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Basic Study

Serelaxin increases the antifibrotic action of rosiglitazone in a model of hepatic fibrosis

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Abstract

AIM

To determine the effect of combined serelaxin and rosiglitazone treatment on established hepatic fibrosis.

METHODS

Hepatic fibrosis was induced in mice by carbon tetrachloride administration for 6 wk, or vehicle alone (nonfibrotic mice). For the final 2 wk, mice were treated with rosiglitazone, serelaxin, or both rosiglitazone and serelaxin. Serum liver enzymes and relaxin levels were determined by standard methods. The degree of liver collagen content was determined by histology and immunohistochemistry. Expression of type I collagen was determined by quantitative PCR. Activation of hepatic stellate cells was assessed by alpha-smooth

muscle actin (SMA) levels. Liver peroxisome proliferator activated receptor-gamma coactivator 1 alpha (PGC1 α) was determined by Western blotting.

RESULTS

Treatment of mice with CCl₄ resulted in hepatic fibrosis as evidenced by increased liver enzyme levels (ALT and AST), and increased liver collagen and SMA. Monotherapy with either serelaxin or rosiglitazone for 2 wk was generally without effect. In contrast, the combination of serelaxin and rosiglitazone resulted in significantly improved ALT levels ($P < 0.05$). Total liver collagen content as determined by Sirius red staining revealed that only combination treatment was effective in reducing total liver collagen ($P < 0.05$). These results were supported by immunohistochemistry for type I collagen, in which only combination treatment reduced fibrillar collagen levels ($P < 0.05$). The level of hepatic stellate cell activation was modestly, but significantly, reduced by serelaxin treatment alone, but combination treatment resulted in significantly lower SMA levels. Finally, while hepatic fibrosis reduced liver PGC1 α levels, the combination of serelaxin and rosiglitazone resulted in restoration of PGC1 α protein levels.

CONCLUSION

The combination of serelaxin and rosiglitazone treatment for 2 wk was effective in significantly reducing established hepatic fibrosis, providing a potential new treatment strategy.

Key words: Relaxin; Peroxisome proliferator-activated receptors; Liver cirrhosis; Liver diseases; Fibrosis

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Core tip: Hepatic fibrosis is a chronic condition that can lead to cirrhosis, but treatment options are limited and ineffective. Agonists of peroxisome proliferator-activated receptor gamma (PPAR γ), such as rosiglitazone have shown limited efficacy. The hormone relaxin has antifibrotic effects, and increases the activity of PPAR γ , leading to the hypothesis that combination treatment may be more effective. Mice with established hepatic fibrosis were treated with relaxin and rosiglitazone alone or in combination. Combination treatment reduced liver fibrosis, and increased the level of a PPAR γ coactivator. These results suggest that relaxin and PPAR γ co-therapy could be a more effective treatment for hepatic fibrosis.

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INTRODUCTION

Relaxin is a polypeptide hormone of the insulin/relaxin superfamily^[1]. One important action of relaxin is the widespread remodeling of extracellular matrix, which involves altered secretion and degradation of matrix components^[1,2]. The case for a role for relaxin as a general protective agent against fibrosis was dramatically strengthened by observations made using the relaxin-null mouse. These mice spontaneously developed age-related pulmonary, cardiac, dermal, and renal fibrosis^[2-4]. This has led to the use of relaxin in the treatment of experimentally-induced pulmonary and renal fibrosis in rodents, which could be reversed by systemic relaxin treatment^[5,6].

