Hepatoprotective Effects of Insulin-like Growth Factor I in Rats With Carbon Tetrachloride–Induced Cirrhosis

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Background & Aims: **Bioavailability of insulin-like growth factor (IGF-I) is reduced in liver cirrhosis. The aim of this study was to analyze the effect of IGF-I on liver histopathology and function in experimental cirrhosis.** ***Methods:*** **Rats received CCl_4 inhalations for 11 or 30 weeks (protocols 1 and 2, respectively) and were treated with 2 $\mu g \cdot 100$ g body $wt^{-1} \cdot day^{-1}$ IGF-I (group CI + IGF) or saline (group CI) on weeks 13 and 14 (protocol 1) or on weeks 28–30 (protocol 2). Normal rats were studied in parallel.** ***Results:*** **Serum albumin and total protein levels were reduced in CI but not in CI + IGF rats compared with normal rats. Clotting factors II, VII, and X were significantly greater in CI + IGF than in CI rats. Liver lipid peroxidation products were significantly increased in CI but not in CI + IGF rats, and liver fibrosis was less pronounced in CI + IGF than in CI animals. The activities of antioxidant enzymes and mitochondrial transmembrane potential were reduced compared with normal animals in CI but not in CI + IGF rats.** ***Conclusions:*** **IGF-I improves liver function and reduces oxidative liver damage and fibrosis in rats with compensated or advanced liver cirrhosis. Improved mitochondrial function could play a role in the hepatoprotective effect of this hormone.**

Cell necrosis, hepatocellular regeneration, and fibrogenesis are processes involved in the development of liver cirrhosis. Manifestations of hepatic insufficiency and protein-calorie malnutrition occur in advanced stages of the disease.[1] It has been proposed that reduced hepatic production of insulin-like growth factor I (IGF-I) in advanced liver disease[2–6] may be one of the factors contributing to malnutrition in cirrhotic patients.[7] This idea stems from the fact that IGF-I, which is mainly produced by hepatocytes under the stimulus of growth hormone, is a substance that has a wide range of anabolic activities.[8]

We have shown previously that low doses of IGF-I improve nutritional status in cirrhotic rats.[7] In the present study, we investigated the effects of IGF-I administration on hepatic biosynthetic functions and on the development of histopathologic changes in the liver of rats with CCl_4-induced cirrhosis. In this experimental model of cirrhosis, cell damage by free radicals is the predominant mechanism of hepatotoxicity.[9] The ensuing oxidative stress leads to lipid peroxidation, mitochondrial dysfunction, and depletion of adenosine triphosphate (ATP).[10–14] In turn, injured mitochondria and the products of lipid peroxidation are capable of perpetuating cell damage by generation of free radicals or cytotoxic substances.[10,11,15] Lipid peroxidation products may stimulate the expression of collagen genes[16] and the activity of hepatic prolyl 4-hydroxylase[17]; these effects seem to be relevant in the development of fibrosis.[16–19] Antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase[20–22] play an important role in cell defense against oxidative stress.[20–23] Thus, we have extended our studies to analyze the effect of IGF-I on lipid peroxidation, mitochondrial function, and activity of antioxidant enzymes in liver tissue.

Materials and Methods

Induction of Liver Cirrhosis

All experimental procedures were performed in conformity with *The Guiding Principles for Research Involving Animals*.[24] Male Wistar rats (3 weeks old, 130–150 g) were subjected to two different study protocols. In protocol 1, liver cirrhosis was induced in 20 rats by inhalation of CCl_4 (Merck, Darmstadt, Germany) administered twice a week for 11 weeks, with a progressively increasing exposure time from 1 to 5 minutes,[25] and phenobarbital (Luminal; Bayer, Leverkusen, Germany) was added to drinking water (400 mg/L).[25,26] In protocol 2, liver cirrhosis was induced in 20 rats as in protocol 1, but animals continued receiving CCl_4 once per week (3-minute inhalation) to complete 30 weeks of CCl_4 administration. In each protocol, healthy control rats which did not receive phenobarbital or

Abbreviations used in this paper: CI, cirrhotic (rats); CI + IGF-I, IGF-treated cirrhotic (rats); CO, control (rats); FAU, arbitrary units of fluorescence after logarithmic to lineal transformation; GSHPx, glutathione peroxidase; IFLg, mean value of green fluorescence on a log scale; IGF-I, insulin-like growth factor I; MDA, malondialdehyde; MTP, mitochondrial transmembrane potential; rhIGF, recombinant human IGF; SOD, superoxide dismutase.

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CCl_4 were studied in parallel. Rats were housed in cages placed in a room with 12-hour light-dark cycle and constant humidity and temperature (20°C). Both food (standard semipurified diet for rodents; B. K. Universal, Sant Vicent dels Horts, Spain) and water were given ad libitum.

Study Design

Protocol 1. The study period (period of administration of saline or IGF-1) was initiated 10 days after stopping CCl_4 administration (day 0). Rats were randomly assigned to receive either saline (group CI, n = 10) or recombinant human IGF-I (rhIGF-I; 2 μg IGF-I · 100 g body $wt^{-1} \cdot day^{-1}$ in two divided doses) (group CI + IGF, n = 10) subcutaneously for 14 days. Animals were killed by decapitation 24 hours after receiving the last dose (day 15). Biochemical parameters were determined on days 0 and 15 and prothrombin time and clotting factors on day 15. Blood samples were taken from retroocular venous plexus with capillary tubes (70 mm; Marienfeld, Germany), divided into aliquots, and stored at −20°C until used. Livers were weighted, and a tissue sample from the left major liver lobe was processed (fixed in Bouin's solution) for histological examination. Tissue specimens were immediately frozen by immersion in liquid N_2 and stored at −80°C until assaying. All animals included in the groups receiving CCl_4 had altered liver function test results at baseline (day 0); liver biopsy specimen on day 15 showed established cirrhosis with fibrous septa delimiting regenerative nodules.

