Published in final edited form as:

Liver Int. 2014 March ; 34(3): 416–426. doi:10.1111/liv.12247.

Relaxin Decreases the Severity of Established Hepatic Fibrosis in Mice

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Abstract

Background—Hepatic fibrosis is characterized by excess collagen deposition, decreased extracellular matrix degradation, and activation of the hepatic stellate cells. The hormone relaxin has shown promise in the treatment of fibrosis in a number of tissues, but the effect of relaxin on established hepatic fibrosis is unknown.

Aims—The aim of this study was to determine the effect of relaxin on an *in vivo* model after establishing hepatic fibrosis.

Methods—Male mice were made fibrotic by carbon tetrachloride treatment for 4 weeks, followed by treatment with two doses of relaxin (25 or 75 ug/kg/day) or vehicle for 4 weeks, with continued administration of carbon tetrachloride.

Results—Relaxin significantly decreased total hepatic collagen and smooth muscle actin content at both doses, and suppressed collagen I expression at the higher dose. Relaxin increased the expression of the matrix metalloproteinases MMP13 and MMP3, decreased expression of MMP2 and tissue inhibitor of metalloproteinases 2 (TIMP2), and increased the overall level of collagen degrading activity. Relaxin decreased TGF β -induced Smad2 nuclear localization in mouse hepatic stellate cells.

Conclusions—The results suggest that relaxin reduced collagen deposition and HSC activation in established hepatic fibrosis despite the presence of continued hepatic insult. This reduced fibrosis was associated with increased expression of the fibrillar collagen-degrading enzyme MMP13, decreased expression of TIMP2, and enhanced collagen degrading activity, and impaired TGF β signaling, consistent with relaxin's effects on activated fibroblastic cells. The results suggest that relaxin may be an effective treatment for the treatment of established hepatic fibrosis.

Keywords

Liver disease; relaxin family peptides; cirrhosis; hepatic stellate cells; collagen

Introduction

Liver injury from a variety of causes results in a repair response, resulting in remodeling of the extracellular matrix from one composed predominantly of basement membrane-type collagens to one with increased fibrillar collagens at the injured area (1). This remodeling is due to changes in the types of secreted collagens, matrix metalloproteinases (MMPs), and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (1,2). When the cause of the injury is removed, the extracellular matrix is returned to the normal state by clearance of the fibrillar collagens and restoration of the normal extracellular matrix components. However, in the case of persistent injury, the fibrillar collagen accumulates, resulting in fibrosis characterized by scarring and eventual impairment of liver function. Ultimately, if the fibrotic process is not reversed, the scarring will progress to cirrhosis. Currently, few options are available for the treatment of hepatic fibrosis.

The primary cells responsible for the production of fibrillar collagen in hepatic fibrosis are the hepatic stellate cells (HSCs) (3). In the normal or quiescent state, HSCs function as lipid storing cells, with prominent lipid droplets containing retinoids. Upon liver injury, HSCs transactivate into myofibroblastic cells, with a loss of lipid droplets, and an increase in the expression of α -smooth muscle actin (SMA), increased expression of fibrillar collagen and basement membrane-degrading MMPs such as MMP2 and MMP9, and increased expression of TIMPs. The result is a net accumulation of fibrillar collagen such as collagen I. After cessation of the cause of injury, the number of activated HSCs is decreased, either by apoptosis or by reversion to the quiescent phenotype (1,4). However, in the face of persistent injury, continual HSC activation results in increased scarring and loss of liver function. Therefore, strategies aimed at decreasing HSC activation are an area of great interest.

