

## Insulin-Like Growth Factor and Epidermal Growth Factor Treatment: New Approaches to Protecting Steatotic Livers against Ischemia-Reperfusion Injury

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Hepatic steatosis is a major risk factor in ischemia-reperfusion (I/R). IGF-binding proteins (IGFBPs) modulate IGF-I action by transporting circulating IGF-I to its sites of action. Epidermal growth factor (EGF) stimulates IGF-I synthesis *in vitro*. We examined the effect of IGF-I and EGF treatment, separately or in combination, on the vulnerability of steatotic livers to I/R. Our results indicated that I/R impaired IGF-I synthesis only in steatotic livers. Only when a high dose of IGF-I (400  $\mu\text{g}/\text{kg}$ ) was given to obese animals did they show high circulating IGF-I:IGFBP levels, increased hepatic IGF-I levels, and protection against damage. In lean animals, a dose of 100  $\mu\text{g}/\text{kg}$  IGF-I protected nonsteatotic livers. Our results indicated that the combined administration of IGF-I and EGF resulted in hepatic injury parameters in both liver types similar to that obtained by IGF-I and EGF separately. IGF-I increased *egf* expression in both liver types. The beneficial role of EGF on hepatic I/R injury may be attributable to p38 inhibition in nonsteatotic livers and to PPAR $\gamma$  overexpression in steatotic livers. In conclusion, IGF-I and EGF may constitute new pharmacological strategies to reduce the inherent susceptibility of steatotic livers to I/R injury. (*Endocrinology* 150: 3153–3161, 2009)

**H**epatic steatosis is a major risk factor after liver surgery because steatotic livers show poor tolerance to ischemia-reperfusion (I/R) (1). Operative mortality associated with steatosis exceeds 14% after major resection compared with 2% for healthy livers (2, 3). In the case of transplantation, steatotic grafts are associated with a primary nonfunction rate of 60% compared with less than 5% for nonsteatotic grafts (4, 5). Therefore, developing protective strategies to minimize the adverse effects of I/R injury in steatotic livers is an urgent need.

IGF-I is member of the IGF superfamily and participates in numerous pathophysiological processes (6–9). The IGF-binding proteins (IGFBPs), which are abundant in the bloodstream, organs, and tissues act as major modulators of IGF-I action by transporting IGF-I to its sites of action (7, 10, 11). Interaction between growth factor families is a field of grow-

ing interest. Various *in vitro* studies indicate that epidermal growth factor (EGF) stimulates IGF-I synthesis in renal cells and isolated hepatocytes (12–14).

To our knowledge, the beneficial effects of IGF-I on hepatic I/R injury have been reported only in nonsteatotic livers (15), whereas the effects of EGF on hepatic I/R injury remain unknown. The first purpose of this study was to evaluate whether treatment with IGF-I and EGF could improve the poor tolerance of steatotic livers to I/R. Given that EGF enhances *igf1* expression in several cell types (12–14), the second purpose of this study was to evaluate whether EGF provides a stimulus for *igf1* expression in hepatic I/R. Finally, the third purpose of the present study was to evaluate the involvement of p38 MAPK (p38) and peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) in the effects of IGF-I and EGF on hepatic I/R injury in steatotic and nonsteatotic livers.

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Abbreviations: EGF, Epidermal growth factor; IGFBP, IGF-binding protein; I/R, ischemia-reperfusion; Ln, lean; Ob, obese; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ .

This approach was based on 1) previous studies from our group revealing the key role of both p38 and PPAR $\gamma$  in nonsteatotic and steatotic livers, respectively, under I/R conditions (16, 17) and 2) data reported in the literature indicating that IGF-I and EGF affect p38 and PPAR $\gamma$  in several different conditions (18–21). Our findings could lead to the design of new pharmacological strategies in liver surgery for reducing hepatic I/R injury.

## Materials and Methods

### Experimental animals

Homozygous (obese, Ob) and heterozygous (lean, Ln) Zucker rats (Iffa-Credo, Lábresle, France) aged 16–18 wk were used. Ob Zucker rats have a mutated leptin receptor and, as a consequence, are hyperphagic, obese, and hyperinsulinemic, because they are insulin resistant, but have normal blood glucose levels. Ob Zucker rats do not develop diabetes. Ln Zucker rats maintain a lean phenotype throughout life, with normal blood insulin and glucose levels (22, 23). Control experiments from our group confirmed that none of the drugs included in the present study altered the plasma insulin or glucose levels in Ob Zucker rats. Ob Zucker rats showed severe macrovesicular and microvesicular fatty infiltration in hepatocytes (60–70% steatosis). Ln Zucker rats showed no evidence of steatosis. The animals were anesthetized with ketamine and xylazine (100 and 8 mg/kg, respectively) (17). The study followed European Union regulations (ECC Directive 86/609) governing animal experiments.