Protocol 2. Rats received treatment with either rhIGF-I (2 μg · 100 g body $wt^{-1} \cdot day^{-1}$ in two divided doses; group CI + IGF, n = 10) or vehicle (group CI, n = 10) during the last 21 days of exposure to CCl_4 (weeks 28, 29, and 30). On day 22, animals were killed and liver and blood samples were collected. In this protocol, all animals receiving CCl_4 showed advanced liver cirrhosis with ascites at the end of the study. Serum biochemistry (with the exception of clotting factors) was performed as in protocol 1.

Analytical Methods

Liver function tests were determined by routine laboratory methods using a Hitachi 747 autoanalyzer (Boehringer Mannheim, Mannheim, Germany). Prothrombin time was measured using standard thromboplastin (OrthoRecombiplastin; Ortho Diagnostics, Raritan, NJ) with a Koagulab 16S (Ortho Diagnostics). Fibrinogen was assessed according to Von Clauss's method.[27] Activity levels of clotting factors II, VII, and X were measured using factor-deficient plasma (Organon Tecnika Corp., Durham, NC). All values were referred to a standard curve obtained with different dilutions of normal plasma, where 1:10 dilution represented 100% activity for each factor.

Histological Degree of Fibrosis and Liver Collagen Content

In liver sections stained with Masson's trichrome, semiquantitative assessment of fibrosis was blindly performed using a numerical scoring system based on the number, length, and thickness of fibrous septa. The length of the septa (examined at 80× magnification) was assessed as follows: 1 point, minimal grade fibrosis that can be observed in normal livers; 4 points, septa confluent between portal tracts and between portal tracts and central veins; and 2 or 3 points, intermediate lengths of septa observed. The width of the fibrous septa was calculated at 150× magnification scoring 6 points when the mean value of the thickness of 9 septa (3 periportal, 3 perivenous, and 3 perinodular), measured in four different fields, oscillates around 90–125 μm; score 4, 70–50 μm; and score 2, ~40–30 μm. The number of septa was scored as 4 points when there were numerous septa extending into the nodules, thus dissecting a small number of hepatocytes forming micronodules; 2–3 points when septa penetrating into nodules were less numerous surrounding bigger nodules; and 1 point when there was no formation of micronodules inside macronodules. Four fields from each preparation were evaluated twice by two different observers, receiving a maximum of 14 points each time. The arithmetical mean of the two scores was taken as the final score.

Liver collagen content was measured by a dye-binding procedure as described by Jimenez et al.[28] in 14-μm-thick sections using Fast green FCF (no. 42053; Fluka Chemie AG, Buchs, Switzerland) and Sirius red F3B (no. 34149; Gurr BDH Chemicals Ltd., Poole, England). The values were expressed as micrograms of collagen per milligram of protein.

Malondialdehyde Levels and Activities of Antioxidant Enzymes in Liver Tissue

Malondialdehyde (MDA) was used as an index of lipid peroxidation and was measured after heating samples at 45°C for 60 minutes in acid medium. It was quantitated by a colorimetric assay using LPO-586 (Bioxytech; OXIS International Inc., Portland, OR), which, after reacting with MDA, generates a stable chromophore that can be measured at 586 nm (Hitachi U2000 Spectro; Boehringer Mannheim). Determinations were performed in homogenates of liver tissue in Tris-HCl solution (1 g of liver tissue/10 mL) centrifuged at 3000*g* during 10 minutes at 4°C.

Activities of the antioxidant enzymes SOD (E.C. 1.15.1.1), catalase (E.C. 1.11.1.6) and GSHPx (E.C. 1.11.1.9) were measured in liver tissue. Liver samples were homogenized in Tris-HCl buffer (20 mmol/L, pH 7.4; 1 g tissue/10 mL) at 0°C and centrifuged at 25,000*g* for 30 minutes at 4°C. All measurements were performed in the supernatant. SOD activity was determined at 37°C using a commercial kit (Ransod; Randox Laboratories Ltd., Crumlin, England) and an autoanalyzer (Cobas Mira; Roche Diagnostic System, Basel, Switzerland). Catalase activity was determined at 25°C by measuring the changes of absorbance using final concentrations of 10 mmol/L H_2O_2 and 50 mmol/L phosphate buffer (pH 7.0) at 240 nm during the time interval 15–30 seconds after addition of the sample.[22] The activity was calculated as velocity constant k. Values were expressed as k per milligram of protein. GSHPx was measured at 37°C with the Ransod commercial kit using the Cobas Mira autoanalyzer. The activity of GSHPx is expressed in units (1

U equals 1 μmol substrate turnover per minute) per milligram of protein. Total protein was determined using Bradford's method.[29]

Total Mitochondrial Protein and Mitochondrial Transmembrane Potential

Mitochondria were obtained after homogenization of liver tissue with 8 mL of isolation medium (250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid, and 10 mmol/L HEPES, pH 7.4) per gram of liver.[30] Liver was submerged in ice-cold buffer and homogenized using a glass Teflon, motorized Potter–Elvejhem homogenizer (B. Braun, Melsungen, Germany). Large cell debris and nuclei were pelleted by centrifuging the homogenate during 10 minutes at 800*g*. The supernatant was then centrifuged for 15 minutes at 8000*g*. The mitochondrial pellet was washed twice in isolation buffer by centrifugation at 8000*g* for 15 minutes. The entire procedure was performed at 4°C using a Beckman Centrifuge model J2-21 M/E and JA 20 rotor (Beckman Instruments, Palo Alto, CA). Total mitochondrial protein was determined in isolated mitochondria suspensions using Bradford's method.[29]

