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Depletion of β-Arrestin2 in Hepatic Stellate Cells Reduces Cell Proliferation Via ERK Pathway

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ABSTRACT

β-Arrestins are multifunctional adaptor proteins. Recently, some new roles of β-arrestins in regulating intracellular signaling networks have been discovered, which regulate cell growth, proliferation, and apoptosis. Though, the role of β-arrestins expression in the pathology of hepatic fibrosis remains unclear. In this study, the possible relationship between the expression of β-arrestins with the experimental hepatic fibrosis and the proliferation of hepatic stellate cells (HSCs) were investigated. Porcine serum induced liver fibrosis was established in this study. At five time points, the dynamic expression of β-arrestin1, β-arrestin2, and α-smooth muscle actin (α-SMA) in rat liver tissues, was measured by immunohistochemical staining, double immunofluorescent staining, and Western blotting. This study showed that aggravation of hepatic fibrosis with gradually increasing expression of β-arrestin2 in the hepatic tissues, but not β-arrestin1. Further, as hepatic fibrosis worsens, β-arrestin2-expressing activated HSCs accounts for an increasingly larger percentage of all activated HSCs. And the expression of β-arrestin2 had a significant positive correlation with the expression of α-SMA, an activated HSCs marker. In vitro studies, the dynamic expression of β-arrestin1 and β-arrestin2 in platelet derived growth factor-BB (PDGF-BB) stimulated HSCs was assessed by Western blotting. The expression of β-arrestin2 was remarkably increased in PDGF-BB stimulated HSCs. Furthermore, the small interfering RNA (siRNA) technique was used to explore the effect of β-arrestins on the proliferation of HSCs and the activation of ERK1/2. Transfection of siRNA targeting β-arrestin2 mRNA (siβ-arrestin2) into HSCs led to a 68% and 70% reduction of β-arrestin2 mRNA and protein expression, respectively. siβ-arrestin2 abolished the effect of PDGF-BB on the proliferation of HSCs. In addition, siβ-arrestin2 exerted the inhibition of the activation of ERK1/2 in HSCs. The present study provided strong evidence for the participation of the β-arrestin2 in the pathogenesis of hepatic fibrosis. The β-arrestin2 depletion diminishes HSCs ERK1/2 signaling and proliferation stimulated by PDGF-BB. Selective targeting of β-arrestin2 inhibitors to HSCs might present as a novel strategy for the treatment of hepatic fibrosis. J. Cell. Biochem. 114: 1153–1162, 2013.

KEY WORDS: β-ARRESTIN; HEPATIC STELLATE CELL; LIVER FIBROSIS

Liver fibrosis is a common end-result of a wide variety of chronic liver diseases following various types of injuries, such as viral infection, alcohol, drugs, and chemical toxicity. It is often associated with severe morbidity and significant mortality, for example, resulting in the necessity of liver transplantation. The pathogenesis of liver cirrhosis is characterized by the excessive production and deposition of extracellular matrix (ECM) components that lead to tissue scarring and destruction of the normal liver parenchyma [Fallowfield, 2011; Hernandez-Gea and Friedman, 2011].

Liver fibrosis could be considered as a model of wound-healing response to chronic liver injury, and the activation of hepatic stellate

Wu-Yi Sun and Yang Song contributed equally to this work.

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cells (HSCs) plays an essential role in the fibrosis progression. The activation of HSCs involves the transdifferentiation from a quiescent state to myofibroblast-like cells with the appearance of α-smooth muscle actin (α-SMA) and loss of cellular vitamin A storage. The activated HSCs are distinguished by accelerated proliferation and enhanced production of ECM components [Moles et al., 2010; Pinzani and Macias-Barragan, 2010]. At the cellular level, platelet derived growth factor (PDGF) is one of the best characterized fibrogenic and proliferative cytokines for HSCs. Furthermore, hepatic injury is associated with both increased autocrine PDGF and up-regulation of PDGF receptor [Pinzani, 2002; Kastanis et al., 2011]. Currently, clinical reports suggested that advanced liver fibrosis is potentially reversible [Povero et al., 2010]. Thus, it is essential to develop therapeutic strategies to counteract liver fibrosis.

The arrestin family has four members: β-arrestin1, β-arrestin2, x-arrestin, and s-arrestin [Ma and Pei, 2007; Coffa et al., 2011]. Extensive studies have been performed on ubiquitously expressed β-arrestin1 and β-arrestin2, whereas x-arrestin and s-arrestin are found exclusively in the visual system. The classical functions of β-arrestins are to mediate desensitization, sequestration, and recycling of G protein-coupled receptors (GPCRs) [Spurney, 2003; Wang et al., 2003]. Mounting evidence suggests that, in addition to regulation of GPCRs signals, β-arrestins also serve as modulators in a number of intracellular signaling pathways, including extracellular regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K-Akt), which play important roles in the regulation of various cellular functions, cell growth, proliferation, and apoptosis [McDonald et al., 2000; Pyne et al., 2004; Revankar et al., 2004; Golan et al., 2009].

Although β-arrestins can transduce multiple signals in cells, little is known about their participation in the modulation of the hepatic fibrosis in mammals. To date, the role of β-arrestins in liver fibrosis has not been investigated. This study was designed to investigate the role of β-arrestins in liver fibrosis and the proliferation of HSCs. To that end, we have used porcine serum (PS) induced liver fibrosis model in rats and investigated the role of β-arrestins in PDGF-BB promoted HSCs proliferation, believed to be a critical component of hepatic fibrosis. The data herein indicate that aggravation of hepatic fibrosis led to gradually increasing expression of β-arrestin2 but not β-arrestin1. β-Arrestin2 depletion in HSCs reduces the PDGF-BB promoted activation of ERK1/2 pathway, thereby inhibited the proliferation of HSCs. Altogether, the data indicate that the β-arrestin2 depletion diminishes HSCs mitogenic signaling and proliferation stimulated by PDGF-BB. Targeting β-arrestin2 may therefore be therapeutically useful for liver fibrosis.