Relaxin is a polypeptide hormone of the insulin/relaxin superfamily (5). The earliest function attributed to relaxin was in pregnancy, where it regulates a number of critical events including softening of the cervix and vagina (6,7). An important mechanism in these effects is the widespread remodeling of extracellular matrix involving altered secretion and degradation of matrix components (5). These observations have been extended to nonreproductive tissues, where a role has emerged for relaxin as a general antifibrotic hormone (8). In cultured skin, lung, kidney and cardiac fibroblasts, relaxin promoted a matrix degrading phenotype by promoting MMP production and suppressing TIMP levels (9-13). Relaxin has proven effective in the treatment of experimental models of fibrosis *in vivo*, including pulmonary, renal, dermal, and cardiac fibrosis (13-17). 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. In addition to the expected difficulties with reproduction, these mice spontaneously developed pulmonary, cardiac, dermal, and renal fibrosis (8,18).

The role of relaxin in liver fibrosis has been less well-studied. The relaxin-null mouse develops elevated liver weight, but it is currently unclear if this is due to increased collagen deposition (19). The major target of relaxin in the liver is the HSCs. Relaxin treatment of HSCs results in a decrease in markers of the myofibroblastic phenotype, including expression and secretion of collagen I and TIMPs (20,21). In one study, relaxin treatment of HSC decreased SMA expression and increased MMP13 expression and activity (21), while another study found no effect of relaxin on these factors (20). The increase in MMP13 is potentially important for the resolution of fibrosis, because MMP13 is the major fibrillar collagen-degrading enzyme in rodents. The HSCs also express the cognate receptor for relaxin, the relaxin family peptide receptor 1 (RXFP1). In the quiescent state, HSC express low levels of RXFP1, but upon activation in vitro and in vivo, expression of RXFP1 is dramatically increased (22). These data suggest that relaxin may target activated HSC to decrease collagen I deposition and promote matrix degradation in hepatic fibrosis. In a prevention model, relaxin administered concomitantly with the start of liver injury by carbon tetrachloride resulted in a decrease in the total liver collagen content (20). But to be clinically useful, candidate agents for the treatment of hepatic fibrosis must be effective after fibrosis has already been established. In a preliminary report, we provided evidence that short-term relaxin treatment improved some parameters of established hepatic fibrosis induced by chronic carbon tetrachloride exposure (23). In this study, we examined the effect of longer-term treatment of relaxin on established fibrosis in this model. Furthermore, due to concerns about the potential of immune responses to human relaxin in mice, we sought to determine the effect of lower doses of relaxin in this model.

Material and Methods

All animals received humane care and all methods were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, and were approved by the Institutional Animal Care and Use Committee. Hepatic fibrosis was induced in male C57BL/6J mice (Jackson Laboratories, using CCl₄ as described (24). Briefly, CCl₄ was diluted 1:7 in sunflower oil and injected intraperitoneally twice per week at a dose of 1μl/g body weight (0.125μl/g CCl₄) for 4 weeks. Subcutaneous osmotic pumps (Alzet model 1004, Durect, Cupertino CA) were then implanted to deliver recombinant human relaxin-2 (generously provided by Dr. Dennis Stewart, Corthera, San Carlos, CA) at 25 or 75 μg/kg/day, or vehicle alone, for a further 4 weeks, with continued CCl₄ injections. There were 5 mice per group. Three days following the last injection, the mice were killed and liver and blood were harvested. Final serum human relaxin levels were determined by an ELISA kit that does not cross-react with murine relaxin (R&D Systems, Minneapolis, MN).

Liver hydroxyproline content

Hepatic hydroxyproline levels were used to estimate total collagen content as described (25). Portions of liver were digested in 6N HCl at 110°C for 18 hours, then filtered and dried under vacuum. After resuspension and neutralization, samples were subject to oxidation with chloramine-T, then hydroxyproline was derivatized with Ehrlich's reagent, and absorbance was read at 558nm. The hydroxyproline levels were calculated from a standard

curve of L-hydroxyproline processed as described above. The data are expressed as μg hydroxyproline per gram liver wet weight.