Relaxin also has effects in the liver. Relaxin treatment of rats caused acute changes in the hepatic microcirculation^[7], and morphological changes were detected in nonparenchymal sinusoidal cells^[8]. In addition, the relaxin-null mouse developed increased liver weight^[9]. Work by our laboratory and others showed that relaxin had antifibrotic effects on activated hepatic stellate cells (HSC), which are the major collagen-producing cells in liver injury. Relaxin treatment of activated HSC had numerous effects, including decreased total collagen deposition, collagen synthesis, and collagen-I secretion, and decreased smooth muscle actin expression, but had no effect on HSC proliferation or apoptosis^[10,11]. Relaxin promoted a matrix degrading phenotype in HSCs by increasing matrix metalloproteinase expression and activity, and inhibiting secretion of the tissue inhibitors of metalloproteinases^[10,11]. The effects of relaxin were mediated by activation of the relaxin family peptide 1 (RXFP1) receptor, which is expressed predominantly in the HSC in liver^[12,13]. Finally, using *in vivo* models of experimental hepatic fibrosis, relaxin prevented hepatic collagen content^[10,14], and was effective in treating established hepatic fibrosis^[13,15]. Therefore, there is considerable evidence to support a functional role for relaxin effects in the liver.

A second critical regulatory element in HSC activation is the PPAR γ pathway. PPAR γ is a transcription factor activated by the antidiabetic thiazolidinedione (TZD) drugs, such as rosiglitazone and pioglitazone, and some prostaglandins^[16]. Expression of PPAR γ is detectable in quiescent HSC, but is lacking in activated HSC and myofibroblasts^[17]. Restoration of PPAR γ expression, either by treatment of activated HSC with PPAR γ ligands or by forced expression of PPAR γ , induced a reversion of the HSC to a state that closely resembled the quiescent phenotype, as shown by decreased proliferation, reduced SMA, collagen and TIMP expression, increased MMP-13 expression, and restoration of lipid-storage^[18]. Importantly, treatment of experimentally-induced fibrosis with PPAR γ ligands

prevented hepatic fibrosis in some *in vivo* models^[19-21]. However, recent studies have suggested that TZD treatment may be ineffective for established fibrosis in rodents, casting some doubt on the utility of using TZDs alone for this purpose^[22-24].

As discussed above, PPAR γ has numerous anti-fibrotic effects, and relaxin reduced many of the same markers reported for PPAR γ agonists in HSC in culture and *in vivo*. We reported that relaxin activates PPAR γ transcriptional activity in cells expressing RFXP1 in a manner that did not require the addition of exogenous PPAR γ ligands^[25]. More recently, we identified the mechanism for this stimulation^[26]. Relaxin increased the expression of a coactivator protein in activated HSC, known as PPAR γ coactivator 1 α (PGC1 α) through cAMP and p38-MAPK dependent pathways, and that these pathways were intact in the human hepatic stellate cell line LX2. Therefore, relaxin treatment may enhance the response to TZDs in hepatic fibrosis. To test this hypothesis, we compared the effectiveness of the recombinant form of relaxin (serelaxin), rosiglitazone, or their combination, in the treatment of established models of hepatic fibrosis.

MATERIALS AND METHODS

Mouse model of hepatic fibrosis and treatment

Fibrosis was induced in male C57BL/6 mice (20-24 g, Charles River Laboratories, Wilmington, MA) as described^[15]. Briefly, mice received twice-weekly intraperitoneal injections of CCl $_4$ (diluted 1:7 in sunflower oil) at 1 mL/kg body weight, for a total of 6 wk to induce hepatic fibrosis. Control (nonfibrotic) mice received oil alone. For the final 2 wk of treatment, mice were randomly assigned to receive implantation of subcutaneous osmotic pumps (model 1002, Durect, Cupertino CA) to deliver serelaxin (generously provided by Dennis Stewart, Novartis) at 150 μ g/g per day, or vehicle (citrate buffer). Rosiglitazone (4 mg/kg per day Enzo Life Sciences, Farmingdale, CA) or vehicle (5% DMSO in phosphate buffered saline) was also administered daily by oral gavage for the final 2 wk. Each group contained 5 mice. Mice were sacrificed 72 h after the final CCl $_4$ injection, and liver and blood were collected. Mice were maintained at 22 °C under 12-h light/dark cycles, and had free access to food and water throughout the study. All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals^[27], and were approved by the VA Nebraska Western-Iowa Institutional Animal Care and Use Committee.