MATERIALS AND METHODS

REAGENTS

PDGF-BB was the product of Peprotech Company. Antibodies for β-arrestin1, β-arrestin2, α-SMA, β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK, anti-phosphorylated ERK antibodies were from Cell Signaling Technology. SuperSignal west femto maximum sensitive substrate was purchased from Pierce Chemical (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM) and porcine serum were obtained from Gibco Co. (CA). MTT was purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals used in the experiment were analytical grade from commercial sources.

ANIMALS AND TREATMENT

Male Wistar rats (120 ± 10 g) were provided by the Shanghai BK Experimental Animal Center (Grade II, Certificate No. D-65). All animals were housed in conventional cages under control conditions of temperature ($23 \pm 3^{\circ}C$) and relative humidity ($50 \pm 20\%$), with light illumination for 12 h/day. The animals were allowed access to food and tap water ad libitum throughout the acclimatization and experimental periods. All experiments were approved by the Ethics Review Committee for Animal Experimentation of the Institute of Clinical Pharmacology, Anhui Medical University.

The rats were randomly divided into two groups including porcine serum (PS) model group and normal controls. The PS model group was intraperitoneally (i.p.) injected with 0.5 ml/rat of PS twice a week for up to 16 weeks [Okuno et al., 2001]. The normal controls were i.p. injected with saline in a dose of 0.5 ml/rat. At 3, 6, 9, 12, and 16 weeks, six rats of PS injection group were sacrificed by euthanasia method. The samples were used for histopathological examination and Western blotting.

IMMUNOHISTOCHEMISTRY

Each liver section was deparaffinized by xylene and rehydrated with graded alcohols. The sections were retrieved in a microwave oven (300 W) in citrate buffer (pH 6.0) for 10 min at 100°C, and then incubated in absolute methanol containing 3% hydrogen peroxide for 10 min at room temperature. The sections were sequentially pre-incubated with 10% normal rabbit serum for 10 min at room temperature and they were then incubated with the primary antibody β-arrestin1, β-arrestin2, α-SMA. Immunoreactivity was visualized using the streptavidin/peroxidase (SP) method (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) and diaminobenzidine (DAB) as the chromagen according to the manufacturer's protocol. The nuclei were lightly counterstained with hematoxylin solution. The primary antibodies were replaced with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin as a negative control. Images were obtained using a Nikon video microscope, and a semiquantitative analysis was conducted using the Image-Pro Plus software: five random fields were analyzed per slide, and the relative intensity of β-arrestin1, β-arrestin2, and α-SMA was reflected by optical density value. The sections were analyzed by two independent pathologists.

IMMUNOFLUORESCENCE DOUBLE-LABELING ASSAY

All sections were firstly blocked with 10% normal serum blocking solution species the same as the secondary antibody, containing 3% BSA and 0.1% Triton X-100 and 0.05% Tween-20 for 2 h at room temperature in order to avoid unspecific staining. Then the sections were incubated with both mouse monoclonal primary antibody for β-arrestin2 and rabbit polyclonal primary antibody for α-SMA (a marker of activated HSC). Briefly, sections were incubated with both primary antibodies overnight at 4°C, followed by a mixture of Cy3-

labeled anti-rabbit and FITC-labeled anti-mouse secondary antibodies for 2 h at 4°C. The sections were washed a final time and then mounted with glycerin. The co-expression of β-arrestin2 and α-SMA was observed using a confocal laser scanning microscope. β-Arrestin2-positive expression appeared as green fluorescent foci, α-SMA-positive expression appeared as red fluorescent foci and colocalization of the two markers appeared as yellow fluorescent foci.

CULTURE OF HSCs AND PROLIFERATION ASSAY

In vitro studies were performed on an immortalized rat HSC cell line (HSC-T6). The HSC-T6 line was cultured at 37°C in an atmosphere of 5% CO_2 in DMEM containing 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, and 5,000 IU/ml penicillin/5,000 g/ml streptomycin for 1–2 days before starting experiments. HSC-T6 had a pseudomyofibroblastic phenotype, characteristic cell shape, and expression of α-SMA [Vogel et al., 2000].

MTT assay was used to evaluate HSCs proliferation. Briefly, HSC-T6 were plated at a density of 5×10^4 cells/ml in 96-well culture plates. The confluent cells were growth-arrested in DMEM containing 0.5% FCS for 24 h. Subsequently, cells were incubated with 50 ng/ml PDGF-BB and either vehicle or siRNA. The cultures were incubated at 37°C, 5% CO_2 for 48 h. Four hours before the termination of culture, MTT (5 mg/ml) 20 μl was added to each well. After incubation at 37°C for an additional 4 h, the cells were centrifuged at 760*g* for 10 min and all the supernatants were aspirated without disturbing the pellet. The formazan crystals were dissolved by the addition of 150 μl dimethylsulfoxide (DMSO) and oscillated for 30 s. The absorbance (A) was measured at 490 nm by using enzyme-linked immunosorbent assay reader and the results were expressed as mean of six wells.

SILENCING β-ARRESTIN2 GENE EXPRESSION IN HSC BY SMALL INTERFERING RNA (siRNA)

siRNA duplexes (GenePharma Co., China) with sequences specifically targeting β-arrestin2 RNA was sense GACCGACUGCUGAAGAAGUTT and antisense ACUUCUUCAGCAGUCGGUCTT. A scrambled RNA duplex was used as a negative control. A day before siRNA treatment, cells were seeded in 6-well plates at 5×10^5 cells/well. siRNA duplexes were then transfected into HSCs using Lipofectamin2000 Reagent (Invitrogen, CO) for 48–72 h before experiments. Knockdown of expression of the target by siRNA was confirmed by RT-PCR and Western blotting.

