Acta Pharmacologica Sinica (2014) 35: 1385–1392
 1671-4083/14 $32.00
www.nature.com/aps

Original Article

Selective α_{1B}- and α_{1D}-adrenoceptor antagonists suppress noradrenaline-induced activation, proliferation and ECM secretion of rat hepatic stellate cells *in vitro*

Ting-ting LIU[1, 2], Ti-long DING[2], Yong MA[1, 2, *], Wei WEI[1, *]

[1]*Institute of Clinical Pharmacology, Anhui Medical University, Key Laboratory of Anti-inflammatory and Immune Medicine (Anhui Medical University), Ministry of Education, Hefei 230032, China;* [2]*Department of Liver Diseases, 123th Hospital of PLA of China, Bengbu 233015, China*

Aim: To explore the effects of noradrenaline (NA) on hepatic stellate cells (HSCs) *in vitro* and to determine the adrenoceptor (AR) subtypes and underlying mechanisms.
Methods: The distribution and expressions of α_{1A}-, α_{1B}-, and α_{1D}-ARs in HSC-T6 cells were analyzed using immunocytochemistry and RT-PCR. Cell proliferation was evaluated with MTT assay. The expression of HSC activation factors [transforming factor-β_1 (TGF-β_1) and α-smooth muscle actin (α-SMA)], extracellular matrix (ECM) secretion factors [tissue inhibitor of metalloproteinase-1 (TIMP-1) and collagen-I (ColI)] and PKC-PI3K-AKT signaling components (PKC, PI3K, and AKT) in the cells were detected by Western blotting and RT-PCR.
Results: Both α_{1B}- and α_{1D}-AR were expressed in the membrane of HSC-T6 cells, whereas α_{1A}-AR was not detected. Treatment of the cells with NA concentration-dependently increased cell proliferation (EC_{50}=277 nmol/L), which was suppressed by the α_{1B}-AR antagonist CEC or by the α_{1D}-AR antagonist BMY7378. Furthermore, NA (0.001, 0.1, and 10 μmol/L) concentration-dependently increased the expression of TGF-β_1, α-SMA, TIMP-1 and ColI, PKC and PI3K, and phosphorylation of AKT in HSC-T6 cells, which were suppressed by CEC or BMY7378, or by pertussis toxin (PT), RO-32-0432 (PKC antagonist), LY294002 (PI3K antagonist) or GSK690693 (AKT antagonist).
Conclusion: NA promotes HSC-T6 cell activation, proliferation and secretion of ECM *in vitro* via activation of G_α-coupled α_{1B}-AR and α_{1D}-AR and the PKC-PI3K-AKT signaling pathway.

Keywords: noradrenaline; hepatic fibrosis; hepatic stellate cell; α_1-adrenoceptor; G_α; PKC; PI3K; AKT

Acta Pharmacologica Sinica (2014) 35: 1385–1392; doi: 10.1038/aps.2014.84; published online 6 Oct 2014

Introduction

Liver fibrosis is a chronic liver damage caused by a variety of pathogenic factors[1]. It is a common pathology and a necessary step on the way to liver dysfunction and cirrhosis[2]. The mechanisms of pathogenesis are the excessive production and low degradation of extracellular matrix (ECM), which leads to ECM deposits in liver and the formation of liver fibrosis[3]. The HSC is the most important cell type involved in liver fibrosis[4]. The activation of hepatic stellate cells (HSCs) is central to liver fibrosis, whereas the apoptosis of HSCs can reverse liver fibrosis[5].

The sympathetic nervous system (SNS) is widely distributed in the body and participates in the regulation of body functions[6]. Liver tissue is rich in autonomic nervous system (ANS) tissue, which is distributed around the Disse cavity[7]. Stoyanova[8] showed that there are many autonomic nerve fibers in the hepatic portal area and hepatic lobule. HSCs express catecholamine biosynthetic enzymes and can synthesize and release noradrenaline (NA), dopamine, 5-hydroxytryptamine (5-HT) and other neurotransmitters[9]. Studies have shown that liver fibrogenesis requires sympathetic neurotransmitters[10] and that SNS blockers significantly reduce liver fibrosis[11]. Numerous evidence is available showing that excessive activation of the SNS is related to the occurrence and development of liver fibrosis[12].

* To whom correspondence should be addressed.
E-mail my4973766@163.com (Yong MA);
wwei@ahmu.edu.cn (Wei WEI)
Received 2014-04-03 Accepted 2014-07-07

Previous studies have indicated that NA promotes the proliferation of HSCs. The non-selective α-AR and β-AR antagonists inhibit the growth of HSCs[13]. We investigated the expression of 3 AR subtypes of α_1-AR in the hepatic stellate cell-T6 (HSC-T6) cell line. We explored the effects of NA on HSCs, the AR subtypes through which NA plays a role and the possible mechanisms through which NA acts on HSCs.

Materials and methods

Drugs and reagents

NA bitartrate was purchased from Harvest (Shanghai, China). The α_{1B}-AR selective antagonist chloroethylclonidine (CEC), the α_{1D}-AR selective antagonist BMY7378 and the PI3K antagonist LY294002 were purchased from Sigma (St Louis, MO, USA)[14]. G_α protein antagonist pertussis toxin (PT) was purchased from Calbiochem (Darmstadt, Hesse, Germany). The PKC antagonist RO-32-0432 and α_{1A}-AR, α_{1B}-AR and α_{1D}-AR primary antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). The AKT antagonist GSK690693 was purchased from Selleckchem (Houston, TX, USA). SABC three-step kit was purchased from Boster (Wuhan, China). Other primary antibodies were purchased from Cell signaling (Boston, MA, USA). Primers were synthesized by Sangon Biotech Co, Ltd (Shanghai, China) (Table 1)[15–17].

Table 1. PCR primers. F, forward; R, reverse.