Histology and immunohistochemistry

Liver tissue was fixed in 4% buffered formalin, embedded in paraffin, and sections were mounted onto slides. Sections were dewaxed and then stained with picrosirius red to visualize total collagen, as described^[28]. For immunohistochemistry, tissues were

subject to antigen unmasking by heating in citrate buffer (Vector Labs, Burlingame CA), then probed overnight at 4 °C with antibodies directed against type I collagen (ab21286, Abcam, Cambridge, MA) at 1:250 dilution, or α -smooth muscle actin (SMA, clone 1A4, Sigma Chemical, St. Louis, MO) at 1:400 dilution. Positive staining was detected using the DAB Envision System (Dako, Carpinteria, CA). Images were captured and analyzed using ImageJ software as described previously^[15].

Gene expression analysis

Total liver RNA was extracted using the Purelink kit (Thermo Fisher, Carlsbad, CA), with on-column DNase treatment as per the manufacturer's instructions. RNA integrity and lack of contaminating genomic DNA was confirmed by visualization on agarose gels, and RNA concentration was determined using the Ribogreen assay (Thermo Fisher, Carlsbad, CA). A total of 2 μ g of RNA was converted to cDNA using the TaqMan High Capacity Reverse Transcription kit (Thermo Fisher, Carlsbad, CA) in a final volume of 20 μ L. Quantitative PCR was conducted using TaqMan hydrolysis probe assays, using 2 μ L of cDNA (diluted 1:15), 10 μ L Taqman universal PCR master mix, 1.0 μ L Taqman primer/probe mix in a final volume of 20 μ L per reaction. The mouse gene expression assays used included procollagen type I α 2 (*Col1a2*; Mm00483888_m1), α SMA (*Acta2*, Mm01546133_m1), and TATA-box binding protein (*Tbp*; Mm01277045_m1). All expression levels were normalized to that of *Tbp* in the same sample, and the data expressed as the expression level relative to nonfibrotic controls, using the $\Delta\Delta C_T$ method.

Serum measurements

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by standard clinical chemistry assays. Human relaxin levels were determined using the Quantikine kit (R&D Systems, Minneapolis MN), which does not detect mouse relaxin or other insulin- and relaxin-related peptides. Mouse adiponectin levels were measured by immunoassay (Alpco, Salem NH).

Western blotting

Lysates were prepared from liver tissue and protein levels were determined by the bicinchoninic acid assay (Thermo Fisher, Carlsbad, CA). A total of 50 μ g protein was applied to 10% SDS-PAGE gel, then transferred to PVDF membranes. The membranes were probed overnight at 4 °C with antibodies directed against PGC1 α (#101707, Cayman Chemical, Ann Arbor, MI, 1:500) or GAPDH (MAB374, Millipore, Temecula, CA, 1:2000). After washing, membranes were probed with fluorescently-labeled secondary antibodies (Li-Cor, Lincoln, NE), and immunoreactive proteins detected using an Odyssey fluorescent

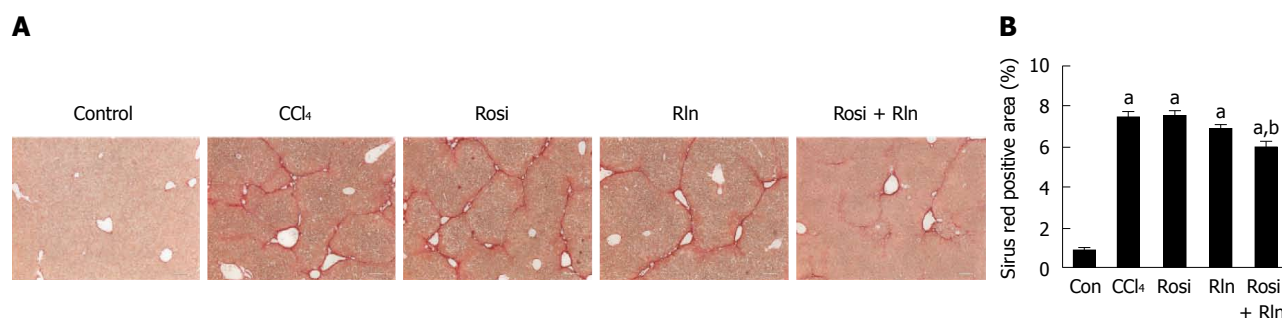


Figure 1 Total liver collagen content. A: Sirius red staining of liver tissue from control (Con), fibrotic (CCl₄), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi + Rln) mice. Bar: 100 μ m. B: Sirius red staining quantified. Data are expressed as mean \pm SE, and analyzed by ANOVA ($n = 5$). ^a $P < 0.001$ vs Con; ^b $P < 0.05$ vs CCl₄, Rosi, or Rln.

