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Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Resistin as an Intrahepatic Cytokine

Overexpression during Chronic Injury and Induction of Proinflammatory Actions in Hepatic Stellate Cells

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Obesity and insulin resistance accelerate the progression of fibrosis during chronic liver disease. Resistin antagonizes insulin action in rodents, but its role in humans is still controversial. The aims of this study were to investigate resistin expression in human liver and to evaluate whether resistin may affect the biology of activated human hepatic stellate cells (HSCs), key modulators of hepatic fibrogenesis. Resistin gene expression was low in normal human liver but was increased in conditions of severe fibrosis. Up-regulation of resistin during chronic liver damage was confirmed by immunohistochemistry. In a group of patients with alcoholic hepatitis, resistin expression correlated with inflammation and fibrosis, suggesting a possible action on HSCs. Exposure of cultured HSCs to recombinant resistin resulted in increased expression of the proinflammatory chemokines monocyte chemoattractant protein-1 and interleukin-8, through activation of nuclear factor (NF)-κB. Resistin induced a rapid increase in intracellular calcium concentration, mainly through calcium release from intracellular inositol triphosphate-sensitive pools. The intracellular calcium chelator BAPTA-AM blocked resistin-induced NF-κB activation and monocyte chemoattractant protein-1 expression. In conclusion, this study shows a role for resistin as an intrahepatic cytokine exerting proinflammatory actions in HSCs, via a Ca^{2+}/NF-κB-dependent pathway and suggests involvement of this adipokine in the pathophysiology of liver fibrosis. ***(Am J Pathol 2006, 169:2042–2053; DOI: 10.2353/ajpath.2006.060081)***

The adipose tissue, previously considered as a passive storage site for excess energy, is now recognized as a hormonally active system, producing numerous molecules, known as adipokines, which exert local, central, and peripheral actions.[1,2] Resistin is a 12.5-kd adipokine belonging to a new family of small cysteine-rich secretory proteins, named FIZZ (found in inflammatory zone) or resistin-like molecules.[3] In rodents, resistin is highly expressed in the adipose tissue and circulating levels are increased during diet-induced or genetic obesity.[4] Lowering plasma resistin concentrations in insulin-resistant mice decreased blood glucose levels and improved insulin sensitivity,[4,5] and treatment of normal mice with recombinant resistin impaired glucose tolerance and insulin actions.[4] On the basis of these observations in rodents, resistin has been proposed as a link between obesity and type 2 diabetes. Nevertheless, the physiological role and sites of synthesis of resistin in humans

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Table 1. Characteristics of the Patients in Whom Intrahepatic Resistin mRNA Levels Were Evaluated (Set C)

	Controls	Acute alcoholic hepatitis	Chronic HCV-related hepatitis
Number	6	23	14
Sex (M:F)	3:3	12:11	10:4
Age [median (range)]	48.5 (37 to 67)	47 (37 to 59)	55 (38 to 63)
BMI (mean ± SD)	28.1 ± 2.2	25.5 ± 4.2*	26.0 ± 2.3

*BMI was not available in one patient.

are still controversial and possibly different from those in rodents.[6,7]

Obesity and insulin resistance are part of the alterations known as the metabolic syndrome, which also includes hypertension and dyslipidemia. Nonalcoholic fatty liver disease is considered the hepatic manifestation of the metabolic syndrome, and in a subset of patients, nonalcoholic steatohepatitis may lead to progressive fibrosis and end-stage liver disease.[8] In addition, obesity and insulin resistance have been shown to accelerate the fibrogenic progression of different types of chronic liver disease, including those caused by hepatitis C virus (HCV) infection, alcohol, or iron overload.[9–11] Despite accumulating clinical evidence, the cellular and molecular mechanisms linking obesity, insulin resistance, and fibrosis are still controversial. Adipokines represent a class of molecules possibly connecting these phenomena via a direct action on the biology of hepatic stellate cells (HSCs).[12] HSCs are key cellular elements involved in liver wound healing and the development of hepatic fibrosis.[13,14] After injury, HSCs undergo activation from a quiescent state to a myofibroblast-like phenotype, which has the ability to proliferate and migrate into areas of injury and to increase the production of extracellular matrix components. In addition, they acquire the capacity to secrete chemotactic factors to recruit leukocytes to the sites of injury, as part of the inflammatory response.[15] Recent studies have demonstrated that adipokines such as leptin or adiponectin differentially modulate the liver wound-healing process and/or HSC biology.[16–20] However, no studies have yet explored the possible significance of resistin in the pathophysiology of liver fibrosis. In this study, we report for the first time that resistin is expressed in human liver tissue and that its expression is up-regulated in conditions of chronic injury. Moreover, we demonstrate that resistin activates a calcium-nuclear factor (NF)-κB signaling pathway resulting in secretion of proinflammatory cytokines by human HSCs.

Materials and Methods

Materials

Phosphorylation-specific antibodies against ERK, IκBα, and p65$^{NF\text{-}\kappa B}$ and polyclonal antibodies against ERK were purchased from Cell Signaling Technology (Beverly, MA). Human recombinant resistin was purchased from Peprotech (Rocky Hill, NJ). Monoclonal antibodies against β-actin and the intracellular Ca^{2+} chelator BAPTA-AM were purchased from Sigma (St. Louis, MO). The rabbit antiserum against human resistin used for immunohistochemistry was purchased from Phoenix Pharmaceuticals (Belmont, CA). Antibodies against α-smooth muscle actin (α-SMA) were from DAKO (Glostrup, Denmark). Antibodies against CD43 were from Santa Cruz Biotechnology (Santa Cruz, CA). The NF-κB inhibitory peptide SN50, the phospholipase C antagonist U73122, and the inositol triphosphate (IP3) receptor antagonist 2-amino-ethoxydiphenyl-borate (2-APB) were from Calbiochem (San Diego, CA). The calcium-sensitive dye FURA-2AM was purchased from Invitrogen (Carlsbad, CA). All other agents were of analytical grade.

Human Tissues

Three different sets of tissues were used. The first set (A) was used to evaluate expression of resistin in normal liver and in patients with end-stage liver disease. Normal liver tissue (four samples) was obtained during surgical liver resection for secondary liver cancer. The tissue was obtained at a minimum of 5 cm from the tumor, and normal histology was assessed by routine examination. Tissue from patients with end-stage liver disease ($n = 5$) was collected at the time of orthotopic liver transplantation. Etiology of chronic liver disease was as follows: biliary atresia ($n = 2$), chronic hepatitis C ($n = 1$), primary biliary cirrhosis ($n = 1$), and primary sclerosing cholangitis ($n = 1$).

Tissue set B was used to compare, by immunohistochemistry, the expression of resistin in normal liver and in patients with different types of chronic liver disease and consisted of paraffin-embedded liver sections obtained from patients with normal liver histology ($n = 5$), severe alcohol-induced liver disease ($n = 5$), nonalcoholic steatohepatitis ($n = 5$), and chronic hepatitis C ($n = 5$).

