Selective α₁B- and α₁D-adrenoceptor antagonists suppress noradrenaline-induced activation, proliferation and ECM secretion of rat hepatic stellate cells in vitro

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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 α₁A-, α₁B-, and α₁D-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-β1 (TGF-β1) 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 α₁B- and α₁D-AR were expressed in the membrane of HSC-T6 cells, whereas α₁A-AR was not detected. Treatment of the cells with NA concentration-dependently increased cell proliferation (EC₅₀=277 nmol/L), which was suppressed by the α₁B-AR antagonist CEC or by the α₁D-AR antagonist BMY7378. Furthermore, NA (0.001, 0.1, and 10 μmol/L) concentration-dependently increased the expression of TGF-β1, 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 Gα-coupled α₁B-AR and α₁D-AR and the PKC-PI3K-AKT signaling pathway.

Keywords: noradrenaline; hepatic fibrosis; hepatic stellate cell; α₁-adrenoceptor; Gα; PKC; PI3K; AKT

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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].
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 α1-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 α1B-AR selective antagonist chloroethylclonidine (CEC), the α1P-AR selective antagonist BMY7378 and the PI3K antagonist LY294002 were purchased from Sigma (St Louis, MO, USA). Gα protein antagonist pertussis toxin (PT) was purchased from Calbiochem (Darmstadt, Hesse, Germany). The PKC antagonist RO-32-0432 and α1A-AR, α1B-AR and α1P-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) |
|------|-----------------|--------------|
| α1A-AR | F GAGAATTCCAGGCAGCTCAGAACCTCCGGCT  | 169 |
|       | R TTAGAATTCTCAGGGGAAAATTGAGCAG  |     |
| α1B-AR | F CTGGGGAAGAGTTGAAGATGCC  | 158 |
|       | R CGGACAGGTGACCAGAGATTT  |     |
| α1P-AR | F TTAGGTCCTACAGACGGACACCCAGACCGAG  | 229 |
|       | R CGGATTTCTAATGGTCACGTCGCCGAG  |     |
| TGF-β1 | F GCCCTGGACACACAACTATTGC  | 324 |
|       | R GAGGCGCAGCAGCATGGTG  |     |
| TIMP-1 | F TCCCGAGAAATATGACACAC  | 329 |
|       | R ATGGTGACCGGGAAACAC  |     |
| Coll | F TACAGCAGCCTTGTTGATG  | 256 |
|       | R TTAGTCTGGGTTGTTGCT  |     |
| PI3K | F GATGTTGGTGACTGAAAG  | 204 |
|       | R CTGGTCTCCTCGGAATAGG  |     |
| GAPDH | F ACACAGCACGAGTTGACG  | 224 |
|       | R TTTGAGGTTGACGACACTT  |     |

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% CO2 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).

Western blot analysis

Proteins were extracted from cells treated with NA in the presence or absence of α1B-AR or α1P-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

Experimental groups and drug delivery

Cultured HSC-T6 cells were divided into 13 groups: Control group; NA (10−6, 10−5, 10−4, 10−3, and 10−2 mol/L) groups; α1P-AR antagonist (CEC) group; α1P-AR antagonist (BMY7378) group; Gα 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 5×10^6 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 α1B-AR and α1P-AR antagonists and with Gα 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 α1A-AR, α1B-AR, and α1P-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 α1B-AR antagonist CEC (10−5 mol/L) or α1P-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 α1P-AR and α1P-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 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 (Biotek 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).

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% CO2 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).
buffer (4:1) (Bio-Rad, Hercules, CA, USA) and heated in boiling water for 10 min. The proteins were separated by 12.5% sodium dodecyl sulfate-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 1:30000 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 method was used to determine the linear regression equation to calculate EC₅₀. The threshold for statistical significance of differences was P<0.05.

