

## Effect of ligand of peroxisome proliferator-activated receptor $\gamma$ on the biological characters of hepatic stellate cells

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### Abstract

**AIM:** To study the effect of rosiglitazone, which is a ligand of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), on the expression of PPAR $\gamma$  in hepatic stellate cells (HSCs) and on the biological characteristics of HSCs.

**METHODS:** The activated HSCs were divided into three groups: control group, 3  $\mu$ mol/L rosiglitazone group, and 10  $\mu$ mol/L rosiglitazone group. The expression of PPAR $\gamma$ ,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and type I and III collagen was detected by RT-PCR, Western blot and immunocytochemical staining, respectively. Cell proliferation was determined with methylthiazolyltetrazolium (MTT) colorimetric assay. Cell apoptosis was demonstrated with flow cytometry.

**RESULTS:** The expression of PPAR $\gamma$  at mRNA and protein level markedly increased in HSCs of 10  $\mu$ mol/L rosiglitazone group ( $t$  value was 10.870 and 4.627 respectively,  $P < 0.01$  in both). The proliferation of HSCs in 10  $\mu$ mol/L rosiglitazone group decreased significantly ( $t = 5.542$ ,  $P < 0.01$ ),  $\alpha$ -SMA expression level and type I collagen synthesis ability were also reduced vs controls ( $t$  value = 10.256 and 14.627 respectively,  $P < 0.01$  in both). The apoptotic rate of HSCs significantly increased in 10  $\mu$ mol/L rosiglitazone group vs control ( $\chi^2 = 16.682$ ,  $P < 0.01$ ).

**CONCLUSION:** By increasing expression of PPAR $\gamma$  in activated HSCs, rosiglitazone, an agonist of PPAR $\gamma$ , decreases  $\alpha$ -SMA expression and type I collagen synthesis, inhibits cell proliferation, and induces cell apoptosis.

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**Key words:** Peroxisome proliferator-activated receptor gamma; Hepatic stellate cell; Rosiglitazone

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### INTRODUCTION

It is well established that hepatic fibrosis is the pathological basis on which chronic liver diseases further progress to hepatic cirrhosis<sup>[1]</sup>. Multiple factors produce hepatic lesions, contributing to hepatic fibrosis<sup>[2]</sup>. Studies show that reversing hepatic fibrosis may block the progression of hepatic cirrhosis<sup>[3]</sup>. Hepatic stellate cell (HSC) is now well established as the key cellular element involved in the development of hepatic fibrosis<sup>[4]</sup>. When the liver is attacked by pathogenic factors, HSCs, a pericyte-like mesenchymal liver cell population, transform from a "quiescent" status ("resting" HSC) into myofibroblast-like cells ("activated" HSC) with the latter synthesizing and secreting extracellular matrix (ECM), tissue inhibitor of matrix metalloproteinases and multiple cytokines, which contribute to collagen deposition, leading to fibrosis<sup>[5-7]</sup>. Recently, it has been reported that resting HSC expresses peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ); the expression of PPAR $\gamma$  gradually decreases during the course of activation of HSC cultured *in vitro*, indicating that certain expression level of PPAR $\gamma$  may be a crucial element that inhibits the natural activation of HSC<sup>[8-14]</sup>. The present study was designed to investigate whether activated HSCs (myofibroblast-like cells) treated by PPAR $\gamma$ -specific ligands were able to enhance the expression of PPAR $\gamma$  and its effect on HSC proliferation, collagen secretion, and cell apoptosis.

### MATERIALS AND METHODS

#### HSCs of rats

HSCs of rats were obtained from HSC strain rHSC-99 produced by the Department of Hepatobiliary Surgery, Peking University People's Hospital and this strain had the characteristics of activated HSCs<sup>[15]</sup>.

#### Rosiglitazone

A high-affinity specific ligand of PPAR $\gamma$ , was purchased from GlaxoSmithKline Investment Co., Ltd.

