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Molecular mechanism of benign biliary stricture inhibition by rosiglitazone-activated peroxisome proliferator-activated receptor gamma

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SUMMARY

OBJECTIVE: The aim of this study was to investigate whether rosiglitazone-activated peroxisome proliferator-activated receptor gamma can inhibit the occurrence of benign biliary stricture and further elucidate the relevant molecular signaling mechanism.

METHODS: The primary cultured rat biliary fibroblasts following experiments were performed using within the fifth generation cells, which were separated from the bile ducts of Sprague-Dawley rats. The primary cultured rat biliary fibroblasts were co-cultured with 10 ng/mL transforming growth factor-beta 1 for stimulating collagen formation. Competent cells were transfected with siRNA that specifically target Smad3 or connective tissue growth factor to inhibit the expression of the corresponding proteins. The cells were incubated with 10 μmol/L rosiglitazone to activate peroxisome proliferator-activated receptor gamma. The cells were incubated with 10 μmol/L GW9662 in the pretreatment session to inactivate peroxisome proliferator-activated receptor gamma. ELISA was used to determine the levels of connective tissue growth factor and type I collagen in the cell supernatant. Western blotting was used to detect the levels of intracellular p-Smad3/t-Smad3.

RESULTS: Rosiglitazone-activated peroxisome proliferator-activated receptor gamma inhibited the secretion of type I collagen induced by transforming growth factor-beta 1. Peroxisome proliferator-activated receptor gamma inhibitor GW9662 could significantly reverse the rosiglitazone-triggered inhibition of transforming growth factor-beta 1-induced type I collagen secretion by suppressing peroxisome proliferator-activated receptor gamma activation ($p<0.01$). Furthermore, we also found that the activation of peroxisome proliferator-activated receptor gamma was accompanied by the inhibition of transforming growth factor-beta 1-induced Smad3 phosphorylation ($p<0.01$), increased connective tissue growth factor expression ($p<0.01$), and production of type I collagen ($p<0.01$), all of which effects elicited by rosiglitazone could be reversed by peroxisome proliferator-activated receptor gamma inhibitor GW9662.

CONCLUSION: Peroxisome proliferator-activated receptor gamma activated by rosiglitazone inhibits the transforming growth factor-beta1 -induced phosphorylation of Smad3 and the increased connective tissue growth factor expression as well as inhibits the secretion of type I collagen in biliary fibroblasts.

KEYWORDS: TGF-beta1. PPARgamma. Signal pathway. Fibroblasts. Collagen.

INTRODUCTION

Benign biliary stricture (BBS) is a postoperative complication of biliary surgery and remains one of the most difficult problems encountered by hepatobiliary surgeons. BBS most often occurs after iatrogenic bile duct injury or reconstruction of the bile duct after liver transplantation[1]. Importantly, the occurrence of BBS severely diminishes the quality of life of patients and constitutes an economic burden to the country and the patient's family[2,3]. With the ultimate purpose of finding preventive and treatment approaches, the search for effective therapeutic targets from the molecular signaling mechanism of BBS formation has always been an ongoing research interest in recent years.

Proteins in the TGF-β/Smad signaling pathway have extensive involvement in the regulation of cell activation, proliferation, and synthesis of ECM such as collagen[4]. A previous study has also confirmed that the local tissues of biliary stricture have higher levels of expression of pro-fibrotic factors, such as TGF-β1, Smad4, and connective tissue growth factor (CTGF)[5]. Our previous studies have also confirmed that TGF-β1 induces the production of CTGF by activating Smad3, thereby promoting the synthesis of type I collagen in biliary fibroblasts[6].

As one of the nuclear hormone receptor super-family, PPAR-γ has been shown to have pleiotropic functions against tumorigenicity, CNS injury and repair, inflammatory response,

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tissue fibrosis and benefit to a series of diseases in animal models and clinic[7-10]. Studies also revealed that activated PPAR-γ inhibits liver fibrosis by inhibiting collagen production stimulated by the TGF-β signaling pathway[11]. However, the ability of PPAR-γ in inhibiting the TGF-β-induced collagen production in biliary fibroblasts so as to inhibit BBS and the underlying molecular signaling mechanism remains to be fully elucidated. In the present study, we determined whether PPAR-γ can inhibit the collagen production of biliary fibroblasts stimulated by TGF-β and delineate its molecular signaling mechanism in a cell culture experiment using primary cultured rat biliary fibroblasts.