With the third set of tissues (set C), we prospectively studied 23 patients with severe acute alcoholic hepatitis (AAH) (Table 1). Diagnostic criteria of AAH were daily alcohol intake >80 g/day, elevated aminotransferases (>100 U/L), and compatible histological features (presence of Mallory's bodies, polymorphonuclear infiltrate, hepatocellular ballooning, and pericellular/sinusoidal fibrosis). A group of patients with chronic HCV-related hepatitis and significant fibrosis ($F \geq 2$ according to Metavir staging[21] was also analyzed (Table 1). Clinical and laboratory data were collected at admission. The protocol was approved by the Ethical Committee of the Hospital Clínic of Barcelona. This set was compared with normal liver tissue obtained from optimal cadaveric liver donors ($n = 3$) or resection for liver metastases ($n = 3$) before vascular clamp (Table 1). All controls had normal serum aminotransferases and normal liver histology.

RNA Isolation and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Isolation of total RNA from liver tissues (set A) was performed using silica membrane filters (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. This method was also used for cultured cells. Two μg of RNA from each sample were reverse-transcribed with TaqMan reverse transcriptase reagents (Applied Biosystems, Foster City, CA). The profile of the one-step, reverse transcription reaction was 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C, in a final volume of 80 μl. One hundred ng of cDNA for each sample were analyzed in duplicate by quantitative PCR, to measure resistin gene expression, using an ABI 7700 sequence detection system (Applied Biosystems) under the following conditions: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The sequence of the primers for human resistin was as follows[22]: forward, 5′-AGCCATCAATGAGAGGATCCAG-3′; reverse, 5′-CCAGGCCAATGCTGCTTA-3′; TaqMan probe, 5′-CGCCGGCTCCCTAATATTTAGGGCA-3′. FAM-labeled probe and primers for the housekeeping gene GAPDH were purchased as Assays-On-Demand (Applied Biosystems) and used as specified by the manufacturer. Results are expressed as $2^{-}\Delta Ct$ (ΔCt = Ct of the target gene minus Ct of the housekeeping gene).

In experiments evaluating resistin expression in AAH, HCV-related hepatitis and control tissues (set C), RNA was obtained from liver specimens using Trizol (Life Technologies Inc., Rockville, MD). RNA integrity and concentration was assessed with a microfluidity glass chip platform (Bioanalyzer 2100; Agilent, Palo Alto, CA). Resistin mRNA levels were measured by quantitative PCR (Applied Biosystems), and data normalized using 18S as housekeeping gene.

Immunohistochemical Studies

Paraffin-embedded liver sections (tissue sets B and C) were incubated with antibodies against resistin (1/2000 dilution), α-SMA (1/1000), or CD43 (1/1000) for 60 minutes. Details of the procedures used have been previously published.[23] As negative controls, all specimens were incubated with an isotype-matched control antibody under identical conditions. Sirius Red staining to identify areas of collagen deposition was performed as described previously.[23] Morphometric assessment was performed using an optic microscope (Eclipse E600; Nikon, Tokyo, Japan) connected to a high-resolution camera (CC12 Soft-Imaging System, Münster, Germany).

Immunofluorescence

A representative liver sample with alcohol-induced liver disease was used to perform double immunostaining with resistin and CD43 antibodies. Liver sections were analyzed using a Leica TCS SL laser-scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany). Primary antibodies were described for immunohistochemistry. Secondary antibodies were anti-rabbit Alexa 647 (Molecular Probes, Leiden, The Netherlands) (represented in green, resistin), and anti-mouse Cy3 (red, CD43; Jackson ImmunoResearch, Cambridgeshire, UK).

Cell Culture

Human HSCs were isolated from wedge sections of normal human liver unsuitable for transplantation by combined digestion with collagenase and pronase, followed by centrifugation on Stractan gradients. Procedures for cell isolation and characterization have been extensively described elsewhere.[24] Cells were cultured on uncoated plastic dishes and used for all of the experiments after complete transition toward an activated myofibroblast-like phenotype.

RNase Protection Assay

Ten μg of total RNA from cultured HSCs were analyzed by RNase protection assay using a commercially available kit from BD Pharmingen (Palo Alto, CA). ^{32}P-labeled complementary RNA was transcribed from a MultiProbe template (BD Pharmingen) according to the manufacturer's instructions. After hybridization, protected fragments were separated on a sequencing gel and autoradiographed, as previously described.[25]

Chemotactic Assay

Monocyte migration was measured in a modified Boyden chamber essentially as previously described.[26] In brief, human blood mononuclear cells were prepared from peripheral blood collected in ACD anticoagulant from healthy volunteers, using a metrizoate/polysaccharide solution (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). The mononuclear cells were washed and resuspended at a concentration of 3×10^6 cells/ml. The assay was performed using polyvinylpyrrolidone-free polycarbonate 5-μm filters and modified Boyden chambers. HSC-conditioned medium was diluted 1:2 to 1:8. After staining with Giemsa, cells migrating to the underside of the filters were quantified as the mean number of cells in 10 high-power fields. In each experiment, the number of cells migrating in the presence of nonconditioned medium was subtracted.

Western Blot Analysis

Confluent, serum-starved HSCs were treated with the appropriate conditions, quickly placed on ice, and washed with ice-cold phosphate-buffered saline. The monolayer was lysed in RIPA buffer [20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 1 mmol/L Na_3VO_4, 1 mmol/L phenyl methyl sulfonyl fluoride, and 0.05% (w/v)

aprotinin] and transferred in microcentrifuge tubes. Insoluble proteins were discarded by a 10-minute centrifugation at 12,000 rpm at 4°C. Protein concentration was measured in triplicate using a commercially available assay. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting as previously described.[27]

Measurement of Chemokine Secretion

Confluent HSCs in 24-well plates were deprived of serum for 24 hours. After replacement with 500 μl of fresh serum-free medium, the cells were treated with resistin for different time points. In some experiments, addition of resistin was performed in the presence or absence of preincubation with 18 μmol/L SN50 or 10 μmol/L BAPTA-AM. At the end of the incubation, conditioned medium was collected and stored at −20°C until assayed. Monocyte chemoattractant protein-1 (MCP-1) concentration in the medium was measured by enzyme-linked immunosorbent assay (ELISA), using a commercially available antibody kit (Biosource, Camarillo, CA). Interleukin (IL)-8 was assayed using a commercially available sandwich ELISA (BLK Diagnostics, Barcelona, Spain).

Intracellular Calcium Concentration

Digital video imaging of intracellular-free calcium concentration ($[Ca^{2+}]_i$) in individual human HSCs was performed as described previously.[28] Human HSCs were grown to subconfluence in complete culture medium on round glass coverslips (25-mm diameter, 0.2-mm thick) and then incubated for 24 hours in serum-free, insulin-free medium. Cells were loaded with 4 μmol/L Fura-2AM and 15% Pluronic F-127 for 30 minutes at 22°C.

$[Ca^{2+}]_i$ was measured in Fura-2-loaded cells in HEPES-$NaHCO_3$ buffer containing 140 mmol/L NaCl, 3 mmol/L KCl, 0.5 mmol/L NaH_2PO_4, 12 mmol/L $NaHCO_3$, 1.2 mmol/L $MgCl_2$, 1.0 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Ratio images (340/380 nm) were collected every 3 seconds, and calibration curves were obtained for each cell preparation. Recombinant resistin was added directly to the perfusion chamber immediately after recording the $[Ca^{2+}]_i$ basal value and maintained throughout the duration of the experiment.

