Kupffer Cells Mediate Leptin-Induced Liver Fibrosis

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BACKGROUND & AIMS: Leptin has profibrogenic effects in liver, although the mechanisms of this process are unclear. We sought to elucidate the direct and indirect effects of leptin on hepatic stellate cells (HSCs).

METHODS: HSCs from Sprague-Dawley rats were exposed to leptin and expression of collagen-I, tissue inhibitor of matrix metalloproteinases-1 (TIMP1), transforming growth factor β1 (TGF-β1), and connective tissue growth factor (CTGF/CCN2) was assessed. The effects of medium from Kupffer cells (KCs) and sinusoidal endothelial cells (SECs) following leptin were evaluated in HSCs; α-smooth muscle actin (αSMA) production and KC signaling were analyzed.

RESULTS: HSCs were not activated by incubation with leptin. However, HSCs cultured with medium taken from KCs that were incubated with leptin had increased expression of collagen I, TIMP1, TGF-β1, and CTGF/CCN2, as well as αSMA protein levels and proliferation. These effects were leptin receptor dependent because conditioned medium from KCs isolated from leptin receptor-deficient Zucker (fa/fa) rats did not activate HSCs. In KCs incubated with leptin, messenger RNA and protein expression of TGF-β1 and CTGF/CCN2 increased. Leptin potentiated signal transducer and activator of transcription 3, AKT, and extracellular signal-related kinase 1/2 phosphorylation in KCs and increased AP-1 and nuclear factor-κB DNA binding. Finally, addition of anti-TGF-β to KC-conditioned medium inhibited HSC expression of collagen I, TIMP1, and CTGF/CCN2, whereas signal transducer and activator of transcription 3 inhibitor attenuated TGF-β1 production by KC.

CONCLUSIONS: Leptin mediates HSC activation and liver fibrosis through indirect effects on KC; these effects are partly mediated by TGF-β1.

Leptin, an adipocyte-derived hormone, has important effects in regulating body weight, metabolism, and reproductive function. Circulating levels of leptin are known to be increased in overweight and obese persons, in individuals with nonalcoholic steatohepatitis,1,2 and in those with alcoholic liver disease and chronic viral hepatitis.3,5 More recently, leptin has been shown to possess direct profibrogenic activity in the liver.6–8 We previously demonstrated that leptin-deficient ob/ob mice failed to develop hepatic fibrosis in a rodent nutritional model of steatohepatitis and in response to chronic CCl4-induced liver injury.8 Restitution of physiologic levels of circulating leptin restored the liver’s “fibrogenic” capacity.6 Similar results have been obtained in models of fibrosis associated with bile duct ligation9 and following the administration of thioacetamide.10 The cellular and molecular mechanisms for this effect, however, have not been fully elucidated.

Because hepatic stellate cells (HSCs) are the main source of extracellular matrix (ECM) during the evolution of fibrosis, the effects of leptin on HSC behavior have been examined, but results are conflicting. One view holds that leptin acts directly on HSCs to trigger downstream response pathways that ultimately lead to ECM deposition.7,11 Others suggest that Kupffer cells (KCs) and/or sinusoidal endothelial cells (SECs) contain a functional leptin receptor, which can stimulate the release of profibrogenic mediators such as transforming growth factor (TGF)-β1 that in turn drives HSC activation.12 To date, there is no evidence to indicate that leptin-primed KCs or SECs exert direct stimulatory effects on HSCs. However, it is well-known that KCs and SECs play important roles in modulating stellate cell behavior by releasing proinflammatory and profibrogenic factors such as TGF-β1 and reactive oxygen species (ROS) upon stimulation by various noxious stimuli. In addition, recent data indicate that KC dysfunction as evidenced by decreased TNF-α production and down-regulation of TGF-β1 gene expression occurs in leptin receptor-deficient Zucker rats and may account for the attenuation of liver fibrosis in these rodents following the administration of pig serum.13

Abbreviations used in this paper: αSMA, α-smooth muscle actin; collagen I, collagen 1α1; CTGF/CCN2, connective tissue growth factor; ERK1/2, extracellular signal-related kinase 1 and 2; KCs, Kupffer cells; HSCs, hepatic stellate cells; SECs, sinusoidal endothelial cells; TGF-β1, transforming growth factor β-1; TIMP1, tissue inhibitor of matrix metalloproteinases-1; STAT3, signal transducer and activator of transcription-3.

