Adiponectin inhibits leptin signalling via multiple mechanisms to exert protective effects against hepatic fibrosis

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INTRODUCTION

Hepatic fibrosis results from excess deposition of ECM (extracellular matrix) proteins, such as type I collagen, and is characteristic of chronic liver injury, regardless of aetiology [1]. The accumulation of excess ECM leads to the formation of fibrous scar tissue that causes portal hypertension and ultimately chronic liver failure. Hepatic fibrosis is considered part of the wound-healing programme in response to liver injury and may be reversed if the initiating insult is resolved sufficiently early, but protracted fibrosis, specifically that which accompanies NAFLD (non-alcoholic fatty liver disease), may lead to cirrhosis and potentially hepatocellular carcinoma [2,3].

HSCs (hepatic stellate cells) in the healthy liver are normally quiescent, residing in the space of Disse. They serve as storage repositories for retinoic acid in the form of retinyl esters. However, during the wound-healing response to chronic liver injury, HSCs are ‘activated’, becoming proliferative and increasing their production of ECM proteins [4]. Although other hepatic cell types may also display fibrogenic properties, the activated HSC appears to be the primary actor responsible for excessive ECM present in the fibrotic liver [3].

Leptin, the 16 kDa adipocytokine product of the ob gene, known largely for its role in influencing hypothalamic control of appetite, insulin secretion and glucose metabolism, displays multiple profibrogenic properties [5,6]. Synthesized and secreted by white adipose tissue, leptin promotes expression of α2 (I) collagen [7,8], the excessive deposition of which is a key feature of hepatic fibrosis. Leptin also suppresses the expression and activity of the collagen-degrading MMP-1 (matrix metalloproteinase-1) [9–11], and promotes the expression of TIMP-1 (tissue inhibitor of metalloproteinases-1) [9], an important negative regulator of this MMP. Leptin also supports maintenance of HSCs in the ‘activated’ phenotype by stimulating their proliferation and suppressing apoptosis [12]. Evidence supporting a role for leptin in promoting hepatic fibrogenesis is provided by the observation by Saxena et al. [8] that lean mice, when compared with their leptin non-producing ob/ob counterparts, display elevated deposition of collagen in the CCL4 (carbon tetrachloride) model of hepatic fibrosis, whereas the CCL4-treated ob/ob mice failed to develop liver fibrosis.

Leptin signal transduction is conducted via the JAK2 (Janus kinase 2)/STAT3 (signal transducer and activator of transcription 3) tyrosine kinase pathway, initiated by leptin binding at the cell surface to the long form leptin receptor Ob-Rb [13,14]. Leptin binding to Ob-Rb results in activation of the receptor-associated JAK2 by autophosphorylation [15,16]; JAK2 subsequently activates Ob-Rb by phosphorylation at Tyr1138 and Tyr201 [17]. Phosphorylation of Ob-Rb at Tyr1138 is required to induce binding by STAT3 [17], which is then also phosphorylated...
and activated by JAK2 [18]. Once activated, STAT3 can form homodimers and translocate to the nucleus, where it acts in concert with other cellular and microenvironment-specific factors, ultimately regulating transcription of respective target genes. Leptin-stimulated transcriptional activation of both type I collagen and TIMP-1 involve JAK/STAT signal transduction [9].

Adiponectin is a 30 kDa protein that, similar to leptin, is synthesized and secreted primarily by white adipose tissue. Adiponectin circulates in the blood as low-, medium- or high-molecular-mass oligomers, and its serum levels correlate inversely with body fat [5,19]. Two receptors that propagate signal transduction in response to adiponectin have been identified: AdipoR1, expressed primarily in skeletal muscle; and AdipoR2, expressed abundantly in liver [19]. Adiponectin signal transduction in the liver is conducted primarily through activation of AMPK (AMP-activated protein kinase) by phosphorylation at Thr172, although there is also evidence of a role for PPARα (peroxisome-proliferator-activated receptor α)-mediated signalling in response to adiponectin [20,21]. LKB1 has been identified as an upstream activator of AMPK, but the molecular events involved in adiponectin signal transduction have not been completely defined [22], particularly as related to biological actions not related to the regulation of cellular energy stores and insulin sensitivity.

Although leptin generates profibrogenic effects, multiple studies indicate that adiponectin is anti-fibrogenic, although the mechanisms of this protective effect have not yet been described [5]. Overexpression of adiponectin in rat HSCs suppresses proliferation and reduces expression of PCNA (proliferating cell nuclear antigen) and proliferation and reduces expression of PCNA (proliferating cell nuclear antigen) and αSMA (α-smooth muscle actin), a marker for HSC activation [23]. Treatment with adiponectin also stimulates apoptosis in activated, but not quiescent, HSCs, suggesting that adiponectin may act to maintain HSCs in the quiescent state, thereby reducing their fibrogenic actions [23]. In humans, reduced serum adiponectin levels are associated with several negative physiological consequences, including poor liver histology, inflammation and fibrosis [5,24–26]. Moreover, mice overexpressing adiponectin via adenoviral delivery are less vulnerable than lac z-expressing mice to CCl4-induced hepatic fibrosis [27]. Extending this paradigm, recent experiments demonstrate that adiponectin-knockout mice (Ad+/−) are more susceptible to CCl4-induced hepatic fibrosis than wild-type mice, and that Ad−− mice are also more vulnerable to leptin-induced fibrosis [6,10]. A potential clue from these experiments suggests that a plausible mechanism explaining the increased vulnerability of the Ad−− mice to leptin-mediated hepatic fibrosis is their comparatively decreased expression of the SOCS-3 (suppressor of cytokine signalling 3) protein, an important negative regulator of leptin signalling. These results imply a functional loss of the ability of Ad−− mice to inhibit leptin signalling, leading to the enhanced hepatic fibrosis observed therein. Although leptin and adiponectin clearly represent a mutually antagonistic paradigm regulating hepatic ECM deposition, very little is known about the molecular mechanisms of cross-talk between these two important pathways. In the present study, we investigated mechanisms by which adiponectin can antagonize leptin signalling to provide protection against leptin-stimulated hepatic fibrosis.