Histology and Immunohistochemistry

To visualize collagen accumulation, sections of paraffin-embedded liver tissue were subject to picrosirius red staining (26). For immunohistochemical detection of α -smooth muscle actin (SMA), sections were heated in antigen unmasking solution (Vector Labs, Burlingame, CA), then probed with a SMA-specific antibody (Clone 1A4, Sigma, St. Louis MO) at 1:400 dilution, and visualized using the Envision system (Dako, Carpinteria, CA). Images were captured using a Nikon Eclipse 80i microscope and digital camera. Staining intensity was quantified by histomorphometry with ImageJ software using image deconvolution and threshold functions (27,28). At least 10 nonoverlapping fields per low magnification (20 $\times$) section were analyzed.

Gene expression

Gene expression was determined using quantitative PCR. Total RNA was extracted from liver tissue using the Purelink system (Invitrogen, Carlsbad, CA), treated with DNase, and then visualized on agarose gels to confirm RNA integrity. The RNA concentration was determined using the Ribogreen assay (Molecular Probes, Carlsbad, CA), and equal amounts of RNA were converted to cDNA using the TaqMan High Capacity Reverse Transcriptase system (Applied Biosystems, Carlsbad, CA). Quantitative PCR was performed using TaqMan mouse gene expression assays (Table 1), all using intron-spanning primers (Applied Biosystems, Carlsbad, CA). The levels of target genes were normalized to that of TATA box binding protein (*Tbp*) in the same sample, and the gene expression relative to control (nonfibrotic) samples calculated using the C_T method.

In situ collagen zymography

The hepatic collagen-degrading activity in liver tissue was analyzed using *in situ* positive fluorescent collagen I zymography (29,30) with modifications. This technique involves the use of quenched fluorescein isothiocyanate- (FITC-) labeled collagen type I (DQ-collagen I, Life Technologies, Carlsbad, CA) that exhibits a dramatic increase in fluorescence when degraded by matrix metalloproteinases. A 1% low melting point agarose solution was prepared in 50mM Tris, pH 7.5, containing 150mM NaCl and 5mM CaCl_2 , and cooled to 37°C. DQ-collagen I (100 $\mu\text{g}/\text{ml}$) and 4',6-diamidino-2-phenylindole (DAPI, 1 $\mu\text{g}/\text{ml}$) were added to the agarose mixture to allow simultaneous detection of collagenase activity and nuclei, respectively. Cryopreserved sections of liver (7 μm) mounted on glass slides were rinsed briefly in saline, and then overlaid with 100 μl of the DQ-collagen-agarose mixture. A glass coverslip was quickly applied, and the slides were cooled to 4°C to allow the agarose to set. The slides were then incubated in a humidified chamber at room temperature for 16 hours. Fluorescent images were captured, and the amount of FITC-fluorescence was quantified by histomorphometry with ImageJ software using the threshold function. At least 10 nonoverlapping fields per low magnification (20 $\times$) section were analyzed.

HSC isolation, nuclear extraction and Western blotting

Primary mouse HSC were isolated by collagenase/pronase perfusion and density gradient purification as described (22). The cells were maintained in culture until they completely adopted the myofibroblastic phenotype, and were used within 5 passages. Cells were treated with relaxin and/or TGF β as described in the figure legend, then nuclear extracts were prepared using the NE-Per kit (Pierce, Rockford IL). Protein levels were determined using the bicinchoninic acid assay (Pierce, Rockford IL). Equal amounts of nuclear protein (25 μ g) were analyzed by Western blotting using antibodies to Smad2 (Cell Signaling Technologies, Danvers MA), Smad3 or TATA binding protein (Abcam, Cambridge MA), and IR-Dye labeled secondary antibodies, and imaged using an Odyssey two-color infrared scanner (Li-Cor, Lincoln NE).

Statistical analysis

Statistical analysis for all studies was performed using analysis of variance (ANOVA) with Newman-Keuls post-test for parametric data or Dunn's post-test for nonparametric data using GraphPad Prism5 software (GraphPad, La Jolla, CA).