METHODS

Culture of primary rat biliary fibroblasts

Sprague-Dawley rats, each weighed 150–200 g, were used in this experiment. The bile ducts were separated from rats and obtained on a sterile operating table. The inner membrane was scraped, and the remaining tissue was cut into small pieces and incubated in a mixture containing 1 mg/mL collagenase (Sigma) and elastase (Sigma) for 20 min. After centrifugation of the suspension, the cells were collected and then cultured in DMEM high glucose supplemented with FBS.

Identification of cultured primary rat biliary fibroblasts by immunofluorescence

The purified primary rat biliary fibroblasts were incubated with vimentin (Sigma) labeled with fluorescein isothiocyanate. Prior to any interventions, the cells were cultured in a FBS-starved medium (10 mL/L) for 12 h. TGF-β1 (Sigma) was used to stimulate biliary fibroblasts to induce the secretion of type I collagen. PPAR-γ inhibitor GW9662 (Sigma) was utilized to inhibit the activation of PPAR-γ, whereas rosiglitazone was used to activate PPAR-γ.

Enzyme-linked immunosorbent assays

The experiment was carried out in accordance with the manufacturer's instructions for the CTGF Detection Kit and Type I Collagen Detection Kit (MyBioSource, San Diego, CA, USA).

Determination of protein levels using Western blotting

The total concentration of intracellular protein in the collected supernatant was determined using the BCA Detection Kit (Pierce). The protein was loaded into each well of SDS-PAGE gel. After electrophoresis, the protein bands were transferred to the nitrocellulose membrane. Subsequently, blocking on the membrane was performed. Rabbit-derived antibodies against p-Smad3, t-Smad3, and GAPDH were used as primary antibodies, and HRP-labeled goat anti-rabbit IgG was used as secondary antibody. Scion NIH image analysis software was used to detect the expression level of the target proteins.

siRNA transfection

Liposome™ 2000 (Invitrogen) was used to transfect specific siRNAs targeting Smad3 and CTGF into the cultured competent primary fibroblasts. When the cell confluence reached about 50%, a transfection reagent that was prepared by mixing Lipofectamine™ 2000 with siRNA at a ratio of 1:1 was used to transfect the cells prior to intervention.

Detection of peroxisome proliferator-activated receptor gamma activation

PPAR-γ Transcription Factor Assay Kit (Cayman Chemical), based on the ELISA detection method, was adopted as per the supplied protocol.

Statistical analysis

The obtained data are expressed as mean±standard deviation. Statistical Package for Social Sciences (SPSS) data analysis software, version 17, was used for data analysis. One-way analysis of variance (one-way ANOVA) was used to compare the data between different groups. $p<0.05$ indicates that the difference is statistically significant.

RESULTS

Transforming growth factor beta1 stimulates collagen production in primary cultured rat biliary fibroblasts in a dose-dependent manner

At 48 h after incubation with TGF-β1, the results showed that TGF-β1 induced the secretion of Col I from biliary fibroblasts in a dose-dependent manner (Table 1A).

Connective tissue growth factor mediates the secretion of Col I from biliary fibroblasts induced by transforming growth factor beta1

The p-Smad3 level was significantly higher than that of the control group (Figure 1A, $p<0.01$). Next, the cells were transfected with siRNA specifically targeting Smad3. The cells were collected 24 h later and the t-Smad3 level was detected, suggesting that Smad3-specific siRNA significantly inhibited the expression of the target protein (Figure 1B, $p<0.01$).

Biliary fibroblasts were transfected with Smad3- or CTGF-specific siRNA for 24 h before stimulation by TGF-β1, and the culture supernatant was collected after 24 or 48 h of TGF-β1 incubation for the detection of CTGF or Col I level. Our results showed that the specific inhibition of Smad3 expression can significantly suppress the production of CTGF protein (Table 1B, p<0.01). We also found that CTGF-specific siRNA interference of target cells significantly inhibited the expression of the target protein (Table 1C, p<0.01). Our results further showed that Smad3- and CTGF-specific siRNAs can inhibit the secretion of Col I stimulated by TGF-β1 to the same extent (Table 1D, p<0.01). This indicates that TGF-β1 upregulates the expression of CTGF by activating Smad3, thereby stimulating the secretion of Col I in biliary fibroblasts.

