Rosiglitazone prevents nutritional fibrosis and steatohepatitis in mice

YUE-MIN NAN1, NA FU1, WEN-JUAN WU1, BAO-LI LIANG1, RONG-QI WANG1, SU-XIAN ZHAO1, JING-MIN ZHAO2 & JUN YU3,4

1Department of Traditional and Western Medical Hepatology, the Third Hospital of Hebei Medical University, Shijiazhuang, China, 2Department of Pathology, Beijing 302 Hospital, Beijing, China, 3Institute of Digestive Disease and Li Ka Shing Institute of Health Sciences, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, and 4Department of Gastroenterology, the First Hospital of Hebei Medical University, Shijiazhuang, China

Abstract

Objective. Currently, no agent has been confirmed as preventing the fibrosing progression of non-alcoholic steatohepatitis (NASH). In this study, rosiglitazone was used in the clinical treatment of insulin resistance in patients with type 2 diabetes mellitus. However, its protective effect on non-alcoholic fibrosing steatohepatitis is not clear. The study aimed to elucidate the effect and the mechanism of rosiglitazone in inhibiting nutrition-related fibrosis in mice.

Methods. C57BL/6J mice were fed a high fat, methionine-choline deficient (MCD) diet for 8 weeks to induce hepatic fibrosis, and rosiglitazone was given in the treated group. The effect of rosiglitazone was assessed by comparing the severity of hepatic fibrosis in liver sections, the activation of hepatic stellate cells (HSCs) and the expression of TGF-β1 and connective tissue growth factor (CTGF).

Results. At week 8, MCD-diet-induced fibrosing NASH models showed increased serum ALT and AST levels, severe hepatic steatosis, and infiltration of inflammation and fibrosis which, associated with down-regulated PPARγ mRNA and protein expression, up-regulated α-SMA protein expression and enhanced TGF-β1, CTGF mRNA and protein expression. Rosiglitazone significantly lowered serum ALT and AST and it reduced MCD-induced fibrosis by repressing levels of α-SMA protein expression and pro-fibrosis factors TGF-β1 and CTGF. It also restored expression of PPARγ.

Conclusions. The present study provides clear morphological and molecular biological evidence of the protective role of rosiglitazone in ameliorating nutritional fibrosing steatohepatitis. Rosiglitazone may ameliorate hepatic fibrosis by activating PPARγ, which can inhibit HSC activation and suppress TGF-β1 and CTGF expression.

Key Words: Fibrosing steatohepatitis, hepatic stellate cells, non-alcoholic steatohepatitis, peroxisome proliferator activated receptor gamma, rosiglitazone

Introduction

The pathological progression of non-alcoholic fatty liver disease (NAFLD) includes simple hepatic steatosis, steatohepatitis, hepatic fibrosis and cirrhosis. Up to now, the pathogenesis of non-alcoholic steatohepatitis with hepatic fibrosis remains unclear. Hepatic stellate cells (HSCs) are the primary source of excessive production of extracellular matrix (ECM), and activation of HSCs is the critical event in hepatic fibrosis [1]. HSCs belong to the non-parenchymal cells in Disse space. Many profibrogenic cytokines can activate quiescent HSCs into activated HSCs which transdifferentiate to a myofibroblast phenotype characterized by cell proliferation, loss of vitamin A storing capability, expression of α-smooth muscle actin (α-SMA) and overproduction of ECM components. Among profibrogenic cytokines, transforming growth factor beta 1 (TGF-β1) and its downstream effective factor, connective tissue growth factor (CTGF), are the master factors for promoting HSC activation, ECM synthesis and secretion of other profibrogenic factors [2–4].