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0016-5085/09/$36.00
In this study, we undertook detailed in vitro experiments to clarify the cellular and molecular mechanisms whereby leptin exerts profibrogenic effects on the liver. Using primary cell culture models of hepatic nonparenchymal cells alone and in coculture, we demonstrate that the principal profibrogenic effects of leptin are mediated via direct effects on KCs leading to the release of soluble mediators including TGF-β1 and connective tissue growth factor (CTGF)/CCN2.

Materials and Methods

Animals

Male Sprague-Dawley rats were obtained from the Animal Resources Centre (Perth, WA, Australia). Zucker rats (fa/fa) and their lean littermates (Fa/Fa) were obtained from Professor Greg J. Barritt (Flinders University, Adelaide, Australia). All animals were maintained under 12-hour light/dark cycles with food and water ad libitum. Experimental protocols were approved by the Sydney West Area Health Service Animal Research Ethics Committee.

Materials

Rat recombinant leptin was purchased from Sigma–Aldrich (St. Louis, MO). Phospho-STAT3, pp38, pERK1/2(44/42), pAKT, and pJNK mouse monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Recombinant TGF-β1, monoclonal TGF-β antibody, platelet-derived growth factor (PDGF) BB, and PDGF ELISA Kit were purchased from R&D Systems (Minneapolis, MN). AP-1 and nuclear factor (NF)-κB consensus double-stranded oligonucleotides were purchased from Promega (Madison, WI). Signal transducer and activator of transcription (STAT) 3 inhibitor peptide (5730956), the MAP kinase kinase (MEK) inhibitor PD98059, and the phosphoinositide-3 kinase (PI3K) inhibitor LY294002 were obtained from Calbiochem (San Diego, CA). Soluble TGF-β receptor (sTGFβR) fusion protein was a gift from Biogen Inc.

Nonparenchymal Cell Isolation and Culture

HSCs were isolated by 2-step (collagenase B and pronase E) perfusion. KCs and SECs were further obtained and purified by elutriation. HSCs were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin and plated on 6-well plates at a density of 0.8 × 10^6 cells/well. Viability was routinely over 95% for all experiments. Purity was 95% as determined by morphology, vitamin A autofluorescence, and desmin positivity.

KCs were identified by their ability to phagocytose latex beads; viability was > 96% and purity > 98%. The viability of SECs was > 98% and purity at least 94% as determined by morphology (cobblestone appearance) and absence of latex bead phagocytosis. KCs were cultured in 10% FCS/DMEM/1% penicillin-streptomycin in 6-well plates. SECs were cultured in M199 with 20% FCS, 1% penicillin-streptomycin, insulin (20 mU/mL), heparin (10 U/mL), VEGF (5 ng/mL), and dexamethasone (10 μmol/L). SEC culture wells were precoated with type I collagen (Nalge Nunc International, Rochester, NY).

Immunoblot Assays for Protein Expression

Culture media was removed, and cells were washed with phosphate-buffered saline (PBS) and then lysed on ice in a buffer containing 20 mmol/L Tris, 0.5 mmol/L MgCl₂, 1 mmol/L DTT, 3 mmol/L NaNO₃, with protease and phosphatase inhibitors. Cell lysates were disrupted with a sonicator on ice. Immunoblots were performed as previously described. Immuno blotting was performed for TGF-β1 protein in culture medium after concentrating the media using Microcon YM-10 Centrifugal Filters (Millipore, Billerica, MA).

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total cellular RNA was prepared from HSCs, KCs, and SECs using TRI REAGENT (Molecular Research Center). Complementary DNA (cDNA) was synthesized from 1 μg RNA using SuperScript III reverse transcriptase and 0.5 nmol of random primers (Invitrogen, Mount Waverly, VIC, Australia). Real-time quantitative reverse-transcription polymerase chain reaction (qPCR) was performed using SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich). The relative amount of messenger RNA (mRNA) was calculated by reference to a calibration curve. Each sample was normalized to the respective 18S value.

Cell Proliferation

HSC proliferation was assessed by using the Cell Proliferation Reagent WST-1 according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany).