### Experimental design

All animals were randomly sorted into groups.

#### Protocol 1: effects of IGF-I and EGF administration on hepatic I/R

Animals were divided into five groups: 1) sham (n = 12, six Ln and six Ob), in which hepatic hilum vessels were dissected; I/R (n = 12, six Ln and six Ob), in which 60 min partial (70%) hepatic ischemia was followed by 24 h reperfusion (17); 3) subgroup 3.1, IGF-I (100) (n = 12, six Ln and six Ob), same as group 2 but treated with IGF-I at doses of 100  $\mu$ g/kg (every 12 h for two doses, starting immediately before surgical procedure) (24, 25), and subgroup 3.2, IGF-I (400) (n = 6 Ob), same as group 2 but treated with IGF-I at doses of 400  $\mu$ g/kg (every 12 h for two doses, starting immediately before surgical procedure) (24, 25); 4) EGF (n = 12, six Ln and six Ob), same as group 2 but treated with EGF at doses of 100  $\mu$ g/kg (every 8 h for three doses, starting immediately before surgical procedure) (26); and 5) IGF-I+EGF (n = 12, six Ln and six Ob), same as group 2 but treated with IGF-I at doses of 100  $\mu$ g/kg in Ln and 400  $\mu$ g/kg in Ob and EGF at doses of 100  $\mu$ g/kg for Ln and Ob. The pretreatment times for IGF-I and EGF were as described above (24–26).

Preliminary studies from our group based on dose response demonstrated that the doses of IGF-I (100  $\mu$ g/kg for Ln and 400  $\mu$ g/kg for Ob) and EGF (100  $\mu$ g/kg for Ln and Ob) were the most effective in protecting the two liver types against I/R damage.

#### Protocol 2: role of p38 in the effects of EGF on hepatic I/R injury

Animals were divided into three additional groups: 6) p38 inhibitor (n = 6 Ln), same as group 2 but treated with SB203580, a p38 inhibitor, at a dose of 1 mg/kg 24 h before surgical procedure (27); 7) p38 activator (n = 6 Ln), same as group 2 but treated with anisomycin, a p38 activator, at a dose of 0.1 mg/kg 24 h before surgical procedure (27); and 8) EGF+p38 activator (n = 6 Ln), same as group 2 but treated with EGF at doses of 100  $\mu$ g/kg and anisomycin, a p38 activator, at a dose of 0.1 mg/kg 24 h before surgical procedure (27). The pretreatment times for EGF were as described above (26).

#### Protocol 3: role of PPAR $\gamma$ in the effects of EGF on hepatic I/R injury

Again, animals were divided into three additional groups: 9) PPAR $\gamma$  antagonist (n = 6 Ob), same as group 2 but treated with GW9662, a PPAR $\gamma$  antagonist, at a dose of 1 mg/kg before surgical procedure (17); 10) PPAR $\gamma$  agonist (n = 6 Ob), same as group 2 but treated with rosiglitazone, a PPAR $\gamma$  agonist, at a dose of 1.5 mg/kg before and after surgical procedure (17); and 11) EGF+PPAR $\gamma$  antagonist (n = 6 Ob), same as group 2 but treated with EGF at a dose of 100  $\mu$ g/kg and GW9662, a PPAR $\gamma$  antagonist, at a dose of 1 mg/kg before surgical procedure (17). The pretreatment times for EGF were as described above (26).

Plasma and liver samples were collected after hepatic reperfusion.

### Quantitative RT-PCR

Quantitative real-time PCR analysis was performed by the pre-made Assays-on-Demand TaqMan probes (Rn00710306\_m1 for *igf1*, Rn00563336\_m1 for *egf*, and Rn00667869\_m1 for  $\beta$ -actin) (Applied Biosystems, Foster City, CA). The TaqMan gene expression assay was performed according to the manufacturer's protocol (Applied Biosystems).