Results

Hepatic fibrosis was established by administration of CCl₄ for 4 weeks prior to treatment with relaxin. Livers from some mice were assessed by Sirius Red staining to visualize the amount of collagen content (Fig. 1) at the 4 week period. The mice receiving CCl₄ had markedly more hepatic collagen deposition than control mice, confirming that fibrosis had been established. The remaining mice then received subcutaneous osmotic pumps to deliver vehicle (Control), or relaxin at 25 or 75 ug/kg/day for 4 additional weeks, with continued CCl₄ treatment. No statistically significant differences were seen in total body or liver weights between any of the groups (Table 2). When liver weight was analyzed as a percentage of total body weight, CCl₄ treatment resulted in a significant increase, but in relaxin-treated mice there was no difference when compared to nonfibrotic mice. To confirm delivery of relaxin, serum levels were determined at the end of the study. No human relaxin was detectable in the serum of mice receiving vehicle alone. Treatment with 25 or 75 ug/kg/day relaxin resulted in serum levels of 4.8 and 14.7 ng/ml, respectively, at the end of the study.

The effect of treatments on hepatic collagen content was assessed by Sirius red staining of hepatic tissue. Treatment with CCl₄ caused a large increase in collagen deposition compared to that in mice treated with vehicle only (Fig. 2A). Relaxin treatment at either 25ug/kg/day or 75ug/kg/day resulted in a decrease in the amount of Sirius red staining. When quantified by histomorphometry, relaxin was found to cause a significant decrease in Sirius red staining at both doses (Fig. 2B). There was no difference in efficacy between the two relaxin doses. These findings were supported by determination of hepatic hydroxyproline content, a major constituent of collagen protein. While CCl₄ caused a significant increase in the hydroxyproline level, relaxin treatment at both doses significantly reduced this effect to approximately the same level (Fig. 2C). Finally, the rate of collagen I gene expression was determined using real-time quantitative RT-PCR. A significant increase in collagen I gene

expression was induced by CCl₄ treatment (Fig. 2D), and this effect was significantly decreased by the higher relaxin dose.

Previous studies have identified HSC as the major relaxin-responsive cell type in the liver. Relaxin treatment of HSC in culture results in a reduction of markers of the HSC myofibroblastic phenotype, such as expression of SMA (21). Compared with control mice, CCl₄ treatment caused an increase in SMA protein in activated HSC in the liver (Fig. 3A). Relaxin treatment at either dose decreased the amount of SMA protein. This reduction was statistically significant at the both relaxin doses (Fig. 3B). Similarly, SMA gene expression was significantly decreased with relaxin treatment in a dose-dependent manner (Fig. 3C).

A major mechanism regulating collagen levels is proteolysis. Collagen and other extracellular matrix proteins are degraded by matrix metalloproteinases (MMPs), which are in turn regulated by their endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs). To determine if relaxin affects expression of MMPs and TIMPs, real-time RT-PCR was performed (Fig. 4). Treatment with CCl₄ caused an increase in the expression of both TIMP1 and TIMP2. Relaxin significantly reduced TIMP2 expression at both doses, and reduced TIMP1 expression at the highest dose, although this decrease did not reach statistical significance. The expression of the gelatinases MMP2 and MMP9 was increased by CCl₄ treatment. Relaxin at the higher dose resulted in a small but significant decrease in MMP2 expression, but had no effect on MMP9 expression. In contrast, CCl₄ had no significant effect on MMP3 expression, but relaxin increased expression at both doses. Finally, CCl₄ caused an increase in MMP13 expression, the major fibrillar collagen-degrading MMP in rodents. Relaxin treatment at the higher concentration potentiated this effect.