**EXPERIMENTAL**

**Antibodies and chemical reagents**

Recombinant human adiponectin was from Biovendor. DMEM (Dulbecco’s modified Eagle’s medium), trypsin/EDTA and penicillin/streptomycin were all from Invitrogen. FBS (fetal bovine serum) was from HyClone. Puromycin, polybrene, recombinant human leptin and antibodies against β-actin were from Sigma. Anti-SOCS-3, anti-Ob-Rb (K-20), anti-[phospho-Ob-Rb (Ty[385]) and anti-[phospho-Ob-R (Ty[1138])] antibodies were from Santa Cruz Biotechnology. Antibodies against JAK2 and phospho-JAK2 (Ty[1007/Ty[1008]]) were from Cell Signaling Technology; the antisera against phospho-JAK2 (Ser[527]) was a gift from Dr Martin Myers (Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, U.S.A.). The anti-PTP1B (protein tyrosine phosphatase 1B) antibody was from Abcam. HRP (horseradish peroxidase)-conjugated secondary antibodies were from GE Healthcare.

**Isolation of HSCs**

Quiescent HSCs were isolated as described previously [8,28]. Sprague–Dawley rats were purchased from Charles River. All rats received humane care, and the Emory University Institutional Animal Care and Use Committee approved the HSC isolation protocol. In brief, *in situ* perfusion of the liver with 20 mg/dl pronase (Boehringer Mannheim) was followed by collagenase (Crescent Chemical) perfusion. Dispersed cell suspensions were layered on a discontinuous density gradient of 8.2 % and 15.6 % Accenduz (Accurate Chemical and Scientific). The resulting upper layer consisted of more than 95 % HSCs. Cells were cultured in Medium 199 containing 20 % (v/v) FBS (Flow Laboratories). Purity of activated HSCs was assessed by immunolocalization of αSMA in the monolayer and by intrinsic autofluorescence in freshly isolated HSCs. HSC viability was verified by propidium iodide exclusion and for *in vitro* experiments was greater than 95 %. Sub-confluent activated HSCs in culture, 10 days after isolation and initial plating, were washed twice with PBS and were cultured in DMEM. Growth medium was replaced every other day, and activated HSCs were passaged 1 in 3 every 7 days by trypsinization. Only cells between passage 2 and 5 were used in experiments.

**In vitro leptin and adiponectin treatments**

Culture-activated HSCs were treated with adiponectin (10 μg/ml), leptin (100 ng/ml), both or neither after 16 h of serum deprivation in DMEM supplemented with 2.5 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin. Treatment durations are indicated in the appropriate Figure legends.

**Animal care and in vivo studies with CCl4 and recombinant leptin**

Ad−− mice, generated as described previously [29], were a gift from the laboratory of Dr Kenneth Walsh (Boston University School of Medicine, Boston, MA, U.S.A.). The animals were cared for in accordance with protocols approved by the Animal Care and Use Committee of Emory University. Animals were housed in a temperature-controlled environment (20–22 °C) with a 12 h/12 h light/dark cycle, and fed *ad libitum* with Purina Laboratory Chow (Ralston Purina) and water. The studies included three cohorts of mice: control mice being administered sterile saline, another group being administered only CCl4 for the duration of the study, and a third group that was administered CCl4 for the duration of the study and recombinant human leptin for the final 6 weeks. Male littermates (6 weeks old) of Ad−− mice and wild-type mice of the same background were administered CCl4 (2 ml/kg of body weight) with olive oil (1:1 ratio) twice a week by gavage for 8 weeks. Leptin was administered concomitantly by intraperitoneal injection every
36 h for 6 weeks at a dosage of 5 mg/kg of body weight. All mice were killed and liver tissue was collected for molecular analysis as described below.

**Protein lysate production**

At the end of the in vitro experiments, HSCs were washed in PBS and suspended in ice-cold RIPA buffer [10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P40, 0.5% sodium deoxycholate and 0.1% SDS containing 20 μl/ml protease inhibitor cocktail (Research Products International)] for 30 min on ice. Lysates were centrifuged at 12,000 × g for 30 min at 4°C. Supernatant was collected and protein concentrations were determined using the Bradford reagent (Sigma) [30].

At the conclusion of the in vivo studies, 100 mg of harvested liver, previously frozen at the time of necropsy, was cut into 0.5 cm × 0.5 cm pieces, allowed to thaw at 4°C in 3 ml of lysis buffer (RIPA buffer containing 20 μl/ml protease inhibitor cocktail) per g of tissue, and sonicated twice for 30 s at 4°C (Branson sonifier 1500) to enhance tissue dispersion. Each sample was then incubated on ice for 30 min and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was collected and centrifuged a second time for 10 min at 10,000 × g; the resulting supernatant constituted the lysates used in subsequent analyses.

**Immunoblotting**

Equal amounts of protein were resolved by SDS/PAGE (4–20% gels) [31] and immobilized on to PVDF membranes by wet transfer. After blocking for 30 min in 5% (w/v) non-fat dried skimmed milk powder in TBS (Triss-buffered saline)-TWEEN 20 (20 mM Tris/HCl, pH 7.5, 137 mM NaCl and 0.05% Tween 20), the membranes were exposed overnight to primary antibody at 4°C and then for 2 h at room temperature (25°C) with the corresponding HRP-conjugated secondary antibody. Equal protein loading was controlled by immunoblotting β-actin. Immunoreactive proteins were visualized using the HyGlo Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific) and exposure to X-ray film (Kodak). Band density was analysed using AlphaEase FC Software, version 4.0.1 (Alpha Innotech).