Peroxisome-proliferator-activated receptor gamma (PPARγ) is a soluble transcription factor. Recent studies have demonstrated that PPARγ expression decreases markedly with the activation of HSCs, which indicates that PPARγ is prominent in...
maintaining the quiescent phenotype of HSCs [5]. This implies that PPARγ may be a potential target in preventing hepatic fibrogenesis and the selective PPARγ agonist may be used as a therapeutic agent for liver fibrosis. Rosiglitazone, an agonist of PPARγ, may protect the pathogenesis and progression of fibrotic disease by activating PPARγ. The aim of this study was to investigate the effect of rosiglitazone on activation of HSCs and expression of PPARγ, TGF-β1 and CTGF in mice with hepatic fibrosis induced by the MCD diet [6].

Material and methods

Animals and treatments

Eight-week-old male C57BL6/J mice with body weights between 20 and 25 g were obtained from the Experimental Animal Center of the Chinese Academy of Medical Sciences and were housed in a 22°C-controlled room under a 12-h light-dark cycle. They had free access to water and were allowed to adapt to their food and environment for 1 week before the start of the experiment. The C57BL6/J mice were divided into 3 groups (10/group) and fed a control diet (ICN, Aurora, Oh., USA), a methionine and choline deficient (MCD) diet (ICN) or an MCD diet supplemented with rosiglitazone (50 mg kg⁻¹ d⁻¹). During the experiments, their body weights and rate of diet consumption were recorded. After 8 weeks, all of the animals were killed and blood collected from the femoral artery. Livers were weighed and fixed in 10% formalin for histological analysis or snap-frozen in lipid nitrogen followed by storage at −80°C in a freezer until required. All the protocols and procedures were carried out following the guidelines of the Hebei committee for care and use of laboratory animals, and were approved by the Animal Experimentation Ethics Committee of the Hebei Medical University.

Measurement of serum ALT and AST

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using spectrophotometric assay kits (Sigma, St. Louis, Mo., USA) with an automatic biochemical analyser ( Olympus UA2700, Japan).

Histological examination

The fat on the frozen liver sections (5 µm thick) was detected with Sudan IV stain, a conventional method for fatty tissue in histology. Haematoxylin and eosin-stained paraffin-embedded liver tissues isolated from mice (5 µm thick) were graded for hepatic steatosis, necroinflammation and fibrosis in accordance with the 2006 Guidelines for Diagnosis and Treatment of Non-Alcoholic Fatty Liver Diseases [7] and the 2001 programme of prevention and cure for viral hepatitis [8] issued by the Chinese Society of Hepatology of the Chinese Medical Association.

Immunohistochemistry

Immunostaining for CTGF and α-SMA was performed in paraffin-embedded liver sections using the specific antibodies (Santa Cruz, Calif., USA) and an avidin-biotin complex (ABC) immunoperoxidase method. Briefly, endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide. After blocking with 10% non-immunized goat serum, the primary specific antibodies for CTGF (dilution 1:200) and for α-SMA (dilution 1:200) were applied. Primary antibodies were omitted and non-immunized goat serum was used for negative controls. After extensive rinsing, the biotinylated secondary antibody and ABC complex/HRP were applied. Peroxidase activity was visualized by applying dianinobenzidine to the sections, which were then counter-stained with haematoxylin. Quantitative analysis of CTGF and α-SMA-stained liver sections was performed by morphometric analysis.

Determination of hepatic PPARγ, TGF-β1 and CTGF mRNA expression

Total RNA was isolated with TRIzol reagent (Saibaisheng Biological Technique Institute, Beijing, China). For RT-PCR, 5 μg total RNA was reverse-transcribed with M-MLV in accordance with the manufacturer’s instructions. cDNAs were amplified using specific sets of primers for PPARγ (sense, 5′-ggtgacacagagatgccattc-3′; antisense, 5′-ggcttctctaggccgtg-3′), TGF-β1 (sense, 5′-caagccgctcatctgaagaaacc-3′; antisense, 5′-actgccgtacaactccagtga-3′) and CTGF (sense, 5′-caagcaccctctgaaaaacctc-3′; antisense, 5′-ggctaattgtattcctccatg-3′). The PCR procedure for PPARγ consisted of 30 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, extension at 72°C for 5 min, with initial denaturation of sample cDNAs at 94°C for 5 min before PCR and an additional extension period of 10 min after the last cycle. The different procedure for TGF-β1 and CTGF is annealing at 60°C for 45 s. In parallel, PCR reactions were performed with primers coding for the housekeeping gene β-actin (sense, 5′-gacaggatccaagggattactg; antisense, 5′-ggatctctggtgggagga-3′) to control for equal amounts of template cDNAs. A quantity of 6 µl of
PCR products was analysed in an 8% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a 100-bp DNA marker. Densitometric analysis of PCR products was performed by computer software and standardized by β-actin.