Immunocytochemistry for CTGF/CCN2

CTGF/CCN2 protein expression in KCs was assessed as previously described.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay

KC nuclear protein preparation and electrophoretic mobility shift assay (EMSA) were performed as previously described.

Sirius Red Staining and Quantification of Collagen in HSCs

These experiments were performed as previously described.
Enzyme-Linked Immunosorbent Assay for PDGF

PDGF concentration in KC media was determined according to the manufacturer’s directions (R&D Systems).

H$_2$O$_2$ Generation by KCs

Intracellular H$_2$O$_2$ production in KCs was assessed as previously described.$^{20}$

Statistical Analysis

The results are expressed as mean ± SD. Comparisons between 2 groups was analyzed using the Student t test. A 2-sided P value < .05 was used to connote significance.

Results

Leptin Has Minimal Direct Effects on Profibrotic Gene Expression in HSCs In Vitro

As shown in Figure 1A, there was no change in the expression of TIMP1, TGF-β1, and CTGF/CCN2 mRNA in HSCs after treatment with either 10 nmol/L or 100 nmol/L leptin. Similarly, leptin did not affect TGF-β1 protein expression in 2-day cultured HSCs (Figure 1B). αSMA protein was unaltered following leptin treatment in 2-day (Figure 1C) and 6-day HSC cultures (Figure 1D). However, exposure to high-dose leptin (100 nmol/L) was associated with a minimal (~30%) increase in collagen I gene expression in 2-day cultured HSCs (Figure 1A) but not in HSCs cultured for 6 days (Figure 1A).

Leptin (10 nmol/L and 100 nmol/L) and TGF-β1 (1 ng/mL and 10 ng/mL) alone or the combination of leptin and TGF-β1 did not enhance αSMA protein expression in 2-day (Figure 1C) or 6-day HSC cultures (Figure 1D). TGF-β1 treatment was associated with up-regulation of collagen I and TIMP1 mRNA expression (Figure 1E). However, coadministration of TGF-β1 (10 ng/mL) with leptin (10 nmol/L or 100 nmol/L) in HSCs failed to have any synergistic effects on profibrotic gene expression (Figure 1E).

Leptin Enhances Stellate Cell Proliferation

In contrast to its minimal direct profibrotic effects, leptin significantly and in a dose-dependent manner enhanced the proliferation of HSCs after both 2 (Figure 2A) and 6 days in culture (Figure 2C). Because

![Figure 1. Direct effects of leptin on profibrotic gene and αSMA protein expression in HSCs. (A) Collagen I, TIMP1, TGF-β1, and CTGF/CCN2 mRNA expression in HSCs determined by qPCR. HSCs were cultured for 48 hours and 6 days before leptin (10 or 100 nmol/L) was added to the medium for 24 hours. (B) TGF-β1 protein expression in HSCs (cultured for 48 hours) treated with leptin (10 or 100 nmol/L) for 24 hours, as determined by immunoblot on cell lysates. (C and D) αSMA protein expression in HSCs (48 hours and 6 days in culture, respectively) treated with leptin (10 nmol/L or 100 nmol/L) and/or TGF-β1 (1 or 10 ng/mL). (E) mRNA expression of collagen I and TIMP1 in HSCs (cultured for 48 hours) treated with leptin (10 or 100 nmol/L) and/or TGF-β1 (10 ng/mL) for 24 hours. Mean ± SD for at least 3 experiments performed on 3 cell preparations, except for Figure 1A (day 2 HSC) where results from 7 different cell isolations were pooled. *P < .05; **P < .01 compared with control HSCs.]
PDGF is known to be the most potent mitogen for HSCs, we assessed whether leptin facilitates PDGF-induced HSC proliferation. As shown in Figure 2A and C, leptin did not have additional effects on HSC proliferation above that mediated by PDGF in cultured HSCs.

We next determined whether leptin indirectly affects HSC proliferation. KC-conditioned medium in the absence of leptin increased HSC proliferation by 2.3-fold compared with control medium, whereas pretreatment of KC with leptin (100 nmol/L) enhanced HSC proliferation 3.1-fold (Figure 2B). SEC-conditioned medium did not affect HSC proliferation (Figure 2B). Similar results were obtained when KC-conditioned medium was applied on activated HSCs, ie, after 6 days in culture (Figure 2C). Low-dose lipopolysaccharide (LPS) to activate KCs enhanced the proliferative effects of KC-conditioned medium on HSCs but, together with leptin, had no additional effect on proliferation (Figure 2B).
PDGF concentration and PDGF receptors were assessed. As shown, neither PDGF concentration in leptin-treated KC-conditioned medium (Figure 2D) nor PDGF receptor (α and β subtypes) mRNA expression in HSCs that had been exposed to leptin-treated KC-conditioned medium (Figure 2E) were altered. These data suggest that PDGF and its receptors are not the principal mediators of the HSC proliferative effects of KC-conditioned medium.