Adenoviral Infection and NF-κB-Responsive Luciferase Assay

Human HSCs were infected with a recombinant adenoviral vector expressing a luciferase reporter gene driven by NF-κB transcriptional activation (Ad5NF-κBLuc), as previously described.[29] Cells were infected with Ad5NF-κBLuc or control adenovirus (Ad5GFP, MOI 500) for 12 hours in medium containing 0.5% fetal calf serum. After infection, the medium was changed to fresh medium with 0.5% fetal calf serum, and the culture was continued for an additional 8 hours before performing the individual experiments. NF-κB-mediated transcriptional induction was assessed by the luciferase assay system (BD Pharmingen, San Diego, CA). Luciferase activity (relative light units) was normalized by protein concentration. In other experiments, HSCs were infected with a dominant-negative mutant form (S32A/S36A) of IκB (IκB super-repressor) or with control adenovirus using the same protocol outlined above.[30]

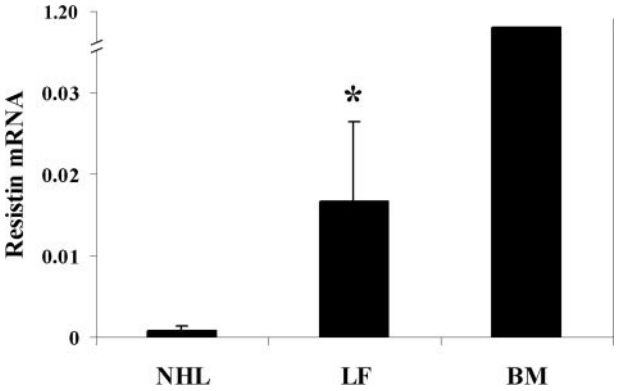

Figure 1. Resistin gene expression in human liver tissue. Expression of resistin mRNA was analyzed by real-time PCR, as described in Materials and Methods, in normal human liver tissues (NHL, $n = 4$) and in liver tissue of patients with end-stage fibrosis (LF, $n = 5$). mRNA isolated from bone marrow (BM) was used as a positive control for resistin expression. Resistin mRNA (mean + SD) is expressed as $2^{-\Delta Ct}$ (ΔCt = Ct of the target gene minus Ct of the housekeeping gene). *$P < 0.05$ versus NHL.

Results

Intrahepatic Expression of Resistin

Several adipokines have been found to be expressed in the liver. We first investigated whether gene expression of resistin is detectable in human liver tissue. Quantitative RT-PCR analysis demonstrated that resistin mRNA was barely detectable in normal liver (Figure 1). However, when tissue obtained from patients with end-stage liver disease was analyzed, a marked up-regulation of resistin expression was observed, indicating increased intrahepatic expression of this adipokine in conditions of chronic damage and repair (Figure 1).

We next analyzed resistin expression in the liver at the protein level, using immunohistochemistry. In normal human liver, low levels of specific signal for resistin were observed in the portal tract and in surrounding hepatocytes (Figure 2A). In contrast, in patients with HCV-induced chronic hepatitis, resistin expression was increased, particularly in areas of inflammation and ongoing fibrogenesis (Figure 2B). Likewise, in patients with alcoholic liver disease, intense staining for resistin was present in the areas of active fibrogenesis, and specific signal decorated the typical chicken-wire pattern observed in this setting (Figure 2C). In nonalcoholic steatohepatitis, the increase in resistin expression was less evident. However, the pattern of staining indicated a clear redistribution to areas of inflammatory infiltration (Figure 2D).

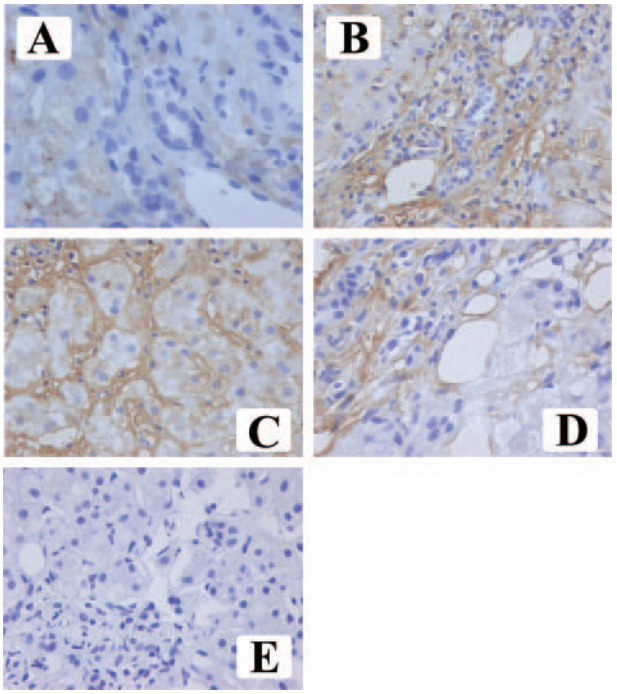

Figure 2. Immunohistochemistry for resistin in human liver tissue. Representative photomicrographs of resistin immunostaining (brown color) in specimens from patients with normal liver (**A**), chronic hepatitis C (**B**), alcohol-induced liver disease (**C**), and nonalcoholic steatohepatitis (**D**). **E:** A negative control slide (alcohol-induced liver disease). Original magnifications, ×400.

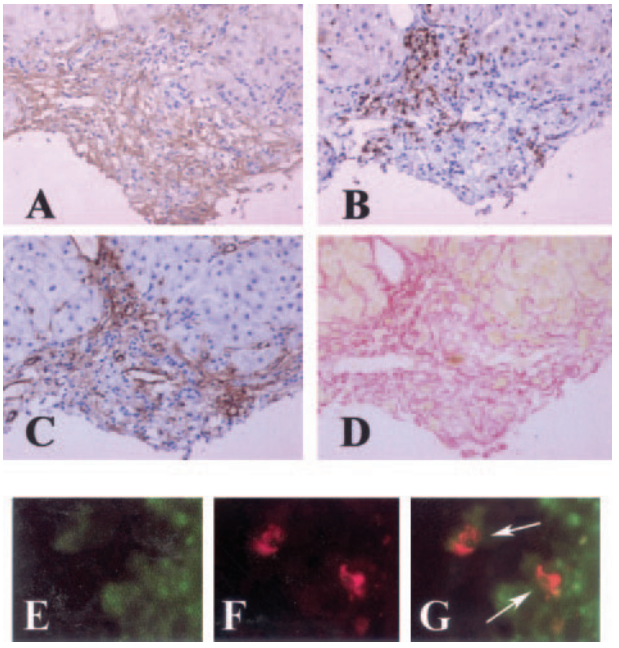

Figure 3. Comparison of resistin expression with that of specific cellular markers or collagen deposition. **A–D:** Serial sections of liver tissue from a patient with severe alcohol-induced liver disease were analyzed by immunohistochemistry for resistin (**A**), CD43 (inflammatory cells, **B**), α-SMA (fibrogenic myofibroblasts, **C**), or stained with Sirius Red (collagen deposition, **D**). **E–G:** Liver tissue from a patient with severe alcohol-induced liver disease was analyzed by immunofluorescence for resistin (**E**, green) or CD43 (**F**, red). **G:** Merged images, with double-positive cells indicated by **arrows**. Original magnifications: ×200 (**A–D**); ×800 (**E–G**).