Table 1 Serum measurements in control and fibrotic mice

	Control	CCl ₄ only	Rosi	Rln	Rosi + Rln
Body weight (g)	26.7 \pm 0.9	25.6 \pm 0.8	24.8 \pm 1.0	24.9 \pm 1.0	24.7 \pm 0.9
Liver weight (g)	1.7 \pm 0.1	1.7 \pm 0.1	1.5 \pm 0.1	1.6 \pm 0.1	1.4 \pm 0.1
Liver (% body wt)	6.3 \pm 0.1	6.7 \pm 0.3	6.1 \pm 0.3	6.4 \pm 0.4	5.8 \pm 0.2
ALT	91.8 \pm 5.7	2656 \pm 538 ^a	3892 \pm 676 ^a	1755 \pm 610 ^{a,b}	3227 \pm 313 ^a
AST	368 \pm 33	1621 \pm 282 ^a	2496 \pm 339 ^a	1278 \pm 277 ^{a,b}	1863 \pm 165 ^a
Human relaxin (ng/mL)	ND	ND	ND	28.6 \pm 7.2	20.5 \pm 7.4
Adiponectin (ug/mL)	35.0 \pm 3.4	32.6 \pm 3.9	86.0 \pm 12.2 ^c	32.4 \pm 2.2	147.5 \pm 18.7 ^c

^a $P < 0.05$ vs control; ^b $P < 0.05$ vs rosiglitazone (Rosi); ^c $P < 0.05$ vs control, CCl₄ only or relaxin (Rln). ND: Not detected.

scanner (Li-Cor, Lincoln, NE).

Statistical analysis

Statistical analysis was performed using Prism5 software (GraphPad, La Jolla, CA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) with the Newman-Keuls post-test. Data are expressed as mean \pm SE of means.

RESULTS

Serum levels of serelaxin were analyzed by a specific assay that does not detect mouse relaxin. Serelaxin was successfully delivered, as evidenced by detectable human relaxin in treated mice, but not control mice (Table 1). As expected, rosiglitazone treatment caused an increase in serum adiponectin levels, confirming successful treatment and bioactivity of rosiglitazone. Fibrotic mice (CCl₄ group) had significantly elevated levels of ALT and AST. None of the treatments resulted in a significant change in ALT or AST levels compared with CCl₄ treatment alone. A significant difference was detected between Rosi and Rln treatments alone, due to opposite but statistically insignificant differences caused by each treatment individually. There was no significant difference in body or liver weight under any treatment condition (Table 1).

The level of total collagen deposition determined by Sirius red staining was markedly increased with CCl₄ treatment, confirming development of hepatic fibrosis (Figure 1). As demonstrated previously^[14], 2

wk treatment with relaxin alone did not reduce Sirius red staining. Similarly, rosiglitazone alone had no significant effect on the total collagen deposition. In contrast, the combination of relaxin and rosiglitazone significantly reduced the degree of Sirius red staining (Figure 1). To more precisely assess the relative levels of fibrillar collagen, immunohistochemistry for type I collagen was performed (Figure 2). Consistent with the Sirius red staining, only the combination of relaxin and rosiglitazone reduced the overall level of type I collagen.