**HSCs and KCs Express OB-Rb but Demonstrate Differential Expression Patterns in Culture**

Quiescent HSCs and KCs express OB-Rb (Figure 2F); however, receptor expression was dramatically down-regulated during the process of HSC activation in vitro. Thus, OB-Rb expression was reduced to 10% by day 3 and to 4% at day 7 of culture when compared with the levels in quiescent HSCs (day 1). In contrast, the expression of Ob-Rb remained stable in KC cultures.

**Leptin Promotes Stellate Cell Activation by Acting on KCs**

Given the minimal direct effects of leptin on profibrotic gene expression in HSCs, we examined whether leptin might exert these actions indirectly, by modulating KC and/or SEC behavior. For these studies, 24-hour leptin-treated KC- or SEC-conditioned medium was transferred onto 3-day-old HSCs in culture. After 24-hour incubation in conditioned medium, collagen I, TIMP1, TGF-β1, and CTGF/CCN2 mRNA expression in HSCs were examined. The expression of all profibrogenic genes was elevated at least 2-fold (P < .05) in HSCs incubated with KC- but not with SEC-conditioned medium (Figure 3A). Consistent with these data, collagen protein was also augmented (Figure 3B).

To exclude the possibility that this effect was due to endotoxin contamination of the recombinant leptin protein, we treated KCs with 0.16 ng/mL LPS. LPS-treated KC-conditioned medium was then transferred onto HSCs. We observed no increase in collagen I, TIMP1, TGF-β1, or CTGF/CCN2 mRNA expression in HSCs (Supplementary Figure 1). Additional experiments showed that leptin-treated Zucker (fa/fa; Ob-Rb receptor deficiency) rat KC-conditioned medium failed to elicit any profibrogenic effects when transferred onto wild-type (WT) HSCs (Figure 3C). In contrast, KC-conditioned medium from leptin-treated lean littersmates (Fa/Fa) reproduced the profibrogenic effects that were observed when using leptin-treated WT KC-conditioned medium (Figure 3A and C). This confirmed that the observed effects were specifically because of leptin acting through its receptor.

We next determined whether leptin indirectly affects HSC αSMA protein expression. Leptin-treated WT KC-conditioned medium significantly increased αSMA protein expression in HSC (Figure 3D). SEC-conditioned medium, however, did not result in increased αSMA expression (Supplementary Figure 2).

Collectively, these data suggest that leptin activates KCs to release soluble profibrogenic mediators that are transferred in the conditioned medium to HSCs. These factors promote the activation, proliferation, and profibrogenic activities of HSCs.

**Leptin Up-Regulates TGF-β1 and CTGF/CCN2 Expression in KCs**

TGF-β1 and CTGF/CCN2 are major profibrogenic cytokines in the development of liver fibrosis. Leptin (100 nmol/L) treatment for 24 hours significantly up-regulated TGF-β1 and CTGF/CCN2 mRNA expression in KCs (Figure 4A). As expected, TGF-β1 protein was elevated in culture medium of WT KCs treated with leptin (100 nmol/L) (Figure 4B), and CTGF/CCN2 protein was also expressed at higher levels in leptin-treated
KS than in control (Figure 4C). Considering that CTGF/CCN2 is induced via TGF-β1-dependent pathways, we analyzed the inhibitory effects of pan-TGF-β blockade on CTGF/CCN2 protein expression by using a sTGFβR fusion protein. Coexposure of KCs to leptin and sTGFβR significantly attenuated CTGF/CCN2 protein expression induced by leptin, whereas human immunoglobulin G as a control did not (Figure 4C). In additional studies, we demonstrated that on leptin treatment, Zucker (fa/fa) rat KCs failed to augment TGF-β1 and CTGF/CCN2 mRNA expression (Figure 4D), whereas the results in lean (Fa/Fa) rat KCs as expected were similar to that in WT rat KCs (Figure 4D). These results further corroborate our data that the effects of
leptin in inducing TGF-β1 and CTGF/CCN2 are indeed leptin receptor dependent.