### Western blotting of IGF-I, EGF, IGFBP-3, p38, PPAR $\gamma$ , and $\beta$ -actin

Western blotting was performed as described elsewhere (16, 17), using the following antibodies: IGF-I, EGF, and IGFBP-3 (Santa Cruz Biotechnology, Santa Cruz, CA) total p38 and phosphorylated p38 (Cell Signaling Technology Inc., Beverly, MA), PPAR- $\gamma$  (Abcam, UK), and  $\beta$ -Actin (Sigma Chemical Co., St. Louis, MO).

### Biochemical determinations

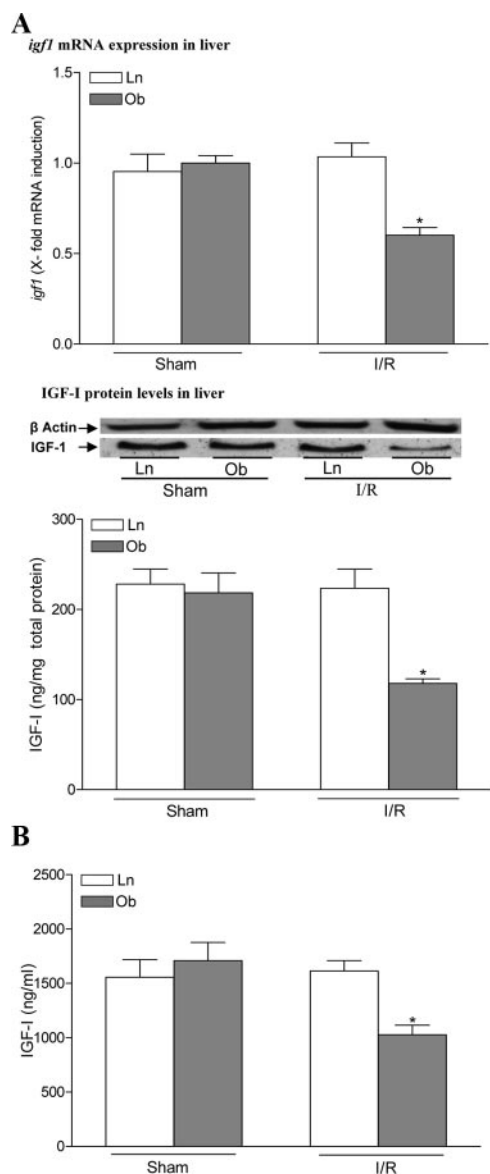
Transaminases were evaluated according to standard procedures. The concentration of total IGF-I in plasma was determined by a modified acid-ethanol (0.25 N HCl/87.5% ethanol) procedure with cryoprecipitation. Then, total IGF-I in plasma was determined by ELISA kit from Immunodiagnostic Systems (Fountain Hills, AZ) according to the manufacturer's instructions. The plasma concentration of free IGF-I was determined by centrifugal ultrafiltration. Briefly, the plasma samples were diluted 1:5 with Krebs-Ringer bicarbonate buffer (pH 7.4, with 5% BSA) and prefiltered through a 0.22- $\mu$ m filter (Millex-GV; Millipore, Molsheim, France) to remove debris. The prefiltered samples were then added to Amicon YMT 30 membranes and MPS-1 supporting devices (Amicon Division, W. R. Grace, Beverly, MA) and centrifuged at 300  $\times$  g at 37 C for 100 min. The ultrafiltrate was collected from 40–100 min centrifugation and used for the determination of IGF-I by an ELISA kit from Immunodiagnostic Systems following manufacturer's guidelines (28, 29). IGFBP-3 levels in plasma were determined by using an ELISA kit from Diagnostic Systems Laboratories (Webster, TX), according to manufacturer's instructions (30).

### Histology and red-oil staining

Hematoxylin- and eosin-stained sections were evaluated as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (31, 32). Steatosis in liver was evaluated by red-oil staining on frozen specimens, according to standard procedures.

### Statistics

Data are expressed as means  $\pm$  SE and were compared statistically by ANOVA, followed by Student-Newman-Keuls test.  $P < 0.05$  was considered significant.