Mitochondrial transmembrane potential (MTP) was determined by flow cytometry in freshly isolated liver mitochondria after incubation with rhodamine 123, a membrane-potential–sensitive dye.[30,31] Washed mitochondria (20 μg protein/mL) were analyzed for fluorescence after staining with aqueous rhodamine 123 (Sigma Chemical Co., St. Louis, MO) at a concentration of 50 ng/mL (at this concentration the dye was not at saturation) in an Epics XL MCL cytofluorometer (Coulter Electronics, Hialeah, FL) with confocal optics and an argon laser (Spectra-Physic 2025-05; Mountain View, CA). For rhodamine 123, excitation was at 488 nm and 400 mW. Filters used were a 488-nm blocking filter, a 550-nm long-pass dichroic filter, and a 525-band pass filter. Each sample was flowing during 3 minutes, at a velocity of 25 μL/min. Measurements were made after 30-minute incubation in the presence of rhodamine 123. For the fluorescence expression, it was used the pulse integral on a four decade log scale.[30,31] Results are expressed as mean value of green fluorescence on a log scale [IFLg].[30,31]

In other experiments, kinetic determinations of rhodamine 123 uptake were performed by adding the dye to the mitochondria suspension already placed in the cytometer. The maximal rate of rhodamine 123 uptake is expressed as fluorescence arbitrary units (FAU) per minute after logarithmic to lineal transformation.[30,31] Mitochondrial autofluorescence was tested in the absence of dye in these experiments.

Statistical Analysis

Data are expressed as mean ± SEM. To assess the homogeneity among the three groups of rats, a Kruskall–Wallis test was used, followed by multiple post hoc comparisons using Mann–Whitney *U* tests with Bonferroni adjustment. Spearman's rank correlation coefficient was used to assess the relationship between fibrosis score and liver collagen content. A regression model was fitted considering MDA concentration and GSPHx activity as the dependent and independent variables, respectively. Any P value of <0.05 was considered to be statistically significant. Calculations were performed with SPSSWin v.6.0. program (SPSS Inc., Chicago, IL).

Results

Liver Function Tests

In protocol 1, on day 0 (10 days after interrupting CCl_4 administration), rats from groups CI and CI + IGF had similar values of serum aspartate aminotransferase (AST; 270 ± 34 vs. 284 ± 42 IU/L), alanine aminotransferase (ALT; 273 ± 48 vs. 278 ± 43 IU/L), bilirubin (1.01 ± 0.2 vs. 1.07 ± 0.19 mg/dL), alkaline phosphatase (763 ± 134 vs. 740 ± 136 IU/L), and glucose (119 ± 5 vs. 113 ± 4 mg/dL). In the two groups, these values were significantly different from those found in normal rats (55 ± 5 IU/L, 26 ± 2 IU/L, 0.48 ± 0.05 mg/dL, 310 ± 43 IU/L, and 221 ± 15 mg/dL, respectively, for the above mentioned parameters). At the end of the study (24 days after stopping CCl_4 inhalation), serum aminotransferase and bilirubin levels had decreased to a similar extent in both groups of cirrhotic rats, reaching values similar to those observed in healthy controls (Table 1). However, alkaline phosphatase level remained significantly increased at the end of the study in group CI but did not show significant differences from controls in group CI + IGF. Also, on day 15, glycemia was significantly lower in CI than in control rats (CO), but no differences from CO were found in CI + IGF animals ($P < 0.05$, CI vs. CI + IGF) (Table 1).

On day 0, groups CI and CI + IGF had serum albumin (3.1 ± 0.2 and 3.0 ± 0.3 g/dL, respectively) and total serum protein (6.4 ± 0.2 and 6.3 ± 0.1 g/dL, respectively) values that were lower than those of normal rats (3.6 ± 0.1 and 6.9 ± 0.1 g/dL for serum albumin and total proteins, respectively). However, as shown in Table 1, animals receiving IGF-I showed a rapid recovery of these parameters, reaching values similar to those of CO group on day 15, whereas no significant changes occurred in CI animals.

Results of clotting factors are shown in Figure 1. At the end of the study, prothrombin time was significantly prolonged in CI compared with both CO and CI + IGF groups, which showed similar values. Clotting factors II, VII, and X were significantly diminished in CI compared with either CO or CI + IGF; in the latter group, the values were not different from controls. The only exception was fibrinogen, a clotting factor that is also an acute-phase reactant. Its values were similar in the three groups of animals.

Rats included in protocol 2 represent a model of more advanced cirrhosis than rats from protocol 1. Groups CI

Table 1. Biochemical Data After Treatment With IGF-I or Vehicle in the Three Experimental Groups of the Two Protocols

	Protocol 1			Protocol 2		
Parameters	CO (n = 10)	CI (n = 10)	CI + IGF (n = 10)	CO (n = 10)	CI (n = 10)	CI + IGF (n = 10)
AST (*IU/L*)	60 ± 8	98 ± 29	65 ± 15	33 ± 1	142 ± 30[c]	86 ± 22[b]
ALT (*IU/L*)	27 ± 4	41 ± 6	40 ± 5	21 ± 1	69 ± 7[c]	54 ± 6[b]
Glucose (*mg/dL*)	204 ± 7	161 ± 6[c]	193 ± 10[d]	164 ± 5	110 ± 6[c]	139 ± 12
Albumin (*g/dL*)	3.5 ± 0.1	3.0 ± 0.7[a]	3.5 ± 0.2	3.3 ± 0.1	2.5 ± 0.2[b]	2.9 ± 0.1
Total proteins (*g/dL*)	7.1 ± 0.1	6.6 ± 0.3[a]	7.2 ± 0.3	6.7 ± 0.2	5.6 ± 0.4[a]	6.3 ± 0.2
ALP (*IU/L*)	204 ± 13	549 ± 127[a]	396 ± 58	199 ± 10	457 ± 66[b]	350 ± 47[b]
Bilirubin (*mg/dL*)	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	1.2 ± 0.4[a]	0.4 ± 0.1[d]

