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ORIGINAL ARTICLE

# Resistin mediates the hepatic stellate cell phenotype

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## Abstract

**AIM:** To describe the role of resistin in liver fibrosis.

**METHODS:** For the *in vivo* animal study, Sprague Dawley rats were subjected to bile duct ligation (BDL) for 4 wk. Rat liver, adipose tissue (epididymal fat) and serum were analyzed for resistin expression. For the *in vitro* experiment, rat primary hepatic stellate cells (HSCs) and Kupffer cells (KCs) were used. HSCs were exposed to recombinant resistin, and collagen Ⅰ, transforming growth factor β1, α smooth muscle actin, tissue inhibitor of metalloproteinase 1 and connective tissue growth factor expression were analyzed. Resistin gene and protein expression was quantified as was the expression of pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1). The effects of resistin on HSC proliferation, migration and apoptosis were determined. The effects of resistin on KCs were also investigated.

**RESULTS:** Following BDL, rat epididymal fat and serum rather than liver showed higher resistin expression compared to control rats. In liver, resistin was expressed in quiescent HSCs and KCs. Resistin treatment resulted in enhancement of *TNFα*, *IL-6*, *IL-8* and *MCP-1* gene expression and increased IL-6 and MCP-1 protein in HSCs. Resistin activated HSC phospho-MAPK/p38, and p38 inhibition diminished IL-6 and MCP-1 expression. Furthermore, resistin facilitated HSC proliferation and migration, but decreased apoptosis which was *via* an IL-6 and MCP-1 mechanism. Finally, resistin-induced transforming growth factor β1 from KCs enhanced HSC collagen Ⅰ expression.

**CONCLUSION:** Resistin directly and indirectly modulates HSC behavior towards a more pro-fibrogenic phenotype.



**Key words:** Resistin; Hepatic stellate cell; Kupffer cell; Liver fibrosis; Monocyte chemotactic protein-1

**Core tip:** Resistin activated hepatic stellate cells (HSCs) phospho-MAPK/p38, and p38 inhibition diminished interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) expression. Furthermore, resistin facilitated HSC proliferation and migration, but decreased apoptosis which was *via* an IL-6 and MCP-1 mechanism. Finally, resistin-induced transforming growth factor β1 from Kupffer cells enhanced HSC collagen Ⅰ expression. Resistin directly and indirectly modulates HSC behavior towards a more pro-fibrogenic phenotype.

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## INTRODUCTION

Metabolic alterations such as glucose intolerance, increased energy expenditure, and negative nitrogen balance with depletion of fat and skeletal muscle mass are frequently encountered in patients with cirrhosis[1,2]. In particular, glucose intolerance and insulin resistance are almost universal[3,4] and, in part, mediate the progression of fibrosis[5]. However, the mechanisms whereby metabolic alterations mediate disease progression are unclear. Adipose tissue secreted proteins (adipokines) such as leptin and adiponectin modulate metabolic homeostasis and have direct effects on the hepatic fibrogenic cascade. For example, leptin promotes liver fibrosis, while adiponectin is anti-inflammatory and anti-fibrotic[6-9]. Resistin, another adipokine, has been reported to be associated with impaired insulin sensitivity and glucose intolerance[10-13], but its role in hepatic fibrosis has not been adequately delineated[14-17].

Resistin is almost exclusively expressed in the white adipose tissue of rodents, but is expressed in humans predominantly by monocytes/macrophages[18]. Several reports indicate that the serum levels of resistin are elevated in cirrhosis[14-16], increasing progressively with worsening liver function as determined by the Child-Pugh class[17]. Furthermore, in patients with liver disease, resistin levels are correlated with the extent of insulin resistance and with clinical complications and prognosis[16]. In a recent animal study[19], hyperinsulinemia and increased tumor necrosis factor α (TNFα) secretion following bile duct ligation (BDL) were shown to up-regulate adipose tissue resistin gene expression which could subsequently contribute to liver fibrosis. A recent human study noted that resistin expression was low in normal liver, but was increased in severe fibrosis, suggesting that intra-hepatic resistin derived from monocytes/macrophages might contribute to fibrosis[15,20,21].

In the present study, we undertook *in vivo* and *in vitro* studies to elucidate the role of resistin in liver fibrosis. We show that resistin has increased expression in the epididymal fat and serum of cirrhotic rats. Resistin has a pro-inflammatory role in mediating the release of TNFα, interleukin (IL)-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) in hepatic stellate cells (HSCs). Importantly, we demonstrate that resistin directly and indirectly mediates HSC activated phenotype in IL-6/MCP-1 and transforming growth factor (TGF) β1 dependent mechanisms, respectively, indicating that resistin contributes to the pro-inflammatory and pro-fibrotic phenotype of activated HSCs.

## MATERIALS AND METHODS

### Materials

Recombinant mouse resistin protein, recombinant IL-6, and MCP-1 ELISA kits, IL-6, MCP-1 and TGFβ1 antibodies were purchased from RD Systems (Minneapolis, MN, United States). Nycodenz, α smooth muscle actin (αSMA) mouse antibody was purchased from Sigma-Aldrich (St. Louis, MO, United States). Pronase E, DNase I and collagenase B were purchased from Roche Applied Sciences (Indianapolis, IN, United States). Resistin rabbit polyclonal antibody was purchased from Abbiotec TM (San Diego, CA, United States). p-p38, pERK1/2, pJNK, nuclear factor κB (NF-κB), p-p65 and p-p50 mouse monoclonal antibodies, p-p38 inhibitor (SB203580) and pJNK inhibitor (SP600125) were purchased from Cell Signaling Technology, Inc (Beverly, MA, United States). The BrdU ELISA kit was purchased from Roche Diagnostics (Castle Hills, NSW, Australia). Anti-mouse IgG conjugated to horseradish peroxidase was purchased from GE Healthcare Life Sciences (Piscataway, NJ, United States). DMEM medium was obtained from Invitrogen (Carlsbad, CA, United States).

### Animals

Male Sprague Dawley (SD) rats were obtained from the Animal Resources Centre (Perth, Australia). All animals were maintained under 12-h light/dark cycles with food and water *ad libitum*. For the *in vivo* experiment, BDL or a sham surgical procedure was performed on rats. After 4 wk, rat liver, epididymal fat and serum were collected for resistin quantification. All experimental protocols were approved by the Sydney West Area Health Service Animal Research Ethics Committee.