Intrahepatic Resistin Is Up-Regulated in Patients with Severe AAH

To better investigate the role of resistin in conditions of hepatic inflammation and fibrogenesis, we assessed resistin expression at the mRNA and protein levels in a series of patients with AAH (n = 23). This condition is characterized by hepatic inflammation, severe hepatocellular damage, and rapidly progressive fibrosis. First, resistin expression at the protein level was examined using serial sections. Expression of resistin partially co-localized with CD43-positive inflammatory cells and with α-SMA-positive fibrogenic myofibroblasts (Figure 3, A–C). A clear co-localization was also observed comparing slides stained for resistin with those stained with Sirius Red, as an index of collagen deposition (Figure 3D). To further evaluate the cell types contributing to resistin expression in this setting, we performed double-immunofluorescence experiments in slides from patients with AAH, confirming the contribution of CD43-positive cells to resistin expression (Figure 3, E–G). In these experiments, hepatocytes also showed a slight, specific signal for resistin. We also measured hepatic resistin mRNA expression, which was found to be markedly and significantly increased in patients with AAH compared with controls (Figure 4A). In contrast, in a group of patients with chronic HCV-related hepatitis, intrahepatic levels of resistin mRNA were not significantly different from those measured in controls. In patients with AAH, resistin mRNA expression directly correlated with the area stained by CD43 (Figure 4B) as a marker of hepatic inflammation. In addition, resistin mRNA directly correlated with Sirius Red staining (r = 0.505, P = 0.023), or with area stained by antibodies directed against α-smooth muscle actin (r = 0.553, P = 0.009). On the other hand, no significant correlations were found between intrahepatic resistin levels and BMI, even when the two sexes were analyzed separately, or between BMI and the area occupied by inflammatory cells. A weak, negative correlation was found between age of the patients and intrahepatic resistin expression (not shown). Overall, these results suggest that resistin is overexpressed in chronically inflamed livers, in areas of inflammation and repair. In light of these findings, we analyzed the possible contribution of HSCs, the major cell type responsible for fibrogenesis, to resistin expression. However, culture-activated human HSCs did not show resistin transcripts when analyzed by real-time RT-PCR. In addition, resistin was always undetectable in HSC-conditioned media using a specific ELISA assay. Likewise, no immunoreactive resistin could be detected in the cell-conditioned medium of cultured Huh7, human cells of hepatocytic lineage (data not shown).

Resistin Modulates Proinflammatory Actions in HSCs

The observation that resistin accumulates in areas of fibrogenesis prompted us to investigate the possible modulation of HSC biology by this adipokine. Secretion of chemotactic factors for leukocytes is a well-established

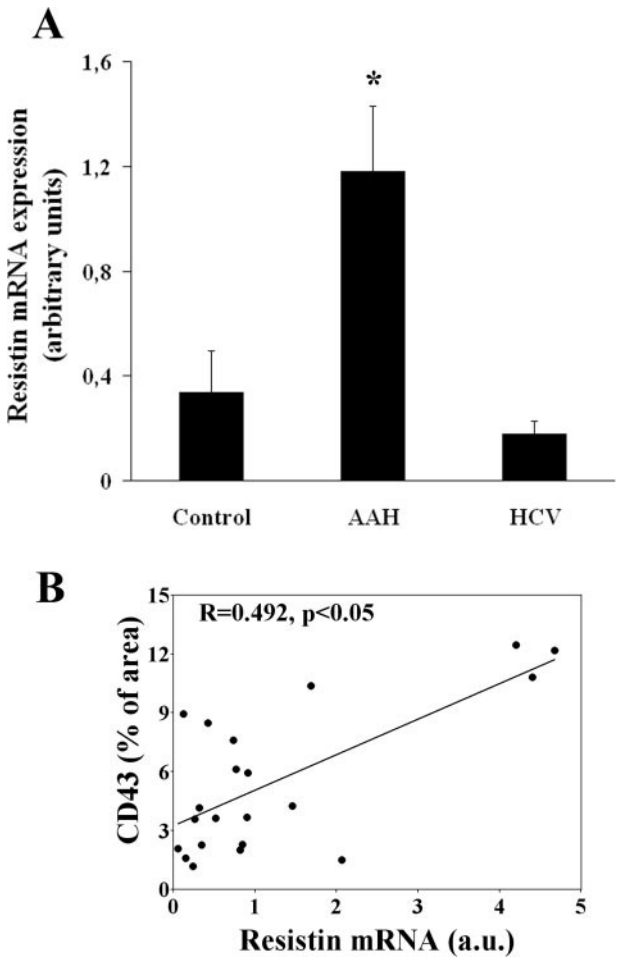

Figure 4. Up-regulated resistin expression in patients with AAH. **A:** Intrahepatic gene expression of resistin (mean + SD), as assessed by quantitative PCR, in patients with AAH ($n = 23$) or with chronic HCV-related hepatitis (HCV, $n = 14$), and controls ($n = 6$). 18S rRNA was used as the housekeeping gene. Resistin mRNA values are given as arbitrary units. $^*P < 0.001$ versus control livers. **B:** Correlation between intrahepatic resistin mRNA expression and CD43-positive area in the group of patients with AAH ($n = 23$). In two patients, data for correlation were not available.

feature of activated HSCs.[31] We evaluated the effects of recombinant resistin on the secretion of MCP-1, a chemokine that modulates the recruitment of monocytes and T lymphocytes.[15] Exposure of serum-deprived HSCs to resistin for 24 to 48 hours induced a significant increase in MCP-1 secretion in the culture medium (Figure 5A). Because stimulation of MCP-1 secretion may be associated with increased gene expression, we next evaluated the steady-state levels of mRNA encoding for MCP-1. Incubation with resistin markedly increased MCP-1 transcript levels, with a stronger effect after 4 hours of stimulation (Figure 5B). To confirm the action of resistin on monocyte chemoattraction, HSC-conditioned media were used in a migration assay using purified human monocytes. Medium obtained from cells exposed to resistin induced a significantly greater monocyte chemotaxis in comparison to medium from unstimulated cells (Figure 5C). Secretion of IL-8, a chemokine with potent chemoattractant activity for neutrophils, was also up-regulated by resistin (data not shown), confirming the ability