NOTE. Values are mean ± SEM. Protocol 1 included animals with compensated cirrhosis, and the CI + IGF group was treated for 2 weeks after discontinuation of CCl_4. In protocol 2, cirrhotic rats had ascitic cirrhosis, and IGF treatment was administered for 3 weeks without stopping exposure to CCl_4.
ALP, alkaline phosphatase.
[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$; CO vs. other groups.
[d]$P < 0.05$; CI vs. CI + IGF.

and CI + IGF from protocol 2 showed similar baseline values of AST (95 ± 18 vs. 101 ± 20 IU/L), ALT (51 ± 5 vs. 53 ± 5 IU/L), alkaline phosphatase (565 ± 83 vs. 575 ± 96 IU/L), bilirubin (1.1 ± 0.4 vs. 1.1 ± 0.3 mg/dL), blood glucose (133 ± 11 vs. 132 ± 12 mg/dL), albumin (2.4 ± 0.2 vs. 2.3 ± 0.2 g/dL), and total proteins (5.71 ± 0.9 vs. 5.68 ± 0.9 g/dL), and all these values were significantly different from those found in healthy controls (35 ± 2, 21 ± 1, 240 ± 17, 0.3 ± 0.1, 161 ± 9, 3.34 ± 0.03, and 6.7 ± 0.1, respectively, for the above-mentioned parameters). In agreement with findings in protocol 1, at the end of the study, CI rats had blood glucose, albumin, and total protein values that were significantly lower than those of CO; in the CI + IGF group, these parameters were not significantly different from those found in normal rats (Table 1). Also, on day 21, CI + IGF animals had serum bilirubin values that were significantly lower than in untreated cirrhotic rats and similar to those of controls (Table 1). Serum transaminase and alkaline phosphatase levels, however, remained significantly elevated compared with CO not only in CI animals but also (although to a lesser extent) in the CI + IGF group (Table 1). It should be considered that in protocol 2 rats were treated with IGF-I during the last 3 weeks of the study without interrupting the exposure to the toxin.

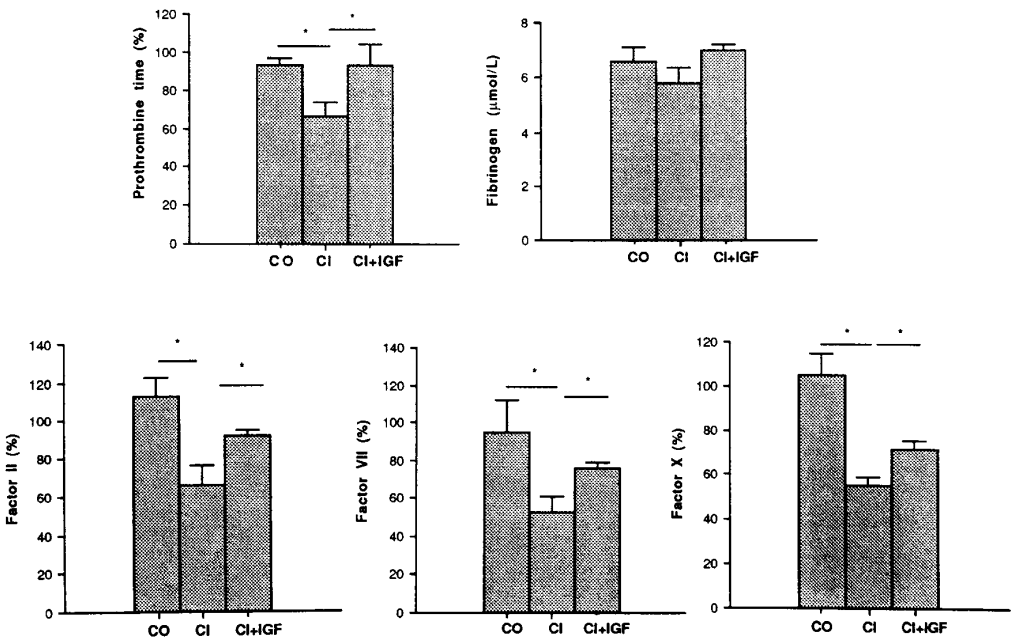

Figure 1. Clotting factors II, VII, and X, fibrinogen level, and prothrombin time in the three groups of animals studied (protocol 1): healthy rats (CO), untreated cirrhotic rats (CI), and cirrhotic rats treated with IGF-I (CI + IGF). *$P < 0.05$.

Liver Histology and Liver Collagen Content

In protocol 1, the histological score of fibrosis was significanly lower in CI + IGF than in CI rats (7.26 ± 0.60 vs. 9.88 ± 0.47; $P < 0.01$) (Figure 2). In accordance with histological findings, the biochemical quantitation of liver collagen content (micrograms of collagen per milligram of protein) was also lower in IGF-I–treated rats than in CI animals (51.39 ± 2.41 vs. 64.22 ± 4.38; $P < 0.01$) and significanly higher in these two groups of rats than in healthy controls (32.67 ± 1.28; $P < 0.001$ for both). As seen in Figure 3, there was a close direct correlation between the histological score of fibrosis and liver collagen content ($r = 0.758$; $P < 0.001$). The histological score of fibrosis was also lower in CI + IGF than in CI rats from protocol 2 (CI + IGF, 11.6 ± 0.4; CI, 13.2 ± 0.3; $P < 0.05$).