### Isolation and culture of rat hepatic stellate cells and Kupffer cells

Rat HSCs were isolated by a two-step (collagenase B and pronase E) perfusion method under ketamine and xylazine anesthesia as reported previously[6]. Briefly, rat liver was perfused through the portal vein using $Ca^{2+}$- and $Mg^{2+}$-free Gey's Balanced Salt Solution (GBSS, Sigma, United States) and then sequentially with pronase E followed by collagenase B (Roche Applied Science, Castle Hills, NSW, Australia). The liver was excised, gently dispersed in GBSS containing 0.01% DNase I and the cell suspension filtered through a sterile nylon mesh and subjected to low-speed centrifugation. The resultant cell pellet was mixed with 30% Nycodenz to obtain an 11% final Nycodenz/cell suspension. After centrifugation at 1400 *g* for 20 min, HSCs were collected, resuspended in culture medium, and plated on 6 well plates with 10% FCS/DMEM at a density of $0.8 \times 10^6$ cells/well. Cell viability was assessed by trypan blue exclusion and was routinely more than 95%. Purity was 95% as determined by morphology, vitamin A autofluorescence and desmin positivity. HSCs were maintained in 95% air and 5% $CO_2$ in DMEM (Gibco, United States) with 10% FCS and 1% penicillin/streptomycin. KCs were further obtained and purified by elutriation[6]. KCs were identified by their ability to phagocytose latex beads; viability was > 96% and purity > 98%. KCs were cultured in 10% FCS/DMEM/1% penicillin-streptomycin.

**Treatments:** For recombinant mouse resistin (RD Systems, Minneapolis, MN, United States), we undertook a dose ranging study based on previous reports[20-23] using 10, 50, 250 and 500 ng/mL. We found that 500 ng/mL

was the optimal dose which was used in all subsequent experiments. Primary rat HSCs and KCs were cultured for the time periods indicated and serum starved (0.2%) for 4 h prior to treatment. Subsequently, control (vehicle) and resistin (500 ng/mL) were added to the culture wells. After 24 h or extended culture as indicated, total RNA and protein were extracted. For the KC-HSC co-culture experiment, control (vehicle) and resistin (500 ng/mL) were added to cultured KCs at day 2 for 24 h, then KCs were washed three times with PBS and fresh medium was added and cultured for another 24 h. Afterwards, KC conditioned medium (KM) was transferred to HSCs at day 4 for 24 h co-culture. In one experiment, lipopolysaccharide (LPS, 50 ng/mL) was used to further activate cultured KCs.

### *Real-time reverse transcription polymerase chain reaction*

Total cellular RNA was prepared from HSCs using TRI@ REAGENT (Molecular Research Center, INC., Cincinnati, OH, United States). Complementary DNA (cDNA) was synthesized from 1 μg RNA using Super-Script Ⅲ reverse transcriptase and 0.5 nmol of random primers (Invitrogen, Carlsbad, CA, United States). Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using SYBR Green Platinum SYBR Green SuperMix (Invitrogen, United States). The synthesized cDNA was amplified using the following sequence specific primers: resistin 5'-CAAGACTTCAGCTCCCTACTGC-3' (forward) and 5'-GACGGTTGTGCCTTCTGG-3' (reverse); collagenα1 (Ⅰ) 5'-TTCACCTACAGCACGCTTGTG-3' (forward) and 5'-TCTTGGTGGTTTTGTATTC-GATGA-3' (reverse); TGFβ1 5'-TCGACATGGAGCT-GG TGAAA-3' (forward) and 5'-GAGCCT-TAGTTTGGACAGGATCTG-3' (reverse); αSMA5'-CGATAGAACACGGCATCATC-3' (forward) and 5'-CATCAGGCAGTTCGTAGCTC-3' (reverse); tissue inhibitor of metalloproteinase 1 (TIMP1) 5'-AAGGGC-TACCAGAGCGATCA-3' (forward) and 5'-GGTATT-GCCAGGTGCACAAAT-3' (reverse); connective tissue growth factor (CTGF) 5'-CGCCAACCGCAAGATTG-3' (forward) and 5'-ACACGGACCCACCGAAGAC-3' (reverse); IL-6 5'- CCCTTCAGGAACAGCTATGAA-3' (forward) and 5'-ACAACATCAGTCCCAAGAAGG-3' (reverse); IL-1 α 5'-ACATCCGTGGAGCTCTCTT-TACA-3' (forward) and 5'-TTAAATGAAC-GAAGTGAACAGTACAGATT-3' (reverse); IL-1β 5'-TACCTATGTC TTGCCCGTGGAG-3' (forward) and 5'-ATCATCCCACGAGTCACAGAGG-3' (reverse); TNFα 5'-GCCCAGACCCTCACACTC-3' (forward) and 5'-CCACTCCAGCTGCTCCTCT -3'(reverse); IL-8 5'-TCTGCAGCTCTGTGTGAAGG-3' (forward) and 5'-AATTTCTGGTT TGGCGCAGT-3' (reverse); MCP-1 5'-AGCATCCACGTGCTGTCTC-3' (forward) and 5'-GATCATCTTGCCAGTGAATGAG-3' (reverse). The relative amount of mRNA was calculated by reference to a calibration curve. The final result for each sample was normalized to the respective β actin value.

**Immunoblotting:** Cell culture media were removed and the cells washed with PBS and lysed on ice in a buffer containing 20 mmol/L Tris, 0.5 mmol/L $MgCl_2$, 1 mmol/L Dithiothreitol (DTT), 3 mmol/L $NaN_3$, and a mixture of protease and phosphatase-inhibitors. Cell lysates were disrupted using a sonicator on ice. After centrifugation at 13000 *g* for 15 min, the supernatant was collected as cytoplasmic protein. Nuclear protein was extracted as described previously[6]. The protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Sydney, Australia). Immunoblotting was performed as previously described with some modifications[6,24]. Total protein (20 μg per lane) was resolved by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) under reducing conditions. The electrophoresed proteins were electrotransferred onto Polyvinylidene difluoride membranes (Immobilin-P, Millipore, Bedford, MA, United States). The membranes were blocked with 5% skim milk (Resistin, TGFβ1, p-p38, pERK1/2, pJNK, NF-κB p-p65, NF-κB p-p50 and αSMA) for 60 min and then incubated overnight with primary antibody (Resistin 1:200, p-p38 1:1000, pERK1/2 1:1000, pJNK 1:1000, p-p65 1:1000, p-p50 1:1000, TGFβ1 1:500, αSMA 1:2000) at 4 ℃. After 3 washes with 0.05% Tween-20/TBS, anti-mouse IgG (peroxidase conjugate) secondary antibody was applied. Blots were visualized by enhanced chemiluminescence (Pierce Perbio, Rockford, IL, United States). All images were quantified by densitometry.

### *Sirius red staining and quantification of collagen in HSCs*

Sirius red staining and collagen quantification were performed according to a previously published protocol[6,25]. Briefly, Sirius red F3BA solution (0.1% in saturated picric acid) was added to cell layers fixed in Bouin's solution. After 1 h the cell layers were washed in tap water and again in 0.01 mol/L HCl to remove unbound dye. For collagen quantification, the dye was dissolved in 0.1 mol/L NaOH and absorbance determined at 450 nm. The amount of collagen was normalized to the protein concentration using the Bradford Reagent. Assays were performed in duplicate.