DETERMINATION OF THE EFFECT OF β-ARRESTIN2 siRNA BY QUANTITATIVE REAL-TIME PCR AND WESTERN BLOT

Quantitative real-time PCR was carried out to examine the inhibitive effect of β-arrestin2 siRNA, and GAPDH served as an internal control. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The real-time PCR was carried out in a Real-time PCR Detection System (ABI 7500) using SYBR GreenER qPCR Supermix Universal Kit (Invitrogen) according to the manufacturer's instructions. The primer sequences for each gene are as follows: GAPDH, forward primer 5′-TCAAGAAGGTGGTGAAGCAG-3′, reverse primer 5′- AGGTGGAAGAATGGGAGTTG-3′. β-arrestin2, forward primer 5′-CCACGTCACCAACAATTCTG-3′, reverse primer 5′-TTGGTGTCTTCGTGCTTGAG-3′. The cycle threshold value was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold. The cycle threshold value of each product was determined and normalized against that of the internal control, GAPDH. β-Arrestin2 protein expression was detected by Western blot analysis as follows.

WESTERN BLOT ANALYSIS

Liver sample and HSC-T6 fractions were isolated and cultured according to the above method. Briefly, liver tissue was homogenized in a homogenizing buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EGTA, 5 mM EDTA, 1.5 mM pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin, and 2 mM dithiothreitol, using a Polytron homogenizer. Cells were washed twice with ice-cold PBS and lysed in lysis buffer (0.5% NP-40, 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin) for 20–30 min on ice. Protein concentration was measured by the Bradford assay. The proteins were resolved by sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat dry milk in 0.05% Tween-20–PBS for 2 h and incubated overnight at 4°C with anti-β-arrestin1, β-arrestin2, α-SMA, β-actin antibodies. Immunoblot was done with the indicated primary antibody followed by the appropriate horseradish peroxidase (HRP)-conjugated goat anti-mouse or rabbit anti-goat IgG and visualized by ECL detection kit (Pierce Chemical). Autoradiographs were scanned using a Image-Pro Plus Imaging analysis software (Media Cybernetics, USA). All the experiments reported in this study were performed three times and the results were reproducible.

STATISTICAL ANALYSIS

Values in tables and figures are given as means and standard deviation of the mean if not otherwise indicated. The analysis of variance (ANOVA) and Student's *t*-test are used in the SPSS software to determine significant differences between groups. The correlation between the Western blot detection result of β-arrestin2 and α-SMA expression in liver tissue was analyzed with Pearson's correlation analysis. Values of $P < 0.05$ were considered to be significant.

RESULTS

DISTRIBUTION OF β-ARRESTIN1 AND β-ARRESTIN2 PROTEIN IN RAT LIVER TISSUES

PS induced liver fibrosis model was established to investigate the changes of β-arrestin1 and β-arrestin2 during the progression of hepatic fibrosis. HE and Masson staining showed that livers in normal control group showed normal lobular architecture with central veins and radiating hepatic cords. After injecting 6 weeks of porcine serum, the liver showed disorderly hepatocyte cords, infiltration of inflammatory cells and collagen deposition extending from central veins or portal tracts, with thick or thin fibrotic septa.