Gene		Sequence (5′→3′)	Product (bp)
α_{1A}-AR	F	GAGAATTCCGAGGCCTCAAGTCCGGCCT	169
	R	TTGAATTCTCGGGAAAACTTGAGCAG	
α_{1B}-AR	F	CTGGGGAGAGTTGAAAGATGCC	158
	R	CCGACAGGATGACCAAGATGTT	
α_{1D}-AR	F	TTGAATTCCTACAGAGACCCACGACCCAG	229
	R	CGGAATTCTTAAATGTCAGTCTCCCGGAG	
TGF-β_1	F	GCCCTGGACACCAACTATTGC	324
	R	GGAGCGCACGATCATGTTGG	
TIMP-1	F	TCCCCAGAAATCATCGACAC	329
	R	ATCGCTGAACAGGGAAACAC	
ColI	F	TACAGCACGCTTGTGGATG	256
	R	TTGAGTTTGGGTTGTTGGTC	
PI3K	F	GATGTGGCTGACGCAGAAAG	204
	R	CTCGTTTCCCTCGCAATAGG	
GAPDH	F	ACAGCAACAGGGTGGTGGAC	224
	R	TTTGAGGGTGCAGCGAACTT	

Cell culture

The HSC-T6 cell line was obtained from the Institute of Clinical Pharmacology, Anhui Medical University. The cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) from Gibco BRL Life Technologies Inc (Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA).

Experimental groups and drug delivery

Cultured HSC-T6 cells were divided into 13 groups: Control group; NA (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} mol/L) groups; α_{1B}-AR antagonist (CEC) group; α_{1D}-AR antagonist (BMY7378) group; G_α protein inhibitor (PT) group; PKC inhibitor (RO-32-0432) group; PI3K inhibitor (LY294002) group and AKT inhibitor (GSK690693) group. HSC-T6 cells in an exponential growth phase were cultured at a density of 5×10^4 cells/well in a 96-well plate for 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) or 1×10^5 cells/well in a 6-well plate for immunocytochemistry, Western blot and reverse transcription-polymerase chain reaction (RT-PCR). The cells were incubated overnight in DMEM containing 10% FBS then starved for serum 24 h before being treated with the compounds. HSC-T6 cells were incubated with α_{1B}-AR and α_{1D}-AR antagonists and with G_α protein, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and AKT inhibitors (1×10^{-5} mol/L) for 12 h. NA at different concentrations was then added to cells for 24 h at 37 °C.

Immunocytochemistry

The cells were cultured in 6-well plates containing cover slips. The cover slips were washed twice with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min. Immunohistochemical staining for α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR were performed according to the standard SABC protocol described in the SABC Reagents Kit (Boster, Wuhan, China). Positive expression was detected as brown or yellow staining. PBS was used as a negative control to replace the primary antibodies.

Cell proliferation assay

Plated HSC-T6 cells were cultured in the presence or absence of α_{1B}-AR antagonist CEC (10^{-5} mol/L) or α_{1D}-AR antagonist BMY7378 (10^{-5} mol/L) for 12 h. Various concentrations of NA were then added[18, 19]. Proliferation was measured with MTT assay (Sigma, St Louis, MO, USA) to determine the effects of NA with or without α_{1B}-AR and α_{1D}-AR antagonists on cell proliferation. After treatment, MTT solution (5.0 mg/mL in PBS) was added (20.0 μL/well), and the plates were incubated for another 4 h at 37 °C. The purple formazan crystals were dissolved in 150.0 μL of dimethyl sulfoxide (DMSO) per well. After 10 min, the plates were read on a microplate reader (Biotech Instruments, NY, USA) at an absorbance of 490 nm. Untreated cells were used as a control. The assays were performed in three independent experiments. The cell proliferation was calculated using the following formula: cell proliferation (%)=[(*OD* of the experimental samples/*OD* of the control)–1] ×100% (n=3, mean±SD).

Western blot analysis

Proteins were extracted from cells treated with NA in the presence or absence of α_{1B}-AR or α_{1D}-AR antagonists and signaling pathway molecule inhibitors using RIPA lysis buffer (Beyotime, Haimen, China) and phenylmethylsulfonyl fluoride (PMSF) (99:1). Each protein sample was mixed with 5× sample

buffer (4:1) (Bio-Rad, Hercules, CA, USA) and heated in boiling water for 10 min. The proteins were separated by 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), and incubated overnight at 4 °C with various primary antibodies at a 1:500 dilution. The secondary antibody [peroxidase (HRP)-labeled goat anti-mouse or goat anti-rabbit] was added and incubated at 1:30 000 dilution. Immunodetection was performed with an enhanced chemiluminescence system (ECL, Pierce, Rockford, IL, USA) using hydrogen peroxide and luminol as a substrate.

RT-PCR

Total RNA was extracted from HSC-T6 cells using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The integrity and concentration of RNA were determined by measuring absorbance at 260 nm and total RNA was stored at -80 °C. RNA was separated for RT-PCR by agarose gel electrophoresis (Thermo, MA, USA). Total RNA from each sample (2 μg) was re-suspended in a final volume of 25 μL of reaction buffer. PCR for GAPDH was performed on each individual sample as an internal control. The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 5 s, 40–60 °C for 30 s and 72 °C for 30 s. After the last cycle of amplification, samples were incubated for 10 min at 72 °C. The amplified RT-PCR products were subjected to electrophoresis at 85 V on 2% agarose gels (Sigma, St Louis, MO, USA) for 30 min. A DNA ladder was used as a molecular marker. Agarose gels were stained with 0.5 mg/mL ethidium bromide (Sigma, St Louis, MO, USA) in TAE buffer. The gel bands were examined using the gel image system (Tanon, Shanghai, China).

Statistical analysis

Statistical analyses were performed using the SPSS 11.0 software program (SPSS Software Products, Chicago, IL, USA). All data are presented as number or mean±SD. Statistical analysis among groups was performed by one-way analysis of variance (ANOVA). Statistical analysis between two groups was performed using Dunnett's method. The Bliss[20] method was used to determine the linear regression equation to calculate EC_{50}. The threshold for statistical significance of differences was $P<0.05$.

Results

The expression of α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR in HSC-T6 cells

The SABC method was used to determine the expression of α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR. α_{1B}-AR and α_{1D}-AR were expressed in the membrane, but α_{1A}-AR was almost undetectable (Figure 1A and 1B). We then measured the expression of α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR mRNA. α_{1B}-AR was expressed at a higher level than α_{1D}-AR ($P<0.05$), and α_{1A}-AR was not expressed (Figure 1C and 1D).