### *Enzyme-linked immunosorbent assay*

The media were collected from cultured HSCs following 24 h stimulation with resistin (500 ng/mL). Levels of IL-6 and MCP-1 in the media were determined according to the manufacturer's instructions (RD Systems). Rat serum was collected and resistin concentration detected using a resistin rat ELISA kit (Boivendor) according to manufacturer's instructions.

**HSC proliferation**: Cell proliferation was analyzed using a BrdU-based enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer's

instructions. HSCs at day 4 were treated with resistin (500 ng/mL) or other agents as indicated for 24 h. The cells were subsequently labeled with BrdU for 2 h at 37 ℃. Cells were then fixed and incubated with a peroxidase-conjugated anti-BrdU antibody for 90 min at room temperature. After adding the peroxidase substrate, 3,3',5,5'-tetramethylbenzidine, BrdU incorporation was determined by measuring optical densities at 450 nm (background 620 nm).

**HSC migration:** HSC migration was assessed both with the wound scratch assay and a modified Boyden chamber. For the wound scratch assay, using a sterile 200 μL pipette tip, three separate wounds were generated through the cell monolayer. HSCs (90% confluence) at day 6 cultured in 12-well plates were treated with resistin (500 ng/mL) or other agents as indicated. The scratch area was photographed immediately and 6 h after scratching and cell migration into the scratch area calculated as the area covered by cells in the percentage of the initial scratch area. For the second method, a cell culture insert (12 well, BD) was used and the porous membrane (pore size 8 μm) of the filter was coated with 30 μg/mL collagen I at 37 ℃ for 30-60 min. HSCs at day 6 were trypsinized and placed into the upper chamber ($10^5$ cells/mL). The lower wells were filled with resistin (500 ng/mL) or other agents as indicated. After 6 h of incubation at 37 ℃, cells adhering to the upper side of the filter were removed with a cotton swab. The filters were then fixed with 100% methanol and stained with HEMA-3. The numbers of HSCs on the lower side of the filter were counted in five randomly chosen microscopic fields at a magnification of × 400 by changing the focus.

**HSC apoptosis:** Annexin-V/PI labeling was used to detect HSC apoptosis. Briefly, trypsinized HSCs were washed twice in PBS, stained with annexin-V (10 μL) and PI (5 μL) for 10 min, and the apoptotic rate quantified by FACS Calibur flow cytometry (Becton Dickinson Inc.) at 488 nm. More than $1 \times 10^4$ cells were detected, and the results were analyzed with FlowJo software (Treestar, United States). The population of apoptotic cells was identified as annexin V+/PI-. The percentage of apoptotic cells was calculated according to total annexin V+/PI- divided by total cells.

### Statistical analysis

The results are expressed as mean ± SD. Comparisons between 2 groups were analyzed using the Student *t* test. For the comparison of more than two groups, we used two-way ANOVA. *P* values < 0.05 were considered statistically significant. All calculations were performed using Statistical Program for Social Sciences (SPSS) software 13.0 (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Resistin expression is up-regulated in cirrhotic rats

Resistin expression in liver, epididymal fat and serum in BDL and sham rats was examined. We noted that resistin expression in epididymal fat was considerably higher than that in liver in the BDL or sham rats (Figure 1A and B, all $P < 0.01$). BDL rat epididymal fat mRNA and protein level were further up-regulated compared to sham rats (Figure 1A and B, both $P < 0.05$). Similarly, BDL rat serum resistin level was also elevated (Figure 1C, $P < 0.05$). However, liver resistin mRNA and protein were unchanged in the BDL and sham groups (Figure 1A and B). These findings suggest that increased adipose resistin rather than liver resistin may play a vital role in resistin-mediated liver injury in rodents. Therefore, we undertook detailed *in vitro* experiments in order to explore the impact of exogenous resistin on HSC activated phenotype.

### Resistin is expressed in quiescent HSCs and KCs

Resistin mRNA was detected in quiescent rat HSCs (Figure 1D) at day 1 and was reduced by 90% ($P < 0.01$) following activation for 8 d on plastic. Resistin mRNA was expressed in quiescent KCs (day 1) and activated KCs (3 d), without significant changes over time. LPS (50 ng/mL for 24 h) stimulation of KCs at day 3 did not enhance resistin expression (Figure 1D). Consistent with the mRNA data, resistin protein expression declined 6-fold in HSCs at day 8 with no change in KCs at day 3 and after LPS stimulation (Figure 1E). These data indicated that autocrine HSC resistin and paracrine KC resistin are unlikely to be of major importance in mediating any effects on activated HSCs.

### Resistin promotes a pro-inflammatory phenotype in HSCs

Pro-inflammatory cytokines and chemokines play a permissive role in liver fibrosis[26-29] and previous reports suggest that resistin increases MCP-1 secretion. We evaluated the expression of TNFα, IL-1α, IL-1β, IL-6, IL-8 and MCP-1 in rat HSCs after stimulation with resistin. As demonstrated, resistin (500 ng/mL) stimulation for 24 h markedly up-regulated the expression of TNFα, IL-6, IL-8 and MCP-1 mRNA (Figure 2A, all $P < 0.05$), but not that of IL-1α and IL-1β. To rule out any potential effects of inadvertent endotoxin contamination, we repeated these studies in the presence of Polymyxin B and noted no difference in the gene expression profile (data not shown). Finally, using trypan blue staining and LDH assays at 24, 48 and 72 h, we excluded the possibility of direct cellular toxicity due to the resistin dose used (data not shown). Since IL-6 and MCP-1 are well documented to play a role in mediating hepatic fibrosis, their protein concentrations were estimated in conditioned medium. As shown in Figure 2B, resistin administration increased IL-6 and MCP-1 concentrations 1.7 and 1.8 fold after 24-h of treatment (both $P < 0.05$).

