

Ghrelin Attenuates Hepatocellular Injury and Liver Fibrogenesis in Rodents and Influences Fibrosis Progression in Humans

Montserrat Moreno,¹ Javier F. Chaves,² Pau Sancho-Bru,¹ Fernando Ramalho,³ Leandra N. Ramalho,¹ Maria L. Mansego,² Carmen Ivorra,² Marlene Dominguez,¹ Laura Conde,⁴ Cristina Millán,¹ Montserrat Marí,¹ Jordi Colmenero,¹ Juan J. Lozano,¹ Pedro Jares,⁴ Josep Vidal,⁵ Xavier Forns,¹ Vicente Arroyo,¹ Juan Caballería,¹ Pere Ginès,¹ and Ramón Bataller¹

There are no effective antifibrotic therapies for patients with liver diseases. We performed an experimental and translational study to investigate whether ghrelin, an orexigenic hormone with pleiotropic properties, modulates liver fibrogenesis. Recombinant ghrelin was administered to rats with chronic (bile duct ligation) and acute (carbon tetrachloride) liver injury. Hepatic gene expression was analyzed by way of microarray analysis and quantitative polymerase chain reaction. The hepatic response to chronic injury was also evaluated in wild-type and ghrelin-deficient mice. Primary human hepatic stellate cells were used to study the effects of ghrelin *in vitro*. Ghrelin hepatic gene expression and serum levels were assessed in patients with chronic liver diseases. Ghrelin gene polymorphisms were analyzed in patients with chronic hepatitis C. Recombinant ghrelin treatment reduced the fibrogenic response, decreased liver injury and myofibroblast accumulation, and attenuated the altered gene expression profile in bile duct-ligated rats. Moreover, ghrelin reduced the fibrogenic properties of hepatic stellate cells. Ghrelin also protected rats from acute liver injury and reduced the extent of oxidative stress and inflammation. Ghrelin-deficient mice developed exacerbated hepatic fibrosis and liver damage after chronic injury. In patients with chronic liver diseases, ghrelin serum levels decreased in those with advanced fibrosis, and ghrelin gene hepatic expression correlated with expression of fibrogenic genes. In patients with chronic hepatitis C, polymorphisms of the ghrelin gene (−994CT and −604GA) influenced the progression of liver fibrosis. *Conclusion:* Ghrelin exerts antifibrotic effects in the liver and may represent a novel antifibrotic therapy. (HEPATOLOGY 2010;51:974-985.)

Hepatic fibrosis is the progressive accumulation of extracellular matrix that occurs in most types of chronic liver diseases. In patients with advanced fibrosis, liver cirrhosis ultimately develops. Currently, the only effective therapy to treat liver fibrosis is to eliminate the causative agent (e.g. successful antiviral therapy in patients with chronic hepatitis C). For those patients in

whom the underlying cause cannot be removed, there are no effective antifibrotic therapies. During recent years, research has focused on molecular and cellular mechanisms involved in liver fibrosis, and many pharmacological interventions have been successfully tested in experimental models of liver fibrosis.¹ However, most of the information derives from the experimental setting,

Abbreviations: α -SMA, α -smooth muscle actin; BDL, bile duct ligation; CCl₄, carbon tetrachloride; Ghrl^{-/-}, ghrelin knockout; Ghrl^{+/+}, ghrelin wild-type; GHS-R, growth hormone secretagogue receptor; HSC, hepatic stellate cell; SEM, standard error of the mean; TNF- α , tumor necrosis factor- α ; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

From the ¹Liver Unit, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), the ⁴Genomics Unit, and the ⁵Endocrinology Unit, Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERdem), Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain; ²Genotyping and Genetic Diagnosis Unit, Research Foundation, Hospital Clínic Universitario de Valencia, Valencia, Spain; and ³Experimental Hepatic Ischemia-Reperfusion Unit, Centro Superior de Investigaciones Científicas, Institut d'Investigacions Biomèdiques de Barcelona, Barcelona, Catalonia, Spain.

Received April 28, 2009; accepted October 17, 2009.

Supported by grants from the Ministerio de Ciencia e Investigación (SAF2005-06245), from the Instituto de Salud Carlos III (FIS2005-050567, FIS 2008-PI08/0237 and PI070497), and from the European Community FP6 (LSHB-CT-2007-036644 - DIALOK) and by fellowships from Institut d'Investigacions Biomèdiques August Pi i Sunyer (to M. M. and M. D.), the Fundación Bilbao Vizcaya Argentaria (to M. D.) and the Fundació Clínic (to P. S. B.).

while translational studies with human samples and clinical trials are scarce. In the current study, we used both experimental and translational approaches to characterize a new potential antifibrotic substance for patients with chronic liver diseases.

Ghrelin is a gut hormone (28 amino acids) firstly discovered as a potent growth hormone secretagogue. Moreover, it plays a major role in the regulation of food intake.² Recently, peripheral effects such as cytoprotection, vasodilatation, regulation of energy balance, and gastrokinesis have been also attributed to ghrelin.³ The primary site of ghrelin synthesis is the stomach, but ghrelin transcripts have been detected in many other organs, including the liver, bowel, pancreas, kidneys, and lungs.⁴ Most ghrelin actions are mediated by growth hormone secretagogue receptor (GHS-R),² which is mainly expressed in the pituitary gland but also in other organs, including the pancreas, spleen, and adrenal gland.⁴ However, ghrelin probably binds to another yet unknown receptor, because cells not expressing GHS-R respond to ghrelin stimulus.⁵

Recent data indicate that ghrelin has protective effects in different organs and cell types including the pancreas, heart, and gastrointestinal tract.⁶⁻⁸ Recombinant ghrelin has been successfully administered to patients with a variety of disorders such as anorexia,⁹ caquexia,¹⁰ and gastroparesis.¹¹ Moreover, ghrelin reduces muscle wasting and improves functional capacity in elderly patients with congestive heart failure and chronic obstructive pulmonary disease.^{12,13} We hypothesize that ghrelin regulates hepatic injury and fibrogenesis. To prove this hypothesis, we investigated the effect of recombinant ghrelin in different models of acute and chronic liver injury. Moreover, we evaluated whether changes in endogenous ghrelin regulate hepatic fibrosis in mice and in patients with chronic liver diseases due to hepatitis C virus infection. We provide evidence that recombinant ghrelin exerts protective and antifibrotic effects in the injured liver. Our results also suggest that endogenous ghrelin plays a role in hepatic fibrogenesis, because ghrelin knockout (*Ghrl*^{-/-}) mice are more susceptible to carbon tetrachloride (CCl₄)-induced liver injury than ghrelin wild-type (*Ghrl*^{+/+}) mice. Moreover, we demonstrate that ghrelin is locally produced in the human liver.