NA promotes HSC-T6 cell proliferation and adrenoceptor antagonists reduce HSC-T6 cell proliferation

We plated HSC-T6 cells in the presence and absence of α_{1B}-AR antagonist CEC (10 μmol/L) or α_{1D}-AR antagonist BMY7378 (10 μmol/L) for 12 h and then added various concentrations of NA. Using the MTT assay to measure the proliferation of HSC-T6 cells, we found that NA significantly induced HSC-T6 cell proliferation in a concentration-dependent manner compared with the control group ($P<0.05$) (Figure 2A). The EC_{50} was 277 nmol/L (Figure 2B). The α_{1B}-AR antagonist CEC and the α_{1D}-AR antagonist BMY7378 both reduced HSC-T6 cell proliferation compared with the equivalent concentration of NA only ($P<0.01$) (Figure 2A). The α_{1B}-AR antagonist may be a competitive antagonist and the α_{1D}-AR antagonist may be a noncompetitive antagonist, because the HSC proliferation curve moved to the right when the cells were cultured with the α_{1B}-AR antagonist. With an increased concentration of NA, the curve did not reach the original E_{max}. However, the HSC cell proliferation curve moved in parallel to the right when the cells were cultured with the α_{1D}-AR antagonist. With an increased concentration of NA, the curve reached the original E_{max} (Figure 2C).

NA promotes the expression of TGF-β_1, α-SMA, TIMP-1, PKC, and PI3K protein and the phosphorylation of AKT but α_1-AR antagonists inhibit their expressions in HSC-T6 cells

To explore the effects and possible mechanisms of action of NA and α_1-AR antagonists on HSC-T6 cells, and to explore the function of the PKC-PI3K-AKT signaling pathway, we measured the expression of HSC activation factors (TGF-β_1 and α-SMA), ECM secretion factors (TIMP-1) and PKC-PI3K-AKT signaling pathway molecules (PKC, PI3K, and AKT) in HSC-T6 cells after treatment with NA with or without α_1-AR antagonists. The results showed that NA significantly promoted the expression of TGF-β_1, α-SMA, TIMP-1, PKC, PI3K protein and the phosphorylation of AKT in HSC-T6 cells ($P<0.05$). The α_{1B}-AR and α_{1D}-AR antagonists significantly inhibited their expression ($P<0.05$) (Figure 3 and 4).

NA promotes TGF-β_1, TIMP-1, ColI, and PI3K mRNA expression and α_1-AR antagonists inhibits their expressions in HSC-T6 cells

The results of RT-PCR showed that NA significantly promoted the mRNA expression of HSC activation factor TGF-β_1, ECM secretion factor TIMP-1 and ColI and PKC-PI3K-AKT signaling pathway molecule PI3K in HSC-T6 cells. These results indicate that NA significantly increased their expression ($P<0.01$). The α_{1B}-AR and α_{1D}-AR antagonists significantly inhibited their expression ($P<0.05$) (Figure 5).

G_α, PKC, PI3K, and AKT inhibitors down-regulate the protein expression of TGF-β_1, α-SMA, and TIMP-1 in NA treated HSC-T6 cells

To explore the mechanisms of the effect of NA on HSC-T6 cells, we plated HSC-T6 cells in the presence and absence of various inhibitors for 12 h. NA was then added, and the expression of TGF-β_1, α-SMA, and TIMP-1 was measured. The results showed that NA significantly increased TGF-β_1, α-SMA, and TIMP-1 protein expressions in HSC-T6 cells ($P<0.01$). Various inhibitors significantly inhibited their