Activated peroxisome proliferator-activated receptor gamma inhibits Col I production in rat biliary fibroblasts stimulated by transforming growth factor beta1 in a dose-dependent manner

The results showed that rosiglitazone can significantly activate the PPAR-γ signal, and its activity increased by 4.02 times compared with the control group (Table 1E, p<0.01), while GW9662 diminished the activity of the rosiglitazone group by 1.74 times (Table 1E, p<0.01). The results demonstrate that rosiglitazone-activated PPAR-γ can be inhibited by GW9662. We further found that TGF-β1-stimulated secretion of Col I in biliary fibroblasts in a dose-dependent manner and pre-incubation of cells with 10 μmol/L rosiglitazone can reduce the secretion of TGF-β1-stimulated Col I to a level of 1.44 times over control group (Table 1F, p<0.01).

Molecular mechanism of inhibition of transforming growth factor beta1-induced Col I secretion in biliary fibroblasts by activated peroxisome proliferator-activated receptor gamma

To delineate the molecular mechanism of activated PPAR-γ inhibiting the TGF-β1-induced secretion of Col I in biliary fibroblasts, the cells were transfected with Smad3- or CTGF-specific siRNA, pretreated with rosiglitazone (10 μmol/L), or pretreated with PPAR-γ inhibitor GW9662 (10 μmol/L) simultaneously at 24 h before TGF-β1 treatment. The results suggest that TGF-β1 significantly activates p-Smad3 (Figure 2, p<0.01), induces CTGF expression (Table 1G, p<0.01), and induces the secretion of Col I from biliary fibroblasts (Table 1H, p<0.01). PPAR-γ activated by rosiglitazone significantly reduced the activity of Smad3 (Figure 2, p<0.01) and inhibited CTGF expression (Table 1G, p<0.01) and Col I secretion

Table 1. Pharmacological activity of various treatment groups (fold of increase over control)[a].

A	
Treatment group	**Collagen I (fold of increase)**
Control	1.0
TGF-β1 (1 ng/mL)	2.2±0.24*
TGF-β1 (3 ng/mL)	3.1±0.32*
TGF-β1 (10 ng/mL)	4.3±0.65*
TGF-β1 (30 ng/mL)	4.6±0.74*
B	
Treatment group	**CTGF (fold of increase)**
Control	1.0
TGF-β1 (10 ng/mL)	2.28±0.36*
Smad3 SiRNA+TGF-β1 (10 ng/mL)	1.42±0.27#
C	
Treatment group	**CTGF (percentage of control)**
Control	100
Con SiRNA	97.03±3.45
CTGF SiRNA	36.38±2.46*
D	
Treatment group	**Collagen I (fold of increase)**
Control	1.0
TGF-β1 (10 ng/mL)	2.80±0.76*
Smad3 SiRNA+TGF-β1 (10 ng/mL)	1.49±0.33#
CTGF SiRNA+TGF-β1 (10 ng/mL)	1.41±0.17#
E	
Treatment group	**PPAR-γ (fold of increase)**
Control	1.0
Rosi (10 μmol/L)	4.02±0.65*
Rosi (10 μmol/L)+GW9662 (10 μmol/L)	1.74±0.52△
F	
Treatment group	**Collagen I (fold of increase)**
Rosi (0 μmol/L)+TGF-β1 (0 ng/mL)	1.0
Rosi (10 μmol/L)+TGF-β1 (0 ng/mL)	0.97±0.12
Rosi (0 μmol/L)+TGF-β1 (10 ng/mL)	2.74±0.53*
Rosi (1 μmol/L)+TGF-β1 (10 ng/mL)	2.11±0.42
Rosi (3 μmol/L)+TGF-β1 (10 ng/mL)	1.73±0.33#
Rosi (10 μmol/L)+TGF-β1 (10 ng/mL)	1.44±0.23#
Rosi (30 μmol/L)+TGF-β1 (10 ng/mL)	1.42±0.26#
G	
Treatment group	**CTGF (fold of increase)**
Control	1.0
TGF-β1 (10 ng/mL)	3.18±0.65*
Rosi (10 μmol/L)+TGF-β1 (10 ng/mL)	1.52±0.27#
GW9662 (10 μmol/L)+Rosi (10 μmol/L)+TGF-β1 (10 ng/mL)	2.23±0.45δ
H	
Treatment group	**Collagen I (fold of increase)**
Control	1.0
TGF-β1 (10 ng/mL)	2.63±0.76*
Rosi (10 μmol/L) + TGF-β1 (10 ng/mL)	1.46±0.31#
GW9662 (10 μmol/L)+ Rosi (10 μmol/L)+TGF-β1 (10 ng/mL)	1.97±0.69δ