The increased MMP13 and MMP3 expression, coupled with the decreased TIMP expression, suggests that relaxin may induce a net increase in fibrillar collagen degrading activity. To address this possibility, *in situ* collagen zymography was performed on liver tissue sections to determine overall collagen-degrading activity (Fig. 5). In nonfibrotic mice, the degree of collagenase activity was very low, and was restricted to the perivenular areas. Treatment with CCl₄ increased the amount of activity, which extended from the areas around the central veins into the parenchyma. Relaxin at both the low and high doses resulted in a further increase in collagenase activity, which corresponded to the areas of CCl₄-induced collagen deposition. Quantification of the zymographs revealed that relaxin caused a dose-dependent increase in fibrillar collagen-degrading activity, consistent with its effects on MMP and TIMP expression.

A major inducer of hepatic fibrogenesis is the cytokine TGF β , mediated through nuclear translocation of its downstream targets Smad2 and Smad3. To determine the effect of relaxin on TGF β signaling in the liver, mouse HSC were treated with relaxin prior to stimulation with TGF β , and then nuclear extracts were subject to Western blotting for Smad2 and Smad3 (Fig. 6). Treatment with TGF β caused a marked increase in nuclear Smad2 content, which was decreased with relaxin pretreatment. Relaxin had no significant effect on nuclear localization of Smad3, suggesting that relaxin's effects on HSC are at least partially due to inhibition of TGF β signaling to activate Smad2.

Discussion

The data presented here represent the first effective treatment of established hepatic fibrosis with relaxin. Relaxin reduced the overall collagen content in the liver, despite the continued administration of carbon tetrachloride. This suggests that cessation of the cause of liver injury is not required for the beneficial effects of relaxin in this model. However, relaxin did not completely restore collagen levels to normal within the 4 week treatment period. Further studies are needed to determine if extended relaxin treatment and/or higher doses of the hormone can completely restore normal liver collagen levels.

The beneficial effects of relaxin were generally dose-dependent, and furthermore, relaxin was effective at 75ug/kg/day, a considerably lower dose than those previously employed using human relaxin (~500ug/kg/day) for other *in vivo* studies of fibrosis (13,31-34). These lower doses were used due to reports that rodents can mount an immune response to higher doses of human relaxin with time (31). The serum relaxin levels reached in our study (5-15µg/ml) approached those attained after 4 weeks treatment with higher doses of relaxin (20-40ng/ml, (31)), and remained proportional to the dose applied. The results suggest that human relaxin may be an effective against hepatic fibrosis at lower doses than previously reported. Consistent with this concept, a recent study described the effect of murine relaxin, which would not trigger the antibody response, on spontaneous fibrosis in the relaxin-null mice (35). In that study, murine relaxin at 100µg/kg/day for an extended period was effective in reducing fibrosis in a number of tissues. Interestingly, the dose used in the recent successful human trial of relaxin to assess its vasodilatory benefits in acute heart failure was 30µg/kg/day (36).

Relaxin asserts many of its effects by triggering extracellular matrix remodeling. Relaxin has effectively reduced collagen deposition in a number of experimental models, including dermal, renal, cardiac, and pulmonary fibrosis (8,18). Importantly, the relaxin-null mouse spontaneously develops multiple fibroses with advanced age (18). Among the prime targets of the antifibrotic actions of relaxin are the myofibroblastic cells producing fibrillar collagen. The primary myofibroblastic cell involved in hepatic fibrosis is the HSC (3). Upon activation, HSC express high amounts of collagen I and SMA. Previous studies using cultured HSC demonstrated that relaxin reduces collagen synthesis and secretion in activated HSC (20,21), and reduced collagen deposition in a prevention model of CCl₄-induced hepatic fibrosis (20). The effect of relaxin had not been previously tested in a model of established hepatic fibrosis. In the present study, 4 weeks treatment with both doses of relaxin reduced total collagen content in the liver tissue as determined by picrosirius red staining and hydroxyproline content. However, only the higher dose of relaxin reduced the gene expression of collagen I. This discrepancy suggests that the decrease in collagen staining and hydroxyproline content might reflect reduced expression of other collagens, such as the fibrillar collagens III and V, or the basement membrane collagen IV. Alternatively, the reduction may be due to enhanced collagen-degrading activity. This is supported by the findings that both doses of relaxin significantly increased MMP3 expression and overall collagen degradation (Figs. 4 and 5). In addition to the effects on collagen levels, relaxin reduced both the protein and gene expression levels of SMA, a strong marker of HSC activation. The major site of relaxin receptor expression in the liver is