#### Experimental groups

Three groups were set up in the study, blank control group, 3  $\mu$ mol/L rosiglitazone, and 10  $\mu$ mol/L rosiglitazone groups, respectively.

### Semi-quantitative RT-PCR detection of PPAR $\gamma$

The extraction and reverse transcription of RNA were performed according to the directions of the kits (Taq PCR MasterMix, TW-Biotech Co.); PPAR $\gamma$  primer was synthesized by Shanghai Bioengineering Co., Ltd, with an upstream fragment of CCCTGGCAAAGCATTGTAT, a downstream fragment of ACTGGCACCCCTTGAAAAATG and an extension fragment of 222 bp. The internal reference  $\beta$ -actin was designed with an upstream fragment of TGGGACG-ATATGGAGAAGAT, a downstream fragment of ATTG-CCGATAGTGATGACCT and an extension fragment of 522 bp. PCR reaction is as follows. PPAR $\gamma$  primers were added to reaction systems, which conditions were set at 95 °C for 5 min; 94 °C for 20 s, 55 °C for 30 s, 72 °C for 40 s, totally 29 cycles; finally at 72 °C for 5 min. The products of PCR were photographed after electrophoresis on 2% agarose gel and the gray scale values determined through image analysis. The sample quantities were adjusted to make the gray scale value in each  $\beta$ -actin group stay at a relatively identical level and then the gray scale values of PPAR $\gamma$  between groups were compared.

### Detection of PPAR $\beta$ protein by western blot

In each electrophoresis lane, a sample of 30  $\mu$ g protein was added and after polyacrylamide gel electrophoresis at a volume fraction of 30%, the electrophoretic proteins were transferred onto nitrocellulose membranes, blocked with 5% fat-free milk in TBST buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.05% Tween 20). The membranes were incubated with the first and second antibody, respectively. Antigen-antibody complexes were visualized using the enhanced chemiluminescence detection system according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc.). Membranes were exposed to autoradiography, and signals were scanned for quantitation. Image analysis was done on the straps to determine the gray scale value. The expression quantity of PPAR $\gamma$  by HSC in each group was expressed relative to that of the control group.

### Detection of PPAR $\gamma$ protein by immunocytochemistry method

After HSCs were cultured on the slides, they were fixed in neutralized 40 g/L formaldehyde, then detected with SP method (Santa Cruz Biotechnology, Inc.) and examined under a microscope. Those cells whose nuclei were brown were defined as positive.

### Effect of rosiglitazone on HSC proliferation

Cells were incubated in a 96-well plate. The cells grew in DMEM culture medium containing a volume fraction of 16% calf serum until the logarithmic growth phase, and were then incubated for 24 h in DMEM culture medium that contained a volume fraction of 1% calf serum, which could stop the growth of cells. After that, cells were incubated in culture media respectively containing a volume fraction of 16% calf serum, as blank control group, and rosiglitazone groups of a final concentration of 3 and 10  $\mu$ mol/L. And the absorbance value of A570 was read on the microplate reader for each well using MTT colorimetric assay at 0, 24, and 48 h.

### Effect of rosiglitazone on the synthesis of $\alpha$ -SMA of HSC, and collagen I and III

HSCs from control group, 3  $\mu$ mol/L rosiglitazone group and 10  $\mu$ mol/L rosiglitazone group were detected by Western blot and immunocytochemistry respectively.

### Effect of rosiglitazone on HSCs apoptosis

The apoptotic index was determined by flow cytometry (Applied Biosystems). A total of  $1 \times 10^6$ /mL cells in a suspension were fixed with precooled 70% ethanol solution and stained, and analyzed by a flow cytometer and the apoptotic index was determined by Multicycles software.