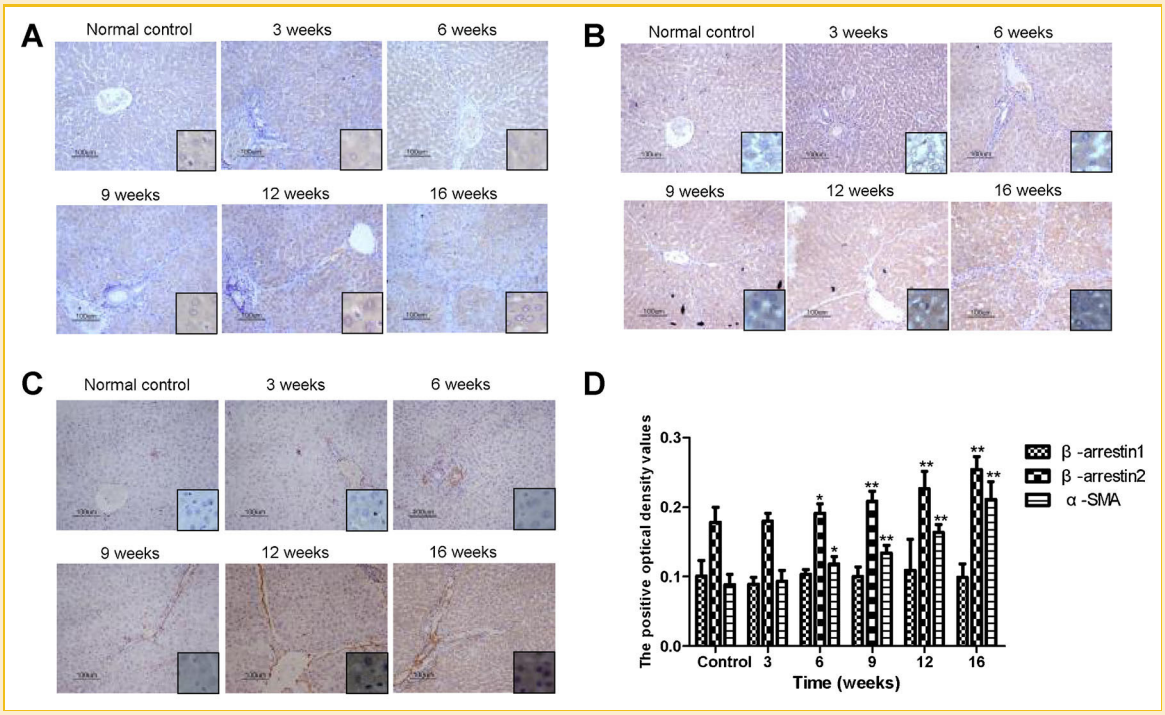

Fig. 1. β-Arrestin1, β-arrestin2, and α-smooth muscle actin (α-SMA) expression in immunohistochemically stained liver tissue of rats (SP × 200). A: β-Arrestin1 expression in liver tissues of normal rats and 3, 6, 9, 12, and 16 weeks after porcine serum injection. B: β-Arrestin2 expression in liver tissues of normal rats and 3, 6, 9, 12, and 16 weeks after porcine serum injection. C: α-SMA expression in liver tissues of normal rats and 3, 6, 9, 12, and 16 weeks after porcine serum injection. Inserts in (A–C) show high magnification images of β-arrestin1, β-arrestin2, and α-SMA staining in the liver tissue. D: Bar graph of the positive optical density values of β-arrestin1, β-arrestin2, and α-SMA in the hepatic tissue of rats at different time points. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group.

From 9 to 16 weeks, prominent fibrotic septa were observed extending in a radial pattern from central veins and portal zones, resulting in the formation of the pseudolobules (data not shown).

To investigate the roles of β-arrestin1 and β-arrestin2 in fibrotic liver tissues, immunohistochemistry was carried out to detect the expression of β-arrestin1 and β-arrestin2 in hepatic fibrosis rats. As shown in Figure 1A,B, β-arrestin1 and β-arrestin2 immunohistochemical examination of hepatic tissue from normal control rats showed staining of β-arrestin1 and β-arrestin2 protein in the cytoplasm, and, to a lesser extent, in the membrane. With the progression of hepatic fibrosis, β-arrestin2 expression in the rat liver correspondingly increased in the portal area, the fibrous septa and the proliferated peripheral cells. Nevertheless, there were no significant changes in the expression of β-arrestin1 protein. At five time points after porcine serum injection, optical density values of β-arrestin2 in rat liver tissue (3, 6, 9, 12, and 16 weeks) increased with each subsequent week. Further, optical density values from porcine serum injection rats after 6 weeks were significantly higher than those from the control group (Fig. 1D).

ACTIVATION AND PROLIFERATION OF HSCs

Given that α-SMA is an activated HSCs marker, immunohistochemical staining and Western blot of α-SMA was used to quantify the activation and proliferation of HSCs. In hepatic tissues of normal control rats, there was only weakly positive expression of α-SMA in the smooth muscle cells of the vessel wall. With the development of hepatic fibrosis, though, the α-SMA-positive cells in the hepatic tissues of rats increased significantly, distributing mainly in the portal area, the fibrous septa, and the hepatic sinusoids (Fig. 1C).

At five time points after porcine serum injection, the optical density values of α-SMA in rat liver tissue increased with each passing week. Further, optical density values from fibrotic rats were all significantly higher than those from the normal control group (Fig. 1D).

β-ARRESTIN2 EXPRESSION IN ACTIVATED HSC FROM PS INDUCED LIVER FIBROSIS RATS

β-Arrestin2 and α-SMA immunofluorescence double-labeled hepatic tissue slices were examined. Single channel scanning displayed β-arrestin2 and α-SMA-positive signal as green and red fluorescence foci, respectively. Alongside the green and red foci, after merging the images of single channel scanning, yellow foci corresponding to colocalized β-arrestin2 and α-SMA were found in the hepatic tissue slices. As in rat liver tissue only activated HSC and a few vascular smooth muscle cells express α-SMA during hepatic fibrosis, the yellow spots marked β-arrestin2-positive expression in activated HSC. Analysis of the images indicated that at five time points after PS injection, the activated HSC of β-arrestin2-positive expression accounted for $12.33 \pm 2.69\%$, $38.56 \pm 5.04\%$, $58.88 \pm 6.58\%$, $78.56 \pm 7.98\%$, and $88.53 \pm 8.92\%$ (3, 6, 9, 12, and 16 weeks, respectively) of the α-SMA-positive expression cells (total activated HSC). Thus, with the development of hepatic fibrosis, the