**Immunoprecipitation**

Equal amounts of protein were incubated with 20 μl of primary antibody for 2 h at 4°C. Protein A/G Plus–agarose (20 ml) (Santa Cruz Biotechnology) was added to the mixture, and the suspension was incubated at 4°C on a rocking platform overnight. The immunoprecipitates were collected by centrifugation at 10,000 × g for 30 s at 4°C, and washed twice in PBS, and centrifugation was repeated following each wash. The supernatant was discarded and the pellet was resuspended in electrophoresis sample buffer. Immunodetection was conducted as described above for immunoblotting.

**Quantitative real-time PCR**

RNA was extracted using RNeasy® (Qiagen). Primers were designed for PTP1B, Adipor1 and Adipor2 using NIH Primer-BLAST, such that the resulting PCR products would be 100–200 base pairs in length and bridge two separate exons. cDNA synthesis was conducted using the iScript™ cDNA Synthesis kit (Bio-Rad), according to the manufacturer’s recommended parameters. First-strand cDNA synthesis was carried out in 20 μl reaction volumes containing 1 μg of total RNA, 4 μl of 5× iScript reaction mix, 1 μl of iScript reverse transcriptase and nuclelease-free water. Quantitative real-time PCR for PTP1B was conducted using IQ™ SYBR® Green Supermix (Bio-Rad) according to the manufacturer’s protocol. PCR was performed in 25 μl reaction volumes containing nuclelease-free water, 1 μl aliquots of cDNA and gene-specific primer pairs, and 12.5 μl of SYBR Green Supermix in a MyIQ™ One Color Real-Time PCR Detection System. The PCR cycle parameters were set at 95°C for 20 s, 55°C for 45 s and 72°C for 30 s, for 40 cycles. Relative amounts of the target cDNA were estimated by the Ct (threshold cycle) number and compared with GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Three independent samples were analysed for each condition in triplicate.

**Lentivirus-mediated knockdown of adiponectin receptors in rat HSCs**

Adipor1 and Adipor2 and non-targeting shRNA (small-hairpin RNA) lentivirus particles were from Santa Cruz Biotechnology. Rat HSCs were seeded at 1 × 10⁵ cells per ml in 100 mm² tissue culture plates. HSCs at 50–70% confluence were incubated for 16 h with lentiviral particles [MOI (multiplicity of infection) = 2] in the presence of polybrene (5 μg/ml). The infected cells were cultured in complete medium for 48 h, and stable clones were selected by culture for several days in complete medium containing 2 μg/ml puromycin. Receptor knockdown was confirmed by RT (reverse transcription)–PCR and Western blot analysis.

**ELISA for MMP-1–TIMP-1 complexes**

The human MMP-1–TIMP-1 Complex ELISA DuoSet® (DY1550) and all assay reagents were purchased from R&D Systems. Primary rat HSCs were treated as described in the appropriate Figure legend for each experiment. Conditioned medium was collected from treated HSC cultures, and the detection of TIMP-1–MMP-1 complexes from rat HSCs was conducted according to the manufacturer’s instructions. Briefly, to immobilize the capture antibody, 160 ng/well of goat anti-MMP-1 was added to a 96-well plate, then the plate was sealed and incubated overnight at room temperature. To pull down MMP-1, 100 μg of protein from the conditioned medium was added per well to the microplate, and the plate was incubated for 1 h. To detect MMP-1–TIMP-1 complexes, 1.8 μg of biotinylated anti-TIMP-1 antibody was added per well to the plate, and the plate was incubated for 2 h at room temperature. Captured anti-TIMP-1 was detected by adding streptavidin-conjugated HRP and an HRP substrate solution (DY999; R&D Systems). The absorbance of each well was determined with a Bio-Tek Synergy 2 plate reader set to 450 nm. The concentration of MMP-1–TIMP-1 complex in each sample was determined from a standard curve constructed using the measured absorbances of known concentrations of MMP-1–TIMP-1 complex.

**Statistical analysis**

Animal experiments were performed with eight animals in each treatment and control group. All in vitro data are reported as the result of three independent experiments including three replicates per experiment. The data are presented as means ± S.E.M. Statistical analysis was performed using Graphpad® Prism 4 software (http://www.graphpad.com), and statistical differences
We also examined JAK2 Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation in livers from Ad$^{-/-}$ and wild-type mice to investigate regulation of leptin signal transduction by adiponectin in vivo. Ad$^{-/-}$ mice displayed greater JAK2 Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation than wild-type mice ($P < 0.05$) in every experimental condition tested, consistent with the implications of our earlier work suggesting that Ad$^{-/-}$ mice are incapable of attenuating leptin signalling, rendering them more vulnerable to hepatic fibrosis [10]. Indeed, the negative consequences of this impaired signalling became more pronounced in mice treated with CCI$_4$, or in mice co-administered CCI$_4$ and leptin. When compared with saline treatment, CCI$_4$, and CCI$_4$/leptin co-administration, increased JAK2 Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation in both wild-type and Ad$^{-/-}$ mice; and, as anticipated, CCI$_4$/leptin co-administration produced an increase in JAK2 Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation greater than CCI$_4$ alone (Figure 1B).

**Adiponectin inhibits leptin activation of Ob-Rb**

The inhibition of leptin-stimulated JAK2 activation by adiponectin would suggest that downstream propagation of the leptin signal might be mitigated. After JAK2 activation, the leptin signal is conducted via JAK2 phosphorylation of Ob-Rb at Tyr$^{985}$ and Tyr$^{1138}$. Phosphorylation of Ob-Rb at Tyr$^{1138}$ recruits binding by STAT3, which is subsequently also phosphorylated by JAK2. We have already reported that adiponectin reduces leptin-stimulated phosphorylation of STAT3 in vitro [10]. It thus follows from the results shown in the present study and elsewhere that adiponectin may also suppress leptin-stimulated phosphorylation of Ob-Rb.