Materials and Methods

Chronic Liver Injury Models in Rodents. Male Wistar rats (250 g) were induced to chronic liver injury and hepatic fibrosis by prolonged bile duct ligation (BDL) as described.¹⁴ Either saline, rat recombinant ghrelin (Phoenix Pharmaceuticals; Burlingame, CA), or ghrelin receptor agonist (Des-Ala³-GHRP-2) (Bachem; Bubendorf, Switzerland) were administered to rats through a subcutaneous osmotic minipump (Alza Corporation; Palo Alto, CA) at a rate of 200 $\mu\text{L}/\text{hour}^{-1}$ throughout the experiment. Doses were chosen from existing data in the literature. Preliminary studies in rats with advanced fibrosis (CCl₄ for 8 weeks) were performed to assess the tolerability of both ghrelin and (Des-Ala³-GHRP-2). The selected doses for the peptides (10 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ for recombinant ghrelin and 30 $\mu\text{g}/\text{kg}^{-1}/\text{d}^{-1}$ for Des-Ala³-GHRP-2) were well tolerated and did not cause arterial hypotension. Experimental groups were as follows (n = 12 per group): rats with BDL or sham-operated rats infused with saline, recombinant rat ghrelin, or the ghrelin receptor agonist (Des-Ala³-GHRP-2). *Ghrl*^{-/-} mice (C57BL/6 background) were obtained from Regeneron Pharmaceuticals (Tarrytown, NY). The generation and characterization of these mice has been described extensively.¹⁵ We used mice aged 8 to 10 weeks. Because C57BL/6 mice develop biliary infarcts early and have a high rate of mortality following BDL,¹⁶ we used a different experimental model to induce chronic liver injury and hepatic fibrosis. CCl₄ (Sigma-Aldrich; St. Louis, MO) was administered intraperitoneally at a dose of 1 mL/kg⁻¹, 12.5% diluted in olive oil (Sigma-Aldrich) twice a week for 4 weeks. Control mice were given olive oil at the same dose. Each group included at least 12 mice. Rats and mice were housed in temperature and humidity-controlled rooms and kept on a 12-hour light/dark cycle. Animal procedures were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Assessment of Hepatic Necroinflammatory Injury and Fibrosis. Paraffin-embedded liver sections were stained with hematoxylin-eosin. Hepatic necroinflamma-

Address reprint requests to: Ramón Bataller, M.D., Liver Unit, Hospital Clínic, Villarroel 170, Barcelona 08036, Spain. E-mail: bataller@clinic.ub.es; fax: (34)-93-451-55-22.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23421

Potential conflict of interest: Dr. Forns received grants from Roche and Schering-Plough. Additional Supporting Information may be found in the online version of this article.

tion was estimated by quantifying the presence of necrosis, hepatocyte ballooning and/or swelling, inflammatory cell infiltration, and lipid droplets. The degree of necro-inflammatory changes was assessed as the percentage of hepatic parenchyma with any of the aforementioned changes: 1, <30%; 2, 30–60%; 3, >60%. Analyses were blindly performed by an expert pathologist (L. N. R.). To assess liver fibrosis, liver specimens were stained with picosirius red (Gurr-BDH Lab Supplies; Poole, England). The positive area stained with picosirius red was quantified using a morphometric method. Briefly, six images per specimen were obtained with an optic microscope (Nikon Corporation; Tokyo, Japan) at a magnification of $\times 40$. Images were imported to an image analysis software (AnalySIS, Olympus; Münster, Germany) and automatically merged.

Acute Liver Injury Model in Rats. Acute liver injury was induced in male Wistar rats (250 g) through a single intraperitoneal injection of CCl_4 (Sigma-Aldrich; 1 mL/kg⁻¹ body weight, 30% diluted in olive oil). Control rats received the same amount of olive oil. Animals were treated with either saline or 20 $\mu\text{g}/\text{kg}^{-1}$ rat recombinant ghrelin (Phoenix Pharmaceuticals) intravenously 1 hour before CCl_4 administration. Rats were divided into three experimental groups ($n = 8$ per group): rats receiving saline and olive oil, rats receiving saline and CCl_4 , and rats receiving ghrelin and CCl_4 . Twenty-four hours after intraperitoneal injection, animals were anesthetized and sacrificed for blood and tissue sample collection. Rats were housed in temperature- and humidity-controlled rooms and kept on a 12-hour light/dark cycle. Animal procedures were conducted in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Human Samples. For analysis of ghrelin serum levels, blood samples from patients with chronic hepatitis C ($n = 67$) and alcoholic hepatitis ($n = 24$) were obtained. Moreover, samples from healthy controls ($n = 24$) matched for age, sex, and body mass index with patients were collected. Blood samples were obtained after an overnight fasting. Hepatic gene expression was assessed in liver specimens obtained by a transjugular approach from patients with alcoholic hepatitis ($n = 37$) and by a percutaneous approach in patients with chronic hepatitis C ($n = 45$) and in patients with nonalcoholic steatohepatitis ($n = 23$). Normal liver specimens ($n = 5$) were obtained from fragments of resections of colon metastases before the vascular clamping as described.¹⁷ For the analysis of the role of variations of the ghrelin gene on the progression of liver fibrosis, DNA from patients with chronic hepatitis C ($n = 284$) was obtained from peripheral blood. The study protocol conformed to the ethical

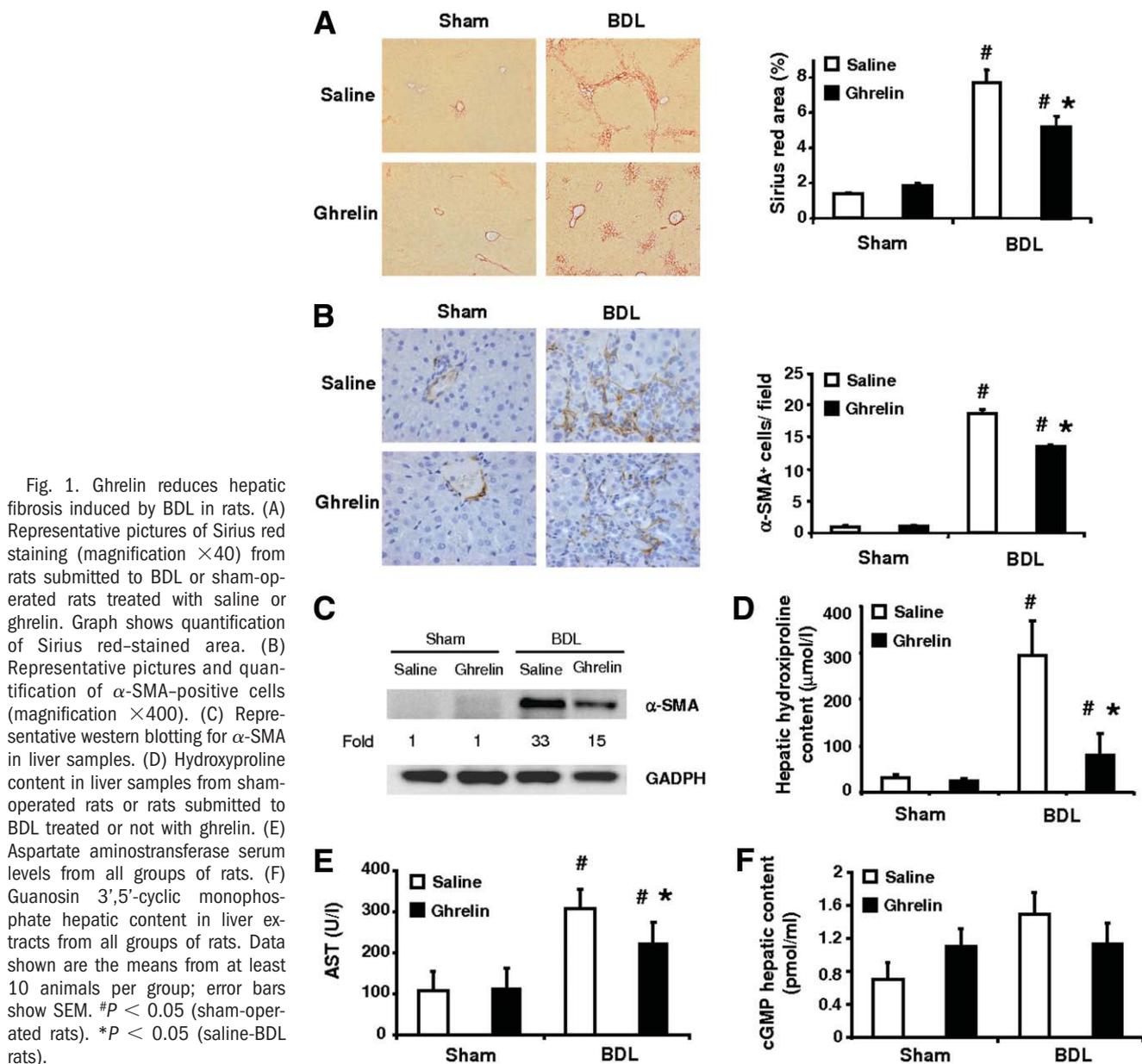
guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Clínic of Barcelona. All patients gave informed consent.