**FIG. 1.** A, *igf1* mRNA expression and IGF-I protein levels in liver. For *igf1* mRNA expression in liver, PCR fluorescent signals for IGF-I were standardized to PCR fluorescent signals obtained from an endogenous reference ( $\beta$ -actin). Comparative and relative quantifications of *igf1* gene products normalized to  $\beta$ -actin and control sham group were calculated by the  $2^{-\Delta\Delta CT}$  method. For IGF-I protein levels in liver, representative Western blot (top) and densitometric analysis (bottom) were performed. B, IGF-I protein levels in plasma. \*,  $P < 0.05$  vs. sham.

## Results

### Effect of I/R on hepatic and circulating IGF-I

In Ln animals, I/R resulted in hepatic *igf1* mRNA and IGF-I protein levels that were similar to those of the sham group (Fig. 1A). However, in Ob animals, I/R reduced hepatic *igf1* mRNA and IGF-I protein levels when compared with the sham group (Fig. 1A). Circulating IGF-I is mainly derived from the liver (33, 34). Our results indicated that IGF-I levels in plasma (Fig. 1B) showed a similar pattern to IGF-I observed in the liver (Fig. 1A).

### Effects of IGF-I administration on hepatic I/R

In Ln animals, I/R resulted in total and free IGF-I in plasma that was similar to that in the sham group (Fig. 2A). In Ob

animals, I/R reduced total and free IGF-I levels in plasma when compared with the sham group. IGF-I administration in Ln and Ob animals increased total and free plasma IGF-I levels when compared with the I/R group (Fig. 2A). IGF-BPs act as major modulators of IGF-I action by transporting circulating IGF-I to their sites of action (7, 10, 11). In Ln animals, I/R resulted in a percentage of IGF-I bound to IGF-BPs (IGF-I:IGFBP complex) in plasma that was similar to that in the sham group (Fig. 2A). In Ob animals, I/R reduced IGF-I:IGFBP complex in plasma when compared with the sham group (Fig. 2A). As previously mentioned, in Ob animals, I/R reduced hepatic IGF-I protein levels when compared with the sham group (Fig. 2B). Transaminase and damage score values of the I/R group were higher in steatotic livers than in nonsteatotic livers (Fig. 2C), indicating that steatotic livers are more vulnerable to hepatic I/R damage. The administration of IGF (100  $\mu$ g/kg) in Ln animals increased the IGF-I:IGFBP complex in plasma compared with the I/R group or the sham group (Fig. 2A). This was associated with increased hepatic IGF-I levels (Fig. 2B) and reduced hepatic injury (Fig. 2C). At the same dose (100  $\mu$ g/kg), IGF-I administration in Ob animals increased IGF-I:IGFBP complex in plasma over that recorded in the I/R group, although levels were similar to those of the sham group (Fig. 2A). This was not associated with increases in hepatic IGF-I levels (Fig. 2B) or protection against hepatic damage (Fig. 2C) when compared with the I/R group. However, the administration of IGF-I (400  $\mu$ g/kg) in Ob animals resulted in a marked increase in circulating IGF-I:IGFBP levels (Fig. 2A), which was associated with increased hepatic IGF-I levels (Fig. 2B) and reduced hepatic injury (Fig. 2C).