To test this hypothesis, we examined the effect of adiponectin on Ob-Rb phosphorylation in rat HSCs and Ad$^{-/-}$ mouse livers using experimental designs identical with those used to investigate JAK2 phosphorylation. In both series of studies, we determined whether or not phosphorylation of Ob-Rb Tyr$^{985}$, one of the tyrosine residues implicated in receptor activation, would be prevented by adiponectin.

As anticipated, leptin stimulated the phosphorylation of Ob-Rb at Tyr$^{985}$ in rat HSCs, whereas adiponectin failed to do so. In contrast, Ob-Rb phosphorylation after leptin and adiponectin co-administration was less than in the presence of leptin alone ($P < 0.05$), and more comparable with the levels associated with adiponectin treatment (Figure 2A). In mice, after CCI$_4$ treatment there was a trend towards increased Ob-Rb Tyr$^{985}$ phosphorylation in both wild-type and Ad$^{-/-}$ mouse livers. Leptin co-administration with CCI$_4$ also produced a trend towards an increase in Ob-Rb Tyr$^{985}$ phosphorylation above that detected with CCI$_4$ alone, but these differences were not statistically significant. Importantly however, leptin/CCI$_4$ co-administration increased Ob-Rb Tyr$^{985}$ phosphorylation significantly more in livers from Ad$^{-/-}$ mice than in livers from wild-type mice ($P < 0.05$, Figure 2B). These results provide evidence that adiponectin negatively regulates leptin-stimulated Ob-Rb activation. We are aware of no other reports that describe adiponectin regulation of Ob-Rb, therefore these results further corroborate the emerging paradigm describing adiponectin as a negative regulator of leptin signal transduction. As implied above, these results are not surprising in light of the observed effects of adiponectin on JAK2 activation and our earlier work showing that adiponectin inhibits leptin-mediated STAT3 phosphorylation [10]. Negative regulation by adiponectin of JAK2, the upstream kinase responsible for activating Ob-Rb, predicts the resulting inhibitory effects on the downstream elements of the leptin signal transduction, including the previously reported inhibition of leptin-mediated STAT3 phosphorylation.

**RESULTS AND DISCUSSION**

**Adiponectin inhibits leptin-stimulated activation of JAK2**

Leptin initiates signalling by binding to its receptor, Ob-Rb, at the cell surface. Ligand binding to the receptor causes activation of the receptor-associated JAK2 by autophosphorylation at Tyr$^{1007}$/Tyr$^{1008}$ [13,32]. JAK2 can also be inhibited by phosphorylation at Tyr$^{770}$ or Ser$^{523}$, but only phosphorylation at Ser$^{523}$ inhibits JAK2-dependent signalling by Ob-Rb [33]. As JAK2 activation represents the most upstream event in leptin signal transduction, we examined first whether adiponectin had any effect on phosphorylation of JAK2 at Tyr$^{1007}$/Tyr$^{1008}$ and Ser$^{523}$. As anticipated, leptin stimulated phosphorylation of JAK2 at Tyr$^{1007}$/Tyr$^{1008}$, whereas adiponectin did not ($P < 0.05$). Importantly, however, adiponectin blocked leptin-stimulated Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation when both adipocytokines were co-administered ($P < 0.05$, compared with leptin treated). In addition, leptin tended to suppress phosphorylation of JAK2 at Ser$^{523}$ compared with untreated cells, but the decrease was not statistically significant. By contrast, adiponectin stimulated Ser$^{523}$ phosphorylation, whether administered alone or in the presence of leptin ($P < 0.05$ compared with untreated). These results indicate that adiponectin can block leptin signal transduction by two upstream mechanisms, inhibiting activation of JAK2 at Tyr$^{1007}$/Tyr$^{1008}$ and suppressing propagation of leptin-mediated downstream signal transduction by promoting the inhibitory phosphorylation of JAK2 at Ser$^{523}$ (Figure 1A).

Figure 1  Adiponectin blocks leptin-mediated activation of JAK2

(A) Upper panels, Western blot of JAK2 Tyr$^{1007}$/Tyr$^{1008}$ or Ser$^{523}$ phosphorylation in lysates prepared from rat HSCs. Lower panel, quantification of immunoblots presented in the upper panel. Leptin treatment for 30 min (Lep, 100 ng/ml) increased JAK2 Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation significantly ($P < 0.05$) compared with untreated samples (Utx). Co-administration of leptin and adiponectin (LA) inhibited leptin-stimulated JAK2 Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation ($P < 0.05$ compared with leptin). Adiponectin (Adi, 10 μg/ml) stimulated JAK2 Ser$^{523}$ phosphorylation whether administered alone or in the presence of leptin ($P < 0.05$ compared with untreated samples). (B) Upper panel, Western blot of JAK2 Tyr$^{1007}$/Tyr$^{1008}$ phosphorylation from liver tissue collected from Ad$^{-/-}$ and wild-type (WT) mice. Lower panel, quantification of immunoblots presented in the upper panels. CCI$_4$ intoxication (2 ml/kg of body weight) and CCI$_4$/leptin co-administration both increased JAK2 phosphorylation significantly over saline-treated control mice. JAK2 phosphorylation was always greater in Ad$^{-/-}$ mice compared with wild-type mice, regardless of treatment ($P < 0.05$ compared with wild-type).
Adiponectin blocks leptin-stimulated Ob-Rb phosphorylation

(A) Upper panels, Western blots of Ob-Rb Tyr985 phosphorylation in lysates prepared from rat HSCs. Lower panel, quantification of immunoblots presented above. Leptin treatment for 30 min (Lep, 100 ng/ml) increased JAK2 Tyr985 phosphorylation significantly (P < 0.05) compared with untreated samples (Utx). Co-administration of leptin (100 ng/ml) and adiponectin (10 μg/ml) (LA) inhibited leptin-stimulated Ob-Rb Tyr985 phosphorylation (P < 0.05 compared with leptin). (B) Upper panels, Western blots of Ob-Rb Tyr985 phosphorylation from liver tissue collected from Adi−/− and wild-type (WT) mice. Bottom panel, quantification of immunoblots presented above. Ob-Rb phosphorylation was significantly increased in CCl4 plus leptin-treated Adi−/− mice compared with similarly treated wild-type mice (P < 0.05 compared with WT). Adi, adiponectin treatment alone.