Data Analysis. Data are representative of at least three independent experiments. Results are expressed as the mean \pm standard error of the mean (SEM). The normality of the data was assessed by the Kolmogorov-Smirnov test. Comparisons between groups were performed using the Student *t* test or nonparametric Mann-Whitney test depending on the normality of data. Statistical analysis of correlations was performed by Spearman rho. *P* values < 0.05 were considered significant. For multiple comparisons, Bonferroni correction was applied to *P* values, with significance set at $P < 0.001$.

Other methods are shown in Supporting Materials and Methods.

Results

Liver Fibrosis is Reduced in Rats Treated with Recombinant Ghrelin. To investigate whether recombinant ghrelin regulates hepatic fibrogenesis following chronic liver injury, a model of secondary biliary fibrosis was induced in rats through prolonged ligation of the common bile duct. Both BDL or sham-operated rats were continuously infused with either saline or recombinant ghrelin through a subcutaneous osmotic pump for 2 weeks. BDL rats infused with saline showed severe septal hepatic fibrosis with a marked disruption of the hepatic architecture (Fig. 1A). Hepatic collagen content was increased over seven-fold compared with control rats. In contrast, BDL rats infused with ghrelin had only mild collagen deposition without formation of bridging fibrosis. Morphometric analysis revealed that ghrelin decreased collagen deposition by about 40%. To uncover the mechanisms underlying this beneficial effect, we first investigated whether ghrelin modulates the accumulation of myofibroblastic fibrogenic cells (α -smooth muscle actin [α -SMA]-positive cells). Myofibroblastic cells accumulated markedly throughout the hepatic parenchyma in BDL rats. Ghrelin treatment reduced the amount of fibrogenic cells by 25% (Fig. 1B). Moreover, ghrelin treatment decreased α -SMA protein expression, as assessed by western blotting (Fig. 1C) and hepatic content of hydroxyproline (Fig. 1D). In addition, ghrelin infusion reduced the elevation of serum aspartate aminotransferase levels, a parameter indicative of hepatocellular damage, induced by BDL (Fig. 1E). Because ghrelin stimulates guanosin 3',5'-cyclic monophosphate production in other tissues,¹⁸ we next studied whether the beneficial effect of ghrelin is associated with increased guanosin



3',5'-cyclic monophosphate hepatic content. We did not find differences between any of the groups (Fig. 1F).

Recombinant Ghrelin Prevents Changes in Hepatic Gene Expression During Liver Fibrogenesis. To explore the effects induced by ghrelin in the fibrotic liver, we analyzed changes in hepatic gene expression by way of complementary DNA microarray analysis. BDL stimulated the hepatic expression of 1,543 genes and repressed the expression of 997 genes compared with sham-operated rats. Ghrelin treatment attenuated changes in the expression of 231 genes including collagen- $\alpha 1(\text{II})$, plasminogen activator-urokinase receptor, matrix metalloproteinase 2 and chemokine receptor 5 (Fig. 2A). A list of all the genes modified by ghrelin treatment is shown in Supporting Table 1. The complete dataset is available at

the National Center for Biotechnology Information's Gene Expression Omnibus public database (<http://www.ncbi.nlm.nih.gov/geo/>), accession number GSE13747. Quantitative polymerase chain reaction confirmed the changes found in microarray analysis in some selected genes (Fig. 2B). Rat liver samples were clustered depending on gene expression profile. Rats were perfectly classified in the different experimental groups. A heatmap of the clustering can be seen in Supporting Fig. 1.

Increased Liver Injury and Fibrogenesis in *Ghrl*^{-/-} Mice. To investigate the role of endogenous ghrelin in liver fibrogenesis, we next analyzed the fibrogenic response in *Ghrl*^{-/-} and *Ghrl*^{+/+} mice. Chronic liver injury was induced by intraperitoneal injections of CCl_4 twice a week for 4 weeks. The extent of liver fibrosis was assessed