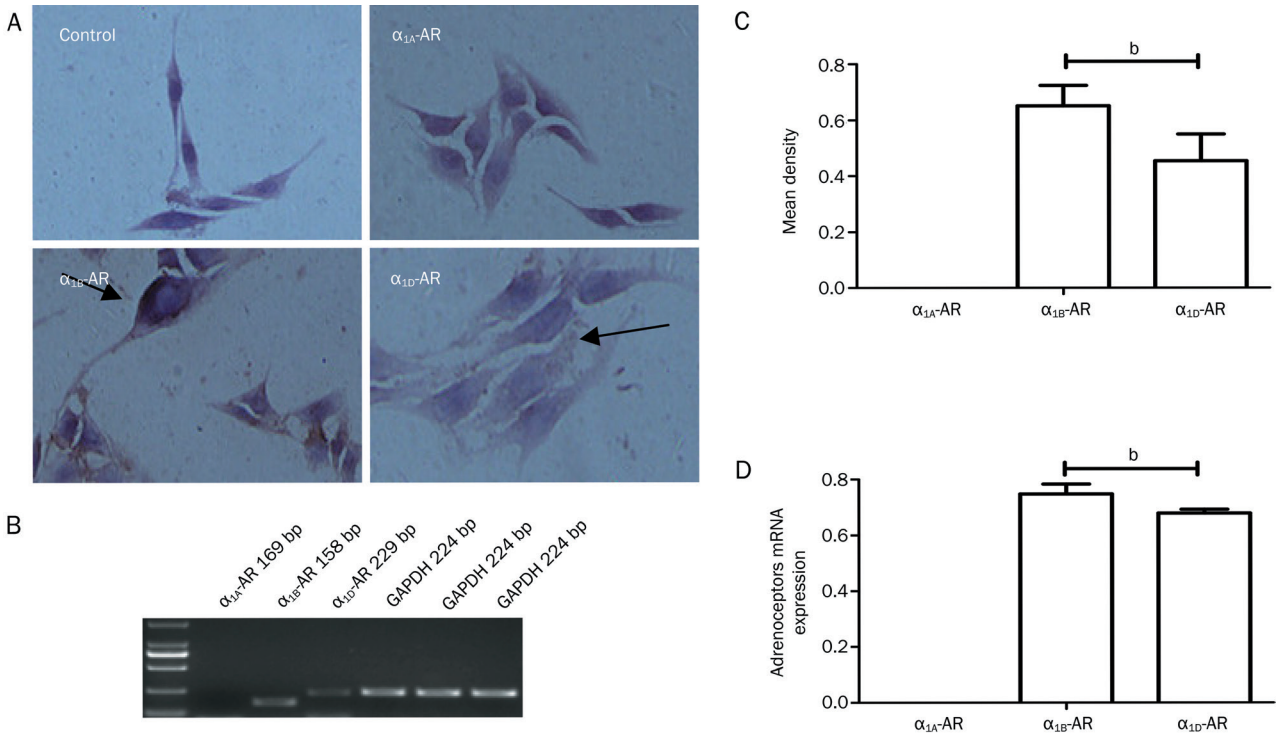

Figure 1. The expression of α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR in HSC-T6 cells. (A) The expression of α_1-AR subtypes in HSC-T6 was analyzed using the SABC method. (B) Bar graphs showed semi-quantitative analysis of their expression. (C) α_1-AR subtype mRNA was analyzed by RT-PCR. GAPDH was used as internal control. (D) Bar graphs showed semi-quantitative analysis of α_1-ARs expression. Data are presented as the mean±SD (n=3). ^bP<0.05 α_{1B}-AR group compared with the α_{1D}-AR group.

expression (P<0.01) (Figure 6).

G_α, PKC, PI3K, and AKT inhibitors down-regulate the mRNA expression of TGF-β_1, TIMP-1, and Coll in NA treated HSC-T6 cells

The RT-PCR results showed that NA significantly increased TGF-β_1, α-SMA, and TIMP-1 mRNA expression in HSC-T6 cells (P<0.05). Various inhibitors significantly inhibited their expression (P<0.05) (Figure 7).

Discussion

Studies have shown that the SNS is involved in regulation in the liver and plays an important role in the development of liver fibrosis. The HSC could be the target of SNS regulation[21]. Oben *et al*[11] suggested that leptin deficient *ob*/*ob* mice have the characteristic of fibrosis resistance in chronic liver injury, because the expression of NA is low and the activation of the SNS is suppressed in these mice. Drugs that have effects on the SNS may provide new strategies for the clinical treatment of liver fibrosis. We are interested in understanding the effects and mechanisms of SNS action on HSC cells and determining the AR subtypes that play a role in this process. We are interested in finding alternative therapeutic targets to increase drug effectiveness and reduce adverse reactions.

Studies have suggested that sympathetic nerve neurotransmitters promote the repair of liver injuries. They also promote the activation of HSCs by coupling with ARs[22]. Sancho-Bru *et al*[23] confirmed that liver tissue expressed α_{1A}-AR, α_{1B}-AR, α_{2A}-AR, α_{2B}-AR, β_1-AR, and β_2-AR. HSCs also express a variety of adrenoceptor subtypes such as α_{1A}-AR, α_{2B}-AR and β_2-AR. However, Oben *et al*[18] showed that HSCs express α_{1B}-AR, α_{1D}-AR, β_1-AR, and β_2-AR. Currently, the distribution and function of adrenoceptor subtypes in liver tissue and HSCs are controversial and need further research. Our study examined this issue further, and we observed the expression of three α_1-AR subtypes (α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR) in HSCs. We found that α_{1B}-AR and α_{1D}-AR are expressed in cell membranes but α_{1A}-AR not. Previous studies have shown that NA promotes HSC proliferation and inhibits apoptosis *in vitro*, mainly through α-AR and β_2-AR[13]. Other results suggested that α_1-AR and β_2-AR expression increased in the liver tissue of rats with liver fibrosis[24]. Duan *et al*[25] also suggested that NA, α_1-AR, and β_2-AR were more highly expressed in rat liver tissue with liver fibrosis. α_1-AR plays important roles in many physiological processes[26]. We studied the various subtypes of α_1-AR to further define the mechanism of action of the SNS in the development of liver fibrosis. The results showed that blocking either α_{1B}-AR or α_{1D}-AR down-regulated the activation, proliferation and secretion of NA treated HSC cells.

The SNS acts through neurotransmitters interacting with different adrenoceptor subtypes, and then activating downstream signaling pathways. α-AR can activate multiple signaling pathways including the phosphoinositide-calcium signal-