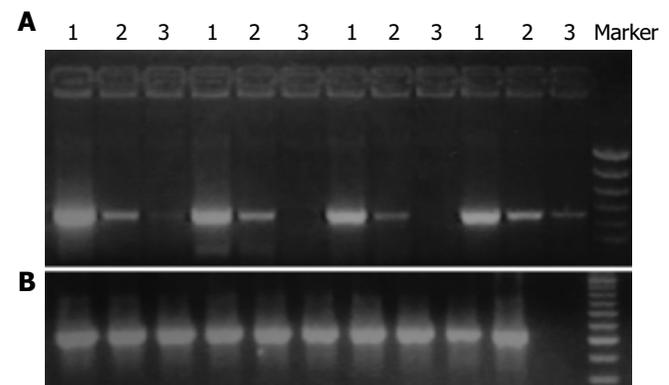
### Statistical analysis

*t*-Test and  $\chi^2$  test were employed to analyze all the data concerned.  $P < 0.05$  was taken as significant.

## RESULTS

### 10 $\mu$ mol/L rosiglitazone enhanced the expression of HSC PPAR $\gamma$ mRNA

At 3  $\mu$ mol/L of rosiglitazone, no significant difference was seen in the expression of PPAR $\gamma$  mRNA in HSCs compared with the control group. There was a significant increase in the expression of PPAR $\gamma$  mRNA in HSCs by 10  $\mu$ mol/L rosiglitazone compared with the control group ( $t = 10.87$ ,  $P < 0.01$ , Figure 1). Meanwhile obvious expression of all  $\beta$ -actin was observed.



**Figure 1** PPAR $\gamma$  mRNA level. **A:** Expression of PPAR $\gamma$  mRNA. Lane 1, 10  $\mu$ mol/L rosiglitazone group; lane 2, 3  $\mu$ mol/L rosiglitazone group; lane 3, control group; **B:** Expression of  $\beta$ -actin.

### Western blot demonstrated that the expression of PPAR $\gamma$ protein increased by 10 $\mu$ mol/L rosiglitazone

At 3  $\mu$ mol/L rosiglitazone, no significant difference was seen in the expression of PPAR $\gamma$  protein in HSCs compared with control group. The expression of PPAR $\gamma$  protein in HSCs by 10  $\mu$ mol/L rosiglitazone was significantly increased compared with that of the control group ( $t = 4.627$ ,  $P < 0.01$ , Figure 2).

### Immunocytochemistry demonstrated enhanced expression of PPAR $\gamma$ protein in HSCs in 10 $\mu$ mol/L rosiglitazone group

In the blank control group and 3  $\mu$ mol/L rosiglitazone

group, no evident expression of PPAR $\gamma$  protein was found by immunocytochemistry. In the 10  $\mu\text{mol/L}$  rosiglitazone group, there was a relatively increased expression of PPAR $\gamma$  protein in the nucleus of most HSCs and in the cytoplasm of some HSCs (Figure 3A). The expression of PPAR $\gamma$  protein seen in the cytoplasm in some of the HSCs might be proteins not entering into the cell nucleus, and the precursor of PPAR $\gamma$  proteins in cytoplasm.

#### 10 $\mu\text{mol/L}$ rosiglitazone markedly inhibited HSCs proliferation

There was no significant difference in the proliferation activity of HSCs between 3  $\mu\text{mol/L}$  rosiglitazone group and the control group. After a 24 and 48 h culture period, the proliferation activity of 10  $\mu\text{mol/L}$  rosiglitazone group was lower than that in both the control group ( $t$  value = 5.542 and 19.293, respectively,  $P < 0.01$  in both) and the 3  $\mu\text{mol/L}$  rosiglitazone group ( $t$  value = 6.880 and 10.502 respectively,  $P < 0.01$  in both, Table 1).