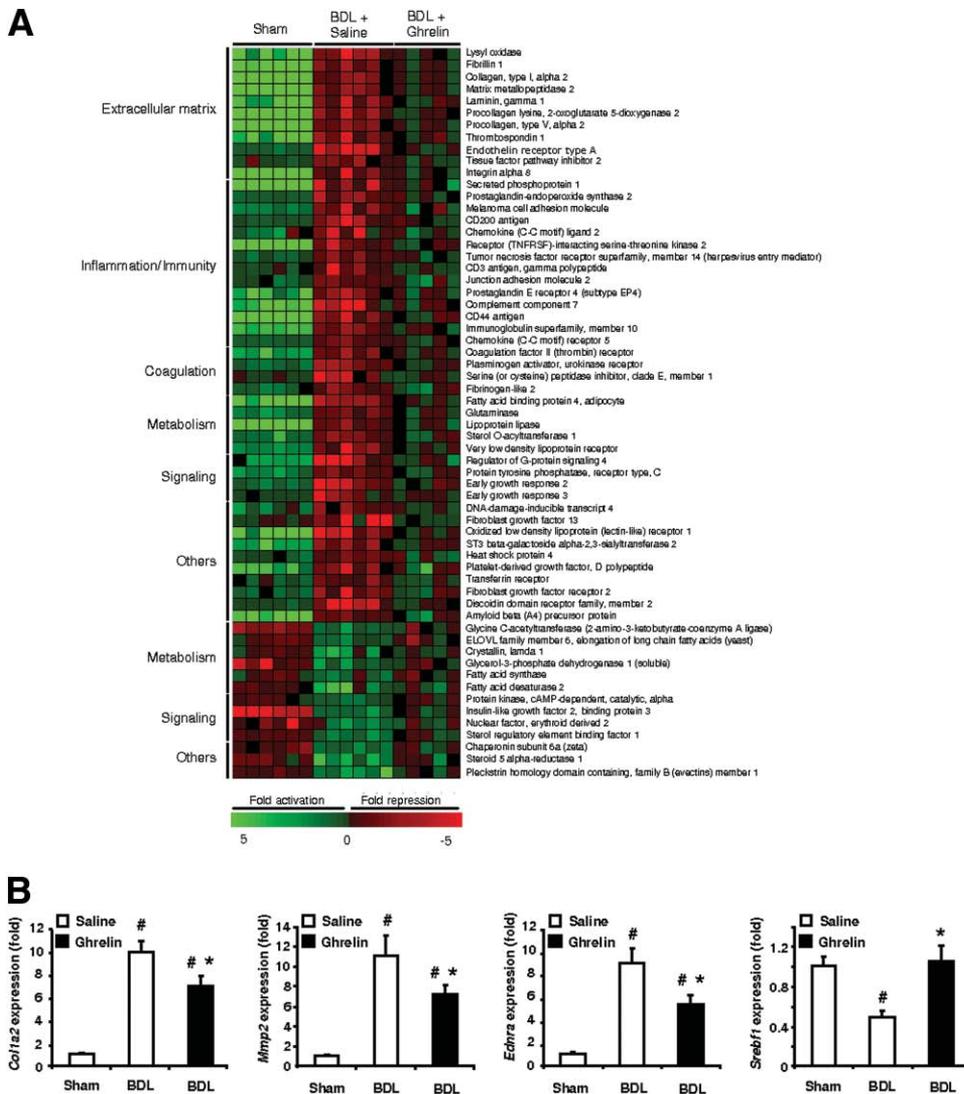


Fig. 2. Hepatic gene expression in rats submitted to sham operation or BDL. (A) Microarray data from hepatic complementary DNA. Expression of key genes was modified by BDL. Ghrelin treatment attenuated changes in gene expression profile. All genes have a false discovery rate < 0.2 and are deviated from the control by at least ± 1.8 -fold. (B) Quantitative polymerase chain reaction confirmed the results obtained in the microarray analysis in procollagen- $\alpha 1$ (II) (*Col1a2*), matrix metalloproteinase 2 (*Mmp2*), endothelin receptor type A (*Ednra*), and sterol regulatory element-binding factor 1 (*Srebf1*). Data shown are expressed as the mean from at least 5 animals per group; error bars show SEM. # $P < 0.05$ (sham-operated rats). * $P < 0.05$ (saline-BDL rats).

in both groups of mice. We found that *Ghrl*^{-/-} mice were more susceptible to CCl₄-induced liver fibrosis and liver injury than *Ghrl*^{+/+} mice, as indicated by increased collagen deposition (Fig. 3A,B) and increased necroinflammatory score (Fig. 3C). Moreover, *Ghrl*^{-/-} mice treated with CCl₄ showed a reduced weight gain compared with *Ghrl*^{+/+} mice (Fig. 3D). In addition, procollagen- $\alpha 2$ (I) and TIMP1 expression were overexpressed in *Ghrl*^{-/-} mice treated with CCl₄ compared with *Ghrl*^{+/+} littermates (Fig. 3E,F).

A GHS-R Agonist Attenuates Liver Fibrosis. We first analyzed the expression of GHS-R in human and rat liver samples by way of polymerase chain reaction. We found transcripts of GHS-R in both human and rat livers (Fig. 4A,B). Specifically, we detected GHS-R expression in human hepatocytes and activated hepatic stellate cells (HSCs) but not in quiescent HSCs (Fig. 4B). To investigate whether stimulation of GHS-R attenuates liver fibrosis new groups of rats were submitted to BDL or sham

operation in the presence or absence of a GHS-R agonist (Des-Ala³-GHRP-2) for 2 weeks. We found that the degree of liver fibrosis was reduced in rats treated with the GHS-R agonist, as indicated by decreased collagen deposition (Fig. 4C,D).

Recombinant Ghrelin Reduces Hepatocellular Injury in a Model of Acute Liver Injury in Rats. The results in BDL rats suggest that ghrelin may attenuate fibrosis by exerting a hepatoprotective effect. To prove this hypothesis, we analyzed the effects of ghrelin in a model of acute liver injury in rats (single intraperitoneal administration of CCl₄). Ghrelin or vehicle were administered to rats intravenously 1 hour before CCl₄. Pretreatment with ghrelin, but not saline, strongly reduced the hepatocellular injury induced by CCl₄, as indicated by decreased necroinflammatory score (Fig. 5A) and aspartate aminotransferase serum levels (170 and 90 IU/L in CCl₄-damaged rats in the absence and the presence of ghrelin, respectively, $P < 0.05$). This beneficial effect was

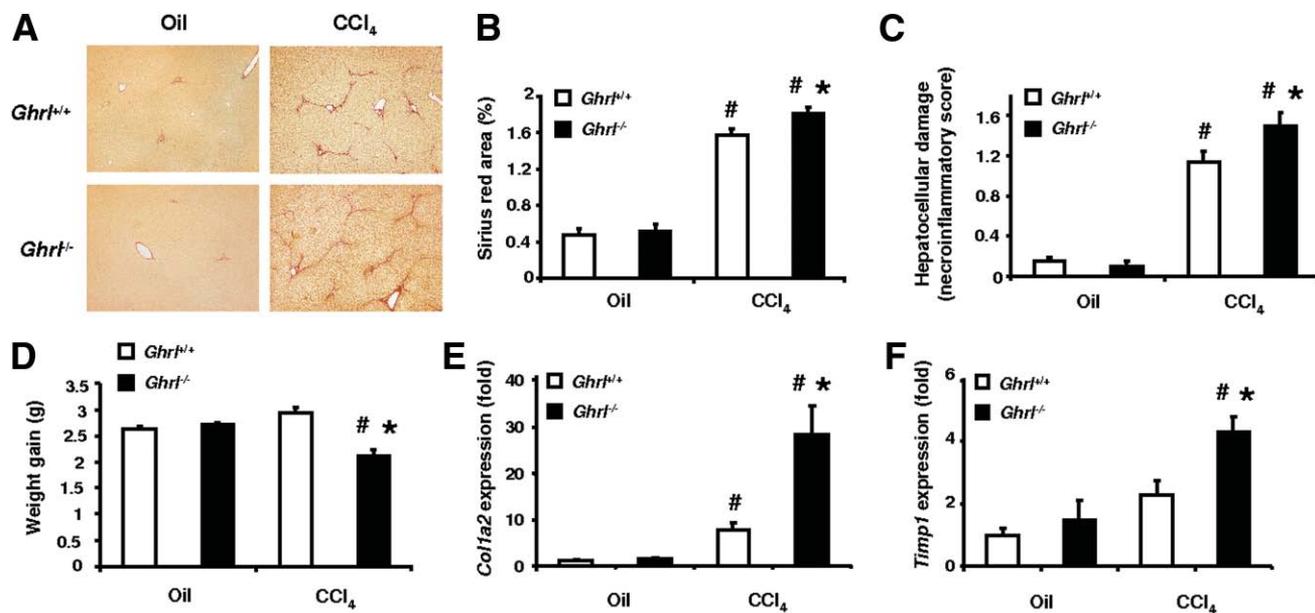


Fig. 3. Role of endogenous ghrelin in liver fibrosis in mice. *Ghrl*^{+/+} and *Ghrl*^{-/-} mice were induced to liver fibrosis by administration of CCl₄ for 4 weeks. *Ghrl*^{-/-} mice show a modest increase in the extent of liver fibrosis and increased liver damage after chronic liver injury induced by CCl₄ compared with *Ghrl*^{+/+}. (A) Representative pictures and (B) quantification of Sirius red staining (magnification $\times 40$) from *Ghrl*^{+/+} and *Ghrl*^{-/-} mice treated chronically with oil or CCl₄. (C) Necroinflammatory score of liver samples from *Ghrl*^{+/+} and *Ghrl*^{-/-} mice chronically treated with oil or CCl₄. (D) Weight increase during the 4 weeks of CCl₄ treatment in all groups of mice. (E,F) Gene expression of genes involved in fibrogenesis. Procollagen- $\alpha 2$ (I) and tissue inhibitor of metalloproteinases (TIMP-1) were overexpressed in *Ghrl*^{-/-} mice induced to liver fibrosis when compared with *Ghrl*^{+/+} mice. Data shown are expressed as the mean from at least 10 animals per group; error bars show SEM. #*P* < 0.05 (oil-treated mice). **P* < 0.05 (CCl₄-wild-type mice).