Western blot analysis of hepatic proteins of PPARγ and TGF-β1
Hepatic tissues (10% w/vol) were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and aprogin, leupeptin, soybean trypsin inhibitors (1 mg/ml each). Total protein was extracted and concentration was measured by the Bradford method (DC protein assay; Bio-Rad, Hercules, Calif., USA) as previously described [9]. A quantity of 100 μg protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred onto equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. Membranes were blocked with 5% skim milk in 1×TBST for 1 h, then incubated with specific antibodies against PPARγ, TGF-β1 or β-actin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) overnight at 4°C. After incubation with secondary antibody, proteins were detected by enhanced chemiluminescence (ECL; Amersham Corporation).

Statistical analysis
The Statistical Package for Social Science software (v. 13.0; SPSS Inc., Chicago, Ill., USA) was used for statistical analysis. The data are presented as means±SD. Statistical analysis was carried out by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test (for evaluating differences between groups). A p-value of <0.05 was considered statistically significant.

Table 1. The effect of MCD diet with or without rosiglitazone on serum characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCD</th>
<th>MCD + Rosiglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>49.67±7.64</td>
<td>591.50±71.60*</td>
<td>270.70±85.05*</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>138.33±31.64</td>
<td>1271.8±118.05*</td>
<td>554.55±163.79*</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>0.76±0.29</td>
<td>0.34±0.81</td>
<td>0.47±0.18</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>0.48±0.18</td>
<td>0.41±0.15</td>
<td>0.42±0.15</td>
</tr>
</tbody>
</table>

NOTE. Data are mean±SD (n=10/group).
*P<0.05 relative to mice fed the control diet.
#P<0.05 relative to mice fed the MCD diet.

Results

Effect of rosiglitazone on liver inflammatory injury
As indicated in Table 1, mice fed the MCD diet showed significantly higher serum ALT and AST levels compared with control mice, indicating hepatic injury (p<0.05), and a significant reduction was noted after rosiglitazone treatment (p<0.05). However, serum triglyceride and cholesterol concentrations were not significantly altered.

Effect of rosiglitazone on liver histology
Compared to the controls (Figure 1A and 2A), the liver section from mice fed the MCD diet exhibited disordered lobule structure, macrosteatosis in Zone 3, spot or focal hepatocyte necrosis and inflammatory infiltration (Figure 1B) and portal and perisinusoidal fibrosis (Figure 2B). Treatment with rosiglitazone markedly improved hepatic steatosis, inflammatory infiltration (Figure 1C) and fibrosis (Figure 2C).

Effect of rosiglitazone on body weight and liver weight changes
Similar to what has been reported previously [10], administration of the MCD diet caused body weight loss and liver weight gain (Table 2). Administering rosiglitazone significantly increased body weight by up to 7% (p<0.05) under the MCD diet and reduced liver weight (p<0.05).