Figure 3 Adiponectin promotes PTP1B expression

(A) Western blot and (B) quantitative real-time PCR analysis of PTP1B expression in Adi−/− and wild-type (WT) mice demonstrate reduced PTP1B expression in Adi−/− mice compared with wild-type mice (P < 0.05 compared with wild-type). (C) Results from quantitative real-time PCR showing PTP1B expression in rat HSCs after treatment with leptin (Lep, 100 ng/ml), adiponectin (Adi, 10 μg/ml), both (LA) or neither (Utx). Leptin suppressed PTP1B mRNA expression, whereas adiponectin stimulated PTP1B mRNA expression, whether administered alone or in the presence of leptin (P < 0.05 compared with untreated).

Adiponectin signalling via AdipoR1 promotes PTP1B expression and activity

PTP1B dephosphorylates and thus deactivates JAK2, acting upstream of Ob-Rb and STAT3 to negatively regulate leptin signal transduction [38,39]. PTP1B is an important inhibitor of leptin signalling in the hypothalamus, where PTP1B participates in the regulation of glucose and fat metabolism [33]. Although the mechanisms and effects of hypothalamic PTP1B activity on metabolism have been well studied, much less is known about its role in non-metabolic functions, including hepatic fibrogenesis. As PTP1B plays a critical role in the negative regulation of JAK/STAT signalling in other tissues, we examined whether adiponectin’s anti-fibrogenic effects involve PTP1B.

Compared with liver lysates from wild-type mice, we detected significantly less PTP1B protein (Figure 3A) and PTP1B mRNA expressed (Figure 3B) in livers from Adi−/− mice (P < 0.05). PTP1B mRNA expression was also significantly increased in rat HSCs exposed to adiponectin (P < 0.05). The stimulatory effect of adiponectin, which was evident at 1 h and 24 h, occurred whether adiponectin was administered alone or in the presence of leptin. By contrast, leptin tended to suppress PTP1B expression in HSCs, although the difference was not statistically
PTP1B activity and expression when AdipoR2 was silenced

the cells for 1 h with leptin (100 ng/ml), adiponectin (10 \( \mu \)g/ml), both (LA) or neither (Utx). Adiponectin stimulated PTP1B activity whether administered alone or in the presence of leptin. *\( P < 0.05 \) compared with untreated samples.

In addition to promoting PTP1B expression, adiponectin treatment also stimulated PTP1B activity in HSCs. Leptin treatment alone in vitro did not change basal PTP1B activity, but co-administration of adiponectin and leptin increased PTP1B activity significantly above that measured in control samples (\( P < 0.05 \)). Although the PTP1B activity detected in the presence of leptin and adiponectin was appreciably less than in the presence of adiponectin alone, the difference was not statistically different (Figure 4B). We also examined the regulation of hepatic PTP1B activity by adiponectin and leptin within the framework of the CCl4 in vivo experimental model. CCl4 gavage suppressed PTP1B activity in the livers of both wild-type and Ad \(-/-\) mice, but the suppression was significantly greater in the knockout mice (\( P < 0.05 \), Figure 4A). There was also significantly less PTP1B activity in liver lysates from wild-type and Ad \(-/-\) mice co-administered CCl4 and leptin, when compared with saline-treated (control) mice or mice gavaged with CCl4 alone (\( P < 0.05 \)). Importantly, PTP1B activity in leptin-treated Ad \(-/-\) mice was significantly lower than the PTP1B activity measured in liver from leptin-treated wild-type mice (\( P < 0.05 \)). These results provide new insights to explain why Ad \(-/-\) mice are exquisitely susceptible to fibrogenic stimuli when compared with wild-type mice. The results indicate that, although leptin may suppress PTP1B activity, adiponectin stimulates hepatic PTP1B expression and activity. Taken together, these results strongly suggest that adiponectin inhibits leptin signalling at least partially by enhancing PTP1B activity, thus promoting JAK2 desphosphorylation and preventing Ob-Rb activation. Such a mechanism would represent a novel molecular link accounting for the hepatoprotective inhibition of leptin signal transduction by adiponectin in the context of hepatic fibrosis.

To further elucidate the molecular events involved in attenuation of hepatic fibrosis by adiponectin, we also investigated the role of the adiponectin receptors in regulating PTP1B expression and activity. In these studies, we silenced the expression of the adiponectin receptors by transfection of culture-activated rat HSCs with non-targeting shRNA, or shRNA targeting AdipoR1 or AdipoR2. After confirming knockdown (Figure 5A), we treated the cells for 1 h with leptin (100 ng/ml), adiponectin (10 \( \mu \)g/ml), both together or neither. Consistent with the results from experiments using untransfected HSCs, adiponectin increased PTP1B expression (Figure 5C) and activity (Figure 5B) in HSCs transfected with non-targeting shRNA (\( P < 0.05 \) compared with control shRNA without adiponectin). Adiponectin also stimulated PTP1B activity and expression when AdipoR2 was silenced (\( P < 0.05 \) compared with control shRNA without adiponectin).