Lipid Peroxidation and Activities of Antioxidant Enzymes

In the two experimental protocols, the hepatic levels of the lipid peroxidation products (estimated as nanomoles of MDA per gram of tissue) were increased in CI rats compared with healthy controls (protocol 1: CI, 138 ± 25; CO, 39.0 ± 2.4; $P < 0.05$; protocol 2: CI, 133.0 ± 26.9, CO, 61.8 ± 1.6; $P < 0.05$). This finding is in accordance with the known toxic effect of CCl_4 that causes oxidative damage in the liver. At first glance it seems paradoxical that liver MDA levels per gram of tissue are similar in CI rats from protocols 1 and 2 because there was a longer exposure to the toxic in the last protocol. However, it must be considered that because the severity of liver fibrosis is higher in rats from protocol 2, the amount of parenchyma per gram of liver tissue should be expected to be reduced in these animals. Interestingly, we found that MDA values in CI + IGF animals from the two protocols were not significantly different from those observed in normal rats (CI + IGF-I: protocol 1, 70 ± 9.4; protocol 2, 92.2 ± 6.6). In protocol 1, MDA values in CI + IGF rats were significantly reduced compared with those obtained in CI animals ($P < 0.05$) (Figure 4*A*). In protocol 2, CI + IGF rats also tended to have less lipid peroxidation than CI animals, but differences did not reach statistical significance (Figure 4*B*).

On the basis of these results it seems that IGF-I administration reduces lipid peroxidation in rats with experimental cirrhosis. In additional experiments, we also analyzed whether IGF-I might also reduce liver MDA levels in healthy normal rats. Ten normal rats from protocol 1 were treated with IGF-I for 2 weeks, and MDA levels were measured in liver tissue. We observed a significant reduction in liver MDA values in this group of animals (28.5 ± 1.6) compared with untreated healthy controls (39.0 ± 2.4; $P < 0.05$).

Because the protective effects of IGF-I against oxidative damage are apparent both in protocol 1 (CCl_4 discontinued 10 days before initiation of treatment) and in protocol 2 (treatment with IGF-I during the last 3 weeks of CCl_4 administration), it seems that IGF-I does not act by modifying CCl_4 metabolism but rather by modulating the generation of free radicals by the damaged liver and/or by stimulating cell defenses against oxidative stress. Table 2 shows activities of the antioxidant enzymes SOD, GHSPx, and catalase in the liver in healthy control rats, cirrhotic rats, and cirrhotic rats treated with IGF-I. As can be seen, these values were significantly higher in CI + IGF rats than in CI animals in the two protocols, with the sole exception of catalase in protocol 2. Interestingly, a close inverse correlation was found between MDA levels (reflecting oxidative damage) and GSHPx activity (protection against oxidative stress) both in protocol 1 ($r = -0.91$; $P < 0.001$) and in protocol 2 ($r = -0.89$; $P < 0.001$) (Figures 5*A* and *B*). IGF-I treatment showed no effect on the activity of antioxidant enzymes in normal livers (data not shown).

MTP and Protein Content of the Mitochondrial Fraction

Because it has been shown that injured mitochondria may generate free radicals and thus perpetuate cell damage in the diseased liver,[10,12,20,32,33] we investigated whether IGF-I treatment of cirrhotic rats could influence mitochondrial function. Thus, we analyzed protein content of the mitochondrial fraction and MTP, a sensitive marker of mitochondrial integrity, in rats from protocol 2.

As shown in Table 3, total protein content of the mitochondrial fraction was significantly reduced in untreated cirrhotic rats compared with controls, whereas CI + IGF-I animals had significantly higher values than untreated cirrhotic rats and values similar to those of healthy rats. Similarly, MTP, as estimated by the intensity of fluorescence after 30-minute incubation with rhodamine 123 suspension (IFLg; Table 3), was significantly decreased in CI rats but was within normal values in CI + IGF animals. In addition, the uptake of rhodamine 123 was significantly faster in the CI + IGF group than in CI animals (Table 3). These findings indicate disturbed mitochondrial function in rats with CCl_4-induced cirrhosis and improved mitochondrial integrity in the group of cirrhotic rats treated with IGF-I.

Discussion

This study analyzed the effect of IGF-I on liver damage and function in rats with CCl_4-induced cirrhosis.