associated with decreased infiltration of inflammatory cells, as assessed by quantification of infiltrating leukocytes (CD43-positive cells) in liver sections (*P* < 0.05, Fig. 5B). Because oxidative stress mediates CCl₄-induced hepatocellular injury, we also explored whether ghrelin reduces this pathogenic event by quantifying 4-hydroxynonenal protein adducts. As shown in Fig. 5C, ghrelin attenuated the accumulation of 4-hydroxynon-

enal in hepatocytes. We next explored the effects on hepatocyte cell death by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis. Ghrelin diminished the number of TUNEL-positive hepatocytes, indicating that it reduces cell apoptosis (Fig. 5D). This effect was associated with decreased activation of nuclear factor κ B, as assessed by p65 nuclear translocation (Fig. 5E). Moreover, ghrelin treatment at-

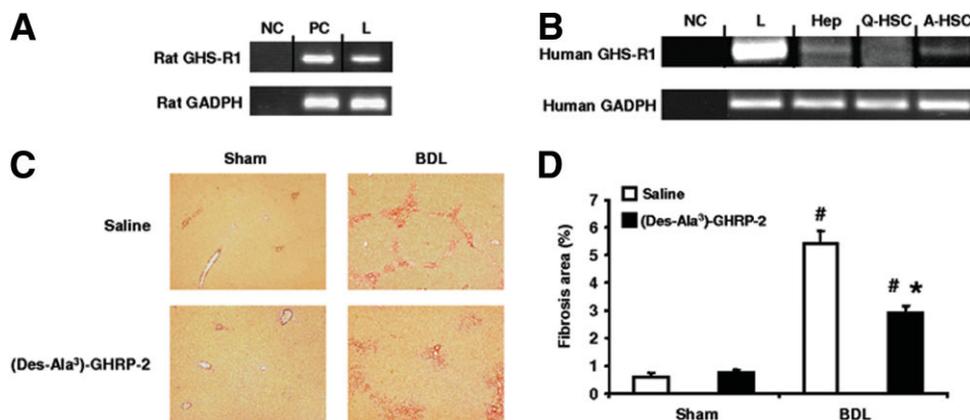


Fig. 4. Effects of a GHS-R agonist, (Des-Ala₃)-GHRP-2, on experimental liver fibrosis. Expression of GHS-R was detected in (A) rat and (B) human livers. NC, negative control; PC, positive control; L, liver; Hep, hepatocytes; Q-HSC, quiescent HSCs; A-HSC, activated HSCs. A GHS-R agonist, (Des-Ala₃)-GHRP-2, was infused in sham-operated rats and rats with BDL during the 2 weeks of the experiment. (C) Representative pictures and (D) quantification of the area stained by Sirius red (magnification $\times 40$). Data shown are expressed as the mean \pm SEM from 8 rats per group. #*P* < 0.05 (sham-operated rats). **P* < 0.05 (saline-BDL rats).