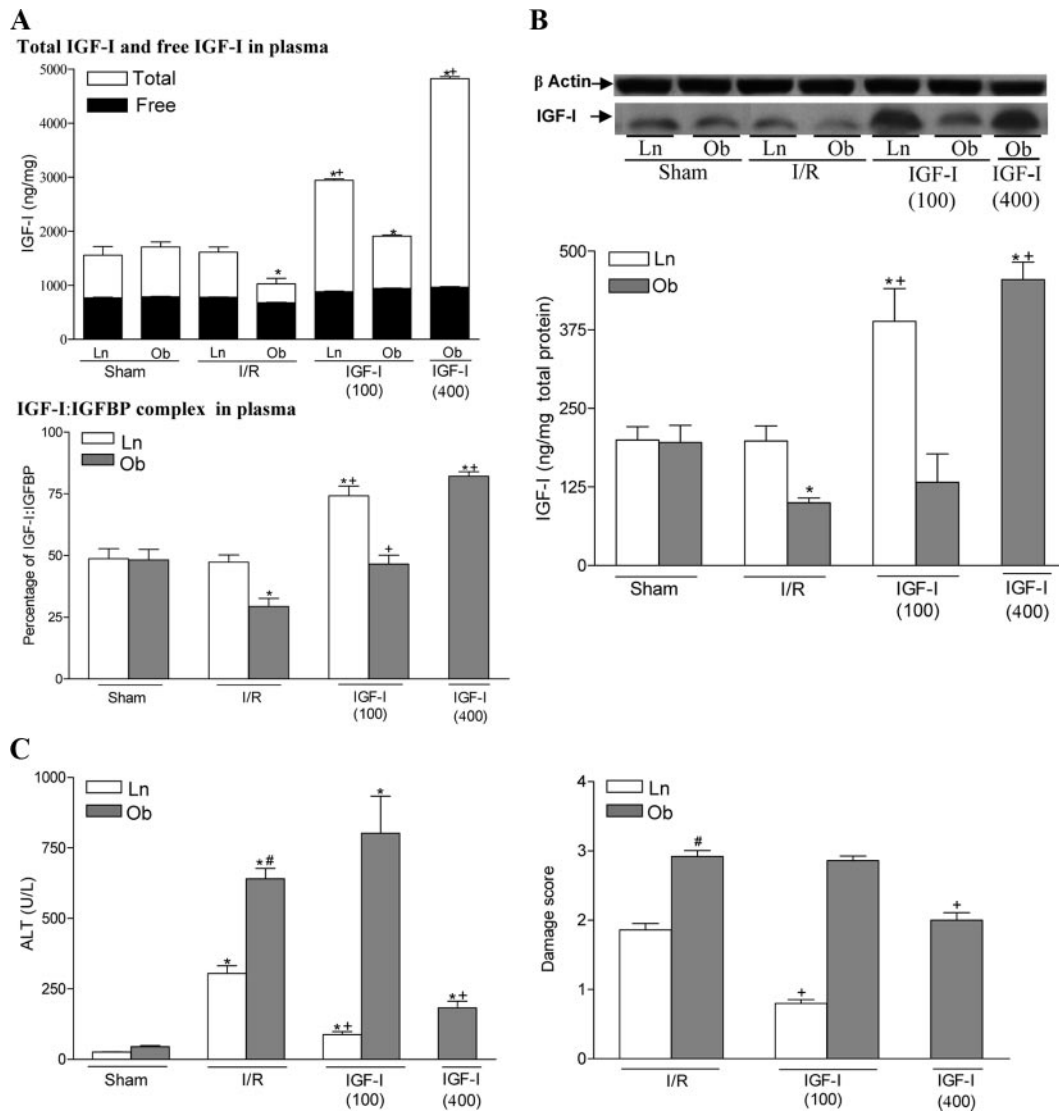
### Effect of IGF-I on circulating and hepatic IGF-BP-3

IGFBP-3 binds the majority of circulating IGF-I (~90%) (33, 35).

In both Ln and Ob animals, I/R led to circulating IGF-BP-3 levels similar to those of the sham group (Fig. 3A). The administration of IGF-I at the effective dose (100 and 400  $\mu$ g/kg in Ln and Ob animals, respectively) reduced IGF-BP-3 in plasma when compared with the I/R group. In both Ln and Ob animals, I/R led to hepatic IGF-BP-3 levels similar to those of the sham group (Fig. 3A). IGF-I administration at the effective dose in both Ln and Ob animals increased hepatic IGF-BP-3 protein levels when compared with the I/R group (Fig. 3A) but did not alter hepatic *igfbp3* mRNA levels. Thus, the *igfbp3* values (expressed as x-fold mRNA induction) in nonsteatotic livers were  $0.89 \pm 0.24$  and  $0.88 \pm 0.25$  for I/R and IGF-I groups, respectively. The *igfbp3* values in steatotic livers were  $0.80 \pm 0.32$  and  $0.87 \pm 0.34$  for I/R and IGF-I groups, respectively.

### Effect of IGF-I on hepatic EGF

Pretreatment with EGF (at the same dose, 100  $\mu$ g/kg in Ln and Ob) reduced hepatic injury in both liver types when compared with the I/R group (Fig. 3B). The combined administration of IGF-I (at the effective dose, 100 and 400  $\mu$ g/kg in Ln and Ob, respectively) and EGF (100  $\mu$ g/kg in Ln and Ob) resulted in similar hepatic injury parameters in both liver types to those obtained by IGF-I and EGF administration, separately (Fig. 3B). EGF administration in both Ln and Ob animals did not alter hepatic IGF-I, because the *igf1* mRNA and IGF-I protein levels



**FIG. 2.** A, Total IGF-I, free IGF-I, and IGF-I:IGFBP complexes in plasma. B, IGF-I protein levels in liver. Representative Western blot (top) and densitometric analysis (bottom) are shown. C, Hepatic injury (Alanine Aminotransferase levels and damage score). \*,  $P < 0.05$  vs. sham; +,  $P < 0.05$  vs. I/R. Transaminase levels and damage score are significantly higher in the untreated Ob group than in the untreated Ln group (#,  $P < 0.05$ ).