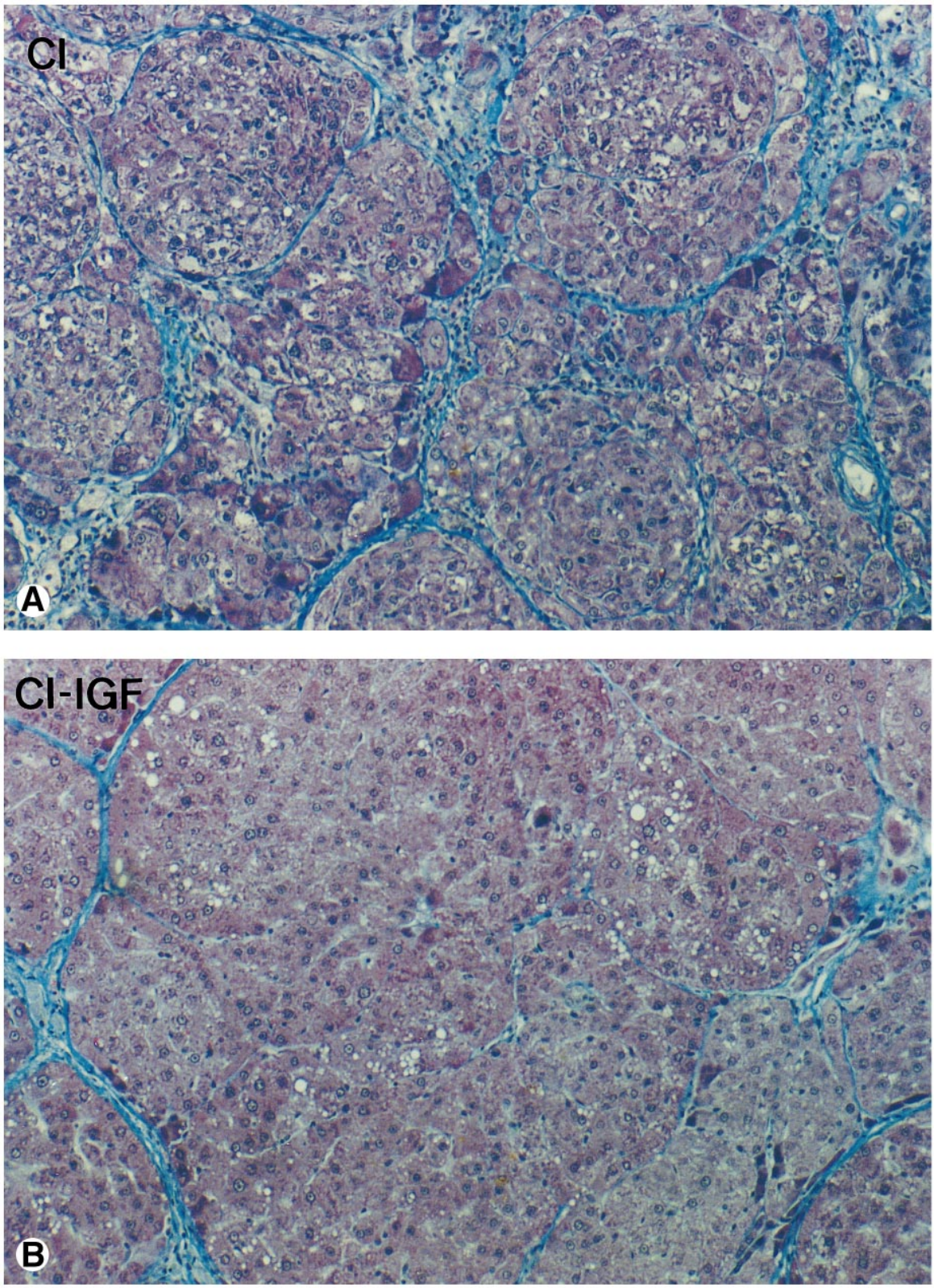

Figure 2. Histopathologic findings in liver biopsy from rats included in protocol 1 (4-μm sections; Masson's trichrome stain; original magnification 150x). (*A*) CI, untreated cirrhotic rat; (*B*) CI + IGF, cirrhotic rat treated with IGF-I. A more intense degree of fibrosis is observed in the CI group. Liver collagen content in these two animals was 77.35 and 53.70 μg collagen/mg protein for CI and CI + IGF, respectively.

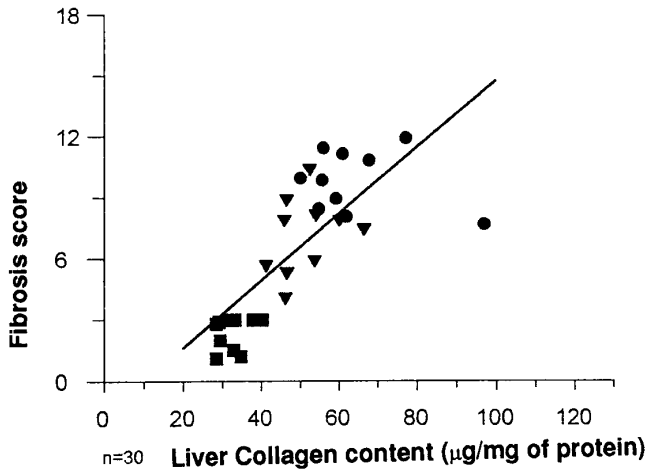

Figure 3. Correlation between the histological score of fibrosis and the quantitative estimation of liver collagen content in rats from protocol 1. ■, Healthy rats; ●, untreated cirrhotic rats; ▼, cirrhotic rats treated with IGF-I ($r = 0.758$; $P < 0.001$).

Two different protocols were used: rats from protocol 1 showed compensated cirrhosis and were treated with IGF-I for 2 weeks starting 10 days after interruption of CCl_4, and rats from protocol 2 showed advanced cirrhosis and IGF-I was given during the last 3 weeks of the study without interrupting CCl_4 administration. In the two protocols, IGF-I at low doses (2 μg/100 g body wt daily) was found to improve liver biosynthetic functions, to exert protection against oxidative liver damage, and to reduce hepatic fibrosis.

In the two protocols, untreated cirrhotic rats showed a significant reduction of serum albumin, total protein, and blood glucose levels, whereas in CI + IGF animals these parameters did not differ from healthy controls. Moreover, in compensated cirrhotic rats, IGF-I treatment significantly increased clotting factor activity and normalized prothrombin time. These results are in accordance with the wide range of anabolic activities described for IGF-I,[8] but the reduction in liver fibrosis observed in cirrhotic rats treated with IGF-I observed in the two protocols was an unexpected finding.

The in vivo antifibrogenic effect of IGF-I in CCl_4-induced cirrhosis is in apparent contrast with the reported role for IGF-I as stimulator of Ito cell proliferation in vitro[34–37] and with the observation of increased expression of IGF receptors during the process of fibrogenesis.[38] The combined effects of improving liver function and reducing collagen deposition prompted us to analyze the influence of IGF-I therapy on peroxidative liver damage, because this process is the essential mechanism of cell injury in CCl_4-induced cirrhosis[9]; on the other hand, lipid peroxidation products are important inducers of collagen gene expression.[16]

Oxidative insult causes lipid peroxidation of cell and organelle membranes, oxidation of critical thiol groups, and disruption of cytoskeletal integrity leading to cell necrosis.[9,10] Mitochondria are recognized as the main sites at which peroxidation of membrane lipids takes place.[32,33] Damaged mitochondria, in turn, generate toxic amounts of reactive oxygen species,[20,32] thus contributing to cell damage even after cessation of the initial toxic insult.[9,23]

Our results suggest that the observed cytoprotective effect of IGF-I in animals with CCl_4-induced liver injury may be partly caused by decreased peroxidative cell damage. In the two protocols, the levels of lipid peroxidation products in livers from CI rats were significantly higher than in normal controls but they were not different from normal in CI + IGF group. The antioxidant effect of IGF-I was more apparent in protocol 1, in which the toxic substance had been stopped days before the initiation of IGF-I administration, than in protocol 2, in which CCl_4 was not withdrawn.