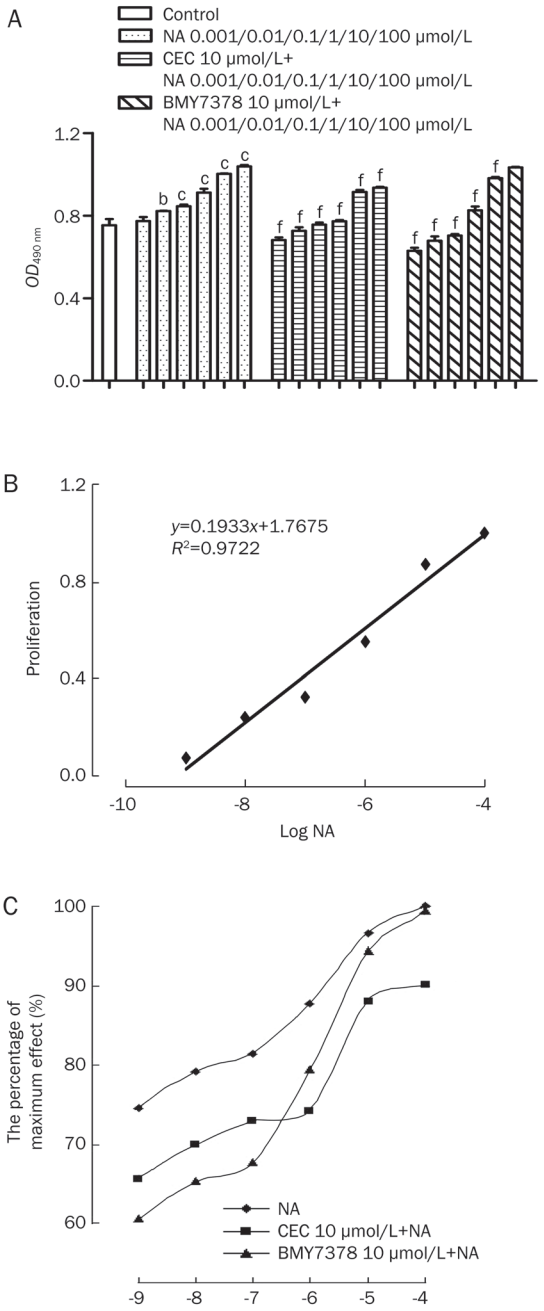

Figure 2. The selective α-AR agonist NA promoted HSC-T6 cell proliferation and the α_{1B}-AR antagonist CEC and the α_{1D}-AR antagonist BMY7378 inhibited the growth of HSC-T6 cells. (A) NA promoted HSC-T6 cell proliferation and adrenoceptor antagonists inhibited the growth of HSC-T6 cells. Data are presented as the mean±SD (n=3). ^{b}P<0.05, ^{c}P<0.01 compared with the control group. ^{f}P<0.01 compared with the corresponding concentration of NA. (B) Using logarithmic NA concentration as the X-axis and cell proliferation rate as the Y-axis to perform linear regression, the regression equation is y=0.1933x+1.7675, R^2=0.9722. There was an obvious linear relationship. The EC_{50} of NA was 277 nmol/L. (C) The concentration-effect curve of NA and α_1-AR antagonists on HSC-T6 cell proliferation.

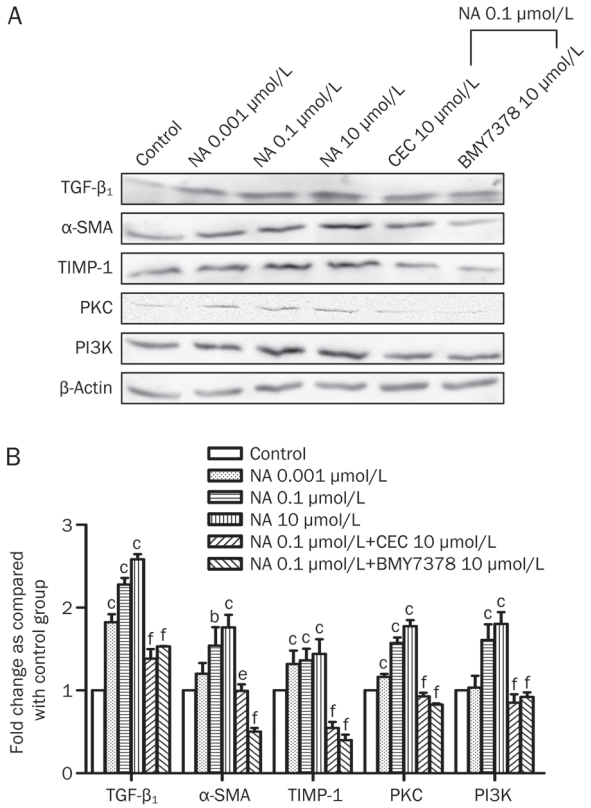

Figure 3. α_1-AR antagonists down-regulated the protein expression of TGF-β_1, α-SMA, TIMP-1, PKC, and PI3K in NA treated HSC-T6 cells. (A) Western blot results showed the expression of these proteins in HSC-T6 cells. β-Actin was used as a loading control. (B) Bar graphs showed semi-quantitative evaluation of their expression by densitometry from triplicate independent experiments. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 compared with the control group. ^{e}P<0.05, ^{f}P<0.01 compared with the 0.1 μmol/L NA group.

ing system, and the PKC signaling system. β-AR can activate the G protein-cAMP-PKA signaling system. Various receptor subtypes also have different characteristics in coupling with G protein. α_1-AR couples with $G_{\alpha q}$ protein and α_2-AR couples with $G_{\alpha i}$ protein. β_1-AR only couples with $G_{\alpha s}$ protein but β_2-AR couples with $G_{\alpha s}$ and $G_{\alpha i}$ proteins[27].

Studies of heart failure have found that SNS regulates the apoptosis of myocardial cells through β-AR coupling with G protein[28]. β_1-AR promoted apoptosis through the mitogen activated protein kinase (MAPK) signaling pathway and β_2-AR inhibited apoptosis through the PI3K signaling pathway[29]. The PI3K signaling pathway is important in cell proliferation[30]. Studies of this pathway are important for elucidating the mechanisms of action of the SNS in the development of liver fibrosis. We would like to identify new methods for the effective treatment of liver fibrosis.

The PKC-PI3K-AKT signaling pathway regulates platelet derivation growth factor (PDGF) to promote HSC proliferation and secretion[31]. Blocking this pathway can inhibit HSC proliferation and ECM expression, leading to an improvement in patients with liver fibrosis[32]. Marra *et al*[33] showed that the activation of the PKC-PI3K-AKT signaling pathways promoted the mitosis and chemotaxis of HSC cells. Our