Results
The expression of α₁A-AR, α₁B-AR, and α₁D-AR in HSC-T6 cells
The SABC method was used to determine the expression of α₁A-AR, α₁B-AR, and α₁D-AR. α₁A-AR and α₁D-AR were expressed in the membrane, but α₁A-AR was almost undetectable (Figure 1A and 1B). We then measured the expression of α₁A-AR, α₁B-AR, and α₁D-AR mRNA. α₁B-AR was expressed at a higher level than α₁D-AR (P<0.05), and α₁A-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 α₁B-AR antagonist CEC (10 µmol/L) or α₁D-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 EC₅₀ was 277 nmol/L (Figure 2B). The α₁B-AR antagonist CEC and the α₁D-AR antagonist BMY7378 both reduced HSC-T6 cell proliferation compared with the equivalent concentration of NA only (P<0.01) (Figure 2A). The α₁D-AR antagonist may be a competitive antagonist and the α₁D-AR antagonist may be a noncompetitive antagonist, because the HSC proliferation curve moved to the right when the cells were cultured with the α₁D-AR antagonist. With an increased concentration of NA, the curve did not reach the original E₅₀. However, the HSC cell proliferation curve moved in parallel to the right when the cells were cultured with the α₁B-AR antagonist. With an increased concentration of NA, the curve reached the original E₅₀ (Figure 2C).

NA promotes TGF-β₁, α-SMA, TIMP-1, PKC, and PI3K protein and the phosphorylation of AKT but α₁A-AR antagonists inhibit their expressions in HSC-T6 cells
To explore the effects and possible mechanisms of action of NA and α₁A-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-β₁ 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 α₁A-AR antagonists. The results showed that NA significantly promoted the expression of TGF-β₁, α-SMA, TIMP-1, PKC, PI3K protein and the phosphorylation of AKT in HSC-T6 cells (P<0.05). The α₁D-AR and α₁B-AR antagonists significantly inhibited their expression (P<0.05) (Figure 3 and 4).

NA promotes TGF-β₁, TIMP-1, Coll, and PI3K mRNA expression and α₁A-AR antagonists inhibit their expressions in HSC-T6 cells
The results of RT-PCR showed that NA significantly promoted the mRNA expression of HSC activation factor TGF-β₁, ECM secretion factor TIMP-1 and Coll 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 α₁D-AR and α₁B-AR antagonists significantly inhibited their expression (P<0.05) (Figure 5).

Gα, PKC, PI3K, and AKT inhibitors down-regulate the protein expression of TGF-β₁, 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-β₁, α-SMA, and TIMP-1 was measured. The results showed that NA significantly increased TGF-β₁, α-SMA, and TIMP-1 protein expressions in HSC-T6 cells (P<0.01). Various inhibitors significantly inhibited their
expression (P<0.01) (Figure 6).

Gα, PKC, PI3K, and AKT inhibitors down-regulate the mRNA expression of TGF-β1, TIMP-1, and Coll in NA treated HSC-T6 cells

The RT-PCR results showed that NA significantly increased TGF-β1, 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 α1A-AR, α1B-AR, α2A-AR, α2B-AR, β1-AR, and β2-AR. HSCs also express a variety of adrenoceptor subtypes such as α1A-AR, α2A-AR and β2-AR. However, Oben et al [18] showed that HSCs express α1B-AR, α1D-AR, β1-AR, and β2-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 α1-AR subtypes (α1A-AR, α1B-AR, and α1D-AR) in HSCs. We found that α1B-AR and α1D-AR are expressed in cell membranes but α1A-AR not. Previous studies have shown that NA promotes HSC proliferation and inhibits apoptosis in vitro, mainly through α-AR and β2-AR [13]. Other results suggested that α1-AR and β2-AR expression increased in the liver tissue of rats with liver fibrosis [24]. Duan et al [25] also suggested that NA, α1-AR, and β2-AR were more highly expressed in rat liver tissue with liver fibrosis. α1-AR plays important roles in many physiological processes [26]. We studied the various subtypes of α1-AR to further define the mechanism of action of the SNS in the development of liver fibrosis. The results showed that blocking either α1B-AR or α1D-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-
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. α1-AR couples with Gαq protein and α2-AR couples with Gαi protein. β1-AR only couples with Gαs protein but β2-AR couples with Gαs and Gαi proteins[27].

Studies of heart failure have found that SNS regulates the apoptosis of myocardial cells through β-AR coupling with G protein[28]. α1-AR only couples with Gαs protein but β1-AR couples with Gαs and Gαi proteins[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
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\cite{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...
down-regulated the effect of NA.

In conclusion, our study demonstrated that, through $\alpha_{1B}$-AR and $\alpha_{1D}$-AR, NA activates $G_\alpha$ 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.

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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.

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