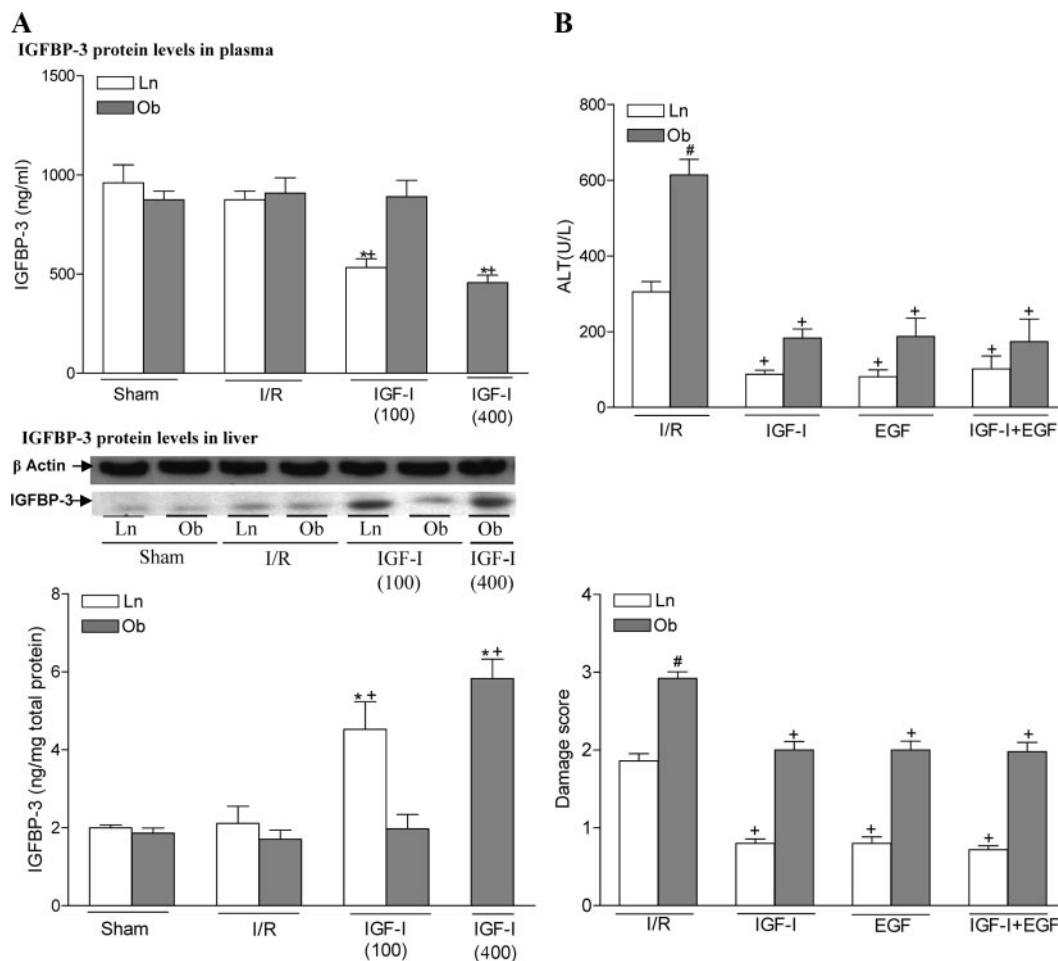
observed in both liver types after EGF treatment were similar to those of the I/R group (Fig. 4A). However, IGF-I administration in both Ln and Ob animals (at the effective dose, 100 and 400  $\mu\text{g}/\text{kg}$  in Ln and Ob, respectively) increased hepatic *egf* mRNA expression and EGF-I protein levels over those of the I/R group (Fig. 4B).