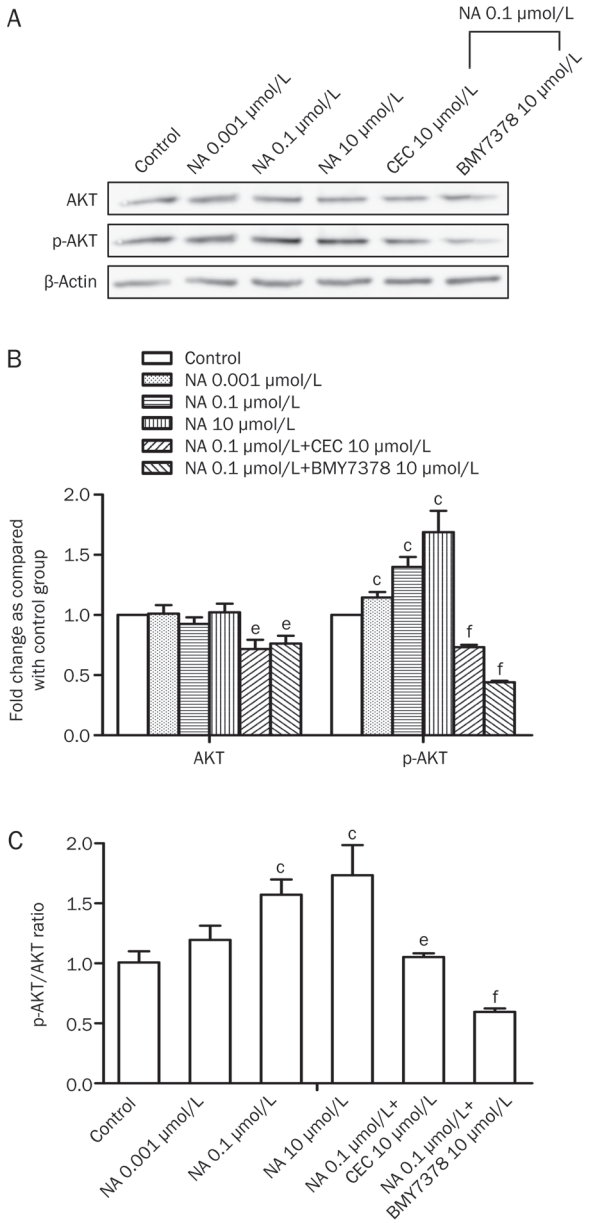

Figure 4. α_1-AR antagonists down-regulated the phosphorylation of AKT in NA treated HSC-T6 cells. (A) Western blot results showed the expression of AKT and p-AKT in HSC-T6 cells. β-Actin was used as a loading control. (B) Bar graphs showed semi-quantitative analysis of their expression by densitometry from triplicate independent experiments. (C) Bar graph shows p-AKT/AKT ratio. Mean±SD. $^cP<0.01$ compared with the control group. $^eP<0.05$, $^fP<0.01$ compared with the 0.1 μmol/L NA group.

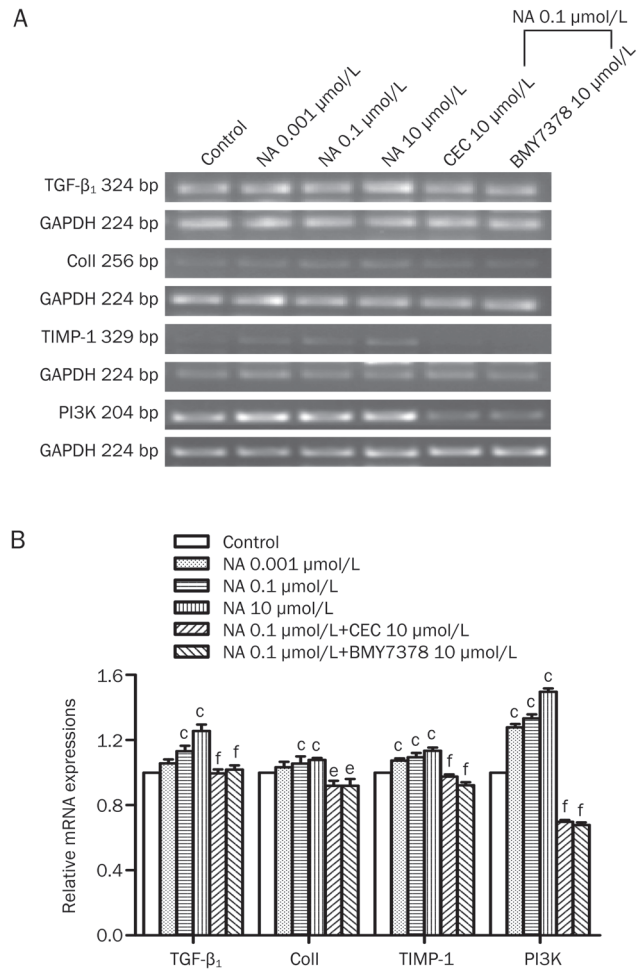

Figure 5. α_1-AR antagonists down-regulated the mRNA expression of TGF-β_1, Coll, TIMP-1 and PI3K in NA treated HSC-T6 cells. (A) RT-PCR results showed the expression levels of them in HSC-T6 cells. GAPDH was used as an internal control. (B) Bar graphs showed semi-quantitative analysis of their expression by densitometry from triplicate independent experiments. Mean±SD. $^cP<0.01$ compared with the control group. $^eP<0.05$, $^fP<0.01$ compared with the 0.1 μmol/L NA group.

experiments studied the PKC-PI3K-AKT signaling pathway in depth. We measured the expression of signaling molecules as well as HSC activation and secretion in the presence of a variety of signaling molecules inhibitors. This research illuminated the function of the PKC-PI3K-AKT signaling pathway in liver fibrosis. Blocking this pathway can down-regulate the activity of NA on HSCs.

Previous experiments have shown that NA promotes HSC proliferation[34]. We demonstrated this action by MTT and performed further experiments. We found that NA promoted the proliferation of HSCs in a concentration-dependence manner. The EC_{50} was 277 nmol/L. Understanding the EC_{50} of drugs is important in guiding clinical use of the drug. And 277 nmol/L is approximately 10^{-7} mol/L. In our experiments, we used 10^{-7} mol/L of NA in co-culture with antagonists or inhibitors to study the effect of NA on HSCs after blocking receptors or signaling pathways. Based on the concentration-effect curves, we demonstrated that the α_{1D}-AR antagonist is competitive and the α_{1B}-AR antagonist is noncompetitive in HSCs. In addition to HSC proliferation, our experiments studied the activation and secretion of HSC-T6 cells. The results showed that NA promoted the activation and secretion of HSC-T6 cells and that the selective α_{1B}-AR and α_{1D}-AR antagonists and the inhibitors of PKC-PI3K-AKT signaling pathways