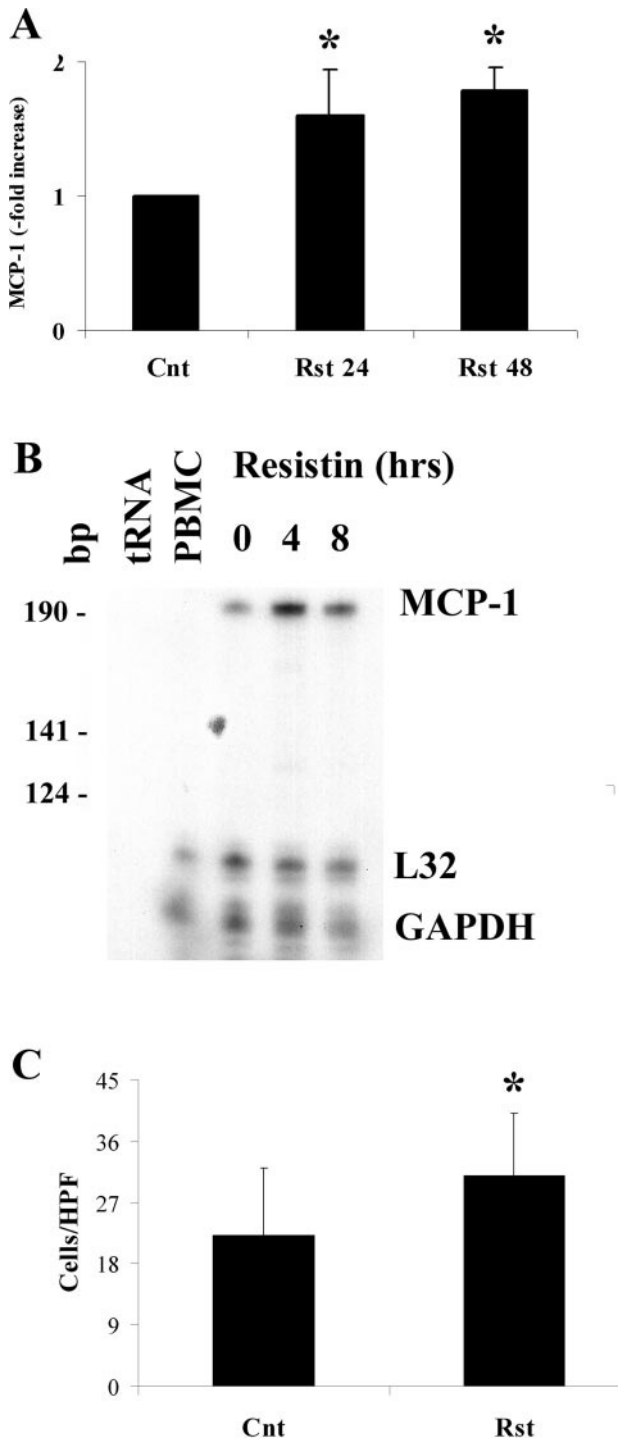

Figure 5. Resistin stimulates MCP-1 expression in HSCs. **A:** Serum-starved HSCs were exposed for 24 or 48 hours to 500 ng/ml resistin, as indicated. At the end of incubation, conditioned medium was collected, and MCP-1 concentrations were measured by ELISA. $^*P \leq 0.05$ versus control. Data are expressed as mean ± SE of three independent experiments. **B:** Serum-starved HSCs were stimulated with 250 ng/ml resistin for the indicated time points. Total RNA was analyzed for MCP-1 gene expression by RNase protection assay, as described in Materials and Methods. Transfer RNA (tRNA) and peripheral blood mononuclear cell RNA (PBMC) were used as negative and positive controls for hybridization, respectively. **C:** HSCs were incubated in the presence or absence of 500 ng/ml resistin for 48 hours. Aliquots of cell supernatant were analyzed in chemotactic assays using human peripheral blood mononuclear cells, as described in Materials and Methods. The number of cells migrating to the underside of the filters was quantified in 10 high-power fields (HPFs). Results are the mean ± SE of four different experiments.

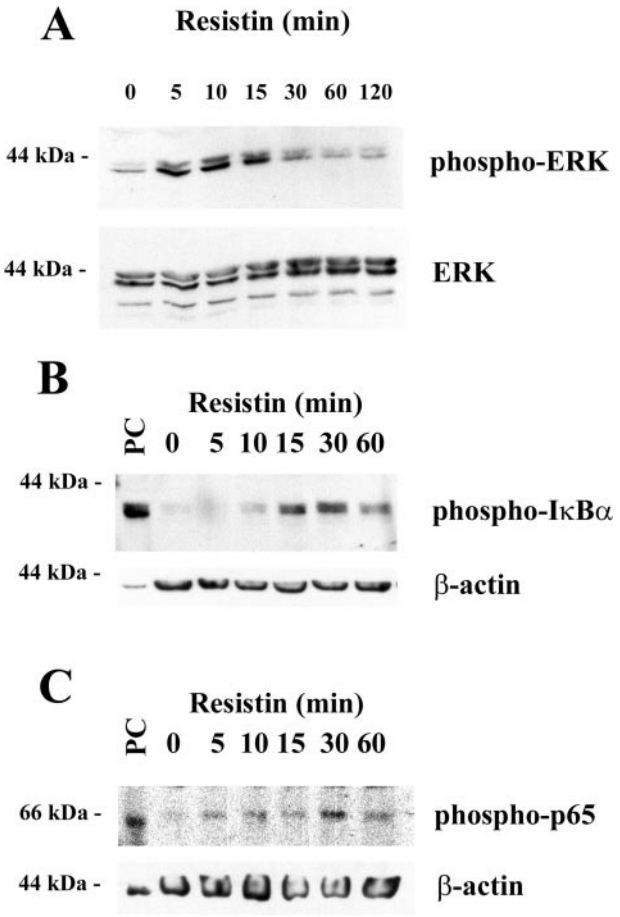

Figure 6. Resistin activates different intracellular signaling pathways in HSCs. **A:** Serum-deprived HSCs were exposed to 500 ng/ml resistin for different time points, as indicated. Ten μg of total cell lysate were separated by SDS-PAGE and sequentially immunoblotted with antibodies specifically recognizing the phosphorylated form of ERK (top) or total ERK (bottom). **B:** Serum-deprived HSCs were incubated with 500 ng/ml resistin at the indicated time points. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting using antibodies specifically recognizing phosphorylated IκBα (top) or β-actin (bottom). A lysate from HepG2 cells exposed to 200 ng/ml TNF-α for 15 minutes was used as positive control (PC). **C:** The experiment was conducted exactly as described in **B**, but the membranes were blotted with antibodies against phosphorylated $p65^{NF\text{-}\kappa B}$ (top) or β-actin (bottom). Migration of the molecular weight markers is shown on the left.

of this adipokine to induce proinflammatory actions in HSCs. We also evaluated the possible modulation of other features of activated HSCs. However, resistin did not increase HSC proliferation, as assessed by incorporation of tritiated thymidine, nor did it modify gene expression of type I procollagen or transforming growth factor-β1 (data not shown).

Resistin Activates Intracellular Signaling in HSCs

The observation that resistin is able to exert biological actions on HSCs suggests that this adipokine may activate intracellular signaling pathways in HSCs. We first explored the ability of resistin to activate the ERK pathway, which is responsive to several soluble factors and regulates different biological functions in HSCs.[32,33] Exposure of serum-deprived HSCs to resistin for different periods of time resulted in increased phosphorylation of