Effect of rosiglitazone treatment on CTGF expression
As shown in Figure 3A, CTGF, a cysteine-rich, matrix-associated, heparin-binding protein, was dramatically increased mostly in fibrous tissue (Figure 3A2) compared to CTGF in the control mice (Figure 3A1), but was significantly reduced by rosiglitazone treatment (Figure 3A3) as determined by a computerized morphometric analysis (Figure 4).
Effect of rosiglitazone treatment on HSC activation

To provide further evidence of the anti-fibrotic property of rosiglitazone in MCD feeding mice, we determine the effects of rosiglitazone on hepatic α-SMA protein expression, a well-established marker of HSC activation during liver fibrogenesis. As shown in Figure 3B and 4, and as compared to MCD mice, rosiglitazone significantly suppressed α-SMA expression.

Analysis of transcripts and proteins of genes associated with HSC activation and liver fibrosis

Both mRNA and protein expression of PPARγ were dramatically decreased in mice fed MCD compared with that in mice fed the control diet. Rosiglitazone restored the expression of PPARγ (Figure 5). The mRNA and protein expression of TGFβ1 was increased in MCD-feeding mice and was significantly blunted by rosiglitazone treatment (Figure 5). Consistent with the protein expression determined by immunohistochemistry (Figure 3A), treatment with rosiglitazone significantly down-regulated the mRNA expression of CTGF induced by MCD feeding (Figure 5).

Discussion

A high fat, methionine-choline-deficient diet in mice results in hepatic steatosis, inflammation and fibrosis. This is distinct from the high fat...
Diet-induced liver injury, but resembles the hepatic pathohistological alteration in human non-alcoholic steatohepatitis. We used this representative experimental model of fibrosing steatohepatitis to investigate the mechanism behind rosiglitazone preventing the progress of hepatic fibrosis.

Following the MCD diet for 8 weeks, mice rapidly and consistently developed a severe pattern of steatohepatitis with liver fibrosis, resulting in characteristic pathology of steatosis, mixed inflammatory cell infiltration hepatocyte necrosis, and fibrosis in the pericellular, perisinusoidal and portal area. Concomitant with increased levels of serum ALT and AST, administering rosiglitazone in MCD-fed mice resulted in attenuation of the fibrosing steatohepatitis, as evidenced by decreased ALT and AST levels.
and diminished histologic evidence of fibrosis. In keeping with our finding, Galli et al. [11] demonstrated that rosiglitazone could prevent liver fibrosis induced by dimethyl nitroxide (DMN), CCl4 and bile duct ligation (BDL) in murine. Our results were also supported by the evidence that rosiglitazone protected mice from MCD diet-induced steatohepatitis [12] and patients with NASH with reduced inflammation and liver injury [13,14]. Restoration of the body weight of mice fed the MCD diet treated with rosiglitazone, with a reduction of liver weight and hepatic fat content and serum ALT level, suggests a shipment of fat from the liver to adipose tissue. The roles of adipose tissue and its PPARγ on hepatic fat accumulation have been demonstrated previously in loss-of-function studies, where it has been shown that genetically engineered mice with no adipose tissue or adipose-tissue-specific PPARγ knockout automatically acquired fatty liver [15–17]. On the other hand, activation of PPARγ by rosiglitazone promotes lipogenesis in adipose tissue. Besides enhancing fat redistribution, the ameliorating effect of rosiglitazone was also mediated through modulating adipokines. Other than inflammatory cytokines in liver induced by lipotoxicity of the accumulated fat, adiponectin derived from adipose tissue certainly plays an anti-inflammatory role in the progression of steatohepatitis [18].

Figure 4. Effect of rosiglitazone on quantitative protein expression of CTGF and α-SMA. The expressions of CTGF and α-SMA were estimated by average area density (areas of positive cells/total areas ×100%) in each scope (200-fold) to observe 10 scopes at every section (2G). Data are shown as mean±SD (n=10/group). *, P<0.05 compared with control mice, #, P<0.05 rosiglitazone-treated MCD-fed compared with the mice fed the MCD diet.