### Resistin enhances HSC proliferation and migration but diminishes HSC apoptosis via an IL-6 and MCP-1 pathway

During the process of chronic liver injury, activated

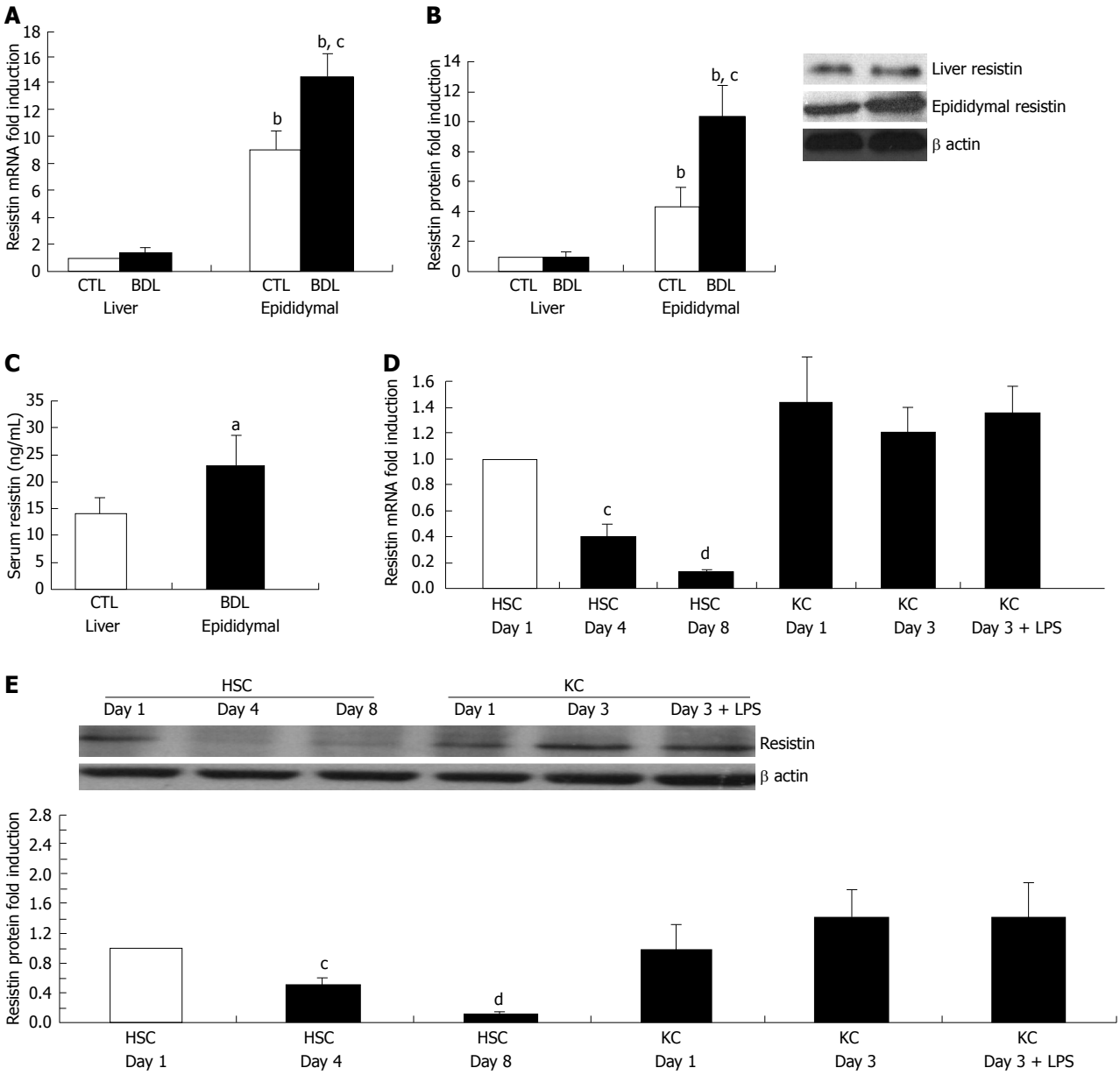


**Figure 1 Rat extrahepatic but not intrahepatic resistin is up-regulated in cirrhosis.** For the *in vivo* animal study, Sprague Dawley rats were subjected to bile duct ligation (BDL) for 4 wk. Liver, adipose tissue (epididymal fat) and serum were collected to determine resistin expression by quantitative polymerase chain reaction (qPCR), immunoblot and enzyme-linked immunosorbent assay. For the cell culture study, rat hepatic stellate cells (HSCs) and Kupffer cells (KCs) were isolated and cultured on plastic. HSC and KC total RNA/protein were extracted at different culture times (day 1, 4 and 8 for HSCs; day 1 and 3 for KCs). One group of KCs at day 2 were treated with Lipopolysaccharide (LPS) (50 ng/mL) for 24 h. qPCR and Immunoblot were performed for quantification of resistin mRNA and protein. β actin was used as an internal control. A: mRNA expression of resistin in liver and epididymal fat; B: Protein expression of resistin in liver and epididymal fat; C: Serum resistin concentrations; D: mRNA expression of resistin in HSCs and KCs on different culture days; E: Protein expression of resistin in HSCs and KCs on different culture days. Results are mean ± SD of at least three independent experiments performed in triplicate. $^{a}P < 0.05$ and $^{b}P < 0.01$ increased *vs* rat liver, HSC control at day 1 or sham rat serum; $^{c}P < 0.05$ and $^{d}P < 0.01$ decreased *vs* liver of control sham rat or HSC control at day 1.

HSCs proliferate and migrate to sites of inflammation and have reduced apoptosis. This phenotype is part of the expected adaptive wound healing response to injury. Hence, we sought to determine the role of resistin in mediating activated HSC behavior. As demonstrated in Figure 3A, resistin enhanced HSC proliferation by approximately 90% compared to the control ($P < 0.01$). Using the wound scratch assay and a modified Boyden chamber, compared to the control, resistin treatment resulted in an approximately 80% and approximately 220% increase in HSC migration, respectively ($P < 0.05$, Figure 3A and B). We next examined the role of resistin on HSC apoptosis. In contrast, resistin significantly reduced HSC apoptosis (56%, $P < 0.05$, Figure 3A), as shown by annexin V/IP flow cytometry. Finally, we determined whether up-regulation of IL-6 and MCP-1 was responsible for the changed HSC phenotype by resistin. As expected, resistin-mediated HSC proliferation, migration and apoptosis were partially, but significantly reversed (all $P < 0.05$, Figure 3A and B) by IL-6 (5 μg/mL) and

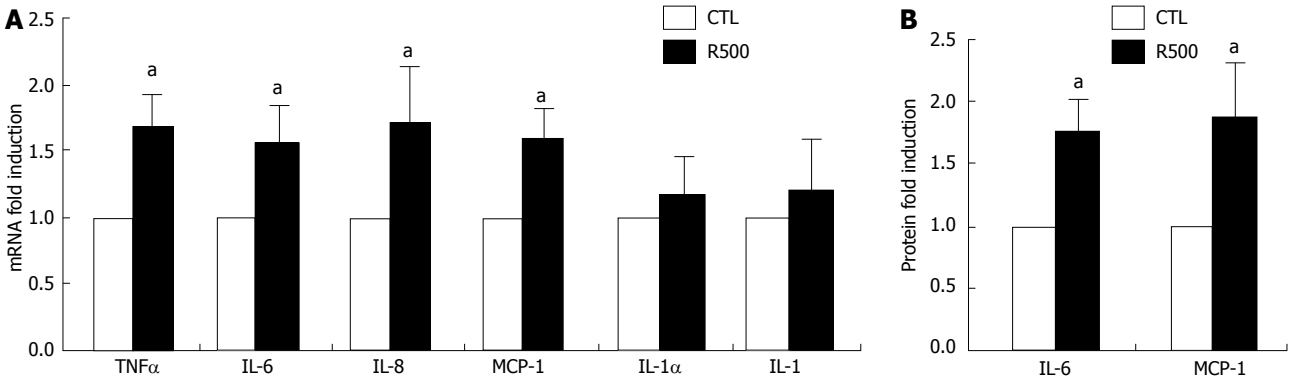


**Figure 2 Resistin enhances the expression of tumor necrosis factor α, interleukin 6, interleukin 8 and monocyte chemotactic protein-1 in hepatic stellate cells.** Rat hepatic stellate cells (HSCs) at day 4 were cultured with resistin (500 ng/mL) (R500) for 24 h. Total RNA was extracted and quantitative polymerase chain reaction was performed to quantify mRNA expression. Media were collected and enzyme-linked immunosorbent assay conducted to determine interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) protein concentrations. A: mRNA expression of tumor necrosis factor α (TNFα), IL-6, IL-8, MCP-1, IL-1α and IL-1; B: IL-6 and MCP-1 protein levels. Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. [a]*P* < 0.05 *vs* controls (untreated).