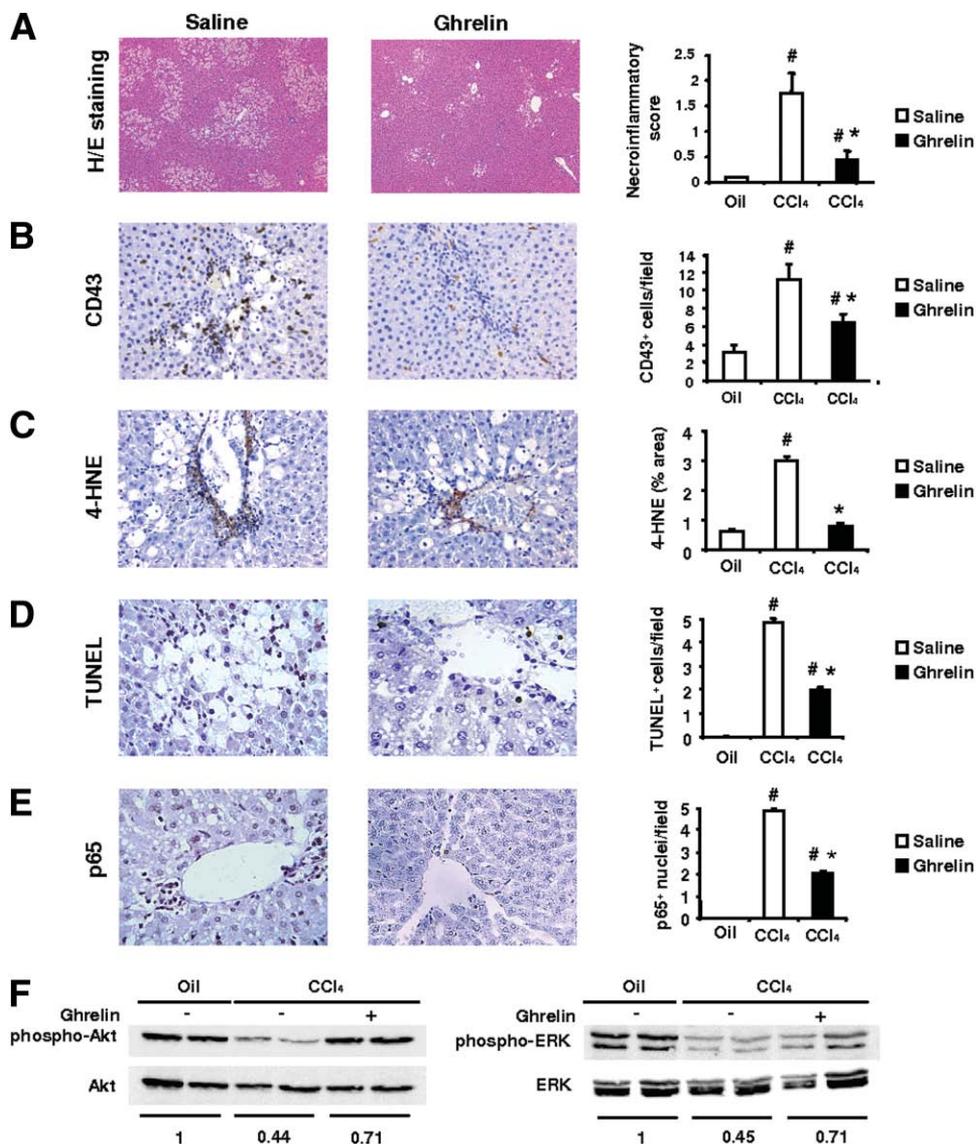


Fig. 5. Ghrelin exerts hepatoprotective effects in rats with acute liver injury induced by CCl₄. Rats received ghrelin (20 μg/kg⁻¹) intravenously 1 hour before CCl₄ administration. (A) Representative pictures of hematoxylin-eosin staining in livers from CCl₄-injured rats treated with saline or ghrelin (magnification ×200). CCl₄ induced hepatocyte ballooning, parenchymal necrosis, and inflammatory infiltrate. Graph shows evaluation of the necroinflammatory score. (B) Representative pictures of CD43 immunostaining in CCl₄-treated rats (magnification ×400). Graph shows quantification of CD43-positive cells per field (magnification ×200). (C) Representative pictures of 4-hydroxynonenal immunostaining in CCl₄-treated rats (magnification ×400). Quantification of the area stained is shown in the graph. (D) Representative pictures of TUNEL immunostaining in CCl₄-treated rats (magnification ×400). Graph shows quantification of TUNEL-positive cells per field (magnification ×400). (E) Representative pictures of p65 immunostaining (magnification ×400). Graph shows quantification of p65-positive nuclei per field (magnification ×400). *P < 0.05 (control). #P < 0.05 (rats receiving saline-CCl₄). (F) Intracellular pathways involved in CCl₄-induced liver damage and ghrelin hepatoprotection. Western blot analyses showing Akt and extracellular signal-regulated kinase phosphorylation in extracts from rat livers. Numbers underneath represent fold expression compared with oil-treated rats. Data are expressed as the mean ± SEM from 8 animals per group.

tenuated the effects of CCl₄ on Akt and extracellular signal-regulated kinase phosphorylation, two intracellular pathways involved in hepatocyte survival and proliferation (Fig. 5F). All together, these results indicate that ghrelin exerts hepatoprotective effects.

Ghrelin Modulates Fibrogenic, But Not Proinflammatory, Properties of Hepatic Stellate Cells. To further elucidate possible mechanisms of the protective effects of ghrelin in the liver, we next investigated whether ghrelin modulates the fibrogenic actions of HSCs, the main fibrogenic cell type in the injured liver.¹ Stimulation of primary cultured HSCs with angiotensin II (0.1 μM), a well-known fibrogenic agonist, resulted in a marked increase in intracellular calcium concentration ([Ca²⁺]_i). Preincubation with ghrelin (0.1 μM) for 10 minutes attenuated angiotensin-II-induced [Ca²⁺]_i increase (Fig. 6A). Ghrelin (0.1 μM) also reduced by 40% the expression of collagen-α1(I) and trans-

forming growth factor-β1 in unstimulated HSCs (Fig. 6B). We then investigated whether ghrelin inhibits the proinflammatory actions of HSCs. Ghrelin did not modulate the activation of nuclear factor κB or the release of interleukin-8 (Fig. 6C and 6D, respectively). These results indicate that ghrelin reduces the fibrogenic but not the inflammatory properties of cultured HSCs.

Serum Ghrelin Levels and Hepatic Ghrelin Expression in Patients with Chronic Liver Diseases. To analyze the potential role of ghrelin in chronic human liver diseases, serum ghrelin concentration was measured in control subjects (n = 24) and in patients with liver fibrosis including alcoholic hepatitis (n = 24) and chronic hepatitis C (n = 67). Serum ghrelin levels were significantly lower in both patients with alcoholic hepatitis and chronic hepatitis C compared with control subjects, after adjusting for age, sex, and body mass index (Fig. 7A).

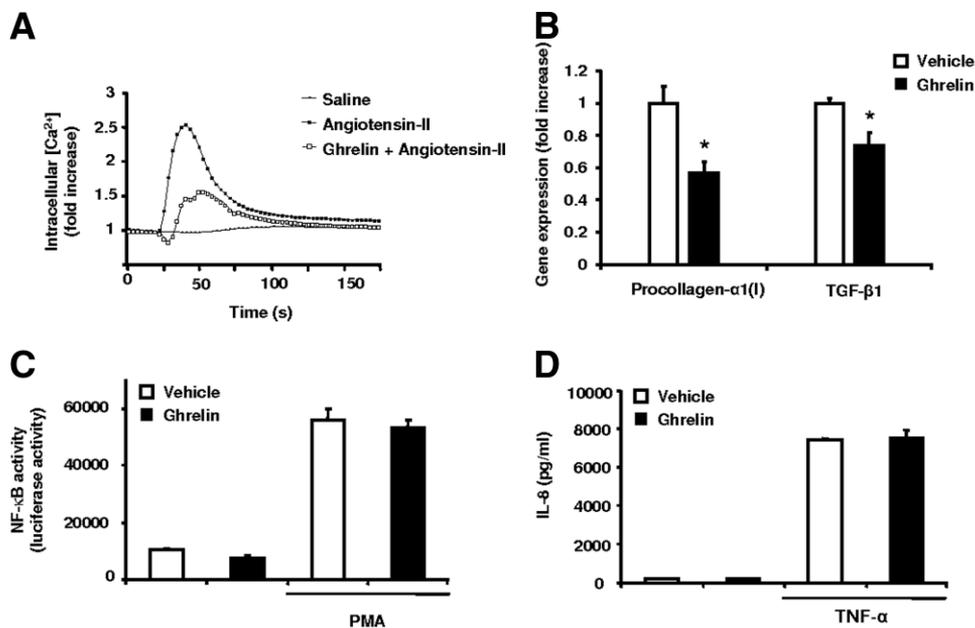


Fig. 6. Effects of ghrelin on profibrogenic and proinflammatory properties in primary human HSCs. (A) Intracellular calcium concentration ($[Ca^{2+}]_i$) as evidenced by Fura 2 intensity in HSCs. Cells were preincubated for 10 minutes with ghrelin ($0.1 \mu\text{mol/L}$) and then challenged with angiotensin II ($0.1 \mu\text{mol/L}$). (B) Expression of procollagen- $\alpha 1(I)$ and transforming growth factor- $\beta 1$ messenger RNA in HSCs exposed to ghrelin ($0.1 \mu\text{mol/L}$) for 24 hours. * $P < 0.05$ (vehicle). (C) Activity of nuclear factor κB assessed by luciferase reporter gene assay. Cells were infected with an adenovirus containing luciferase gene with the promoter region for nuclear factor κB transcription factor and incubated overnight with vehicle, ghrelin, or phorbol 12-myristate 13-acetate. Ghrelin ($0.1 \mu\text{mol/L}$) did not modulate nuclear factor κB activity in HSCs. 12-myristate 13-acetate (1 mg/mL) was used as a positive control. Preincubation of cells with ghrelin for 10 minutes did not modulate the effect of 12-myristate 13-acetate. (D) Cells were incubated with vehicle, ghrelin, or tumor necrosis factor- α (TNF- α) for 24 hours. Medium was collected to analyze interleukin 8 concentration. Ghrelin ($0.1 \mu\text{mol/L}$) did not modulate interleukin 8 release by HSCs to the culture medium. TNF- α (1 ng/mL) was used as a positive control. Preincubation of cells with ghrelin for 10 minutes did not modulate the effect of TNF- α . Data are expressed as the mean \pm SEM from 3 independent experiments.