**Protective mechanisms of EGF in hepatic I/R**

I/R increased phosphorylated p38 levels in both type of livers when compared with the sham group (Fig. 5A). The protein levels of total p38 were unchanged in all groups. EGF administration in Ln animals reduced hepatic phosphorylated p38 levels when compared with the I/R group. However, EGF administration in Ob animals resulted in hepatic phosphorylated p38 levels similar to those of the I/R group (Fig. 5A). The effect of EGF on PPAR $\gamma$  in the presence of steatosis was also evaluated. As previously reported (17), I/R induced changes in PPAR $\gamma$  levels only in steatotic livers (Fig. 5A). EGF administration in Ob animals

increased PPAR $\gamma$  levels when compared with the I/R group. No changes in PPAR $\gamma$  were observed in nonsteatotic liver of any group compared with the sham group (Fig. 5A). Like EGF, IGF-I also reduced p38 levels in nonsteatotic livers and increased PPAR $\gamma$  levels in steatotic livers when compared with the I/R group (data not shown). This may be due to the fact that IGF-I increased EGF.

Next, the relevance of the changes in p38 and PPAR $\gamma$  induced by EGF treatment in nonsteatotic and steatotic livers, respectively, was also evaluated. First we showed that the increase in p38 induced by I/R had injurious effects on hepatic damage in nonsteatotic livers. Administration of p38 inhibitor in Ln animals reduced hepatic injury when compared with the I/R group (Fig. 5B). To test our hypothesis (that EGF exerts its action in nonsteatotic livers through p38 inhibition), we used a p38 activator at a dose that increased p38 levels to the same levels as those of the I/R group but not higher. We carried out control experiments to confirm that in all groups treated with p38 activator,



**FIG. 3.** A, IGFBP-3 protein levels in liver and plasma. For IGFBP-3 protein levels in liver, representative Western blot (top) and densitometric analysis (bottom) were performed. B, Effect of IGF-I and EGF administration on hepatic injury (alanine aminotransferase levels and damage score). \*,  $P < 0.05$  vs. sham; +,  $P < 0.05$  vs. I/R. Transaminase levels and damage score are significantly higher in the untreated Ob group than in the untreated Ln group (#,  $P < 0.05$ ). There were no significant differences in the hepatic injury parameters between IGF-I, EGF, and EGF+IGF groups.

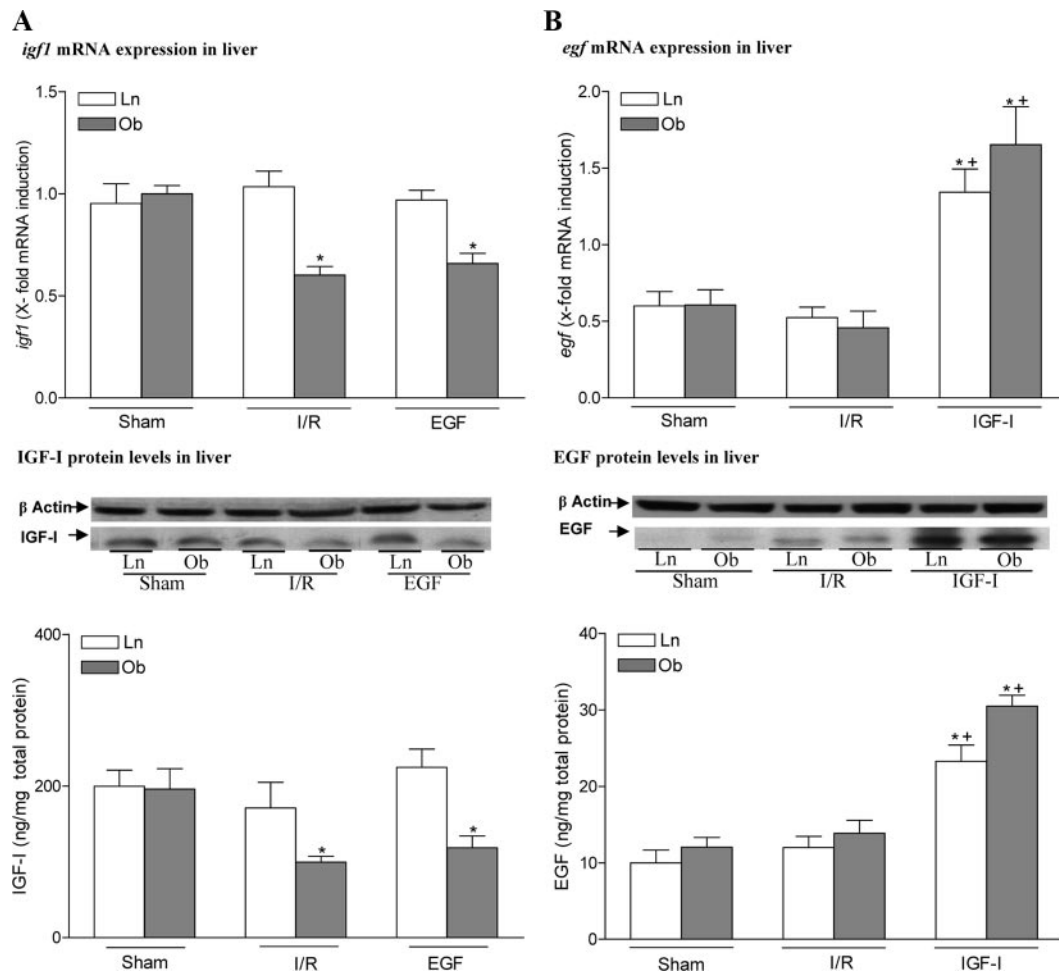
p38 levels were similar to those of the I/R group (data not shown). Thus, the administration of p38 activator in Ln animals (p38 activator group) resulted in similar hepatic p38 levels to those of the I/R group (data not shown) and, consequently, similar parameters of hepatic injury to those of the I/R group (Fig. 5B). As previously mentioned, EGF administration in Ln animals (EGF group) reduced hepatic phosphorylated p38 levels with respect to those of the I/R group (Fig. 5A), and this was associated with reduced hepatic injury (Fig. 5B). However, the administration of both EGF and p38 activator in Ln animals (EGF+p38 group) resulted in hepatic p38 levels similar to those of the I/R group (data not shown), and this was associated with biochemical and histological parameters of hepatic injury that were similar to those of the I/R group (Fig. 5B). Thus, when p38 activator was administered, EGF pretreatment did not protect nonsteatotic livers against hepatic I/R injury.