However, silencing of AdipoR1 blocked the adiponectin-induced increase in PTP1B protein and activity (\( P < 0.05 \) compared with control shRNA plus adiponectin). Taken together, these results suggest that in HCSs signalling via AdipoR1, but not AdipoR2, mediates adiponectin-induced stimulation of PTP1B expression and activity.

Although the question of which specific effectors are acting downstream of AdipoR1 to regulate PTP1B will require further investigation, a recent report by others suggest that AMPK is not involved [40]. AMPK\( \alpha^{-/-} \) mice are as sensitive to CCl4-induced hepatic fibrosis as AMPK\( \alpha^{++} \) are and, although HSCs derived from AMPK\( \alpha^{-/-} \) mice initially show impaired proliferation, they nevertheless become activated in culture and, upon passage, proliferate normally [40]. Moreover, recent work by Miller et al. [41], showing that adiponectin can suppress hepatocyte gluconeogenic gene expression independent of LKB1/AMPK signalling, underscores our limited understanding of the molecular details involved in the regulation of hepatic function. PPAR\( \alpha \) and the identification of several AdipoR-interacting proteins [42,43], particularly APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif) [44,45], provide attractive targets for future investigations.

Delibegovic et al. [46] have generated liver-specific PTP1B-knockout mice that show improved metabolism. Although the authors do not describe the state of hepatic fibrosis in these animals, the improvement in glucose homoeostasis and increase in insulin sensitivity of livers from Alb-Cre-PTP1B \(-/-\) mice initially show impaired proliferation, they nevertheless become activated in culture and, upon passage, proliferate normally [40]. Moreover, recent work by Miller et al. [41], showing that adiponectin can suppress hepatocyte gluconeogenic gene expression independent of LKB1/AMPK signalling, underscores our limited understanding of the molecular details involved in the regulation of hepatic function. PPAR\( \alpha \) and the identification of several AdipoR-interacting proteins [42,43], particularly APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif) [44,45], provide attractive targets for future investigations.

Although experiments directly testing this hypothesis are clearly necessary, some of the results we show in the present study are consistent with a cell-specific response to adiponectin in the context of hepatic fibrogenesis. Although hepatic PTP1B expression was reduced in Ad \(-/-\) mice when compared with wild-type mice (Figures 3A and 3B), there was no significant difference

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**Figure 4 Adiponectin promotes PTP1B activity**

(A) Analysis of PTP1B activity in liver tissue collected from livers of wild-type (WT) and Ad \(-/-\) mice. Administration of CCl4 (2 ml/kg of body weight) reduced PTP1B activity in both wild-type and Ad \(-/-\) mice. Leptin administration (5 mg/kg of body weight) further suppressed PTP1B activity. In both treatments, PTP1B activity was significantly lower in Ad \(-/-\) mice than in wild-type mice. *\( P < 0.05 \) compared with wild-type. (B) In vitro analysis of PTP1B activity in rat HSCs treated for 30 min with leptin (Lep), adiponectin (Adi), both (LA) or neither (Utx). Adiponectin stimulated PTP1B activity whether administered alone or in the presence of leptin. *\( P < 0.05 \) compared with untreated samples.
Adiponectin inhibits hepatic fibrosis via PTP1B

in hepatic PTP1B activity between Ad−/− and wild-type mice when no fibrogenic stimulus was introduced (Figure 4A). As mentioned above and reviewed thoroughly elsewhere [3,4], HSCs are normally quiescent in healthy liver, comprising a relatively small percentage of total liver cells. However, during the wound-healing response to liver injury, HSCs are activated to produce ECM and proliferate, temporarily increasing their percentage of the total hepatic cellular population and presumably their contribution to any hepatic phenotype. Consistent with this paradigm, induction of hepatic fibrosis by CCl4 intoxication suppresses PTP1B activity significantly more (P < 0.05) in Ad−/− mice than in wild-type mice. Moreover, this difference was exacerbated by leptin (Figure 4A). Our results from the present study thus demonstrate an important distinction: in the absence of liver injury, and therefore when HSCs are quiescent, there is no difference in hepatic PTP1B activity between Ad−/− and wild-type mice. However, after a fibrogenic stimulus, with subsequent activation of HSCs, the ability of Ad−/− mice to promote hepatic PTP1B activity is reduced when compared with wild-type mice. The in vitro results further underscore the role of PTP1B in culture-activated rat HSCs. In such experiments our results from the present study show that, in response to adiponectin, PTP1B expression increased 3.5-fold (Figure 3C) and PTP1B activity 200% (Figure 4B). Therefore, although conventional thought suggests that adiponectin serves only as a cellular energy sensor acting via AMPK activation, this mechanism may not be as critical for the role of adiponectin as an agonist to hepatic fibrosis in a myofibroblastic or portal fibroblastic phenotype, i.e., cells associated with fibrosis but not glucose homeostasis or other metabolic functions typically carried out in liver by hepatocytes.

Adiponectin promotes SOCS-3 association with Ob-Rb

Leptin induces expression of SOCS-3, which in turn negatively regulates leptin signalling. Neural cell-specific SOCS-3-knockout mice, as well as mice with SOCS-3 haploinsufficiency, exhibit greater sensitivity to leptin while being resistant to diet-induced obesity, when compared with wild-type mice [50–52]. SOCS-3 inhibits leptin signalling by binding Ob-Rb and preventing JAK2 phosphorylation of STAT3, and by targeting the activated receptor complex for degradation. Bjorbaek et al. [53] showed that SOCS-3 binds specifically to phosphorylated Tyr985 of Ob-Rb and that SOCS-3 fails to inhibit transcriptional activation from an erythropoietin receptor–Ob-Rb chimaera when Tyr985 of the chimaera is mutated.