**Leptin Does Not Induce Intracellular Hydrogen Peroxide Production in KCs**

H2O2 is a well-recognized profibrogenic factor, and leptin is reported to stimulate H2O2 production in HSC cell lines. However, we were unable to demonstrate significant increase in H2O2 production by leptin-treated KCs (Figure 4E). These data suggest that, at least in vitro, leptin’s profibrogenic effects that are mediated via KCs are not due to the production of H2O2.

**TGF-β Neutralization Attenuates the Profibrogenic Effects of Leptin on KCs**

To clarify further whether TGF-β1 is indeed one of the soluble mediators of the profibrogenic effects of leptin on KC, we undertook TGF-β neutralization studies. As shown (Figure 4F), after TGF-β antibody (10 μg/mL) treatment of leptin-treated KC medium, collagen I mRNA was reduced by 67%, TIMP1 by 78%, TGF-β1 by 76%, and CTGF by 102% in HSCs. These data confirm that TGF-β1 is likely to be the principal profibrogenic mediator that is released on leptin treatment of KCs.

**Leptin Activates the Phosphorylation of STAT3, ERK1/2, and AKT and Activates the Transcription Factors AP-1 and NF-κB in KCs**

Leptin acts principally through the long-form OB-Rb leptin receptor and downstream Janus kinase (JAK)/STAT pathways. Leptin also activates mitogen-activated protein kinase (MAPK) and PI3K/AKT. Therefore, we determined whether leptin activates these pathways in KCs to target downstream components leading to profibrotic gene transcription. Exposure of KCs to leptin resulted in increased activation of ERK1/2 (particularly the 44-kilodalton isoform) and AKT after 5 and 10 minutes incubation, respectively, and in a time-dependent manner (Figure 5A and B). A rapid and time-dependent increase in STAT3 phosphorylation was also observed upon leptin treatment of KCs (Figure 5A and B). Leptin did not increase JNK or p38 phosphorylation (Figure 5C).

We speculated that leptin may also activate downstream transcription factors such as AP-1 and/or NF-κB because these transcription factors can be activated by MAPK/ERK1/2 and PI3K/AKT signaling, and AP-1 and/or NF-κB binding motifs are known to be present in the promoter regions of TGF-β1 and CTGF/CCN2. EMSAs were therefore performed on nuclear protein extracted from leptin-treated and control KCs. As shown, leptin, in a dose-dependent fashion, increased AP-1 and NF-κB DNA binding abilities in KCs (Figure 5D).

**Activated STAT3 Mediates TGF-β1 Expression in KCs**

To clarify which of the activated signaling pathways contributes to the observed increase in TGF-β1 gene expression, we undertook studies to inhibit the various signaling pathways and then measured TGF-β1 expression. As shown (Figure 5E), the STAT3 inhibitor but not the MEK (PD98059) or PI3K (LY294002) inhibitors resulted in a significant reduction of TGF-β1 mRNA expression in KCs.

**Discussion**

Leptin not only regulates body weight and metabolism but also exerts pleiotropic effects on other organs such as the liver. Leptin has been shown to accelerate and enhance the process of liver fibrosis induced by various stimuli in vivo, whereas leptin-deficient animals are resistant to fibrosis. A number of studies have assessed whether leptin has direct profibrogenic effects on HSCs, but the results have been conflicting. Saxena et al reported that leptin binds to the signaling form of the leptin receptor (OB-Rb) on primary rat HSCs and HSC-T6 cells, an immortalized rat HSC cell line, and that leptin activates STAT3 to enhance the expression of α2(I) collagen mRNA. However, others have failed to confirm the expression of OB-Rb in activated primary rat HSCs or in the human HSC cell line LX-1, and have failed to demonstrate induction of collagen gene expression in HSCs by leptin. In contrast, expression of the short-form leptin receptor (OB-Ra) in activated primary HSCs and LX-1 cell lines has been established. Therefore, whether leptin directly signals to HSCs via the OB-Rb remains unresolved.