Interestingly, ghrelin serum levels were lower in patients with advanced fibrosis (Metavir score 3-4) than in those with mild fibrosis (Metavir score 0-2) (Fig. 7B). Next, we assessed ghrelin gene (*GHRL*) expression in normal ($n = 5$) and diseased human livers (37 patients with alcoholic hepatitis, 45 patients with chronic hepatitis C, and 23 patients with nonalcoholic steatohepatitis). Ghrelin transcripts were found in both normal and diseased livers. Interestingly, *GHRL* was clearly overexpressed in livers with nonalcoholic steatohepatitis compared with the rest of the groups (Fig. 7C). Moreover, in the whole series of patients with chronic liver diseases, *GHRL* hepatic expression positively correlated with the expression of genes involved in fibrogenesis (Supporting Table 2) as well with body mass index ($r = 0.675$, $P < 0.0001$). At the cellular level, *GHRL* transcripts were found in both hepatocytes and HSCs freshly isolated from human livers as well as in culture-activated human HSCs (Fig. 7D).

Polymorphisms in the Ghrelin Gene Are Associated with the Degree of Fibrosis in Patients with Chronic Hepatitis C. Finally, we investigated whether ghrelin gene polymorphisms are associated with the progression of liver fibrosis in patients with chronic liver diseases. For

this purpose, we analyzed six single nucleotide polymorphisms on the ghrelin gene (Supporting Fig. 2A): $-994CT$, $-604GA$, $-501AC$, Arg51Gln, Met72Leu, and Leu90Gln (GeneBank numbers can be found in Supporting Materials and Methods) in 284 patients with HCV-induced liver disease. One single nucleotide polymorphism in the promoter ($-994CT$) was differently represented between women with advanced fibrosis (F3-F4) and those with mild fibrosis (F0-F2). Moreover, we found that patients with the haplotype $-994T$ and $-604A$ are more susceptible to severe liver fibrosis after adjusting by age and sex (Table 1). These results suggest that variations in *GHRL* modulate the progression of chronic hepatitis C. To investigate the functionality of these polymorphisms, we constructed plasmids containing the promoter of ghrelin with different haplotypes (wild-type and $-994CT -604GA$) bound to the luciferase gene. Plasmids were transfected to Huh7 hepatocytes. The plasmid with the promoter containing the haplotype associated with an increased risk to develop advanced fibrosis was found to be more active than the plasmid containing the wild-type promoter (Supporting Fig. 2B).

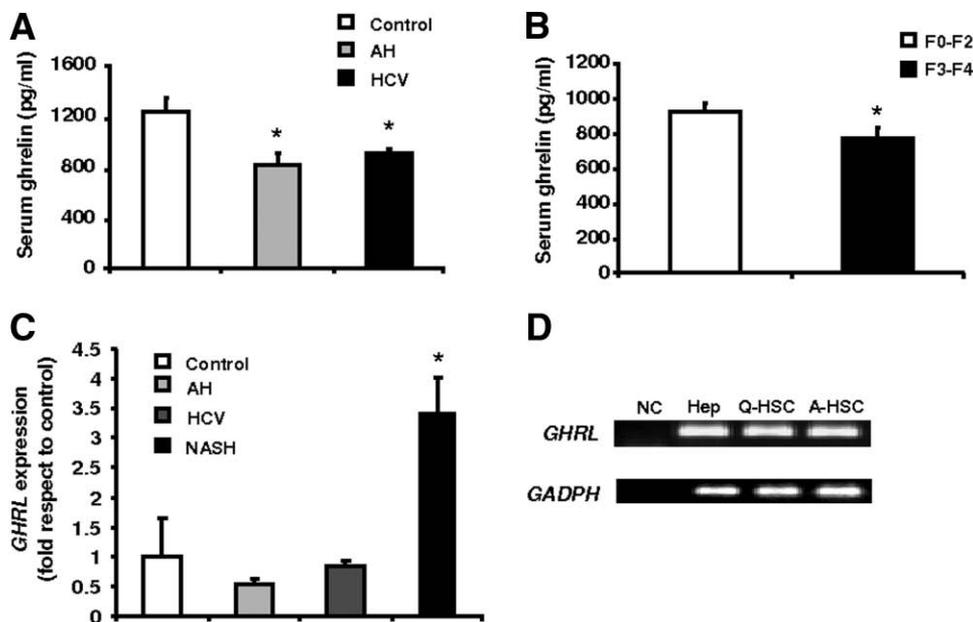


Fig. 7. Ghrelin serum levels and hepatic ghrelin expression in control subjects and in patients with chronic liver diseases. (A) Fasting ghrelin serum levels were analyzed in blood samples from patients with chronic hepatitis C virus infection, alcoholic hepatitis, and healthy controls. Serum ghrelin levels were decreased in all groups of patients. (B) Ghrelin levels were lower in patients with advanced fibrosis compared with those with mild fibrosis. $*P < 0.05$ (control or F0-F2). (C) *GHRL* hepatic expression was analyzed in samples from controls, chronic hepatitis C, alcoholic hepatitis, and nonalcoholic steatohepatitis patients. $*P < 0.05$ (all groups). (D) Ghrelin expression was analyzed in different hepatic cell types. AH, alcoholic hepatitis; A-HSC, human in culture-activated HSCs; HCV, hepatitis C virus; Hep, primary human hepatocytes; NASH, nonalcoholic steatohepatitis; NC, negative control; Q-HSC, quiescent human HSCs.

Discussion

Gut hormones play a major role in food intake and energy homeostasis at different levels, from central regulation of appetite to motility of the gastrointestinal tract. They also regulate inflammatory and fibrogenic processes in a variety of tissues. Ghrelin is a gut hormone that is also produced by extraintestinal tissues and exerts a variety of pleiotropic effects in parenchymal cells.³ We provide extensive evidence that ghrelin exerts antifibrotic and hepatoprotective effects in the injured liver in rodents. We demonstrate that recombinant ghrelin regulates the fibrogenic response of the liver to acute and chronic injury. Moreover, endogenously produced ghrelin also regulates fibrogenesis in mice and humans. The hepatoprotective effects of ghrelin confirm previous studies indicating that ghrelin exerts protective effects in parenchymal cells and in damaged tissues such as the heart and the colon.^{6,19} In the liver, a single study²⁰ suggests protective effects of ghrelin in a model of chronic liver injury. Our study extensively expands this notion by demonstrating a role for ghrelin in liver fibrosis. This new effect of ghrelin has potential therapeutic implications, as discussed later.

The main finding of our study is that ghrelin regulates hepatic fibrosis. Although a number of studies have suggested that ghrelin has protective effects against cell

death,^{5,21} the current study expands this effect by demonstrating that ghrelin also prevents scar tissue formation in chronically injured tissues. Most importantly, we demonstrate for the first time that endogenously produced ghrelin regulates fibrogenesis in the liver. In addition to the effects in experimental models of liver injury (BDL and CCl₄), we used a translational approach to study the potential role of ghrelin in samples from patients with chronic liver injury. First, we analyzed ghrelin hepatic expression in patients with different liver diseases. We found ghrelin expression in both normal and diseased livers. Interestingly, obesity and the presence of nonalcoholic steatohepatitis were associated with increased hepatic expression of ghrelin. This interesting result is probably related to the deregulated energetic metabolism in obese subjects and deserves further investigation. We also analyzed serum ghrelin levels in patients with chronic liver diseases. We found that ghrelin serum levels decreased in patients with advanced fibrosis. Our results apparently differ from a recent report showing that ghrelin serum levels are increased in patients with chronic liver diseases.²² In this latter study, ghrelin serum levels were increased in patients with advanced cirrhosis. This advanced state is associated with profound hepatic failure, caquexia, endotoxemia, and hemodynamic distur-