the HSC (22). Thus, the overall effects of relaxin treatment were consistent with reduced HSC activation.

Relaxin has been shown to regulate the expression and activity of MMPs and TIMPs in a number of fibroblastic cells. Relaxin increased MMP levels in dermal, pulmonary, renal, lower uterine, and cardiac fibroblasts (9-13,37), and decreased TIMP expression in dermal and lower uterine segment fibroblasts (12,37). In culture-activated HSC, relaxin caused decreased TIMP1 and TIMP2 expression but no change in MMP2 or MMP9 levels (20,21). In one study, there was no change in MMP13 expression (20), but in another study relaxin increased expression and activity of MMP13 in HSC cultures (21). Few studies have examined the effect of relaxin on MMPs and TIMPs during the treatment of experimental fibrosis *in vivo*. In two models of cardiac fibrosis, relaxin increased MMP2 levels, but had no effect on MMP9 (11,38). In a recent study, relaxin treatment of renal fibrosis model caused increased MMP2 and decreased TIMP1 and TIMP2 levels early in the disease, but the effect was lost with advanced fibrosis, where relaxin actually caused a decrease in MMP2 (34).

The expression of proteins involved in the clearance of extracellular matrix changes dynamically with HSC activation both *in vitro* and *in vivo* (4). In the early stages of activation to the myofibroblastic phenotype, a transient increase in MMP13 and MMP3 occurs (39,40). With advanced activation, the expression of MMP13 and MMP3 declines, while expression of MMP2 and MMP9 (gelatinase A and B, respectively) increases (41,42). These enzymes are predominantly involved in degradation of basement membrane-type collagen such as collagen IV, although there is evidence that MMP2 has some fibrillar collagen-degrading activity (43). In the present study, relaxin increased the expression of both MMP13 and MMP3. In rodents, MMP13 is the major interstitial collagenase, and would be the most likely MMP involved in clearance of collagen I. Furthermore, MMP3, also known as stromelysin, has been implicated in the activation of other MMPs, including MMP13 (44). In the present study, relaxin significantly increased the degradation of type I collagen in liver tissue, suggesting increased MMP13 activity. This finding is consistent with studies of other *in vivo* models showing enhanced MMP13 levels with relaxin (15,45,46), as well as studies using decidual cells and lower uterine segment fibroblasts, where relaxin increased MMP1 (the human interstitial collagenase) and MMP3 levels (37,47).

Consistent with *in vivo* studies in other organs, relaxin had no effect on MMP9 expression in hepatic fibrosis. Relaxin caused a small but statistically significant decrease in MMP2 expression. This is in contrast to the earlier studies of cardiac fibrosis showing increased MMP2 expression with relaxin treatment (11,38). However, in a unilateral ureteric obstruction model of renal fibrosis, relaxin initially reduced MMP2 levels, but actually decreased MMP2 expression with advanced disease (34). Therefore, the effect of relaxin on MMP2 may change during progression of fibrosis, or there may be model- or tissue-specific differences in this response.