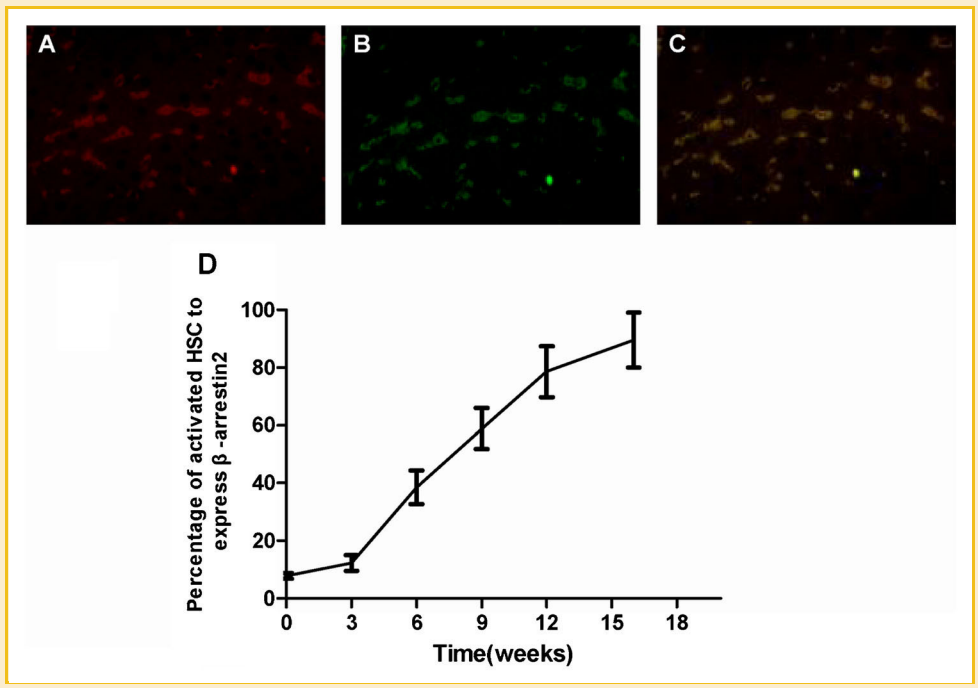

Fig. 2. Immunofluorescent double labeling of β-arrestin2 and α-SMA in liver tissue of rats at 16 weeks after PS injection. A: Single channel scanning showing the location of α-SMA in cells (see red color). B: Single channel scanning showing the location of β-arrestin2 in cells (see green color). C: The colocalization of β-arrestin2 and α-SMA in activated HSCs in fibrogenic liver tissues of rats (see yellow color in the merged image). D: With the progression of hepatic fibrosis, the activated HSCs of β-arrestin2-positive expression accounted for an increasingly larger percentage of total activated HSCs ($n = 6$).

ratio of activated HSC of β-arrestin2-positive expression to total activated HSC significantly increased (Fig. 2).

TIME-COURSE ANALYSIS OF β-ARRESTIN1, β-ARRESTIN2, AND α-SMA PROTEIN EXPRESSION IN LIVER TISSUE OF HEPATIC FIBROSIS RAT

Western blotting at five time points after PS injection showed that the expression levels of β-arrestin2 and α-SMA protein in fibrotic rat liver tissue (6, 9, 12, and 16 weeks) increased significantly with increasing severity of hepatic fibrosis. Further, the values from PS injection rats from 6th week were significantly higher than those from the normal control group (Fig. 3). While, the expression of β-arrestin1 had no significant change compared with the normal control group.

CORRELATION ANALYSIS OF β-ARRESTIN2 AND α-SMA

Pearson's correlation analysis showed that the expression of β-arrestin2 had a significant positive correlation with the expression of α-SMA ($r = 0.860$, $P < 0.01$, Fig. 4), the higher the β-arrestin2 expression, the higher the α-SMA expression and the more severe the hepatic fibrosis.

TIME-COURSE EXPRESSION OF β-ARRESTIN1 AND β-ARRESTIN2 IN HSCs STIMULATED WITH PDGF-BB

PDGF-BB has a growth stimulatory effect on HSCs [Pinzani, 2002]. To further investigate the role of β-arrestin1 and β-arrestin2 in the proliferation of HSCs in vitro, time-course expression of β-arrestin1 and β-arrestin2 in HSCs stimulated with PDGF-BB was observed. As

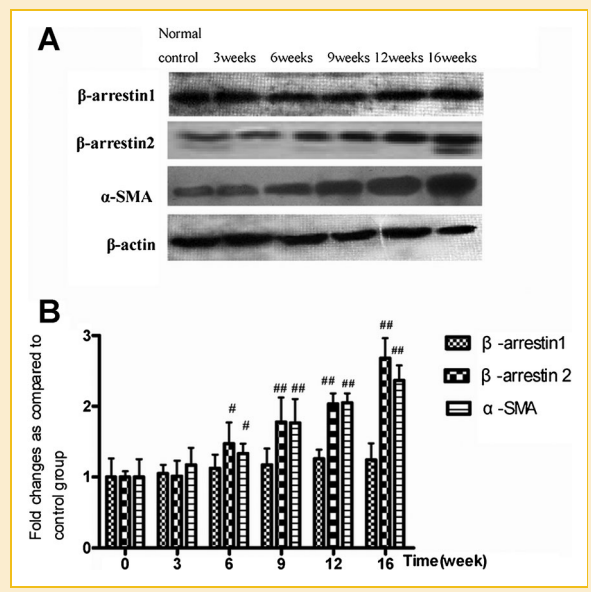

Fig. 3. Western blotting analysis was used for time-course analysis of β-arrestin1, β-arrestin2, and α-SMA protein expression in liver tissue of hepatic fibrosis rat. A: β-Arrestin1, β-arrestin2, and α-SMA protein expression in rat liver tissues of normal control group and hepatic fibrosis at different time points. B: Bar graph of protein quantification plotted from not <3 independent experiments. The band intensity of β-arrestin1, β-arrestin2, and α-SMA was quantified by densitometry and normalized to β-actin. Densitometry values in the histograms were expressed as fold change relative to the normal control, which was assigned a value of 1. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with normal control group.

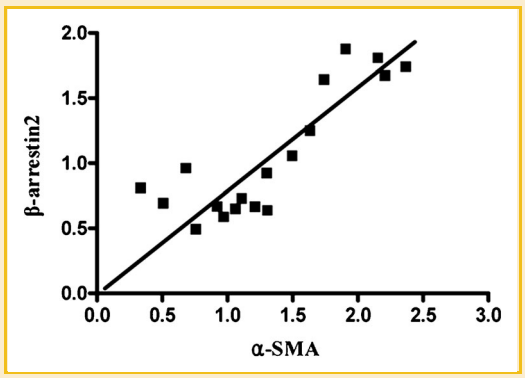

Fig. 4. The scatter plot of the β-arrestin2 and α-SMA expression revealed a strong positive correlation, indicating that the β-arrestin2 expression is related to the proliferation of HSCs ($r = 0.860$, $P < 0.01$).

shown in Figure 5, the expression of β-arrestin2 was gradually increased after stimulation with PDGF-BB for 0.5 h in HSCs and peaked at 2 h. While, the β-arrestin1 had no significant change under the PDGF-BB stimulation.