The primary cell type responsible for the deposition of collagen in fibrosis are the activated hepatic stellate cells (HSC). Using immunohistochemistry for the activated HSC marker α -smooth muscle actin (α SMA), robust induction of HSC activation was induced by CCl₄ treatment (Figure 3). Treatment with rosiglitazone was without effect, while relaxin caused a modest but significant, decrease in α SMA staining. The combination of relaxin and rosiglitazone induced a significant reduction in the level of HSC activation as exemplified by the reduction in α SMA level. These effects were confirmed at the transcriptional level, as similar effects were observed on the gene expression level of type I collagen as determined by qPCR.

Relaxin was previously shown to increase the levels of PGC1 α in cultured hepatic stellate cells^[26]. To determine the effect of serelaxin and rosiglitazone treatment on PGC1 α protein levels in hepatic fibrosis *in vivo*, Western blotting was performed on liver lysates. The level of PGC1 α was decreased after CCl₄ treatment

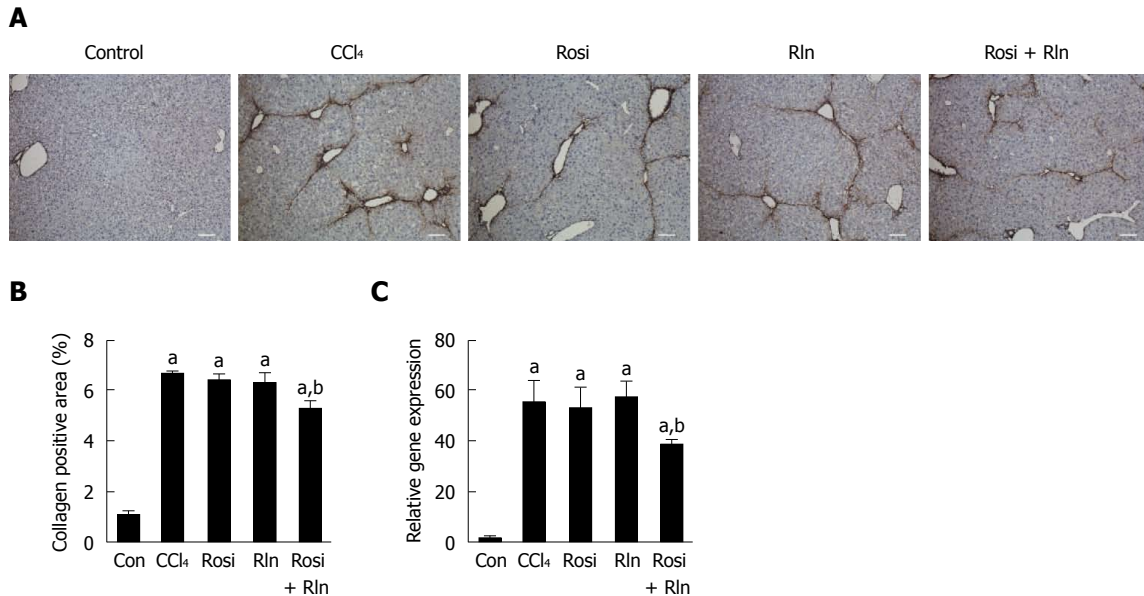


Figure 2 Liver type I collagen content. A: Immunohistochemical staining of liver tissue from control (Con), fibrotic (CCl₄), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi + Rln) mice. Bar: 100 μ m; B: Type I collagen staining quantified; C: Type I collagen gene expression determined by qPCR. Data are expressed as mean \pm SE, and analyzed by ANOVA ($n = 5$). ^a $P < 0.05$ vs Con; ^b $P < 0.05$ vs CCl₄, Rosi, or Rln.