Activation of HSC in culture and *in vivo* is accompanied by increased expression and secretion of TIMP1 and TIMP2 (39,48). Relaxin treatment of hepatic fibrosis significantly

decreased the CCl₄-induced expression of TIMP2, and also caused a modest, but statistically insignificant decrease in TIMP1. At present it is unclear why relaxin failed to significantly decrease TIMP1 levels in hepatic fibrosis, since relaxin treatment of isolated HSCs reduced expression of both TIMP1 and TIMP2 (20,21). A possible answer may be found in the recent report that, in the unilateral ureteric obstruction-induced model of renal fibrosis, relaxin significantly decreased TIMP1 and TIMP2 levels early in the disease, but was ineffective with advanced fibrosis (34). Therefore, it is possible that the effect of relaxin changes with the progression of fibrotic disease. Further studies are needed to determine the response to relaxin during progressing fibrosis.

A major factor in the promotion of fibrogenesis in the liver and other tissues is TGF β (3). In the liver, TGF β promotes perpetuation of HSC activation through phosphorylation and nuclear translocation of its downstream mediators Smad2 and Smad3 (49). Many of relaxin's antifibrotic effects in kidney and heart have been attributed to perturbation of TGF β signaling through inhibition of phosphorylation and nuclear localization of Smad2, but not Smad3 (9,34,50,51). In mouse HSC, relaxin decreased the nuclear content of Smad2 in response to TGF β , but did not significantly affect Smad3, consistent with findings in fibroblasts from other tissues. This suggests that in HSC, like other myofibroblastic cells, relaxin acts to decrease TGF β signaling largely through Smad2.

The search for effective treatments of hepatic fibrosis is an area of intense interest in the field of hepatology. To be clinically useful, such treatments must be effective against established hepatic fibrosis. Ideally, such an agent would not only suppress production of fibrillar collagen, but also enhance its clearance. Relaxin is rapidly emerging as a candidate for treatment of fibrosis in many organ systems. The studies presented here suggest that the liver may be an additional target for the antifibrotic actions of relaxin.

Acknowledgments

This study was supported by funding through The Department of Veterans Affairs Biomedical Laboratory Research & Development Program, NIH NIAAA (AA015509), and the Bly Memorial Research Fund (RGB), and by a University of Nebraska Medical Center Graduate College Fellowship (SS). We thank Dr. Dennis Stewart of Corthera, Inc. for providing recombinant human relaxin.

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Fig. 1. Confirmation of established fibrosis after 4 weeks CCl₄ treatment

Mice were made fibrotic by twice weekly injections of CCl₄ for 4 weeks. Control mice received vehicle (oil) alone. Formalin-fixed paraffin-embedded sections were stained with Sirius Red to visualize collagen. Images were captured at original magnification 20×. Bar: 1mm

Fig. 2. Collagen protein content and gene expression in liver tissue

After the initial 4 week treatment to establish fibrosis, mice were treated with vehicle (control), CCl₄ alone, or CCl₄ and relaxin at 25 or 75μg/kg/day for 4 weeks. **(A.)** Formalin-fixed paraffin-embedded sections were stained with Sirius Red to visualize collagen. Images were captured at original magnification 20× (left) or 100× (right). Bar: 1mm (left) or 100μm (right). **(B.)** Sirius Red staining was quantified by histomorphometry. Data are expressed as percent Sirius Red-positive area. **(C.)** Liver hydroxyproline content was determined to estimate collagen content, expressed as μg hydroxyproline per g liver tissue (wet weight). **(D.)** The gene expression level of procollagen 2α(I) was determined by real-time PCR. Data are represented as the collagen I expression relative to a housekeeping gene (TATA binding protein).

Fig. 3. SMA protein content and gene expression

After the initial 4 week treatment to establish fibrosis, mice were treated with vehicle (control), CCl₄ alone, or CCl₄ and relaxin at 25 or 75 µg/kg/day for 4 weeks. **(A.)** The SMA protein content was determined by immunohistochemical analysis of liver tissue. Images were captured at original magnification 100×. Bar: 100 µm. **(B.)** The SMA staining intensity was quantified by histomorphometry. Data are expressed as percent SMA-positive area. **(C.)** The gene expression level of SMA was determined by real-time PCR. Data are represented as SMA expression relative to that of a housekeeping gene (TATA binding protein).