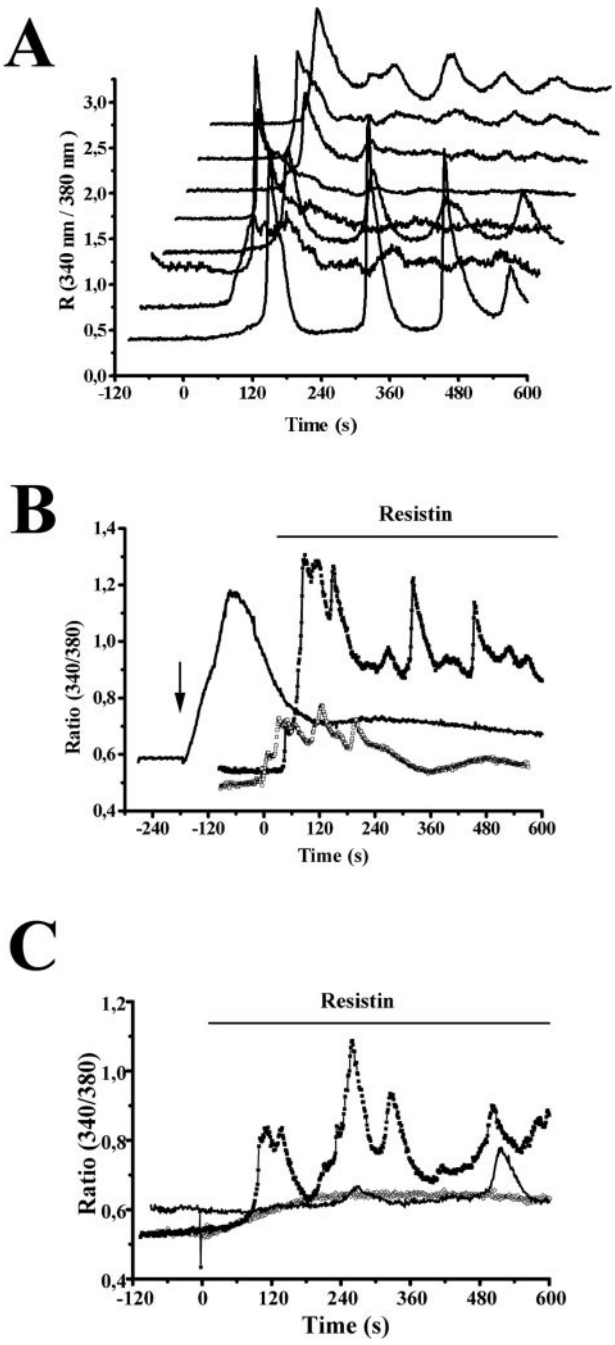

Figure 7. Effect of resistin on intracellular Ca^{2+} concentration. **A:** Subconfluent serum-starved HSCs were loaded with the fluorescent dye Fura-2AM. Intracellular Ca^{2+} concentration was measured by image analysis, as described in Materials and Methods. Resistin (100 ng/ml) was added at time 0. Each trace is the response measured in an individual cell at 3-second time intervals. **B:** The effects of resistin (100 ng/ml, added at time 0) were analyzed in control conditions (black squares), after depletion of intracellular calcium stores with 30 nmol/L thapsigargin (added at the **arrow**, black trace), or in the absence of extracellular calcium (open squares). Traces are the mean of at least seven cells. **C:** The effects of resistin (100 ng/ml, added at time 0) were analyzed in control conditions (black squares), after a 10-minute preincubation with 4 μmol/L U73122 (open diamonds) or after preincubation with 50 μmol/L 2-APB (black trace). Traces are the mean of at least seven cells.

both ERK isoforms on activation-specific residues (Figure 6A). These data confirm the ability of resistin to modulate the biology of human HSCs and demonstrate that HSCs possess functional resistin receptors.

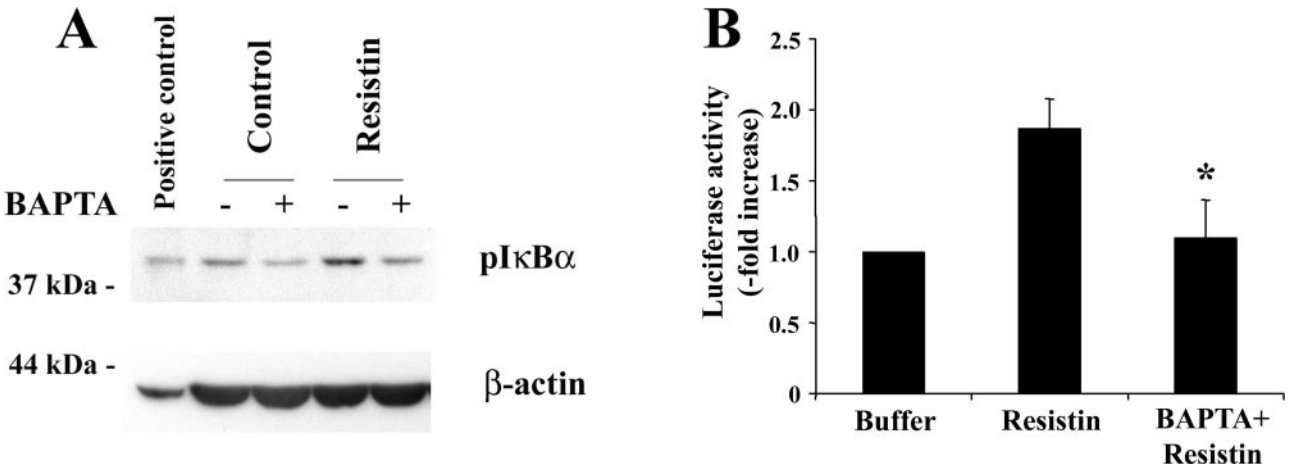

Figure 8. NF-κB activation by resistin requires intracellular calcium. **A:** Serum-deprived HSCs were preincubated with 10 μmol/L BAPTA-AM or vehicle for 30 minutes, before the addition of 250 ng/ml resistin for 15 minutes, as indicated. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting using antibodies specifically recognizing the phosphorylated form of IκBα. Total protein lysate from HepG2 cells exposed to 200 ng/ml TNF-α for 15 minutes was used as positive control. The membrane was stripped and reblotted with β-actin antibody to ensure equal loading. Migration of the molecular weight marker is shown on the left. **B:** Serum-starved HSCs were preincubated with BAPTA-AM for 10 minutes and then exposed to 250 ng/ml resistin. NF-κB-dependent gene expression was assessed by luciferase reporter gene assay as described in Materials and Methods. Results are shown as fold increase with respect to untreated cells. $^*P < 0.05$ versus resistin alone.

Because NF-κB is a critical regulatory mechanism of chemokine expression in many different cells, including HSCs,[31] we hypothesized the involvement of this transcription factor in resistin-induced up-regulation of MCP-1 and IL-8. Phosphorylation at Ser32 and Ser36 residues of the inhibitory protein IκBα is followed by proteasome-mediated degradation, allowing active NF-κB to translocate to the nucleus. Exposure of HSCs to resistin markedly increased IκBα phosphorylation (Figure 6B). To confirm that resistin activates the NF-κB pathway, we also analyzed the ability of this cytokine to induce phosphorylation of the p65 subunit of NF-κB. NF-κB phosphorylation was increased with a time course closely similar to the one observed for IκBα (Figure 6C). These data identify NF-κB as an additional pathway regulated by resistin in HSCs.

Increase in Intracellular Calcium Concentration by Resistin

No receptor for resistin has yet been identified. To confirm the presence of functional resistin receptors in HSCs, we investigated the effects of recombinant human resistin on $[Ca^{2+}]_i$ in HSCs. Shortly after the addition of recombinant resistin, a synchronous calcium peak was observed, followed by a long-lasting plateau (Figure 7A). This increase in $[Ca^{2+}]_i$ was consistently observed at concentrations of resistin ranging between 50 and 500 ng/ml (data not shown). To explore further the mechanisms of calcium increase, we manipulated extracellular and intracellular calcium. In the absence of extracellular calcium, the effect of resistin on $[Ca^{2+}]_i$ was maintained, although the magnitude of the early peak was reduced, and the late plateau phase was abolished (Figure 7B). To evaluate the contribution of internal Ca^{2+} pools to the $[Ca^{2+}]_i$ increase induced by resistin, we used the sarcoendoplasmic Ca^{2+} ATPase inhibitor thapsigargin. Preincubation of cells with 30 nmol/L thapsigargin completely prevented the action of resistin on cytosolic calcium (Figure 7B).