Using leptin at pharmacologic concentrations in a range similar to those of earlier studies, we have shown that only high leptin concentrations (100 nmol/L–1600 ng/mL) marginally increased collagen I mRNA expression in HSCs at day 2. This result is similar to the study by Tang et al demonstrating that only high concentrations of leptin (1000 ng/mL) resulted in increases of collagen I expression in HSCs. In addition, we noted that expression of the other profibrotic genes (TIMP1, TGF-β1, and CTGF/CCN2) and the protein expression of αSMA was not altered by leptin treatment. Similarly, leptin neither enhanced TGF-β1 protein expression nor facilitated TGF-β1-induced collagen I or TIMP1 gene expression. Based on these composite data, the direct profibrogenic effects of leptin on HSCs, if present, appear modest.

As distinct from the above, leptin significantly and directly increased the proliferation of HSCs in a dose-dependent manner. It has been previously reported that leptin facilitates HSC proliferation via activation of the MAPK/ERK1/2 and PI3K/AKT pathways and by increased PDGF receptor expression. Activation of either the functional OB-Rb or OB-Ra receptors is able to
trigger MAPK and PI3K signaling, and it therefore appears that the pro-proliferative effect of leptin on HSCs is more prominent than its profibrogenic effects. In our studies of the indirect profibrogenic effects of leptin that are mediated by KCs, we were unable to demonstrate any increase in PDGF protein in KC-conditioned medium nor any increase in PDGF receptor expression following the addition of the KC medium to HSCs. These data suggest that HSC proliferation by leptin-treated KC medium may be a complex phenomenon and must involve other pro-proliferative factors, the identity of which is unclear. In addition, it should be noted that activated HSCs elaborate leptin, and this could, in part, mediate HSC proliferation.

In contrast to the minimal direct profibrotic effects of leptin on HSCs, conditioned medium from leptin-treated KCs markedly induced gene expression of collagen I, TIMP1, TGF-β1, and CTGF/CCN2 compared with un-

Figure 5. Leptin activated JAK/STAT3, MAPK/ERK1/2, and PI-3K/AKT pathways as well as AP1 and NF-κB transcription factors in Kupffer cells. (A) Leptin enhanced phosphorylation of STAT3, ERK1/2, and AKT in KCs in a time-dependent manner. KCs were exposed to leptin 100 nmol/L for 0, 5, 10, 30, and 60 minutes and cell lysates used for immunoblot analysis. (B) Densitometry analysis of Figure 5A as the ratio of p-protein to total protein (ERK1/2 or AKT) or the ratio of pSTAT3 to β-actin. *P < .05; **P < .01. (C) Leptin did not influence phosphorylation of p38 or JNK in KCs. Cell lysates from KCs exposed to leptin 100 nmol/L for 10 or 60 minutes were used for immunoblotting. (D) Leptin activated AP-1 and NF-κB DNA binding in KCs. Nuclear protein from KCs cultured for 60 minutes with leptin (10 or 100 nmol/L) was used for EMSA. The disappearance of the shifted band in the presence of a molar excess of unlabeled (cold) oligonucleotide confirms the specificity of the binding. (E) TGF-β1 mRNA expression in KCs in the presence or absence of STAT3 inhibitor peptide (50 μmol/L), a MEK inhibitor (50 μmol/L), and a PI-3K inhibitor (25 μmol/L). RNA was extracted after 24-hour treatment. *P < .05 and **P < .01 vs control group. #P < .05 vs leptin treatment group without STAT3 inhibitor peptide. Each experiment was conducted in at least 3 independent sets.
treated KC-conditioned medium. Likewise, leptin-treated KC-conditioned medium augmented collagen and αSMA protein. Conversely, leptin-treated SEC-conditioned medium did not enhance the expression of any of the profibrogenic genes tested. These results suggest that KCs play a major role in mediating the profibrogenic effects of leptin on HSCs by directly signaling to KCs.

OB-Rb expression in HSCs is still controversial. Ikejima et al. noted that KCs harbored functional OB-Rb and that exposure to leptin activated the JAK/STAT signaling pathway of this receptor. Consistent with this result, we confirmed that OB-Rb is highly expressed in quiescent and activated KCs and thus could mediate leptin signal transduction. In contrast, OB-Rb expression in activated HSCs was dramatically decreased compared with that in quiescent HSCs, explaining why leptin treatment was unable to activate these cells. Having established that KCs express Ob-Rb receptors, we determined the downstream signaling pathways activated by leptin treatment. Our studies indicated that leptin treatment of KCs activates JAK/STAT3, MAPK/ERK1/2, and PI3K/AKT and the transcription factors NF-κB and AP-1 in WT KCs.