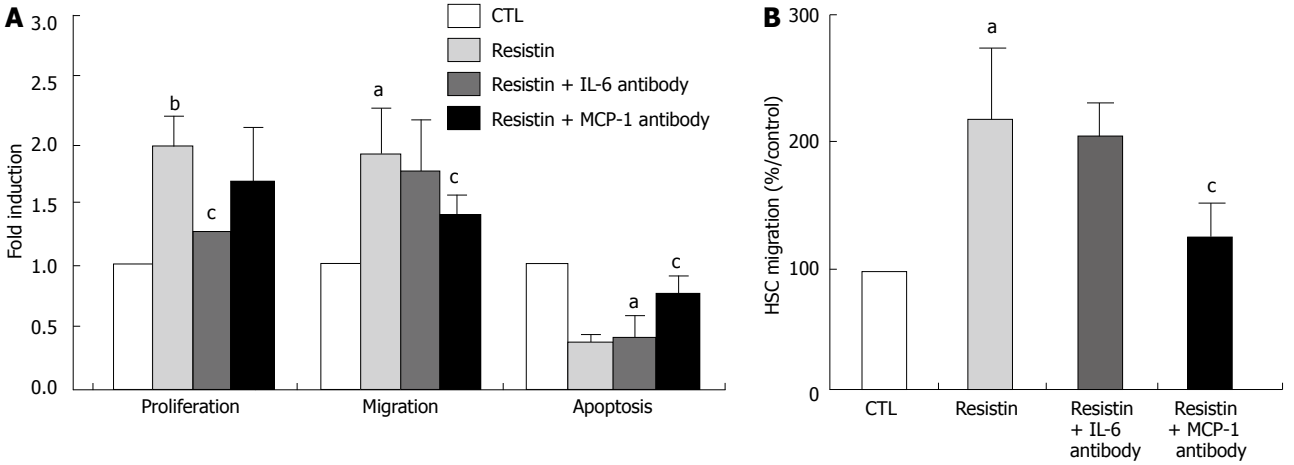


**Figure 3 Resistin promotes hepatic stellate cells proliferation and migration but decreases hepatic stellate cells apoptosis in an interleukin 6 and monocyte chemotactic protein-1 dependent mechanism.** BrdU, enzyme-linked immunosorbent assay, Wound Scratch Assay (or Boyden chamber) and annexin V/IP flow cytometry were performed to determine hepatic stellate cells (HSCs) proliferation, migration and apoptosis, respectively. For the interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) inhibition experiments, IL-6 and MCP-1 neutralizing antibodies (5 μg/mL and 10 μg/mL, respectively) were added to the culture 1 h before resistin (500 ng/mL) administration. Resistin (500 ng/mL) was added to rat HSCs at day 4 for 24 h. Absorbance was measured and apoptosis assessed. For the migration assay, rat HSCs at day 6 were used. After a scratch wound was made, resistin (500 ng/mL) was added and the cells were cultured for 6 h and photographed. For the Boyden chamber assay, the detailed procedure is described in the Materials and Methods section. A: Resistin promoted HSC proliferation and migration, but inhibited HSC apoptosis, while IL-6 and MCP-1 antibodies reversed the resistin-induced HSC phenotype; B: The Boyden chamber assay confirmed that resistin enhanced HSC migration and MCP-1 neutralization reversed this effect. Results are mean ± SD of at least three independent experiments performed in triplicate. [a]*P* < 0.05 and [b]*P* < 0.01 *vs* control (untreated); [c]*P* < 0.05 *vs* resistin treatment alone.

MCP-1 (10 μg/mL) neutralization, respectively. These data suggest that resistin triggered HSC IL-6 and MCP-1 production, thereby modulating HSC phenotype.

### *Resistin activates HSC MAPK/p38 and nuclear NF-κB p65*

Mitogen-activated protein kinases (MAPK) and NF-κB play critical roles in the induction of pro-inflammatory cytokines and chemokines, and regulate cell biological behaviors. Therefore, we determined whether resistin activates HSC MAPK (p38, ERK1/2 and JNK) and NF-κB. Phoshor-p38, ERK1/2 and JNK in the cytoplasm as well as NF-κB p65 and p50 in cytosolic and nuclear extracts were analyzed by immunoblotting. The results showed that cytoplasmic p-p38 and nuclear p-p65 were up-regulated (both $P < 0.05$, Figure 4A and C). Changes in cytosolic pERK1/2, pJNK (data not shown), p65 and cytosolic and nuclear p-p50 (data not shown) were not observed. In the p-p38 inhibition experiment using SB203580, we found that p-p38 activation was responsible for IL-6 and MCP-1 induction in HSCs ($P < 0.05$, Figure 4B). Furthermore, resistin (500 ng/mL) enhanced NF-κB DNA binding ability (luciferase mRNA. $P < 0.05$, Figure 4D). As expected, NF-κB inhibition by pyrrolidine dithiocarbamate (PDTC) (100 μmol/L) attenuated the resistin-induced increase in nuclear p-p65 and NF-κB DNA binding ability ($P < 0.05$, Figure 4C and D). Similarly, PDTC reversed resistin-induced up-regulation of IL-6 and MCP-1 (Figure 4E).