Release of calcium from intracellular stores is dependent on sequential activation of phospholipase C isoforms and the resulting increase in IP3 levels. To establish whether these mechanisms are operating in resistin-stimulated cells, HSCs were preincubated with U73122 or with 2-APB, specific inhibitors of phospholipase C or of the IP3 receptor, respectively (Figure 7C). Both compounds prevented the rise in $[Ca^{2+}]_i$ triggered by resistin. Taken together, these results show that resistin increases cytosolic calcium concentration in HSCs, mainly through phospholipase C activation and release of calcium from intracellular IP-3-sensitive pools, followed by calcium influx from the extracellular milieu.

Calcium Influx Is Necessary for NF-κB Activation and Chemokine Secretion

To explore the existence of a possible connection between the increase in $[Ca^{2+}]_i$ elicited by resistin and NF-κB activation, we first analyzed resistin-induced IκBα phosphorylation in the presence or absence of BAPTA-AM, an intracellular calcium chelator. The increase in IκBα phosphorylation induced by resistin was prevented by preincubation of cells with BAPTA-AM (Figure 8A). To provide a more direct link between resistin, calcium influx, and NF-κB activation, we analyzed luciferase activity after infection with an adenoviral vector encoding a reporter construct under the control of NF-κB (Figure 8B). Incubation with resistin increased NF-κB-dependent transcription by twofold greater than control values, thus confirming the activation of this pathway shown using phosphorylation-specific antibodies. Moreover, the increase in luciferase activity induced by resistin was in-

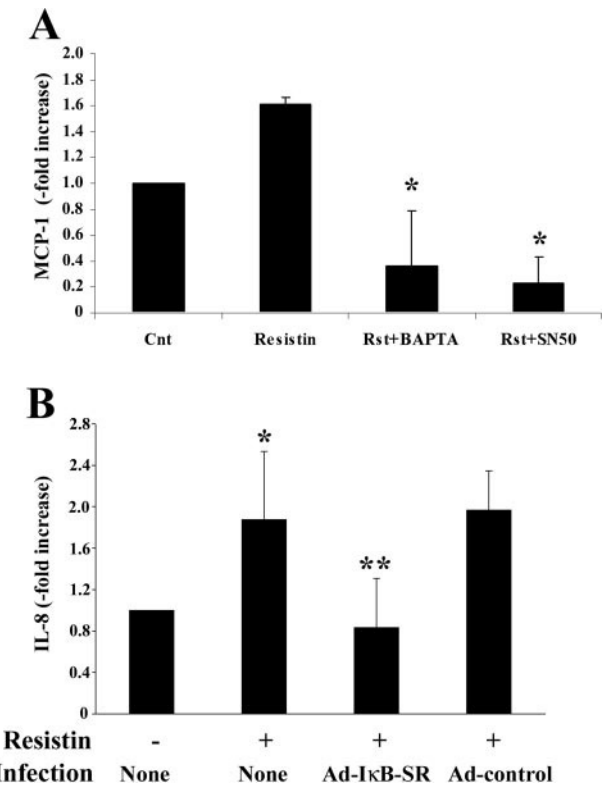

Figure 9. Resistin-induced chemokine secretion is NF-κB- and calcium-dependent. **A:** Serum-starved HSCs were preincubated either with the NF-κB inhibitory peptide SN50 (18 μmol/L) for 15 minutes or with BAPTA-AM (10 μmol/L) for 30 minutes, before exposure for 24 hours to 500 ng/ml resistin. At the end of incubation, MCP-1 concentration in the conditioned medium was measured by ELISA. Results are shown as fold increase with respect to untreated cells. $^{*}P < 0.05$ versus resistin alone. **B:** HSCs were infected with an IκB-superrepressor adenovirus (Ad-IκB-SR) or control adenovirus (Ad-control), as described in Materials and Methods. After infection, serum-deprived HSCs were incubated with 250 ng/ml resistin for 24 hours, as indicated, and IL-8 concentration in the conditioned medium was measured by ELISA. Results are shown as fold increase with respect to untreated cells. $^{*}P < 0.05$ versus buffer; $^{**}P < 0.05$ versus Ad control + resistin.

hibited by 90% in the presence of BAPTA-AM, compared with cells stimulated with resistin alone (Figure 8B).

Given the effect of intracellular calcium depletion on NF-κB activation, we next analyzed MCP-1 secretion in the presence of BAPTA-AM. Chelation of intracellular calcium resulted in a marked inhibition of MCP-1 secretion in the cell-conditioned medium in response to resistin (Figure 9A). In addition, preincubation of HSCs with SN50, a peptide that specifically inhibits NF-κB activation, completely blocked MCP-1 expression induced by resistin, confirming that NF-κB activation is necessary for resistin-induced secretion of this chemokine (Figure 9A).

To define better the relationships between resistin, NF-κB, and the proinflammatory action of HSCs, we analyzed the effect of this adipokine on the expression of IL-8, another chemokine secreted by these cells.[31] Analogously to MCP-1 expression, exposure of HSCs to recombinant resistin for 24 hours increased IL-8 secretion in the culture medium, as assessed by a specific ELISA assay (Figure 9B). Moreover, infection of cells with an adenoviral vector expressing an IκB superrepressor inhibited resistin-induced IL-8 secretion, confirming the critical role played by NF-κB (Figure 9B). The increase in IL-8 secretion stimulated by resistin was also blocked by preincubation with BAPTA-AM (data not shown). Taken together, these data indicate that resistin acts as a proinflammatory molecule in HSCs, stimulating the expression of MCP-1 and IL-8, and that this action is mediated by calcium transients and activation of NF-κB.

Discussion

Accumulating evidence indicates that obesity and insulin resistance are associated with a faster fibrogenic progression of several chronic liver diseases, including alcoholic liver disease, nonalcoholic steatohepatitis, and HCV infection.[9–11,34] Although several mechanisms are likely to contribute to this association, increasing attention has been directed to the role played by soluble mediators predominantly expressed by adipose tissue, collectively known as adipokines.[1,2] The present study identifies two novel aspects of the biology of the adipokine resistin that are relevant to the pathophysiology of chronic liver injury. First, we found that, in humans, expression of resistin is detectable in liver tissue, and is up-regulated by chronic damage. Second, resistin is able to modulate HSC biology, indicating that this cytokine participates in the chronic wound-healing process in human liver.

Intrahepatic staining for resistin was found to be increased in different conditions of chronic injury, including hepatitis C, alcoholic liver disease, and nonalcoholic steatohepatitis. However, intrahepatic mRNA levels of resistin were significantly higher than in controls only in patients with severe AAH, but not in patients with chronic HCV-related hepatitis or nonalcoholic steatohepatitis (data not shown). This observation may be related to the more severe degree of inflammation and fibrogenesis present in the group of patients with alcoholic liver disease. An alternative, and not mutually exclusive, possibility is that circulating resistin is deposited to a higher extent than in control patients because of the presence of an altered matrix environment, as occurs also in patients with viral hepatitis and ongoing fibrogenesis. Several adipokines, whose expression is prominent in adipose tissue, have been shown to be expressed within the liver. Leptin is expressed by activated HSCs, and its role in fibrogenesis and wound healing has been extensively investigated in the last 3 years.[16,17,20] More recently, adiponectin expression has been found in liver tissue, where it is decreased in patients with nonalcoholic steatohepatitis.[35] Therefore, resistin represents an additional adipokine whose expression is detected in the liver. These results imply that resistin may not only become available to liver resident cells, such as HSCs, through the bloodstream, but it may possibly exert biological actions through local production, by paracrine mechanisms. These observations expand the spectrum of conditions in which resistin is potentially implicated. In obese patients, overproduction of resistin attributable to accumulation of visceral fat may result in increased availability through the portal circulation. This possibility is supported by data showing that higher levels of resistin expression have been found in omental and abdominal

subcutaneous fat than in thigh or breast fat.[36] However, resistin availability may be increased even in lean patients with chronic damage, through augmented intrahepatic expression, and/or local accumulation.