Table 1. Effects of Ghrelin Genetic Polymorphisms in the Progress of Fibrosis in Patients with Chronic Hepatitis C

-994 CT Polymorphism						
Sex	Genotype	F0-F2, n (%)	F3-F4, n (%)	Odds Ratio (95% Confidence Interval)	P Value	
All	CC	134 (84.3)	93 (74.4)	1.00		-
	TT + CT	25 (15.7)	32 (25.6)	1.79 (0.96-3.37)		0.068
Females	CC	55 (90.2)	29 (69)	1.00		-
	TT+ CT	6 (9.8)	13 (31)	9.75 (1.34-71.05)		0.010
Males	CC	79 (80.6)	64 (77.1)	1.00		-
	TT+ CT	19 (19.4)	19 (22.9)	1.01 (0.47-2.19)		0.981
Haplotype						
Sex	-994 CT	-604 GA	F0-F2, n (%)	F3-F4, n (%)	Odds Ratio (95% Confidence Interval)	P Value
All	C	A	82 (51.64)	63 (50.4)	1.00	-
	C	G	64 (40.5)	44 (35.6)	0.97 (0.66-1.41)	0.850
	T	A	11 (7.17)	17 (14.0)	2.06 (1.08-3.91)	0.028
Females	T	G	1 (0.7)	0 (0.0)	0.00	1.00
	C	A	32 (51.64)	21 (51.19)	1.00	-
	C	G	26 (43.44)	13 (30.95)	0.96 (0.35-2.66)	0.943
	T	A	3 (4.92)	8 (17.86)	8.47 (1.31-54.84)	0.029
Males	T	G	0 (0.00)	0 (0.00)	0.00	1.00
	C	A	51 (51.82)	42 (50.00)	1.00	-
	C	G	38 (38.49)	31 (37.95)	1.12 (0.61-2.05)	0.712
	T	A	8 (8.39)	10 (12.05)	1.40 (0.48-4.05)	0.54
	T	G	1 (1.31)	0 (0.00)	1.08 (0.00-1088)	0.982

bances, which could influence serum levels of cytokines and vasoactive substances. In our series, the vast majority of patients have mild to moderate degree of fibrosis, which could explain the discrepant results. Finally, we studied the role of ghrelin gene variations in the progression of liver fibrosis in a well-characterized series of patients with biopsy-proven chronic hepatitis C. We analyzed *GHRL* polymorphisms and compared their frequencies in patients with mild fibrosis and patients with advanced fibrosis. We found two single-nucleotide polymorphisms in *GHRL* associated with advanced fibrosis in women but not in men. The fact that polymorphisms affect mainly women is a very intriguing question. It is well known that sex is a major factor influencing ghrelin expression and serum levels.^{23,24} In fact, previous studies indicate that sex markedly influences the effect of ghrelin polymorphisms in different diseases.^{25,26} Therefore, it is not surprising that in our study the influence of ghrelin polymorphisms on liver fibrosis were sex-dependent. Further studies are required to investigate this issue. Moreover, it is well known that fibrosis progression is modulated by estrogens.²⁷

Different mechanisms may explain the antifibrotic effects of ghrelin in the injured liver. First, ghrelin seems to protect hepatocytes from cell death, as indicated by decreased necroinflammatory injury and serum levels of aminotransferases in rats subjected to both acute and chronic liver injury. This effect was related to a reduction

in the number of infiltrating inflammatory cells as well as decreased apoptosis in hepatocytes in the model of acute liver injury. These results confirm published data indicating that ghrelin prevents parenchymal cell death in different injured tissues.^{8,18,28} Interestingly, we found that ghrelin administration to injured rats resulted in increased hepatic expression of hepatoprotective signaling pathways such as phospho-Akt and phospho-extracellular signal-regulated kinase. These results are in keeping with several studies showing that ghrelin induces activation of Akt and extracellular signal-regulated kinase in different cell types.^{5,7,29} Second, we found that ghrelin decreases the extent of oxidative stress in the liver, which is a major pathogenic event in the wound healing response to injury. This antioxidant effect of ghrelin has been shown in other organs.^{30,31} Whether ghrelin reduces the formation of reactive oxygen species or increases the activity of antioxidant defenses is unknown and deserves further investigation. Third, we provide evidence that ghrelin reduces the accumulation of activated HSCs in the liver and it directly reduces collagen synthesis by cultured HSCs. This effect is associated with decreased transforming growth factor- β 1 expression, a major profibrogenic cytokine in the liver. Finally, microarray analysis revealed several potential mechanisms by which ghrelin could exert its antifibrotic effect. Thus, besides reducing expression of genes involved in extracellular matrix synthesis, ghrelin reduced the expression of genes involved in apoptosis

(caspases), inflammation (osteopontin, chemokine receptor 5), and cellular contractility (tropomyosin).

This study has several limitations. First, it is unknown whether locally produced ghrelin or extrahepatic synthesis of ghrelin (e.g., by the stomach) regulate hepatic fibrogenesis. The finding that ghrelin serum levels are decreased in patients with more aggressive fibrosis suggests that extrahepatic sources of ghrelin could be implicated in the progression of fibrosis. Second, further studies using GHS-R antagonists should confirm the involvement of this receptor in the beneficial effects induced by ghrelin. Third, the role of ghrelin in fibrosis resolution and the therapeutic effect of exogenous ghrelin in established cirrhosis should be evaluated. Fourth, because ghrelin requires a posttranslational modification (octanoylation) to be active,³² further analysis of the ghrelin active form should be performed in liver samples and cell types. Fifth, the results in *Ghrl*^{-/-} are less impressive than in rats receiving recombinant ghrelin probably because constitutive knockout mice usually develop strategies to overcome the lack of a given gene. Further studies using ghrelin conditional knockout mice and/or ghrelin receptor knockout mice should clarify this question. Finally, although we provide evidence that ghrelin exerts direct antifibrotic effects in fibrogenic cells, the precise molecular mechanisms by which ghrelin exerts beneficial effects in liver undergoing acute and/or chronic injury should be uncovered in further studies.

The results of our study have potential therapeutic implications. Recombinant ghrelin has been tested in patients with different conditions, including gastroparesis,¹¹ anorexia,⁹ caquexia,¹⁰ and chronic heart failure.¹² In these studies, ghrelin is generally well tolerated and only causes a mild decrease in arterial pressure. Our results suggest that ghrelin could also be useful in patients with liver injury and liver fibrosis. Further studies should evaluate this hypothesis. Moreover, due to the orexigenic properties of ghrelin, ghrelin receptor antagonists have been recently proposed for the treatment of diabetes and obesity.³³ Due to its protective effects, prolonged blockade of ghrelin receptors may cause adverse effects such as accelerated tissue fibrosis, which is commonly seen in the heart and the kidney of patients with metabolic syndrome.

In conclusion, the results of the current study indicate that ghrelin exerts hepatoprotective and antifibrogenic effects in the liver. Further studies should evaluate the safety and efficacy of ghrelin and/or ghrelin agonists in patients with chronic liver diseases.

Acknowledgment: We thank Elena Juez for her excellent technical assistance. We are grateful to M. Sleeman (Regeneron) and T. L. Horvath and M. Shanabrough (Yale University) for providing ghrelin knockout mice.

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