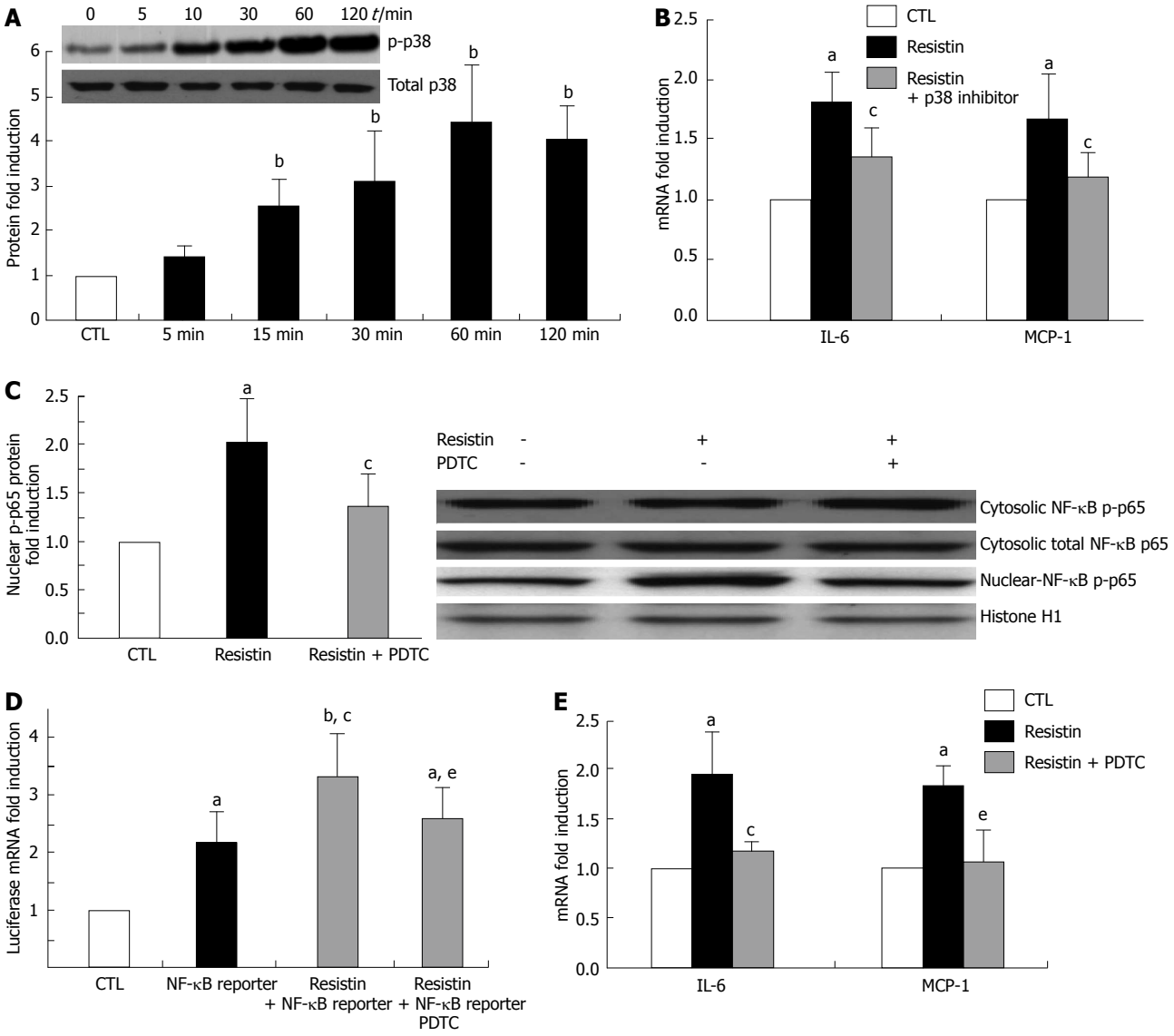


**Figure 4 Resistin activates hepatic stellate cells MAPK/p38 and nuclear factor κB p65.** Rat hepatic stellate cells (HSCs) at day 4 were cultured with resistin (500 ng/mL) for 120 min. Cytosolic and nuclear proteins were extracted and Immunoblot performed to quantify p-p38 and nuclear factor κB (NF-κB) p-p65. For NF-κB DNA binding capacity, 3 × NF-κB /Luc reporter was added to the culture for 24 h and Luciferase mRNA quantified by quantitative polymerase chain reaction. For the p-p38 and NF-κB inhibition experiments, SB203580 (20 μmol/L, p-p38 inhibitor) or pyrrolidine dithiocarbamate (PDTC) (100 μmol/L, NF-κB inhibitor) was added 1 h before resistin treatment. Resistin (500 ng/mL) was added to rat HSCs for 24 h. A: p-p38 was enhanced by resistin; B: p-p38 inhibition (24 h) diminished resistin-induced interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) increase by HSCs; C: Nuclear p-p65 was increased by resistin exposure and decreased by PDTC (120 min); D: Luciferase mRNA was augmented by resistin and diminished by PDTC (24 h); E: PDTC reversed resistin-induced enhancement of IL-1 and MCP-1 (24 h). Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* controls (untreated); $^{c}P < 0.05$ *vs* resistin treatment alone or NF-κB reporter treatment alone; $^{e}P < 0.05$ *vs* combination of resistin and NF-κB reporter.

### *Resistin indirectly promotes HSC collagen I expression through the actions of KCs*

To determine whether resistin affects KCs and whether KCs participate in the process of resistin-mediated HSC phenotype, the appropriate experiments were undertaken. As shown in Figure 5A and B, resistin up-regulated TGFβ1 and CTGF mRNA in KCs and enhanced TGFβ1 protein in KC medium (both $P < 0.05$). Co-culture of HSCs and KC conditioned medium resulted in a significant increase in HSC collagen I and CTGF expression (Figure 5C and D, all $P < 0.05$), however, TGFβ1 (10 μg/mL) neutralization diminished this increase (Figure 5C and D, all $P < 0.05$). Downstream signaling responsible for increased KC TGFβ1 and CTGF expression by resistin were further analyzed. We found that pJNK and p-p38 were activated following exposure to resistin. Furthermore, pJNK and p-p38 inhibition partially, but significantly reversed resistin-induced TGFβ1 and CTGF enhancement (Figure 5E and F, $P < 0.05$ and 0.01). These data suggest that resistin affected HSC activated phenotype by increased TGFβ1 from KCs.

## DISCUSSION

Resistin is suggested to play a pathogenic role in insulin resistance and altered glucose metabolism in rodents[3,4].