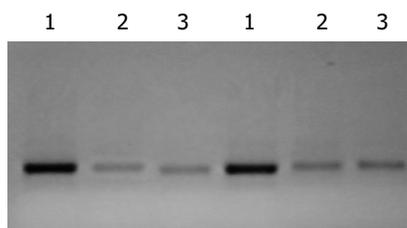
**Table 1** Effect of rosiglitazone on proliferation of rHSC-99

Groups	0 h	24 h	48 h
Controls	0.21 $\pm$ 0.041	0.62 $\pm$ 0.088	1.00 $\pm$ 0.045
3 $\mu\text{mol/L}$	0.22 $\pm$ 0.038	0.63 $\pm$ 0.060	0.93 $\pm$ 0.049
10 $\mu\text{mol/L}$	0.22 $\pm$ 0.064	0.33 $\pm$ 0.085 <sup>b</sup>	0.49 $\pm$ 0.060 <sup>b</sup>

<sup>b</sup> $P < 0.01$  vs controls or 3  $\mu\text{mol/L}$  treatment group.

#### 10 $\mu\text{mol/L}$ rosiglitazone decreased synthesis of $\alpha$ -SMA, collagen I in HSCs

Detection of synthesis of  $\alpha$ -SMA, collagen I and III in HSCs by immunocytochemistry indicated that cytoplasmic



**Figure 2** PPAR $\gamma$  protein was assessed with Western blotting. Lane 1, 10  $\mu\text{mol/L}$  rosiglitazone group; lane 2, 3  $\mu\text{mol/L}$  rosiglitazone group; lane 3, control group.

staining of  $\alpha$ -SMA and collagen I and III in both the blank control group and 3  $\mu\text{mol/L}$  rosiglitazone group was strongly positive. In 10  $\mu\text{mol/L}$  rosiglitazone group, the expression of  $\alpha$ -SMA and collagen I decreased significantly while there was no distinct change in the expression of collagen III. Through gray-scale scanning analysis, the quantity of  $\alpha$ -SMA and collagen I decreased 48% and 42%, respectively compared with the blank control group ( $t$  value = 10.256 and 14.627, respectively,  $P < 0.01$  in both). Western blot detection of  $\alpha$ -SMA showed that there was no significant difference between 3  $\mu\text{mol/L}$  rosiglitazone group and blank control group. The gray-scale value of  $\alpha$ -SMA strap of 10  $\mu\text{mol/L}$  rosiglitazone group significantly decreased compared with the blank control group ( $t = 4.627$ ,  $P < 0.01$ ).

#### 10 $\mu\text{mol/L}$ rosiglitazone accelerated HSC apoptosis

Flow cytometry analysis revealed that HSC apoptosis index in 10  $\mu\text{mol/L}$  rosiglitazone group was 23.8%, significantly higher than that in the control group (3.6%,  $\chi^2 = 16.682$ ,  $P < 0.01$ ).

## DISCUSSION

Excessive production and deposition of extracellular matrix is one of the main characteristics of hepatic fibrosis, in which type I collagen is an essential component<sup>[16]</sup>. Type I collagen mainly comes from activated myofibroblast HSC<sup>[17]</sup>. At present, studies on hepatic fibrosis are increasingly concentrating on HSC, in order to find specific and efficient approaches to inhibit and reverse hepatic fibrosis<sup>[1,2,18,19]</sup>.

Several studies demonstrated that resting HSCs express PPAR $\gamma$  and when resting HSCs are activated to become myofibroblast HSCs, they lose the ability to transcribe and express PPAR $\gamma$ <sup>[8,9,12,20,21]</sup>. PPAR $\gamma$  is a member of nuclear receptor superfamily<sup>[22]</sup>. When combined with its ligand, PPAR $\gamma$  forms heterodimer with retinoid X receptor, which is then combined with peroxisome proliferator response element (PPRE) at the upstream of regulated gene promoter to play a role in transcription and regulation<sup>[23]</sup>. Rosiglitazone is widely used in clinical practice as an insulin sensitizer, and at present, it is identified as a high-affinity specific ligand of PPAR $\gamma$ , which can promote the expression of PPAR $\gamma$  in cells and enhance the transcription activity of PPAR $\gamma$ <sup>[24-28]</sup>. According to the change of blood drug level after rosiglitazone is taken, 2-3  $\mu\text{mol/L}$  rosiglitazone is believed