Recent evidence from our laboratory suggests that adiponectin also regulates SOCS-3 expression positively [10], but whether the mechanism is transcriptional or post-translational is unclear. Ad−/− mice express less hepatic SOCS-3 mRNA and protein than wild-type mice, suggesting a transcriptional mechanism. Conversely, the kinetics of the change in SOCS-3 protein level from adiponectin-treated or leptin/adiponectin-treated rat HSCs suggest that adiponectin may also function to stabilize SOCS-3 protein, at least in vitro. These results, along with the SOCS-3-knockout mouse experiments discussed above, suggest a mechanism whereby an increase in adiponectin increases the available pool of SOCS-3, thereby promoting the binding of SOCS-3 to Ob-Rb, resulting in leptin signalling inhibition. To investigate this hypothesis, we examined the influence of adiponectin on SOCS-3 binding to Ob-Rb.

Consistent with the role of adiponectin as a negative regulator of HSC leptin signalling, less SOCS-3 was bound to Ob-Rb in livers from Ad−/− mice than in livers from wild-type mice (Figure 6A). As demonstrated previously [10], hepatic expression of SOCS-3 protein was also decreased in livers from Ad−/− mice, when compared with liver from wild-type mice (P < 0.05, Figure 6B). The difference in SOCS-3 protein expression presumably accounts for the difference in Ob-Rb-bound SOCS-3 that we observed and is consistent with our hypothesis that, by increasing the available pool of SOCS-3, adiponectin promotes...
binding of SOCS-3 to Ob-Rb, thus inhibiting leptin signal transduction.

**In vitro**, 1 h of adiponectin treatment stimulated SOCS-3 binding to Ob-Rb, even in the presence of leptin. We detected no SOCS3–Ob-Rb association in lysates from untreated HSCs or HSCs treated with leptin only (Figure 7A) for 1 h. In studies using immunoprecipitation of SOCS-3, however, we did indeed detect a SOCS3–Ob-Rb association in lysates from untreated and leptin-only treated HSCs (Figure 7B). We suspect that the discrepancy between the two sets of experiments results from differences in the relative sensitivities of the two assays based on the relative lack of abundance of our bait proteins in these experiments, Ob-Rb and SOCS-3. As Ob-Rb is a low-abundance protein, using it as bait in co-immunoprecipitation studies results in a fairly insensitive assay, hence our inability to detect any binding between Ob-Rb and SOCS-3 in the experiment presented in Figure 7(A). However, because SOCS-3 is a relatively abundant protein, the results represented in Figure 7(B) are not subject to this limitation.

In HSCs, Ob-Rb protein levels were similar in all of the conditions tested, although there was a trend towards a decrease in the presence of adiponectin and a trend towards an increase after leptin, or leptin/adiponectin co-administration. As anticipated, leptin treatment stimulated Ob-Rb phosphorylation at Tyr1138, whereas adiponectin reduced Ob-Rb Tyr1138 phosphorylation (P < 0.05 compared with untreated). Compared with untreated samples, adiponectin and leptin co-administration also increased Ob-Rb Tyr1138 phosphorylation, but the stimulation was attenuated compared with leptin-only treated samples (P < 0.05, Figures 7C and 7D). These results are consistent with both the findings reported in the present study on Ob-Rb Tyr1138 phosphorylation and our earlier work showing that adiponectin inhibits leptin-induced STAT3 phosphorylation [10], and suggests that steady-state Ob-Rb protein levels alone do not account for the difference in SOCS-3–Ob-Rb binding that we observed.

In contrast, SOCS-3 protein levels were again consistent with the increased amount of available pools of SOCS-3, producing increased SOCS-3–Ob-Rb binding. Adiponectin increased SOCS-3 protein levels (P < 0.05), whether it was administered alone or in the presence of leptin (P < 0.05), whereas SOCS-3 protein levels were decreased in HSCs by 1 h of exposure to leptin (P < 0.05, Figures 7C and 7D). The binding characteristics are consistent with the kinetics of SOCS-3 protein expression described in our previous work [10], where we observed stabilization of SOCS-3 as early as 5 min after the administration of adiponectin, followed by sustained high expression for at least 24 h. By contrast, in those studies, leptin-only treatment initially decreased the expression of SOCS-3 protein, which did not begin to recover until 1 h after treatment was begun. These kinetics are consistent with a negative-feedback loop as described in the literature, where prolonged Ob-Rb activation leads to SOCS-3 transcriptional activation [17, 51–53]. In Chinese-hamster ovary cells stably expressing Ob-Rb, SOCS-3 protein levels were maximal after 2–3 h of leptin treatment and remained elevated at 20 h [54]. Indeed, in our studies, the negative-feedback loop was also intact in rat HSCs, as SOCS-3 protein levels were also elevated after 24 h of leptin treatment (results not shown).