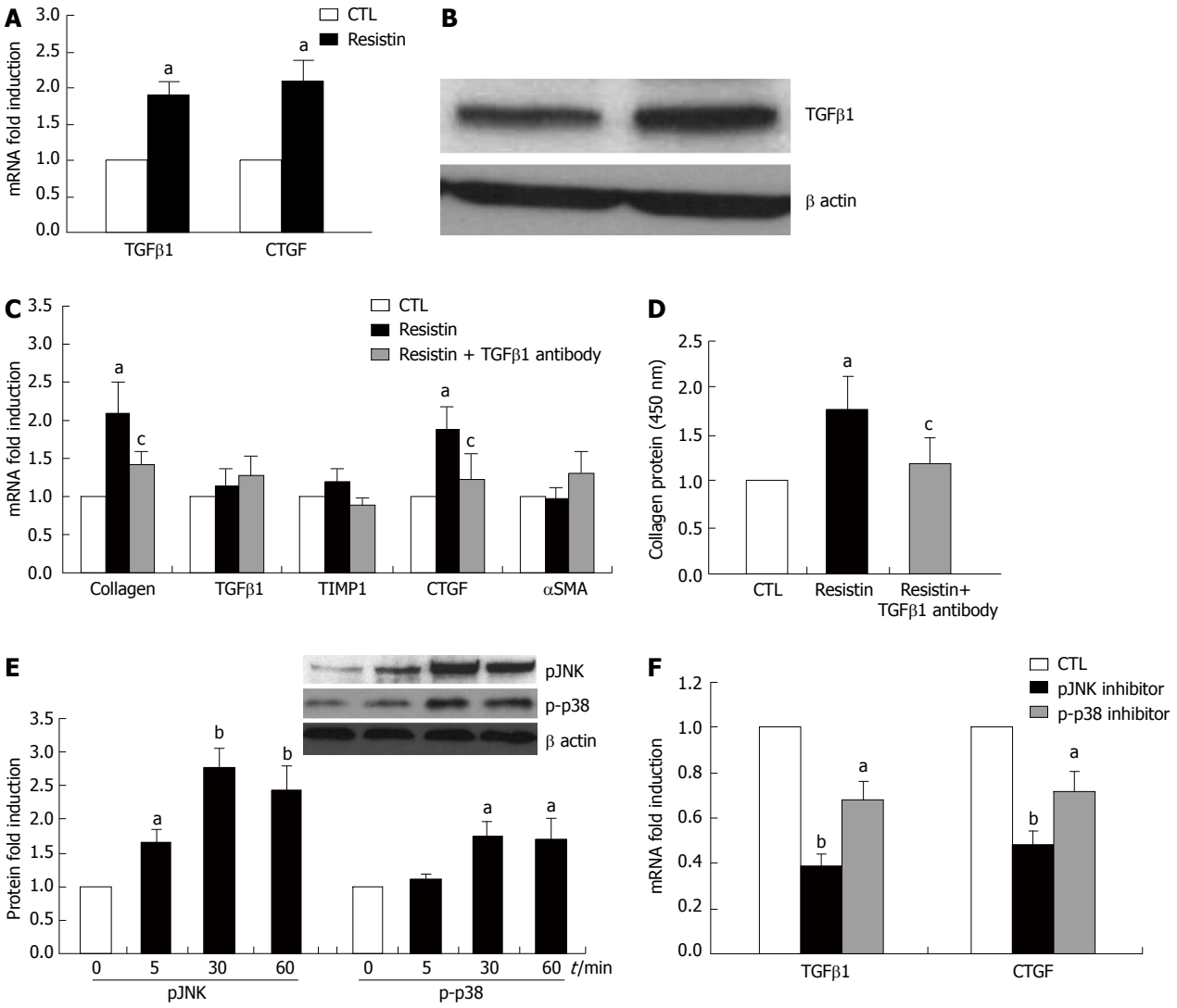


**Figure 5 Resistin indirectly enhances hepatic stellate cells collagen I expression through a transforming growth factor β1 dependent mechanism *via* the action of Kupffer cells.** Rat Kupffer cells (KCs) at day 2 were cultured with resistin (500 ng/mL) for 24 h. Total RNA was extracted and quantitative polymerase chain reaction was performed. Transforming growth factor β1 (TGFβ1) protein expression in KC conditioned medium (KM) was quantified by immunoblotting. Sirius red was used for collagen I protein quantification in the medium. For the KC-hepatic stellate cell (HSC) co-culture experiment, KCs at day 2 were incubated with resistin for 24 h and then washed three times with phosphate-buffered saline, fresh medium was subsequently added to the culture for another 24 h. KM was then transferred to HSCs at day 4 for 24 h. HSC collagen I , TGFβ1, tissue inhibitor of metalloproteinase 1 (TIMP1), connective tissue growth factor (CTGF) and α smooth muscle actin (αSMA) expression were determined. For inhibition experiments, TGFβ1 monoclonal antibody (10 μg/mL), SP600125 (50 μmol/L) and SB203580 (20 μmol/L) were added to KCs for 24 h. A: Resistin promoted KC T*GFβ1* and *CTGF* gene expression; B: Resistin augmented TGFβ1 expression in KM medium; C: HSC collagen I and CTGF mRNA were augmented by resistin conditioned KM reversed by TGFβ1 neutralization (10 μg/mL); D: HSC collagen protein was increased by resistin conditioned KM but reversed by TGFβ1 neutralization (10 μg/mL); E: Resistin increased KC JNK and p38 phosphor-protein; F: pJNK inhibitor (50 μmol/L, SP600125) and p-p38 inhibitor (20 μmol/L, SB203580) partially, but significantly reversed resistin-induced TGFβ1 and CTGF expression by KCs. Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* controls (untreated); $^{c}P < 0.05$ *vs* resistin treatment alone.

For example, lowering plasma resistin in insulin-resistant mice decreases blood glucose levels and improves insulin sensitivity[10,30,31], while treatment of normal mice with resistin impairs glucose tolerance and insulin actions[10,30]. Resistin may also play a pivotal role in inflammation since it up-regulates IL-6 and TNFα expression in human peripheral blood mononuclear cells *via* NF-κB activation[22]. Furthermore, the addition of resistin protein from both mice and humans to macrophages results in enhanced secretion of pro-inflammatory cytokines including TNFα and IL-12[32]. In human cirrhosis, resistin levels in the liver and plasma are elevated and increase further with the severity of liver disease[14-17,33]. This suggests that the pro-inflammatory activities of resistin may modulate liver inflammation and drive disease progression in cirrhosis.

This study provides evidence that in cirrhotic rats, adipose tissue (epididymal fat) and blood resistin are up-regulated, and adipose tissue may be the main source of resistin secretion. Rat liver, HSCs and KCs express resistin, but are unlikely to be important sources of resistin secretion in cirrhosis. Resistin exerts pro-inflammatory activities on HSCs with enhanced secretion of pro-