TGF-β1: transforming growth factor beta; CTGF: connective tissue growth factor. *p<0.01 compared with Con; #p<0.01 compared with TGF-β1 group; △p<0.01 compared with Rosi group; δp<0.01 compared with Rosi+TGF-β1 group. Here a is n=3 in each group.

(Table 1H, p<0.01). However, GW9662 inhibited the activation of PPAR-γ, thereby significantly reversing the abovementioned effects of rosiglitazone (Figure 2, Table 1G,H, p<0.01). This suggests that rosiglitazone-activated PPAR-γ can inhibit the activation of Smad3 stimulated by TGF-β1 and inhibit the expression of its downstream protein CTGF, thereby suppressing the secretion of Col I in biliary fibroblasts.

DISCUSSION

The preferred treatment for BBS is endoscopic retrograde cholangiopancreatography (ERCP) under X-ray combined with biliary stent placement[12]. Based on the clinical guidelines issued by the American Society for Gastrointestinal Endoscopy, the incidence of bile duct restenosis within 2 years after biliary stent placement is as high as 30%, and restenosis should be treated using repeated or multiple ERCP combined biliary stent placement operations[13]. Compared with endoscopic biliary stent placement, biliary reconstruction surgery after surgical management of biliary stenosis is associated with higher difficulty and surgical risk[14]. Therefore, it is particularly important to study the molecular mechanism of BBS formation so as to explore therapeutic targets and drugs that inhibit the formation of BBS. We have developed a degradable polymer stent with good supporting properties as a treatment for BBS[15]. In view of this, we aimed to search for new drugs that inhibit BBS.

As an important pathogenic factor of BBS, specific inhibition of the expression of CTGF is considered to be a potential therapeutic approach to preventing benign stricture of the bile duct[5]. This study further confirmed that CTGF specifically mediates the activation of the TGF-β1/Smad3 signaling pathway and induces the secretion of type I collagen in biliary fibroblasts. The anti-fibrotic effect of activated PPAR-γ has been proven[16,17]. Using biliary fibroblasts, the current study confirmed that the activation of PPAR-γ can inhibit Smad3 phosphorylation and the expression of downstream protein CTGF and suppress TGF-β1-induced type I collagen secretion in biliary fibroblasts, suggesting that activated PPAR-γ may prevent the occurrence of BBS by specifically suppressing the secretion of type I collagen in biliary fibroblasts. Our previous studies also confirmed that PPAR-γ exerts an anti-proliferative effect on biliary fibroblasts, and activated PPAR-γ

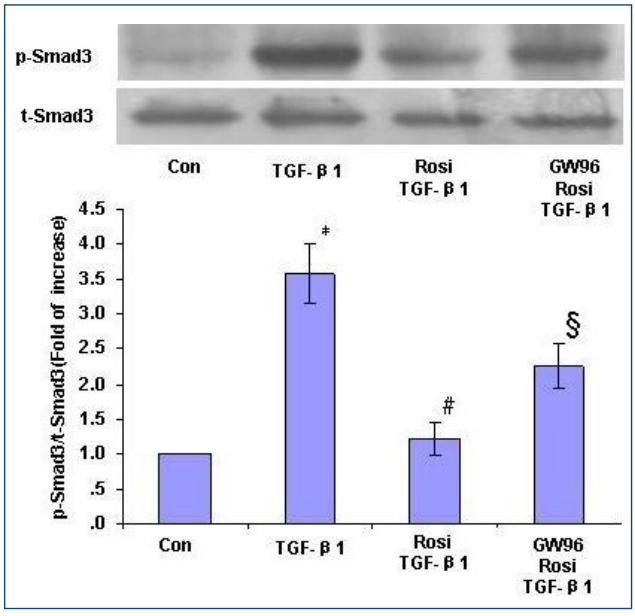

Figure 2. *p<0.01 compared with Con. #p<0.01 compared with the TGF-β1 group. §p<0.01 compared with the Rosi+TGF-β1 group. Activated PPAR-γ suppresses the secretion of TGF-β1-stimulated Smad3 phosphorylation. n=3 in each group. Con, control group; Rosi, rosiglitazone; TGF-β1.