In an attempt to characterize the protection afforded

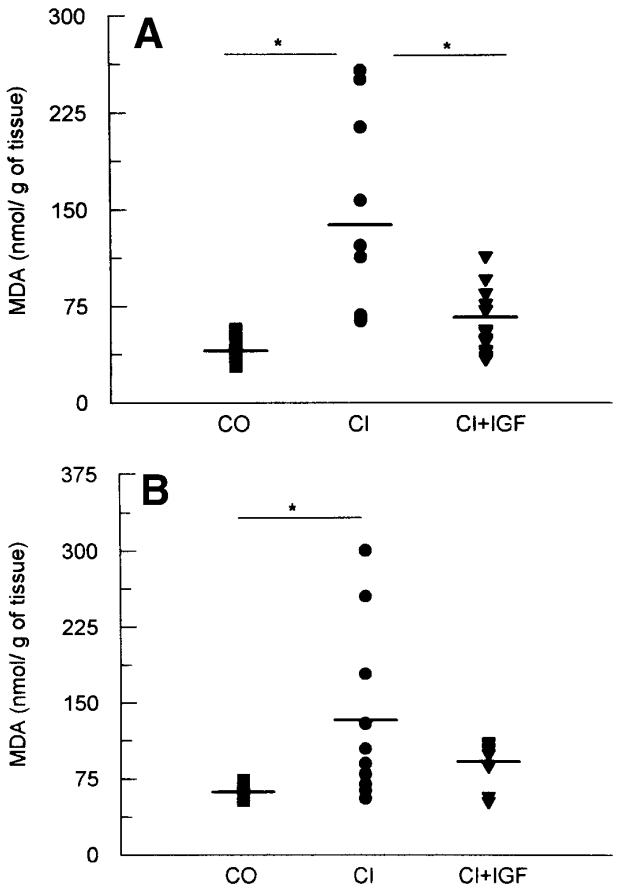

Figure 4. Values of MDA, a marker of lipid peroxidation, in liver tissue from control animals (CO), cirrhotic (CI), and CI + IGF groups with compensated cirrhosis (protocol 1). *$P < 0.05$; n = 10. (*A*) Protocol 1; (*B*) protocol 2.

Table 2. Antioxidant Enzyme Activities in Liver Tissue

Parameters	Protocol 1			Protocol 2		
	CO (n = 10)	CI (n = 10)	CI + IGF (n = 10)	CO (n = 10)	CI (n = 10)	CI + IGF (n = 10)
SOD (*U/mg protein*)	19.8 ± 1.8	16.2 ± 1.7[a]	21.0 ± 2.4[b]	21 ± 1.8	16.2 ± 1.7[a]	20.4 ± 1.9[b]
GSHPx (*U/mg protein*)	2.38 ± 0.10	1.64 ± 0.11[a]	2.01 ± 0.14[b]	2.52 ± 0.06	1.44 ± 0.12[a]	1.92 ± 0.12[a,b]
Catalase (*K/mg protein*)	0.971 ± 0.10	0.517 ± 0.099[a]	0.984 ± 0.10[b]	0.950 ± 0.024	0.413 ± 0.35[a]	0.428 ± 0.039[a]

NOTE. Values are mean ± SEM. Protocols and groups of animals are as in Table 1.
[a]$P < 0.05$, CO vs. other groups.
[b]$P < 0.05$, CI vs. CI + IGF.

by IGF-I against free radical damage, we investigated the effect of IGF-I on antioxidant enzymes and on mitochondrial function, because the former are relevant components of the cell defense against pro-oxidant injury and damaged mitochondria are an important source of free radicals. An additional mechanism to explain the cytoprotective activity of IGF-I against pro-oxidant injury could be through the stimulation of reduced synthesis, as has been described for *S*-adenosylmethionine treatment.[39] However, this last mechanism was not explored in the present study.

In our study, the activities of the antioxidant enzymes SOD, GSHPx, and catalase were reduced in untreated cirrhotic rats but returned to normal (with the exception of catalase in protocol 2) in cirrhotic rats receiving IGF-I. The effect of IGF-I enhancing GSHPx activity in cirrhotic livers may have special pathophysiological importance because this enzyme is considered to be the main enzymatic defense against the oxidative destruction of biomembranes in mitochondria and other organelles; it is also known to participate in the metabolism of lipid hydroperoxides.[21,40] In fact, we have observed a close inverse correlation between hepatic levels of lipid peroxidation products and the activity of GSHPx in liver tissue. It is possible that these changes in SOD, GSHPx, and catalase activities do not reflect a specific effect of IGF-I on antioxidant enzymatic systems but rather the general anabolic properties of the hormone, promoting protein synthesis in damaged liver cells.