We also characterized, at least partially, the cell types responsible for intrahepatic resistin expression in the group of patients with AAH. Because of the very low levels of resistin transcripts, we could not perform *in situ* hybridization coupled with immunohistology for cell-specific markers. Serial sections indicate that resistin is expressed in areas of inflammatory cell accumulation. Moreover, some cells show double staining for resistin and the inflammatory cell marker CD43. Although hepatocytes showed a slight signal for resistin by immunofluorescence, cultured Huh7 did not express immunoreactive resistin by ELISA. In addition, cultured human HSCs failed to show resistin expression both at the mRNA and protein levels. In contrast, high levels of resistin mRNA have been detected in the bone marrow[7] and in circulating macrophages.[6] Thus, it is likely that inflammatory cells represent a major site of intrahepatic resistin production. Nonetheless, future studies are needed to establish whether other cell types, including Kupffer cells, contribute to resistin expression, also considering that different cells may account for intrahepatic resistin expression in different pathological conditions. Of note, marked resistin signals were present in fibrotic areas. This observation, together with the fact that in patients with HCV-related chronic hepatitis, increased resistin immunostaining was not paralleled by higher intrahepatic resistin mRNA, suggesting that resistin may be trapped by extracellular matrix material, which would provide a storage site for this cytokine. This hypothesis is also consistent with data indicating that several cytokines interact with extracellular matrix components accumulated during the fibrotic process.[37,38] Finally, it is also important to consider that the pattern of resistin expression in humans is markedly different from the one observed in rodents where resistin is present almost exclusively in the adipose tissue.[4,39]

The other relevant result of the present study is the observation that resistin stimulates HSCs to secrete the proinflammatory chemokines MCP-1 and IL-8, critical mediators of intrahepatic leukocyte recruitment,[31] thus providing a direct link between resistin and the mechanisms of liver inflammation. A connection between resistin and inflammation is in agreement with recent reports showing that resistin up-regulates inflammation-related molecules in endothelial cells[40,41] and causes arthritis when injected in the joints of healthy mice.[42] Moreover, in human mononuclear phagocytes, resistin expression is strongly increased by proinflammatory cytokines or lipopolysaccharide,[43,44] and serum levels directly correlate with ongoing inflammation.[44,45] Resistin is a member of the FIZZ (found in inflammatory zone) family, a set of proteins showing increased expression in areas of active inflammation.[46] Recently, FIZZ1, also known as resistin-like molecule-α, was found to be overexpressed in experimental lung inflammation and to modulate the biology of pulmonary fibroblasts.[47] Data from the present study identify a role for resistin (FIZZ3) in the pathogenesis of liver inflammation and repair through its action on activated, myofibroblast-like HSCs. Further support to the connection between resistin and liver inflammation and fibrosis is provided by data obtained in patients with AAH. In this condition characterized by intense inflammation and active fibrogenesis, not only was intrahepatic resistin confirmed to be higher than in control patients, but it was also directly correlated with histological parameters of fibrogenesis and inflammatory infiltration. These data are well fitting with those recently reported by Pagano and colleagues,[48] who demonstrated that serum concentration of resistin and adipose tissue mRNA levels are higher in patients with nonalcoholic fatty liver disease than in control lean and obese patients. More important, resistin concentrations directly correlated with the nonalcoholic steatohepatitis score, which comprises necroinflammation and fibrosis. Taken together, these findings identify resistin as a novel modulator of intrahepatic inflammation in conditions of chronic damage. However, further experiments in animal models are clearly needed to elucidate the quantitative contribution of resistin to the pathogenesis of hepatic inflammation and fibrosis in an *in vivo* situation.

Despite the numerous recent studies concerning resistin pathophysiology, no specific receptor for this cytokine has yet been identified, and little is known about the intracellular signaling pathways involved. This study provides novel information about the events that follow the interaction of resistin with responsive cells. In particular, we found, for the first time, that a marked rise in intracellular calcium concentration is detectable shortly after exposure of HSCs to recombinant resistin. Moreover, pharmacological modulation of calcium transients indicated that resistin activates a pathway involving phospholipase C, IP3, and mobilization of calcium from intracellular stores. These lines of information are of general interest in resistin's biology and may be helpful in the process of identification of resistin receptor(s). Calcium flux was accompanied by activation of other pathways relevant for the biology of HSCs. Specifically, resistin caused an increase in NF-κB activation, as indicated by phosphorylation of the inhibitory protein IκBα and by the ability to drive NF-κB-dependent expression of a reporter gene. Interestingly, NF-κB activation was found to be dependent on the increase in cytosolic calcium concentration induced by resistin because an intracellular calcium chelator blocked both NF-κB activation and chemokine secretion. The observation that NF-κB is downstream of calcium elevation is an additional novel finding related to resistin's signaling and is in agreement with recent studies showing that intracellular calcium increase is an upstream event for NF-κB activation in response to different agonists, such as histamine or lysophosphatidic acid, in various cell types.[49,50] Activation of NF-κB has been shown to be critical for expression of proinflammatory genes, such as cell adhesion molecules and chemokines. This concept is confirmed by data reported herein, indicating that the action of resistin on MCP-1 or IL-8 expression was prevented by interference with the NF-κB pathway. Of note, increased NF-κB activation has been documented in human liver tissue from

patients with nonalcoholic steatohepatitis, HCV infection, or alcohol-induced liver disease.[51,52] In rodents with diet-induced or genetic obesity, increased intrahepatic activation of NF-κB is associated with up-regulation of proinflammatory cytokines, and inhibition of NF-κB ameliorates insulin resistance.[53,54] Accordingly, transgenic mice with low-level, liver-specific activation of NF-κB have recently been shown to develop insulin resistance both in the liver and systemically, even in the absence of obesity or steatosis.[54] Based on these observations, it may be speculated that resistin, via NF-κB, participates in the generation of a low-grade inflammatory state within the liver, possibly contributing to the development of insulin resistance associated with obesity and/or chronic HCV infection. Because in the present study data for circulating resistin levels and parameters of insulin resistance were not available, studies designed to investigate a possible relationship between intrahepatic and/or circulating resistin and insulin resistance are warranted. Along these lines, the possible modulation by resistin of other molecules involved in the generation of insulin resistance should also be investigated. Preliminary experiments demonstrate that in HSCs, resistin does not alter the expression of suppressor of cytokine signaling-1 (data not shown), which has been demonstrated to contribute to insulin resistance.[55]

In summary, this study demonstrates that resistin is expressed in human liver and provides evidence for a proinflammatory action of this adipokine on human HSCs. These data indicate, for the first time, resistin as a possible link between the conditions of obesity and insulin resistance and the development of liver inflammation and fibrosis.

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