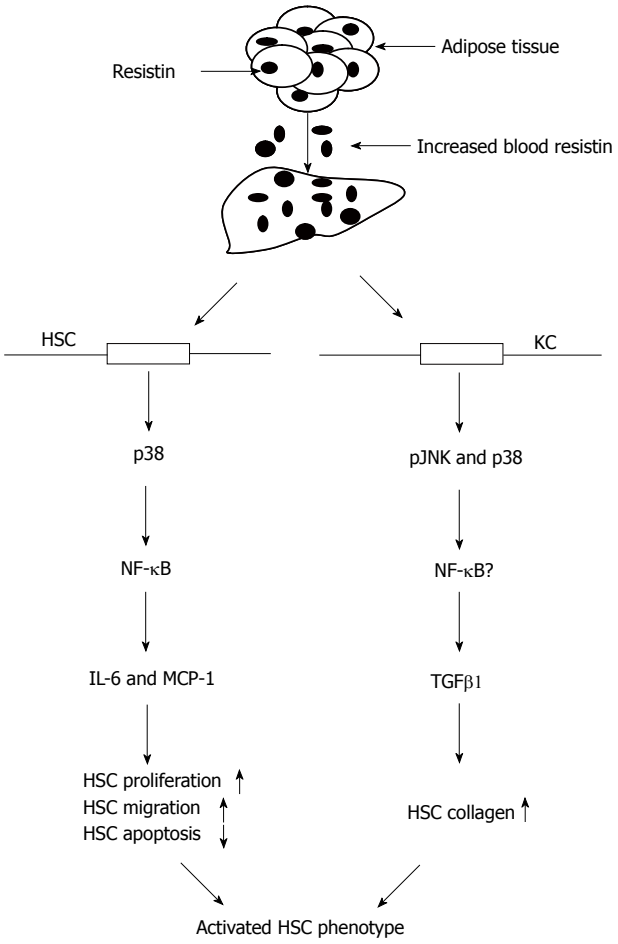


**Figure 6 Schematic diagram illustrating a possible mechanism by which resistin potentiates hepatic stellate cells profibrogenic phenotype.** Adipose tissue is a predominant source of resistin expression and secretion in rodents. Increased adipose resistin released into the bloodstream and liver stimulates HSCs to produce increased amounts of pro-inflammatory cytokines and chemokines (IL-6 and MCP-1), leading to enhanced HSC proliferation and migration, but attenuation of HSC apoptosis. In addition, resistin promotes KC TGFβ1 which subsequently activates HSC by up-regulation of collagen I. Therefore, resistin is considered one of the pro-fibrogenic adipocytokines. TGFβ1: Transforming growth factor; NF-κB: Nuclear factor κB; IL-6: Interleukin 6; MCP-1: Monocyte chemotactic protein-1; HSCs: Hepatic stellate cells.

inflammatory cytokines (TNFα, IL-6, IL-8 and MCP-1). Most importantly, resistin promotes HSC proliferation and migration, while inhibiting their apoptosis *via* an IL-6 and MCP-1 mechanism. KCs participate in this process by up-regulating HSC collagen I through increased TGFβ1. Taken together, our data suggest that resistin promotes the progression of liver injury.

Resistin is almost exclusively expressed by white adipose tissue in rodents, but is expressed by monocytes and macrophages in humans[18,34,35]. Liver infiltrating CD43 cells and KCs have been suggested as key sources of resistin in the liver of cirrhotic patients[20,21], thus resistin was more abundant in adipose tissue than in human liver[20,21]. In this study, although rat quiescent HSCs expressed resistin, it declined markedly on activation. The relevant mechanism is unclear, however, adipogenic transcriptional regulation may be required for maintenance of the quiescent HSC phenotype[26]. KCs also expressed resistin but no change was found on activation or LPS stimulation. Therefore, it is unlikely that resistin derived from HSCs and KCs contributed to the increase in serum resistin in BDL rats, thus HSCs and KCs are non-critical sources of resistin. However, other liver cell types may not represent a likely source of resistin production as hepatocytes and endothelial cells do not express resistin[15]. Thus, adipose tissue, including epididymal fat, could be the predominant source of resistin in liver injured rodents. It has been demonstrated in *in vivo* and *ex vivo* studies, that increased TNFα and insulin in BDL cirrhotic rats stimulate adipose resistin expression[14,15,19].

Why LPS was unable to trigger resistin secretion by KCs is unknown. KCs belongs to the macrophage family, and many studies have shown that LPS exposure induced resistin production in human and rodent macrophages[36,37]. The mechanisms involved require further clarification.

As expected, HSC expression of TNFα, IL-6, IL-8 and MCP-1 mRNAs was increased on resistin exposure, as was IL-6 and MCP-1 protein. Bertolani *et al*[20] reported similar findings in human HSCs and noted that resistin up-regulated human HSC MCP-1 that was dependent on a $Ca^{2+}$/NF-κB-dependent pathway[20]. We further demonstrated that resistin directly augmented HSC proliferation and migration, but reduced HSC apoptosis *via* an IL-6 and MCP-1 mechanism. These novel data imply that IL-6 and MCP-1 inhibition may prevent resistin-induced liver fibrogenesis. The pro-fibrogenic effects of IL-6 and MCP-1 are well documented in the literature[38-40]. Moreover, we found that resistin was able to promote KC activation as it stimulated enhancement of KC TGFβ1 expression. Thus, increased TGFβ1 led to up-regulation of HSC collagen I and HSC activation. This is an important finding, as TGFβ1 is a potent profibrogenic cytokine. Interestingly, this phenomenon is similar to our previous report[6]. We observed that the profibrogenic role of leptin could be achieved at least through TGFβ1 from KCs[6]. Collectively, these data indicate that resistin is able to modulate HSC behaviors towards a more profibrogenic phenotype.

Although many functions of resistin in inflammation and inflammation-related diseases have been described, the relevant intracellular signaling pathway of resistin is not yet completely understood. We further demonstrated that resistin mediated HSC IL-6 and MCP-1 *via* p38 and KC TGFβ1 *via* pJNK and p-p38 (Figure 6). These results may provide evidence to prevent resistin-mediated liver injury/fibrosis using relevant signaling inhibitors.

In summary, this study demonstrates that in rodents, resistin production in the context of liver injury is principally non-hepatic in origin. Extrahepatic resistin could contribute to liver fibrosis by its direct and indirect profibrogenic effects on HSCs. Further studies on resistin knockout and transgenic animals are needed.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Metabolic abnormalities usually cause the progression of liver fibrosis. To date, the mechanism whereby metabolic alterations mediate disease progression are unclear. Resistin, an adipokine, has been reported to be associated with metabolic alterations, however, its role in hepatic fibrosis has not been clearly investigated.

### Research frontiers

Although many functions of resistin in inflammation and inflammation-related diseases have been described, the relevant intracellular signaling pathways of resistin in liver fibrosis are not yet completely understood.

### Innovations and breakthroughs

To date, there have been a limited number of studies regarding the impact of resistin on the phenotype of hepatic stellate cells and how it functions in liver fibrosis. In this study, the authors employed a direct analysis to identify the significant correlation between resistin and hepatic stellate cells (HSCs). The authors confirmed that resistin mediated-HSCs move towards a more pro-fibrotic phenotype which is dependent on interleukin 6/monocyte chemotactic protein and/or transforming growth factor β1.

### Applications

By understanding the mechanism whereby resistin mediates HSC activation, this study may provide evidence to prevent resistin-mediated liver injury/fibrosis using relevant signaling inhibitors.

### Terminology

The serum levels of resistin are elevated in cirrhosis, and the changed phenotype of HSCs play an important role in the pathogenesis of liver fibrosis. Resistin is involved in this process.

### Peer review

The paper reported the effects of the adipokine resistin on the biology of hepatic stellate cells and Kupffer cells. It is well presented.

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