As mentioned, injured mitochondria can be an im-

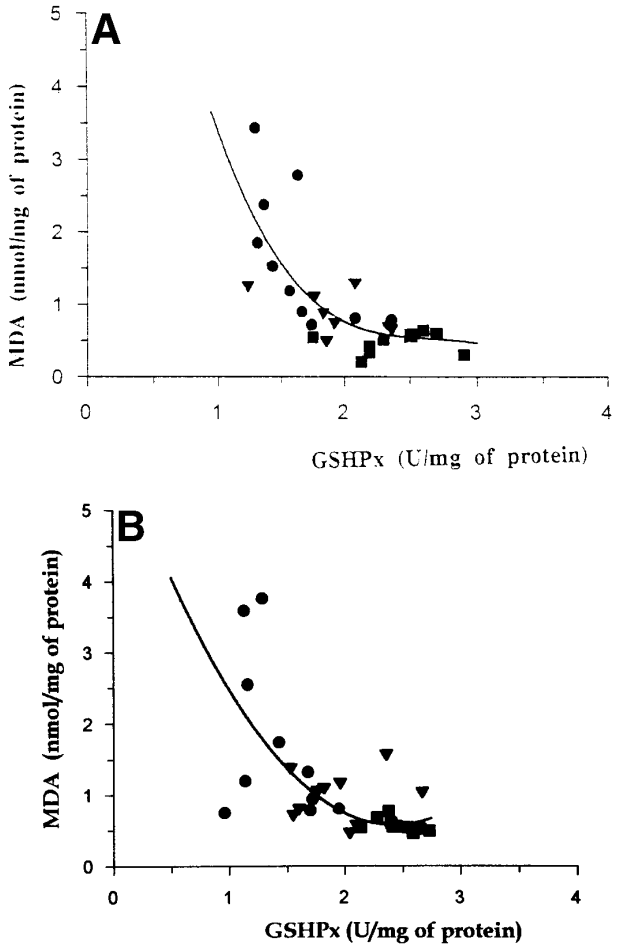

Figure 5. Correlation between concentrations of MDA, a marker of lipid peroxidation, and GSHPx activity (a key antioxidant enzyme) in homogenates of liver tissue in the three groups of rats studied (n = 30). ■, Healthy rats; ●, untreated cirrhotic rats; ▼, cirrhotic rats treated with IGF-I. (*A*) Protocol 1 ($r = -0.91$; $P < 0.001$); (*B*) protocol 2 ($r = -0.89$; $P < 0.001$).

Table 3. Total Mitochondrial Protein Content and Fluorescence Intensity of Washed Mitochondria Stained With Rhodamine 123 and Analyzed by Flow Cytometry in Healthy Control Rats, Untreated Cirrhotic Rats, and Cirrhotic Rats Treated With IGF-I (Protocol 2)

	CO (n = 5)	CI (n = 5)	CI + IGF (n = 5)
IFLg	24.6 ± 2.4	18.4 ± 0.7[a]	24.7 ± 2.8[b]
Rate of Rh123 uptake (*FAU/min*)	17.33 ± 0.64	3.62 ± 0.72[a]	14.7 ± 0.68[b]
Total proteins in the mitochondrial fraction (*mg/g liver*)	22 ± 2	12 ± 5[a]	21 ± 2[b]

Rh123, rhodamine 123.
[a]$P < 0.05$, CO vs. other groups.
[b]$P < 0.05$, CI vs. CI + IGF.

portant source of free radicals and thus contributes to persisting cell damage.[23,32,33] In this study, IGF-I treatment was associated with a recovery of MTP and mitochondrial protein content to normal. It seems that IGF-I can stimulate mitochondrial protein synthesis and improve mitochondrial function. This effect can be an additional mechanism to explain the antioxidant and hepatoprotective activity displayed by this hormone in experimental liver cirrhosis. Further studies are needed to characterize the effects of IGF-I on mitochondrial integrity in toxic liver injury.

Lipid peroxidation has been proposed as a common pathway for fibrogenesis.[16] The significant antifibrogenic effect exerted in vivo by IGF-I in this model of cirrhosis might be related to the observed antioxidant activities of this hormone. Because pro-oxidant hepatocellular injury and resulting fibrogenesis occurs in a diversity of human liver diseases, such as alcoholic hepatitis,[10,12,41] our results provide an experimental basis for further studies aiming at analyzing the potential of IGF-I in the treatment of these conditions.

In type II diabetes mellitus, the use of IGF-I at daily doses of 24 μg/100 g body wt induced decreases in fasting and postprandial blood glucose levels. This hypoglycemic effect, together with the reduction in insulin levels, can attenuate the anabolic effect of IGF-I when administered at these doses.[42] On the other hand, doses of 32–24 $\mu g \cdot 100\ g\ body\ wt^{-1} \cdot day^{-1}$ have also been shown to cause adverse effects such as arthralgia, generalized myalgia, exhaustion, jaw pain, and edema of face and hands.[43] In the present study, the hepatoprotective and antifibrogenic activities of IGF-I were observed at doses as low as 2 $\mu g \cdot 100\ g\ body\ wt^{-1} \cdot day^{-1}$ (12–16-fold inferior to those used in the clinical trial mentioned above). Hypoglycemia was not observed and blood glucose in IGF-I–treated animals did not differ from healthy normal rats. In fact, in CI + IGF rats, blood glucose was significantly higher than in untreated cirrhotic animals, probably reflecting this finding the overall improvement in hepatocellular function. Finally, it should be noted that, as reported in our previous study,[7] doses of 2 $\mu g \cdot 100\ g\ body\ wt^{-1} \cdot day^{-1}$ do not significantly increase circulating levels of IGF-I in rats with nondecompensated cirrhosis but induce significant changes in the pattern of IGF binding proteins, which are critical modulators of the biological actions of IGF-I.[8]

In summary, we describe a previously unrecognized activity of IGF-I on diseased liver. In CCl_4-induced cirrhosis, IGF-I at low doses decreases lipid peroxidation, improves liver function, and reduces collagen deposition. These findings may stimulate further research on the therapeutical potential and mechanisms of action of IGF-I in situations in which hepatocellular damage and fibrogenesis occur as a consequence of pro-oxidant injury to the liver.

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