ESTABLISHMENT OF THE STABLE CELL LINE OF HSCS WITH β-ARRESTIN2 INHIBITION

It is well known that HSCs plays a central role in hepatic fibrogenesis. To further investigate the role of β-arrestin2 in hepatic fibrosis, we used siRNA targeting β-arrestin2 mRNA to block the expression of β-arrestin2 in activated HSCs. The real-time PCR results revealed that there was a 68% decrease of β-arrestin2 mRNA in the stable cell line of HSC-T6 transfected with siRNA as compared with the control (Fig. 6A). In addition, siRNA led to an approximately 70% reduction of β-arrestin2 protein expression by means of Western blot (Fig. 6B). These results clearly demonstrated that the expression of β-arrestin2 mRNA and protein in HSC-T6 transfected with siRNA targeting β-arrestin2 mRNA was inhibited strongly.

EFFECTS OF β-ARRESTIN2 ON THE PROLIFERATION OF THE HSCs

To examine the effect of β-arrestin2 on HSCs proliferation stimulated by PDGF-BB, MTT was performed to detect the proliferation of HSCs transfected with siRNA targeting β-arrestin2. The result revealed that the proliferation of HSCs with PDGF-BB was significantly increased compared with that in HSCs without PDGF-BB. However, the proliferation of HSCs was remarkably inhibited with the transfection of siRNA target β-arrestin2.

SILENCING β-ARRESTIN2 GENE EXPRESSION BY siRNA ATTENUATES ERK1/2 ACTIVATION IN HSCs STIMULATED WITH PDGF-BB

If β-arrestin2 mediated proliferation of HSCs stimulated by PDGF-BB requires downstream interaction between ERK1/2, and then downregulating the level of β-arrestin2 protein in PDGF-BB stimulated cells would impair ERK1/2 activation. Therefore, we

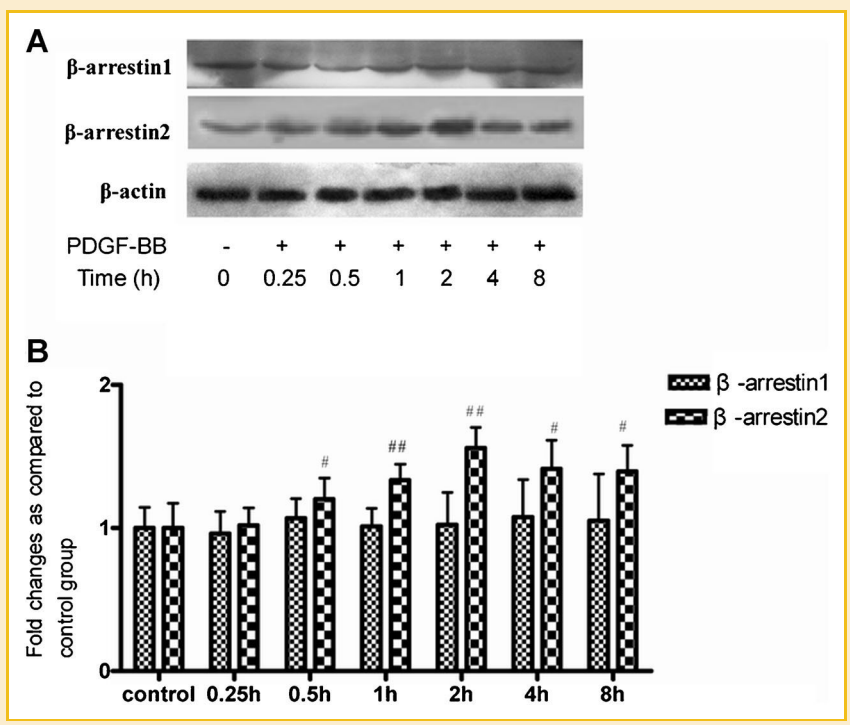

Fig. 5. Time-course expression of β-arrestin1 and β-arrestin2 in HSCs stimulated with PDGF-BB. HSCs were incubated with 50 μg/L PDGF-BB for the times indicated in the figure and protein extracted and analyzed by Western blotting. HSCs without any PDGF-BB stimulation were used as the baseline control. β-Actin immunoblot was included as the internal loading control. A: Representative blot and (B) the histogram corresponding to the quantitative analysis of β-arrestin1 and β-arrestin2 are shown. Densitometry values in the histograms were expressed as fold change relative to the control, which was assigned a value of 1. The data from three independent experiments are shown as means ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control group.