Figure 5. Effects of rosiglitazone on hepatic PPARγ, TGF-β1 and CTGF expression in MCD feeding mice. (A) PPARγ, TGF-β1 and CTGF mRNA expression was analysed by RT-PCR. The level of PPARγ was low and TGF-β1 and CTGF were high in MCD mice. However, rosiglitazone could restore PPARγ and suppress TGF-β1 and CTGF expression. The expression of mRNA was estimated by ratio against β-actin. (B) Protein expression of PPARγ and TGF-β1 was determined by Western blot. The changes in protein were consistent with those of the mRNA. Western blots were scanned by densitometry and the data presented as relative intensity units against β-actin. Each bar represents the mean±SD of four to seven mice. *, P<0.05 compared with control mice, ##, P<0.05, ###, P<0.001 rosiglitazone-treated MCD-fed compared with the mice fed the MCD diet.
HSC activation is key in the early phase of liver fibrosis and activated HSC is accompanied by a high expression of \(\alpha\)-SMA protein. Many kinds of fibrogenic cytokines are involved in HSC activation in keeping HSCs quiescent [5]. We found that reduced expression of PPAR\(\gamma\) and enhanced expression of \(\alpha\)-SMA in fibrosing steatohepatitis in mice fed MCD and rosiglitazone could dramatically up-regulate PPAR\(\gamma\) and down-regulate \(\alpha\)-SMA. Thus, rosiglitazone might delay or inhibit the progression of liver fibrosis through agitating PPAR\(\gamma\), which might be the major mechanism of anti-fibrogenesis in non-alcoholic fatty liver disease.

The important role of TGF-\(\beta\)1 in liver fibrosis has been well documented [19–22]. It has been proposed that TGF-\(\beta\)1 – secreted by fibroblasts, Kupffer cells and T-lymphocytes [23] – is a key mediator in the pathogenesis of liver fibrosis, and has a variety of biological effects that include activation of HSCs [24,25]. It could inhibit secretion of matrix metalloproteinases and promote tissue inhibitor of metalloproteinases, and it could up-regulate platelet-derived proteinases and promote tissue inhibitor of metalloproteinases. TGF-\(\beta\)1 suppressed by treatment with rosiglitazone. This effect might take place at the early stage of HSCs activation [3,26], as activated HSCs secrete TGF-\(\beta\)1, which, combined with its receptor through autocrine or paracrine in turn, activates HSCs and produces collagen. Rosiglitazone may activate PPAR\(\gamma\) to reverse activate HSCs into a quiescent type that reduces the expression of TGF-\(\beta\)1. As another fibrogenesis factor, CTGF is the downstream effective factor of TGF-\(\beta\)1 [27,28]. We found that CTGF expression was consistent with TGF-\(\beta\)1 expression. Rosiglitazone could also inhibit CTGF expression in fibrosing steatohepatitis in mice fed MCD. Several studies have shown that leptin exerts a pro-fibrogenic activity that acts directly on hepatic stellate cells [29,30] to increase expression of TGF-\(\beta\)1 and \(\alpha\)-SMA. The fact that leptin and the TGF-\(\beta\)1 promoter have common binding domains would facilitate activation of TGF-\(\beta\)1 [31,32]. The strong correlation found between TGF-\(\beta\)1 expression, overexpression of leptin receptor and the degree of hepatic fibrosis suggests that TGF-\(\beta\)1 activation may be one of the mechanisms by which the leptin could contribute to the development of hepatic fibrosis [32,33]. The effect of rosiglitazone on leptin in fibrosing steatohepatitis is still unknown and warrants further investigation.

In conclusion, rosiglitazone, a selective PPAR\(\gamma\) agonist, attenuated the development of fibrosing steatohepatitis in mice fed the MCD. The mechanisms of its action could be involved in activating PPAR\(\gamma\) to reverse activated HSCs into a quiescent phenotype and reduce the expression of TGF-\(\beta\)1 and CTGF.

Acknowledgements

This work was supported financially by the Wang Bao-en Foundation of Hepatic Fibrosis, No. 20070021 and by a Research Grants Council Competitive Earmarked Research Grant (CUHK 478207).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