**Figure 3** Immunocytochemistry of rHSC-99  $\times 100$ . **A:** Positive of PPAR $\gamma$  in 10  $\mu\text{mol/L}$  rosiglitazone group; **B:** positive of  $\alpha$ -SMA in control group; **C:** weak

positive of  $\alpha$ -SMA in 10  $\mu\text{mol/L}$  rosiglitazone group.

to be at a relatively high level, but in a preliminary experiment, only 10  $\mu\text{mol/L}$  rosiglitazone displayed a notable effect. Therefore, we set up a blank control group, 3  $\mu\text{mol/L}$  rosiglitazone group, and 10  $\mu\text{mol/L}$  rosiglitazone group. It was shown that 10  $\mu\text{mol/L}$  rosiglitazone markedly increased the transcription activity and expression level of PPAR $\gamma$  in HSC, indicating that using PPAR $\gamma$ -specific ligand was able to recover the expression and transcription activity of PPAR $\gamma$  to a certain extent.

HSC strain rHSC-99 has been established by serial passage of rat HSC. This strain has the characteristics of activated myofibroblast HSC and highly expresses  $\alpha$ -SMA and collagen I and III<sup>[15]</sup>. The results of our experiment demonstrate that while 10  $\mu\text{mol/L}$  rosiglitazone remarkably enhances the transcription activity and expression level of PPAR $\gamma$  in HSCs, the PPAR $\gamma$  inhibits the expression of  $\alpha$ -SMA and collagen I and the proliferation of HSCs.

At present, the mechanism that enhances the transcription activity and expression level of PPAR $\gamma$  in HSC can inhibit the expression of  $\alpha$ -SMA and collagen I and HSC proliferation has not been well elucidated<sup>[30]</sup>. PPAR $\gamma$  has a regulatory effect on multiple genes, but little research has been done regarding the effect of changes in PPAR $\gamma$  expression and transcription activity on related genes of HSCs<sup>[9,12,13,21,29]</sup>. Researches have shown that the enhancement of PPAR $\gamma$  transcription activity can inhibit the expression of TGF- $\beta$ 1 mRNA induced by TGF- $\beta$ 1, suggesting that the activation of PPAR $\gamma$  might block the autocrine loop of TGF- $\beta$ 1 and further reduce TGF- $\beta$ 1 induced secretion of collagen and HSCs proliferation<sup>[30-32]</sup>. In researches on human and mouse macrophages<sup>[33-36]</sup>, it was found that there existed an interaction between PPAR $\gamma$  and AP-1 signal pathway and the activation of PPAR $\gamma$  could inhibit the activity of transcription factor AP-1, STAT, and NF- $\kappa$ B. It is assumed that, there may be similar mechanism in HSC that PPAR $\gamma$ , through inhibiting the activity of transcription factor AP-1, STAT, and NF- $\kappa$ B, further plays an inhibitory role in HSC proliferation and expression of collagen.

HSC apoptosis can reduce the absolute quantity of activated HSC, thus not only reducing the synthesis of collagen, but also weakening the inhibitory effect on collagen degradation through decreasing activated HSCs to reduce the expression of matrix tissue inhibitor of metalloproteinases<sup>[5]</sup>. Therefore, induction of HSC apoptosis has been considered as an efficient countermeasure to postpone or reverse hepatic fibrosis<sup>[19]</sup>. Rosiglitazone, as a specific ligand of PPAR $\gamma$ , can significantly increase HSC apoptosis rate.

The results of our experiment indicate that PPAR $\gamma$ -specific ligand rosiglitazone, through activating the expression of PPAR $\gamma$ , can inhibit the expression of  $\alpha$ -SMA and collagen I in activated HSCs, the proliferation of activated HSCs and induce apoptosis of activated HSCs.

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