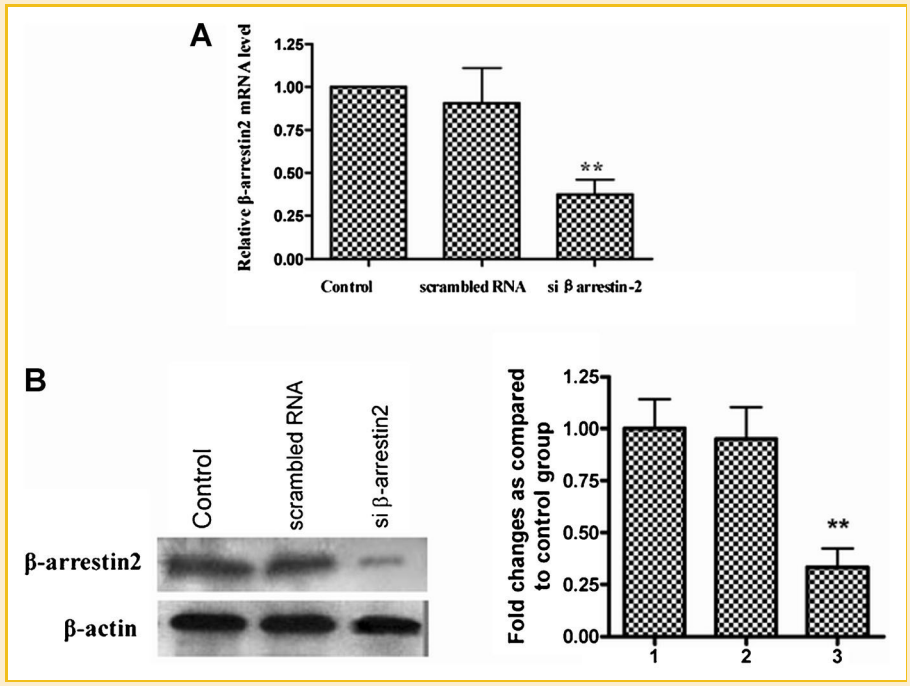

Fig. 6. Expression of the β-arrestin2 in the stable HSC-T6 cells transfected with β-arrestin2 siRNA. A: Transcriptional expression of β-arrestin2. The real-time PCR revealed that there was a 68% decrease of β-arrestin2 mRNA in HSC-T6 cells transfected with β-arrestin2 siRNA compared with the control. GAPDH was used as an internal control. B: Protein level of the β-arrestin2. The expression of β-arrestin2 in HSC-T6 cell transfected with siRNA targeting β-arrestin2 was detected with a Western blot assay. The protein level of β-arrestin2 reduced 70% by β-arrestin2 siRNA. Each sample was tested in triplicate. $^{**}P < 0.01$ compared with control group.

further examined this hypothesis by silencing β-arrestin2 gene expression by siRNA. The silencing of β-arrestin2 gene expression significantly inhibited p-ERK1/2 activity in HSCs treated with β-arrestin2 siRNA plus PDGF-BB compared to cells treated with lipofectamine plus PDGF-BB (Fig. 7).

DISCUSSION

Arrestins are known to promote receptor desensitization and internalization by interacting with the phosphorylated form of GPCRs, thereby preventing their coupling with G protein [Oakley et al., 2001; Liggett, 2011]. Several studies have demonstrated that in addition to GPCR desensitization, arrestins also modulate a variety of intracellular functions, including activation of mitogen-activated protein kinase (MAPK), tyrosine kinase, and growth factors [Morrison and Davis, 2003; Bianchi and Ferrari, 2009; Schreiber et al., 2009; Cervantes et al., 2010]. Mounting evidence has showed that β-arrestin2 expression level was significantly up-regulated in several kinds of tumors, including experimental hepatocarcinogenesis [Rosano et al., 2009; Khan et al., 2011]. Our studies are the first to examine the role of β-arrestin2 in experimental hepatic fibrosis. Here, we proved that β-arrestin2 expression was increased in liver of hepatic fibrosis rats compared with normal controls by results from immunohistochemical staining and Western blotting analysis. In addition, the expression of β-arrestin2 was positively correlated with α-SMA, which was a marker of activated HSCs in rat liver tissues ($r = 0.860$, $P < 0.01$). Furthermore, we found that β-arrestin2 expression was gradually

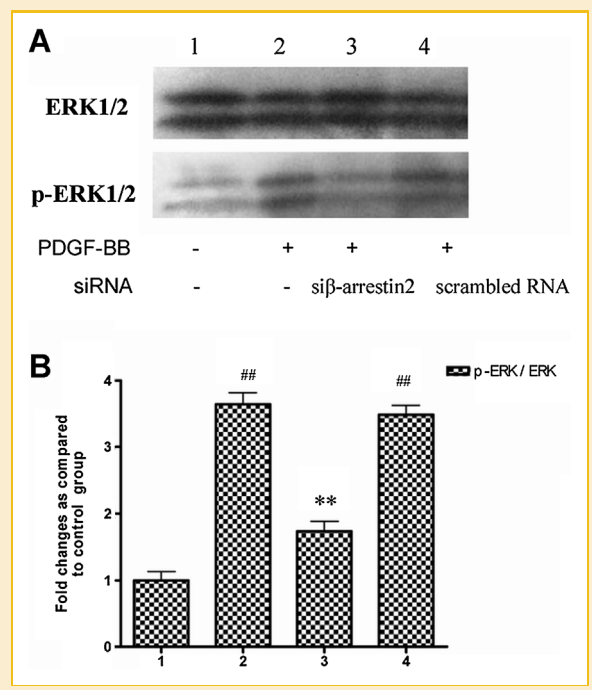

Fig. 7. Effects of silencing HSCs β-arrestin2 gene expression on PDGF-BB-dependent the activation of ERK1/2. A: Down regulation of the β-arrestin2 gene expression by siRNA causes inhibition of ERK1/2 activation. B: The changes in phospho-ERK1/2 are expressed as ratios of phosphorylated/unphosphorylated kinases and are shown as a bar diagram. Densitometry values in the histograms were expressed as fold change relative to the control (Lane 1), which was assigned a value of 1. $^{\#\#}P < 0.01$ compared with Lane 1, $^{**}P < 0.01$ compared with Lane 2.

increased in HSCs stimulated with PDGF-BB. However, the expression of β-arrestin1 was not significantly changed. Transfection of siRNA targeting β-arrestin2 mRNA (siβ-arrestin2) into HSCs abolished the effect of PDGF-BB on the proliferation of HSCs. In addition, siβ-arrestin2 exerted the inhibition of the activation of ERK1/2 in HSCs.

PS-induced rat hepatic fibrosis is a model that shows an intense immune response to the administration of heterogonous serum. Changes in PS-induced hepatic fibrosis in rats have some similarities to those observed in hepatic diseases in human. Thus, the PS induced model is a suitable experimental model of human liver disease and has been used for investigation into hepatic fibrosis pathogenesis and hepatic physiopathology [Baba et al., 2005]. Hepatic fibrosis induced by PS injection causes acute hepatocellular injury and leads to progressive fibrogenesis. Although the mechanism of PS-induced liver injury is not well understood, oxidative stress or proinflammatory cytokinesis plays an important role in the development of both hepatocellular injury and fibrogenesis [Gotardo et al., 2003]. This study selected the PS-induced hepatic fibrosis model, employing immunohistochemical staining and Western blotting to detect dynamic β-arrestin1 and β-arrestin2 expression in rat liver tissue. The results indicated that the protein expression of β-arrestin2 was higher in fibrotic liver tissue of rat than in normal rat liver tissue.