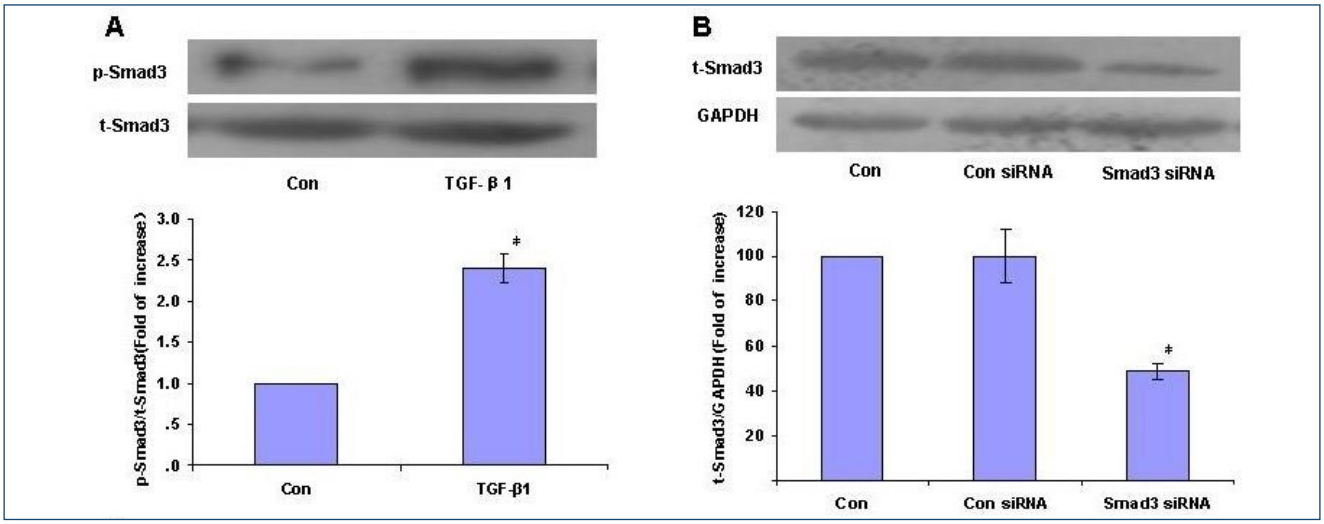

Figure 1. (A and B) TGF-β1 upregulates p-Smad3 activation. n=3 in each group. *p<0.01 compared with Con.

inhibits platelet-derived growth factor (PDGF)-stimulated PI3K/AKT/Skp2 signaling axis to influence AKT phosphorylation and its downstream effects[18].

As a specific PPAR-γ activator, rosiglitazone has been utilized to treat diabetes in the clinic. Many animal or clinic studies have suggested that enhancing the function of PPAR-γ might benefit a lot of other diseases, such as polycystic ovary syndrome in women by affecting insulin-like growth factor-binding protein-3, insulin-like growth factor 1, and insulin resistance[19]. A number of studies including this one further indicate that PPAR-γ activation could inhibit remolding of biliary and has a potential value in the treatment of BBS. Yet, a series of clinical trials are further needed to assure that the treatment mode is effective and safe in the management of BBS[11,20].

CONCLUSIONS

Activated PPAR-γ can inhibit the proliferation of biliary fibroblasts and the deposition of type I collagen by manipulating different molecular signaling mechanisms, indicating the potential of PPAR-γ activator rosiglitazone and other drugs in preventing BBS. This study is expected to provide ideas on new therapeutic approaches for the prevention of BBS.

AUTHORS' CONTRIBUTION

LJ: Conceptualization, Data curation, Formal Analysis, Funding acquisition. **YL:** Investigation, Methodology, Project administration, Resources, Software, Supervision Validation,Visualization. **SJ:** Writing – original draft, Writing – review & editing.

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