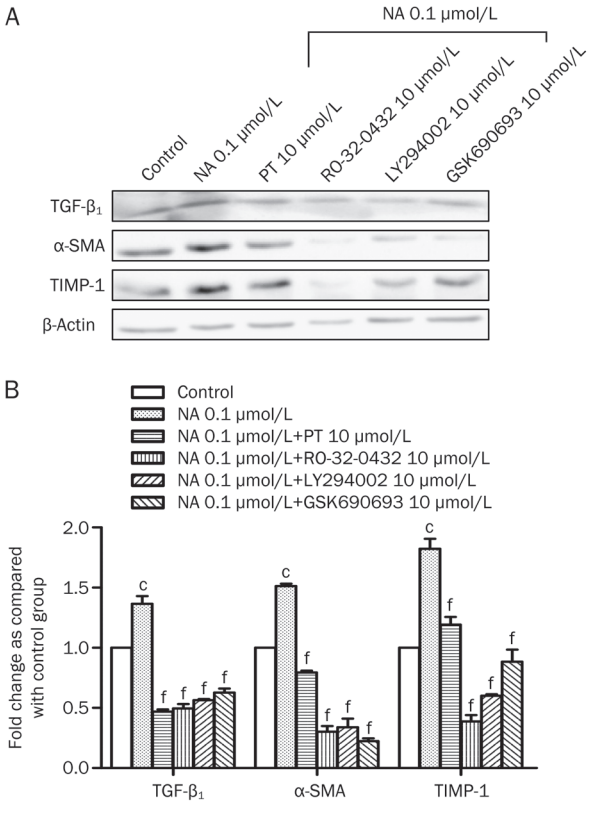

Figure 6. G_α, PKC, PI3K and AKT inhibitors down-regulated the protein expression of TGF-β_1, α-SMA, and TIMP-1 in NA treated HSC-T6 cells. (A) Western blot results showed their expression in HSC-T6 cells. β-Actin was used as a loading control. (B) Bar graphs showed semi-quantitative analysis of their expression by densitometry from triplicate independent experiments. Mean±SD. $^cP<0.01$ compared with the control group; $^fP<0.01$ compared with the 0.1 μmol/L NA group.

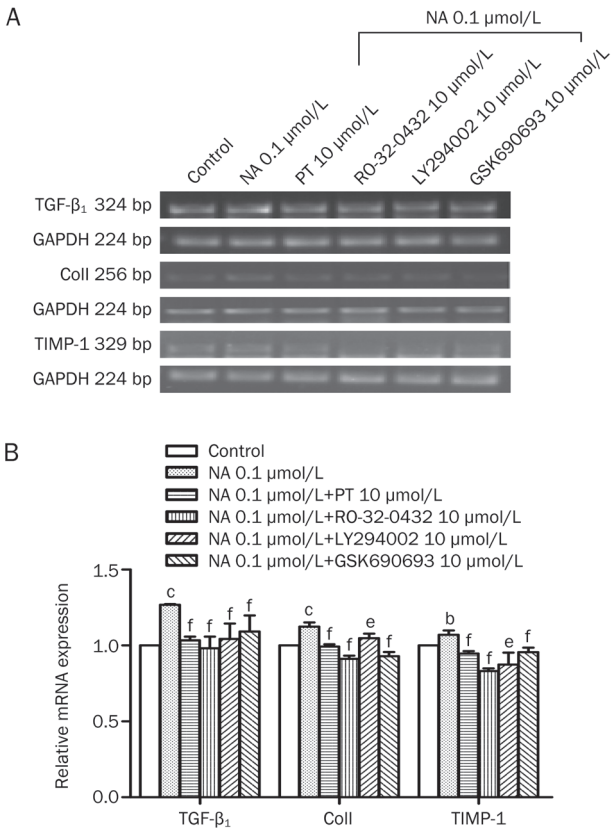

Figure 7. G_α, PKC, PI3K, and AKT inhibitors down-regulated the mRNA expression of TGF-β_1, Coll, and TIMP-1 in NA treated HSC-T6 cells. (A) RT-PCR results showed their expression in HSC-T6 cells. GAPDH was used as an internal control. (B) Bar graphs showed semi-quantitative analysis of their expression by densitometry from triplicate independent experiments. Mean±SD. $^bP<0.05$, $^cP<0.01$ compared with the control group. $^eP<0.05$, $^fP<0.01$ compared with the 0.1 μmol/L NA group.

down-regulated the effect of NA.

In conclusion, our study demonstrated that, through α_{1B}-AR and α_{1D}-AR, NA activates G_α protein coupling with the PKC-PI3K-AKT signaling pathway, which might be one of the mechanisms to promote HSC-T6 activation, proliferation and secretion of ECM.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No 81173075) and the Nanjing Military Medical Science and Technology Innovation Foundation of China (No 10MA037).

Author contribution

Yong MA and Wei WEI designed the experiments; Ti-long DING guided the research; and Ting-ting LIU performed experiments, analyzed data and wrote the paper.

References

1 Yi ET, Liu RX, Wen Y, Yin CH. Telmisartan attenuates hepatic fibrosis in bile duct-ligated rats. Acta Pharmacol Sin 2012; 33: 1518–24.

2 Moreno M, Gonzalo T, Kok RJ, Sancho-Bru P, van Beuge M, Swart J, *et al*. Reduction of advanced liver fibrosis by short-term targeted delivery of an angiotensin receptor blocker to hepatic stellate cells in rats. Hepatology 2010; 51: 942–52.

3 Gong Y. Identifying the targets for treatment of liver fibrosis and hepatocellular carcinoma from both Western medicine and Chinese medicine. Chin J Integr Med 2012; 18: 245–9.

4 Liu Q, Wang X, Zhang Y, Li CJ, Hu LH, Shen X. Leukamenin F suppresses liver fibrogenesis by inhibiting both hepatic stellate cell proliferation and extracellular matrix production. Acta Pharmacol Sin 2010; 31: 839–48.

5 Xu C, Chen X, Chang C, Wang G, Wang W, Zhang L, *et al*. Analysis of gene expression profiles of liver stellate cells during liver regeneration in rats. Mol Cells 2011; 31: 17–23.

6 Mancia G, Bousquet P, Elghozi JL, Esler M, Grassi G, Julius S, *et al*. The sympathetic nervous system and the metabolic syndrome. J Hypertens 2007; 25: 909–20.

7 Fukuda Y, Imoto M, Koyama Y, Miyazawa Y, Hayakawa T. Demonstration of noradrenaline-immunoreactive nerve fibres in the liver. J Int Med Res 1996; 24: 466–72.