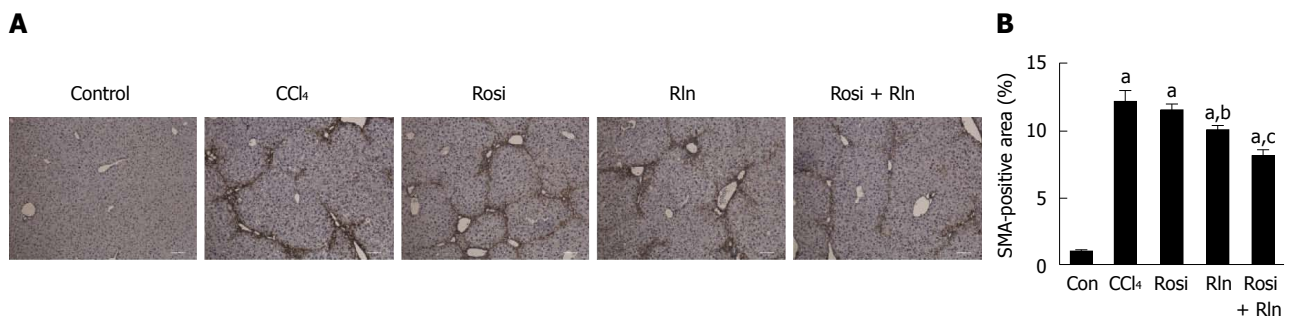


Figure 3 Liver smooth muscle actin content. A: Immunohistochemistry of liver tissue for SMA content from control (Con), fibrotic (CCl₄), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi+Rln) mice. Bar: 100 μ m; B: SMA staining quantified. Data are expressed as mean \pm SE, and analyzed by ANOVA ($n = 5$). ^a $P < 0.001$ vs Con; ^b $P < 0.05$ vs CCl₄; ^c $P < 0.05$ vs CCl₄, Rosi, or Rln.

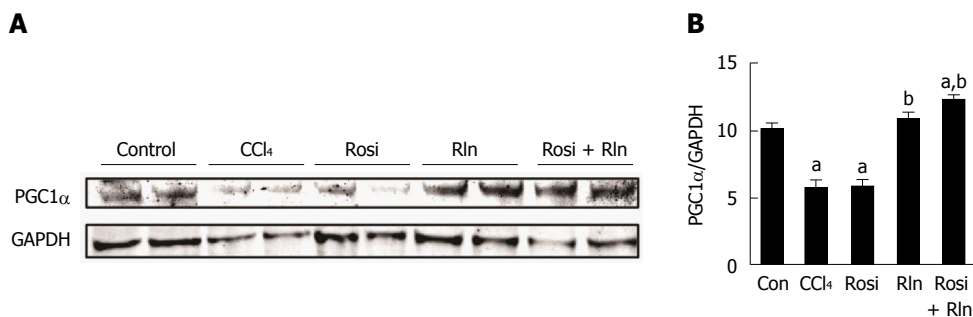


Figure 4 Western blotting of PGC1 α content in livers from control (Con), fibrotic (CCl₄), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi + Rln) mice. A: Liver tissue extracts were analyzed by Western blotting for PGC1 α . The levels of GAPDH are shown as a loading control; B: The levels of PGC1 α relative to GAPDH were determined by densitometry. Data are shown as mean Data are expressed as mean \pm SE, and analyzed by ANOVA ($n = 5$). ^a $P < 0.05$ vs Con; ^b $P < 0.01$ vs CCl₄ or Rosi.

(Figure 4). While single treatment with rosiglitazone was without effect, serelaxin alone or in combination with rosiglitazone restored PGC1 α levels.

DISCUSSION

While hepatic fibrosis is a major health concern worldwide, the options for treatment are limited. The effectiveness of TZDs in the treatment of human liver disease remains to be studied. Early studies of the antidiabetic PPAR γ agonists of the thiazolidinedione class, including rosiglitazone and pioglitazone, reduced the activation of HSCs^[29-31], and had preventive effects in rat models of hepatic fibrosis^[20,21,32,33]. However, in more clinically relevant studies exploring the effectiveness of TZDs in the treatment of established hepatic fibrosis in rats, pioglitazone was only effective when introduced very early in the course of the disease^[23]. Furthermore, pioglitazone was ineffective in reducing the fibrotic phenotype of mouse HSC, and did not prevent CCl₄-induced hepatic fibrosis in mice^[22]. These findings dampened enthusiasm for the utility of TZD treatment of hepatic fibrosis.