Our results imply that, upon ligand binding to the leptin receptor, KCs release profibrogenic factor(s) that are able to activate HSCs. KCs produce a large array of cytokines and other mediators, including tumor necrosis factor-α, TGF-β1, CTGF/CCN2, interleukin-6, and ROS when activated, that may contribute to liver injury and fibrosis. We have shown that leptin-treated KCs increase production of TGF-β1 and CTGF/CCN2, both potent profibrogenic proteins. The increased expression of TGF-β1 is consistent with data from Ikejima et al. and Leung et al., who demonstrated that leptin enhances TGF-β1 expression in KCs and mesothelial cells, respectively. This finding is further supported by Sakaida et al., who noted decreased TGF-β1 expression in KCs with leptin receptor deficiency. As expected, we further demonstrated that CTGF/CCN2 production is TGF-β1 dependent because the sTGFBR fusion protein attenuated CTGF/CCN2 protein expression. Finally, by neutralizing TGF-β, we were able to confirm that this protein is, indeed, a principal mediator of the profibrogenic effects of leptin on KCs. CTGF/CCN2 could also, in part, mediate the fibrogenic effects of leptin-treated KCs. Unfortunately, we were unable to unequivocally confirm this because potent and reliable CTGF/CCN2 blocking antibodies are not currently available. It should be noted that, in our study, TGF-β1 treatment of HSCs did not increase αSMA protein expression, whereas leptin-treated KC-conditioned media did. This suggests that collagen I, TIMP1, and CTGF/CCN2 are at least, in part, regulated by TGF-β1. In contrast, αSMA may be regulated by another soluble factor, possibly including CTGF/CCN2.

Finally, we sought to determine which of the signaling pathways activated by leptin is responsible for augmenting TGF-β1 expression in leptin-treated KCs and were able to demonstrate that STAT3 plays a critical role. Previous studies suggest that STAT3 enhances TGF-β1 production by binding to elements in the TGF-β1 promoter. Because we also observed increased AP-1 and NF-κB DNA binding activities, it is likely that AP-1 and NF-κB, together with STAT3, contribute to the observed increase in TGF-β1 production.

In conclusion, our results suggest that leptin mediates hepatic fibrosis mainly through actions on KCs. Increased TGF-β1 by KCs exposed to leptin is a major mediator of these effects. These mechanisms are likely to be important in mediating liver fibrosis associated with obesity and elevated leptin levels, such as in persons with nonalcoholic fatty liver disease.

**Supplementary Data**

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: [10.1053/j.gastro.2009.04.011](http://10.1053/j.gastro.2009.04.011).

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Received July 16, 2008. Accepted April 8, 2009.

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Acknowledgments
The authors thank N. Subramaniam, J. Sesha, M. Kacevska, H. Yu, and X. Wang for technical assistance.

Conflicts of interest
The authors disclose no conflicts.

Funding
Supported by the National Health & Medical Research Council of Australia (No: 358398), NIH grant (5R01AA016003), and the Robert W. Storr Bequest to the University of Sydney.
Supplementary Figure 1. mRNA expression of collagen I, TIMP1, TGFβ1 and CTGF/CCN2 in HSCs cocultured with KC conditioned medium treated with LPS. To exclude the possibility that LPS contamination of recombinant leptin could be involved in the profibrogenic effects that we demonstrated, the stimulatory effects of LPS on the above four profibrogenic genes in HSCs was examined. LPS (0.16 ng/mL, similar to that in 100 nmol/L recombinant leptin) was added to KCs for 24 h prior to transferring onto HSCs at day 3 in culture. HSC RNA was extracted after 24 h and real time PCR was performed. Three independent experiments were undertaken.

Supplementary Figure 2. αSMA protein expression of HSCs cocultured with SEC medium treated with leptin. Leptin 100 nmol/L was added to SEC (48 hours in culture) medium and incubated for 24 hours prior to transfer onto HSCs at 3 days in culture. Total protein was extracted after 24 hours coculture and Western blotting undertaken. Three independent experiments were performed.