We also examined the phosphorylation status of SOCS-3-bound Ob-Rb in rat HSCs. In the presence of leptin alone, SOCS-3 was bound to Tyr1138-phosphorylated Ob-Rb (Figure 7B), consistent with a previous report describing the phosphorylation of Ob-Rb at Tyr1138 as being required for SOCS-3 binding and feedback inhibition of leptin signal transduction [53]. We detected no SOCS-3 association with Tyr985-phosphorylated Ob-Rb in the presence of leptin alone by immunoprecipitation analysis. Interestingly, regardless of whether adiponectin was administered to HSCs by itself or in the presence of leptin, immunoprecipitation analysis revealed that SOCS-3 was associated with Ob-Rb phosphorylated at both Tyr985 and Tyr1138 (Figure 7B). These studies may explain, with respect to hepatic fibrosis, why the leptin/SOCS-3-feedback loop is insufficient to block the fibrogenic potential of leptin. Mutation studies by Bjorbaek et al. [53] demonstrated that Tyr985 of Ob-Rb is essential for negative-feedback inhibition of leptin signalling, but not essential for STAT3-mediated transcription in response to leptin, as HEK...
(human embryonic kidney)-293 cells expressing a mutant Ob-Rb containing a leucine residue at position 985 (instead of a tyrosine residue) promotes STAT3-mediated transcription in response to leptin as well as cells expressing wild-type Ob-Rb, but they are incapable of producing negative-feedback inhibition of leptin signal transduction. Conversely, several laboratories have reported results showing that mutation of Ob-Rb at Tyr1138 abrogates STAT3-mediated transcription initiated by Ob-Rb [55–57]. The requirement for phosphorylation of Ob-Rb at Tyr1138 for STAT3-mediated transcriptional activation during leptin signalling results from its role as the site for STAT3 binding to Ob-Rb: when STAT3 is not bound to Ob-Rb, it is not phosphorylated by ligand-activated JAK2. Our results from the present study suggest that, by stimulating binding of SOCS-3 to Ob-Rb at both Tyr985 and Tyr1138, adiponectin exploits the distinct importance of each of these phosphorylation sites to inhibit leptin signal transduction. Although inhibiting leptin signal transduction by binding of SOCS-3 to Ob-Rb at Tyr985 is similar to the negative-feedback loop originally described to occur in the hypothalamus, inhibition of leptin signalling by causing SOCS-3 to bind Ob-Rb at Tyr1138 represents a novel mechanism of action not only for adiponectin, but also for SOCS-3. Taken together, these results suggest that adiponectin promotes an SOCS–Ob-Rb association independent of the negative-feedback mechanism originally associated with leptin signalling.

**Adiponectin inhibits leptin-stimulated formation of TIMP-1–MMP-1 complexes**

MMPs regulate ECM homoeostasis by catalysing the degradation of various ECM components [2]. MMP-1, or collagenase, is produced by activated HSCs and catalyses proteolysis of fibrillar collagens. TIMPs, on the other hand, regulate ECM homoeostasis by binding a particular MMP to prevent its activity. Leptin, in addition to its other profibrogenic properties, stimulates TIMP-1 secretion by rat HSCs and decreases extracellular MMP-1 activity [10]. These effects presumably correlate with the increased formation of TIMP-1–MMP-1 complexes, in which MMP-1 would be deactivated because it is bound by the TIMP-1 molecule. Conversely, the findings suggest that the anti-fibrogenic inhibition of leptin signalling by adiponectin may reduce leptin-stimulated formation of extracellular TIMP-1–MMP-1 complexes.

To test these hypotheses, we used an ELISA to measure the amount of TIMP-1–MMP-1 complexes present in conditioned medium from rat HSCs treated with leptin, adiponectin, both or neither. As anticipated, leptin increased the amount of such complexes significantly, nearly 3-fold more than untreated samples ($P < 0.05$). The presence of adiponectin, however, significantly reduced leptin-stimulated formation of TIMP-1–MMP-1 complexes ($P < 0.05$) to levels statistically equal to those measured in the presence of adiponectin alone (Figure 8).

Taken together, these results provide evidence of multiple mechanisms by which adiponectin can inhibit leptin signal transduction to reduce its profibrogenic effects. Our results suggest that, through signalling via AdipoR1, adiponectin targets JAK2 and Ob-Rb directly to inhibit the early events of leptin signal transduction and produces a cellular environment where leptin signalling would be attenuated. By preserving the inhibitory phosphorylation of JAK2 at Ser523 and blocking the activating phosphorylation at Tyr1007/Tyr1008, adiponectin can inhibit leptin signal transduction at the earliest events in the pathway, as supported by the reduced activation of the downstream signalling elements Ob-Rb and STAT3. Understanding the mechanisms of these effects requires further investigation, but the stimulation of PTP1B activity by adiponectin in HSCs provides a plausible explanation for how adiponectin negatively regulates Tyr1007/Tyr1008 phosphorylation. To target Ob-Rb, adiponectin increases SOCS-3 expression and subsequent binding to Ob-Rb in a mechanism that involves Tyr1138 phosphorylation of Ob-Rb. We propose that this mechanism is distinct from the negative-feedback loop of leptin signalling in which SOCS-3 also participates, which requires only Tyr985 phosphorylation of Ob-Rb; however, the specific roles of Ob-Rb phosphorylation at Tyr985 and Tyr1138 in adiponectin regulation of leptin signalling require further investigation. Ultimately, these varied effects converge to reduce the strength of the leptin signal, inhibiting molecular events such as the formation of TIMP-1–MMP-1 complexes, which leptin produces to promote excessive deposition of ECM.

Our understanding of the role that adiponectin plays in stellate cell biology and liver fibrosis is evolving. On the one hand, adiponectin appears to suppress HSC mitosis and increase susceptibility to apoptosis [23], effects that may be mediated by the conventional AMPK signalling axis. The results in the present study, however, coupled with the recent report that AMPK-knockout mice are still exquisitely sensitive to hepatic fibrosis [40], support pleiotropic effects of adiponectin, based on differential receptor and downstream signalling pathway activation. Although one may be involved with regulating the life cycle of activated HSCs, another is involved in the down-regulation of fibrogenic stimuli, including leptin. In either case, the emerging findings provide additional molecular details that must be addressed, while raising the opportunity for targeting molecular therapy in human liver disease.

**AUTHOR CONTRIBUTION**

Jeffrey Handy participated in all aspects of the work. Ping Fu and Pradeep Kumar contributed to the execution of the experiments and acquisition of the data. Jamie Mells participated in the design of the experiments and interpretation of the data. Shvetank Saxena and Frank Anania participated in the experimental design, data interpretation and preparation of the paper. Neeraj Sharma participated in interpretation of the data and preparation of the paper. Jeffrey Handy participated in all aspects of the work. Ping Fu and Pradeep Kumar contributed to the execution of the experiments and acquisition of the data. Jamie Mells participated in the design of the experiments and interpretation of the data. Shvetank Saxena and Frank Anania participated in the experimental design, data interpretation and preparation of the paper.

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