The reason for the failure of mice to respond to thiazolidinediones is unknown, but may be related to the lack of PPAR γ expression in activated mouse HSC^[23]. If this is the case, then strategies to increase PPAR γ signaling might restore responsiveness to TZDs. We previously identified PPAR γ as a downstream target of relaxin signaling through its receptor, RXFP1^[25]. Furthermore, we demonstrated that relaxin activated PPAR γ through a ligand-dependent mechanism mediated by increased expression of the PPAR γ coactivator PGC1 α ^[26]. The co-treatment of cells with relaxin and the PPAR γ agonist rosiglitazone resulted in greater PPAR γ transcriptional activity than either relaxin or rosiglitazone alone, suggesting that relaxin was acting to enhance the activity of PPAR γ ^[25]. The purpose of the present study was to test this relationship using an *in vivo* model of established hepatic fibrosis. In earlier studies, we found that short-term relaxin treatment of hepatic fibrosis (2 wk) was insufficient to significantly reduce collagen deposition, and that 4 wk of treatment was required for significant results^[14,15]. We therefore chose to 2 wk of serelaxin treatment for this study. While serelaxin alone had no effect on total collagen or type I collagen, it did significantly reduce α SMA content and therefore, HSC activation. This suggests that the effects of serelaxin on HSC activation precede the degradation of excess collagen. We also confirmed, using rosiglitazone, the lack of effectiveness of TZD treatment alone on mouse hepatic fibrosis, reported previously using pioglitazone^[23]. However, consistent with our earlier cell culture studies^[25], the combination of relaxin and rosiglitazone significantly decreased collagen content and HSC activation, and reduced AST levels. The effects occurred with only 2 wk of treatment, and in the face of continued delivery

of toxin (CCl₄), suggesting that the combination treatment accelerated the rate of the antifibrotic effect.

Our previous findings showed that relaxin enhanced PPAR γ signaling through increased expression of PGC1 α ^[26]. In the present study, CCl₄ treatment reduced the level of PGC1 α , as shown previously in models of liver injury^[34,35]. Treatment with serelaxin, or the combination of serelaxin and rosiglitazone, restored PGC1 α levels. This finding supports the previous findings suggesting that relaxin acts to enhance PPAR γ activity through increased expression of PGC1 α . However, since relaxin treatment alone for 2 wk failed to reduce collagen levels, induction of PGC1 α alone is not sufficient for resolution of hepatic fibrosis, and the presence of PPAR γ agonists is necessary for maximum effectiveness.

Taken together, these data suggest that the combination of serelaxin and rosiglitazone may be a more effective treatment for hepatic fibrosis than either agent alone. This raises the possibility of new approaches to the treatment of hepatic fibrosis that can exploit the combined effects of both RXFP1 and PPAR γ activation. Further studies are needed to determine if combination therapy can be effective in alternative models of hepatic fibrosis, or extended to extrahepatic fibrotic conditions.

COMMENTS

Background

Hepatic fibrosis is characterized by excess collagen deposition in response to a variety of causes of liver injury. There are currently no effective treatments for hepatic fibrosis and cirrhosis. The hormone relaxin has antifibrotic effects in a number of tissues, and was recently found to increase the activity of peroxisome proliferator-activated receptor γ (PPAR γ).

Research frontiers

Relaxin is quickly emerging as an antifibrotic agent, and in preclinical studies has shown efficacy in the treatment of a variety of fibrosis models. The use of PPAR γ agonists have also been explored for antifibrotic effects, but have had limited success in models of hepatic fibrosis.

Innovations and breakthroughs

This is the first study to explore the effect of combined relaxin and PPAR γ agonist treatment of established hepatic fibrosis. The results suggested that the combination treatment was more effective than either treatment alone.

Applications

The findings provide evidence that combined use of relaxin and PPAR γ agonists may represent a potential new approach for the treatment of hepatic fibrosis.

Terminology

Relaxin is a polypeptide hormone with important roles in pregnancy, cardiovascular function, and extracellular matrix regulation. PPAR γ is a nuclear transcription factor that regulates the expression of target genes.

Peer-review

The authors have provided evidence that combined treatment with relaxin and rosiglitazone was effective in a model of hepatic fibrosis.

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