ras homolog gene family, member A; ROCK1, Rho-associated protein kinase 1; RT-PCR, real-time PCR.
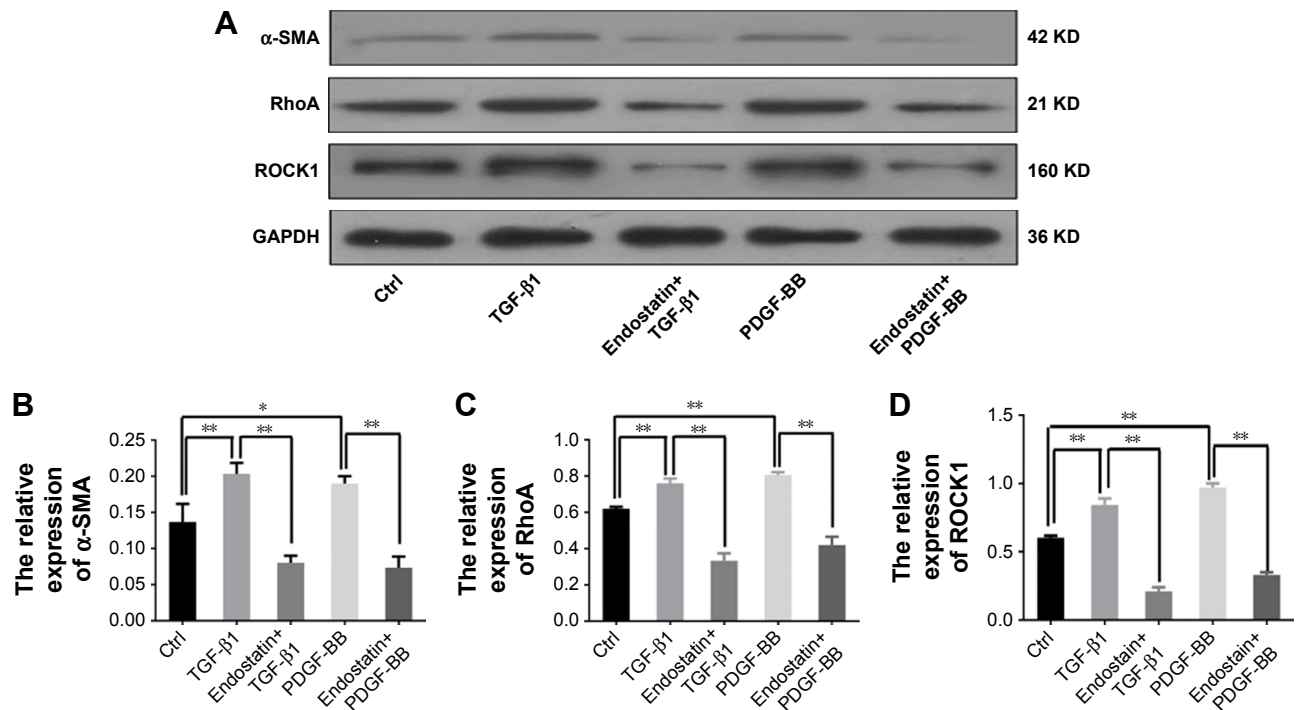
Figure 5 Endostatin inhibits the expressions of α-SMA, RhoA, and ROCK1 at protein level.
Notes: α-SMA, RhoA, and ROCK1 protein was detected by Western blot and normalized to GAPDH. (**A**) The image of immunoblotting. (**B**) Quantification of α-SMA normalized to GAPDH. (**C**) Quantification of RhoA normalized to GAPDH. (**D**) Quantification of ROCK1 normalized to GAPDH. Results are expressed as means ± standard deviation. *P<0.05, **P<0.01 (n=3 per group). PDGF-BB and TGF-β1 upregulated the expressions of α-SMA, RhoA, and ROCK1 protein levels significantly. Endostatin significantly suppressed the expression of α-SMA, RhoA, and ROCK1 at protein (**C**) level in HSC-T6 cells. Results are expressed as mean ± SD. **P<0.01.
Abbreviations: α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSC, hepatic stellate cell; PDGF, platelet-derived growth factor; RhoA, ras homolog gene family, member A; ROCK1, Rho-associated protein kinase 1; TGF, transforming growth factor.

ear model[14] and inhibits fibrosis in human fibroblasts.[8,15] In the present study, endostatin significantly inhibited the upregulated expressions of collagen I, α-SMA, and F-actin induced by PDGF-BB and TGF-β1 in HSCs.

Hepatic fibrosis is a complex pathological process that involves a variety of signaling pathways. RhoA/ROCK1 pathways participated in liver fibrosis.[16] The transformation from fibroblasts into myofibroblasts has a close relationship with the RhoA/ROCK1 signal pathways.[17] RhoA regulates the cell cytoskeleton and activates HSCs.[18] The actin filament production is directed by the RhoA/ROCK1 signal pathways.[19] RhoA/ROCK1 signaling pathways control cell morphology, proliferation, and adhesion. In addition, ROCK1 plays important roles in PDGF-BB-induced cell proliferation in human aortic vascular smooth muscle cells.[20]

We have uncovered here a novel mechanism of endostatin to inhibit HSC activation by selectively modulating the RhoA/ROCK pathways. For the limitations, we investigated only the effect of endostatin on RhoA and ROCK1 expressions in mRNA and protein levels. Further research is needed to clarify the molecular mechanisms behind these effects.

Conclusion

Endostatin attenuates PDGF-BB- or TGF-β1-induced HSC activation via suppressing RhoA/ROCK1 signal pathways.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

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