8 Stoyanova II. Relevance of mast cells and hepatic lobule innervation to liver injury. Rom J Gastroenterol 2004; 13: 203–9.

9 Tian XP, Zhang XL. Sympathetic nervous system in pathogenesis of hepatic fibrosis. Int J Dig Dis 2008; 28: 323–5.

10 Dubuisson L, Desmoulière A, Decourt B, Evadé L, Bedin C, Boussarie L, *et al*. Inhibition of rat liver fibrogenesis through noradrenergic antagonism. Hepatology 2002; 35: 325–31.
11 Oben JA, Roskams T, Yang S, Lin H, Sinelli N, Li Z, *et al*. Norepinephrine induces hepatic fibrogenesis in leptin deficient *ob/ob* mice. Biochem Biophys Res Commun 2003; 308: 284–92.
12 Timmermans JP, Geerts A. Nerves in liver: superfluous structures? A special issue of The Anatomical Record updating our views on hepatic innervation. Anat Rec B New Anat 2005; 282: 4.
13 Liu N, Zhang XL, Liang CD, Yao DM, Liu L, Zhao DQ, *et al*. Dynamic changes of α-AR, β_1-AR, and β_2-AR expression during hepatic fibrogenesis. Chin J Hepatol 2009; 17: 653–6.
14 Koshimizu TA, Tanoue A, Hirasawa A, Yamauchi J, Tsujimoto G. Recent advances in α_1-adrenoceptor pharmacology. Pharmacol Ther 2003; 98: 235–44.
15 Knittel T, Aurisch S, Neubauer K, Eichhorst S, Ramadori G. Cell-type-specific expression of neural cell adhesion molecule (N-CAM) in Ito cells of rat. Am J Pathol 1996; 149: 449–62.
16 Sun DX, Liu Z, Tan XD, Cui DX, Wang BS, Dai XW. Nanoparticle-mediated local delivery of an antisense TGF-β_1 construct inhibits intimal hyperplasia in autogenous vein grafts in rats. PLoS One 2012; 7: e41857.
17 Dun ZN, Zhang XL, An JY, Zheng LB, Barrett R, Xie SR. Specific shRNA targeting of FAK influenced collagen metabolism in rat hepatic stellate cells. World J Gastroenterol 2010; 16: 4100–6.
18 Oben JA, Roskams T, Yang S, Lin H, Sinelli N, Torbenson M, Hepatic fibrogenesis requires sympathetic neurotransmitters. Gut 2004; 53: 438–45.
19 Hansel DE, Eipper BA, Ronnett GV. Neuropeptide Y functions as a neuroproliferative factor. Nature 2001; 410: 940–4.
20 Liu CX, Sun RY. Drug evaluation experiment design and statistical basis. Beijing: Military Medical Science Press, 1999. p 84–6.
21 Püschel GP. Control of hepatocyte metabolism by sympathetic and parasympathetic hepatic nerves. Anat Rec A Discov Mol Cell Evol Biol 2004; 280: 854–67.
22 Van Heeswijk JC, Vianen GJ, van den Thillart GE. The adrenergic control of hepatic glucose and FFA metabolism in rainbow trout (*Oncorhynchus mykiss*): increased sensitivity to adrenergic stimulation with fasting. Gen Comp Endocrinol 2006; 145: 51–61.
23 Sancho-Bru P, Bataller R, Colmenero J, Gasull X, Moreno M, Arroyo V, *et al*. Norepinephrine induces calcium spikes and proinflammatory actions in human hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 2006; 291: G877–84.
24 Zapater P, Gómez-Hurtado I, Peiró G, González-Navajas JM, García I, Giménez P, *et al*. Beta-adrenergic receptor 1 selective antagonism inhibits norepinephrine-mediated TNF-alpha downregulation in experimental liver cirrhosis. PLoS One 2012; 7: e43371.
25 Duan RX, Tang WX, Wu CH, Liu HY, Gao X, Guo Y, *et al*. The effects of sympathetic neurotransmitters and adrenergic receptors on liver fibrosis in murine schistosomiasis. Zhonghua Gan Zang Bing Za Zhi 2008; 16: 352–4.
26 Docherty JR. Subtypes of functional alpha1-adrenoceptor. Cell Mol Life Sci 2010; 67: 405–17.
27 Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T, *et al*. Coupling of β_2-adrenoceptor to G_i proteins and its physiological relevance in murine cardiac myocytes. Circ Res 1999; 84: 43–52.
28 Remondino A, Kwon SH, Communal C, Pimentel DR, Sawyer DB, Singh K, *et al*. β-Adrenergic receptor-stimulated apoptosis in cardiac myocytes is mediated by reactive oxygen species/c-Jun NH_2-terminal kinase-dependent activation of the mitochondrial pathway. Circ Res 2003; 92: 136–8.
29 Hakuno D, Fukuda K, Makino S, Konishi F, Tomita Y, Manabe T, *et al*. Bone marrew-derived regenerated cardiomyocytes (CMG cells) express functional adrenergic and musearinic receptors. Circulation 2002; 105: 380–6.
30 Zhai YP, Lu Q, Liu YW, Cheng Q, Wei YQ, Zhang F, *et al*. Over-production of nitric oxide by oxidative stress-induced activation of the TGF-β_1/PI3K/Akt pathway in mesangial cells cultured in high glucose. Acta Pharmacol Sin 2013; 34: 507–14.
31 Jones SM, Kazlauskas A. Growth-factor-dependent mitogenesis requires two distinct phases of signaling. Nat Cell Biol 2001; 3: 165–72.
32 Parsons CJ, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. J Gastroenterol Hepatol 2007; 22: S79–84.
33 Marra F, Romanelli RG, Giannini C, Failli P, Pastacaldi S, Arrighi MC, *et al*. Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. Hepatology 1999; 29: 140–8.
34 Oben JA, Yang S, Lin H, Ono M, Diehl AM. Norepinephrine and neuropeptide Y promote proliferation and collagen gene expression of hepatic myofibroblastic stellate cells. Biochem Biophys Res Commun 2003; 302: 685–90.