HSCs is the main fibrogenic liver cell type, accounts only for 5–8%. The activation of quiescent HSCs into proliferative, contractile, and fibrogenic cells in response to liver injury appears to be the dominant driving force in fibrosis [Senoo et al., 2010; Hernandez-Gea and Friedman, 2011]. So, it is crucial to examine the relationship of β-arrestin2 up-regulation and HSCs activation and proliferation. HSCs are normally quiescent, so expression of α-SMA in unactivated HSCs is low. During hepatic fibrosis, HSCs are awakened from their quiescent state by cytokines, acetaldehyde, and oxygen-derived free radicals. Once the HSCs activate, they become α-SMA-expressing myofibroblasts and migrate to the site of hepatic injury area. Once there, they proliferate and express various signal transduction proteins, producing both pro-inflammatory cytokines and a great deal of collagen-rich ECM [Choi et al., 2010; Clement et al., 2010]. For this reason, α-SMA can be considered as an indicator of activated HSCs, and the degree of expression of α-SMA in hepatic tissues can reflect the extent of the activation and proliferation of HSCs. This study showed that only a few vascular smooth muscle cells expressed α-SMA in normal rats. With the aggravation of hepatic fibrosis, though, the α-SMA-positive cells in rat liver tissue increased, suggesting an increase in the number of activated HSCs due to accelerated proliferation and activation. This study also found co-expression of β-arrestin2 and α-SMA by immunofluorescence double-labeling assay, indicating that activated HSCs express β-arrestin2. Although the expression of β-arrestin2 in all hepatic tissues is up-regulated during the fibrotic process, this study nevertheless found that β-arrestin2-expressing activated HSC account for an increasingly larger proportion of total activated HSCs as hepatic fibrosis progresses. Statistical analysis showed that β-arrestin2 expression had a positive correlation with the expression of α-SMA. Hence, it is possible that the gradually increased expression of β-arrestin2 during worsening hepatic fibrosis actually influences the activation and proliferation of HSCs and thus the pathogenesis of hepatic fibrosis.

Several growth factors, including PDGF, transforming growth factor β1, insulin-like growth factor, leptin, and connective tissue growth factor, are mitogenic for the HSCs [Kobayashi et al., 2007; Gressner and Gressner, 2008; Gentilini et al., 2009]. It has been demonstrated that in cultured HSCs isolated from normal rat liver, transforming growth factor β1 induces activation of the MAPK pathway [Liu et al., 2011]. Insulin-like growth factor 1 stimulates the proliferation of HSCs and increases type I collagen gene expression and accumulation in HSCs through an ERK- and PI3K-dependent pathway [Svegliati-Baroni et al., 1999]. In addition, leptin-induced HSCs proliferation is also ERK-dependent [Saxena et al., 2004]. PDGF is the most potent mitogen for HSCs in vitro. For the PDGF receptor, there are at least two major pathways of signaling: one involving PI-3 kinase and the other MAPK. PDGF induced proliferation of extracellular matrix-producing cells mediated by stimulating ERK activity [Si et al., 2008; Liu et al., 2009; Atorrasagasti et al., 2011; Kastanis et al., 2011]. Thus, the PDGF-BB stimulated HSCs in vitro was engaged to further investigate the role of β-arrestin2 in the proliferation of HSCs. The results showed that the expression of β-arrestin2 was gradually increased in HSCs under the stimulation of PDGF-BB.

β-Arrestin1 and β-arrestin2, the main members of arrestin family, are expressed ubiquitously. We also measured the expression of β-arrestin1 in hepatic fibrosis rats and normal controls. The results showed that β-arrestin1 expression was not significantly changed in fibrotic liver. And the expression of β-arrestin1 also had no significant change in HSCs stimulated with PDGF-BB, compared with the control group. The structural and functional differences between β-arrestin1 and β-arrestin2 may contribute to the divergence [Nobles, 2007]. Although the structure of β-arrestin1 and β-arrestin2 is highly homological, the N-terminal domain and the conformation of β-arrestin1 are different from that of β-arrestin2 in its activated state [Kumar et al., 2007; Seregin et al., 2010].

β-arrestin2 has been demonstrated as an important modulator of GPCR signaling and plays a role in anti-apoptotic function [Sun and Lin, 2008; Sun et al., 2010]. Little is known regarding role of β-arrestin2 in PDGF-induced cell proliferation. To further investigate the role of the β-arrestin2 in the mitogenic effects on the HSCs, in the present study, we silenced endogenous β-arrestin2 by siRNA to determine the effect of endogenous β-arrestin2 on PDGF -induced cell proliferation in HSCs. Our data showed that PDGF-BB induced HSCs proliferation through an ERK-dependent pathway and siRNA targeting the β-arrestin2 significantly inhibited HSCs proliferation. Importantly, the activation of ERK1/2 was significantly inhibited after β-arrestin2 siRNA compared with that of the PDGF-BB stimulated HSCs by Western blot analysis. Therefore, from the point of view of signal transduction, we infer that β-arrestin2 can inhibit the activation and proliferation of HSCs via ERK1/2 pathways.

In summary, the present investigation provides strong evidence for the involvement of the β-arrestin2 in the progression of hepatic fibrosis. Aberrant expression of the β-arrestin2 contributes to the pathogenesis of hepatic fibrosis. Interfering the expression of β-arrestin2 at the posttranscriptional level could inhibit the

proliferation of HSCs and the activation of ERK1/2, suggesting that β-arrestin2 can be targeted for therapeutic intervention against liver fibrosis. Further studies are required to elucidate the mechanisms and diverse signaling networks of β-arrestin2-related in liver fibrosis.

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