Fig. 4. TIMP and MMP expression levels

The gene expression levels of TIMPs and MMPs in liver tissue from mice treated for 4 weeks with vehicle (control), CCl₄ alone, or CCl₄ and relaxin at 25 or 75 µg/kg/day were determined by real-time PCR. The genes analyzed were TIMP1 (A), TIMP2 (B), MMP2 (C), MMP9 (D), MMP3 (E), and MMP13 (F). Data are represented as gene expression level relative to that of a housekeeping gene (TATA binding protein).

Fig. 5. *In situ* collagen zymography

Collagen-I degrading activity in cryopreserved liver tissue from fibrotic mice were treated with vehicle (control), CCl₄ alone, or CCl₄ and relaxin at 25 or 75 μg/kg/day for 4 weeks was determined by *in situ* collagen zymography. (A.) Degraded collagen was detected by FITC fluorescence (green), and nuclei detected by DAPI staining (blue). Bar: 100 μm. (B.) The collagen-degrading activity was quantified by histomorphometry. Data are expressed as percent FITC fluorescence-positive area.

Fig. 6. Nuclear Smad2/3 content

Mouse HSCs were treated with vehicle (Con) or 1nM relaxin (Rlx) overnight, then stimulated with or without 100pM TGFβ for 30 minutes. Nuclear extracts were prepared and subjected to Western blotting. (A). Western blot for Smad2, Smad3 or TATA binding protein (TBP) as a loading control. (B). Densitometry was performed to determine the levels of nuclear Smad2. Data are expressed as Smad2 relative to TBP in the same sample, normalized to control. *p<.05 by ANOVA, n=3.

Table 1
Gene expression assays

Gene Name (Abbreviation)	Gene Symbol	Primer/Probe ID
Procollagen 1 α 2	<i>Col1a2</i>	Mm00483888_m1
Smooth muscle alpha-actin (SMA)	<i>Acta2</i>	Mm01546133_m1
Matrix metalloproteinase 2 (MMP2)	<i>Mmp2</i>	Mm00439506_m1
Matrix metalloproteinase 3 (MMP3)	<i>Mmp3</i>	Mm00440295_m1
Matrix metalloproteinase 9 (MMP9)	<i>Mmp9</i>	Mm00442991_m1
Matrix metalloproteinase 13 (MMP13)	<i>Mmp13</i>	Mm00439491_m1
Tissue inhibitor of metalloproteinases 1 (TIMP1)	<i>Timp1</i>	Mm01341361_m1
Tissue inhibitor of metalloproteinases 2 (TIMP2)	<i>Timp2</i>	Mm00441825_m1
TATA-box binding protein (TBP)	<i>Tbp</i>	Mm01277045_m1

Table 2
Body and liver weights and serum relaxin levels

After the initial 4 week treatment to establish fibrosis, mice were treated with vehicle (control), CCl₄ alone, or CCl₄ and relaxin at 25 or 75µg/kg/day for 4 weeks. At the end of the treatment period, total body and liver weights were obtained, and liver weight as the percent of total body weight (BW) was calculated. Serum relaxin levels were determined by ELISA.

	Body weight (g)	Liver weight (g)	Liver (% BW)	Relaxin (ng/ml)
Oil	27.2 ± 0.9	1.81 ± 0.06	5.78 ± 0.22	n.d.
CCl ₄	26.4 ± 1.2	1.94 ± 0.14	7.33 ± 0.25*	n.d.
CCl ₄ + RLX (25µg/kg/d)	26.6 ± 1.0	1.83 ± 0.07	6.88 ± 0.12	4.8 ± 1.6
CCl ₄ + RLX (75 µg/kg/d)	25.3 ± 0.8	1.67 ± 0.09	6.87 ± 0.20	14.7 ± 6.6

*
p<.05 compared to oil alone. n.d.: not detected.