Regarding PPAR $\gamma$ , the slight but significant increase in PPAR $\gamma$  levels that occurred in steatotic livers as a consequence of I/R (Fig. 5A) had no effect on hepatic injury, because the administration of PPAR $\gamma$  antagonist in Ob animals (PPAR $\gamma$  antagonist group) resulted in hepatic injury parameters that were similar to those of the I/R group (Fig. 5B). Previous studies (17) reported

that PPAR $\gamma$  agonist pretreatment or strategies that increase PPAR $\gamma$  levels over those found in I/R protect steatotic livers against hepatic I/R injury. PPAR $\gamma$  agonist administration in Ob animals (PPAR $\gamma$  agonist group) reduced hepatic injury when compared with the I/R group (Fig. 5B). As shown above, EGF administration in Ob animals (EGF group) increased hepatic PPAR $\gamma$  levels in steatotic livers over those found in the I/R group (Fig. 5A), and this was associated with reduced hepatic injury (Fig. 5B). The administration of both EGF and PPAR $\gamma$  antagonist in Ob animals (EGF+PPAR $\gamma$  antagonist group) resulted in hepatic injury parameters that were similar to those of the I/R group despite the presence of EGF (Fig. 5B).

## Discussion

Hepatic synthesis and secretion of IGF-I is impaired in inflammatory conditions such as sepsis, endotoxemia, and cirrhosis (36–38). Our results, based on hepatic *igf1* mRNA and IGF-I protein levels, indicate that synthesis of IGF-I is impaired in steatotic livers of Ob animals subjected to I/R. The decrease in circulating IGF-I observed in various liver disorders could result



**FIG. 4.** A, Effect of EGF administration on *igf1* mRNA expression and IGF-I protein levels in liver. B, Effect of IGF-I administration on *egf* mRNA expression and EGF protein levels in liver. For *igf1* and *egf* mRNA expression in liver, PCR fluorescent signals for IGF-I and EGF were standardized to PCR fluorescent signals obtained from an endogenous reference ( $\beta$ -actin). Comparative and relative quantifications of *igf1* and *egf* gene products normalized to  $\beta$ -actin and control sham group were calculated by the  $2^{-\Delta\Delta CT}$  method. For IGF-I and EGF protein levels in liver, representative Western blot (top) and densitometric analysis (bottom) were performed. \*,  $P < 0.05$  vs. sham; +,  $P < 0.05$  vs. I/R.

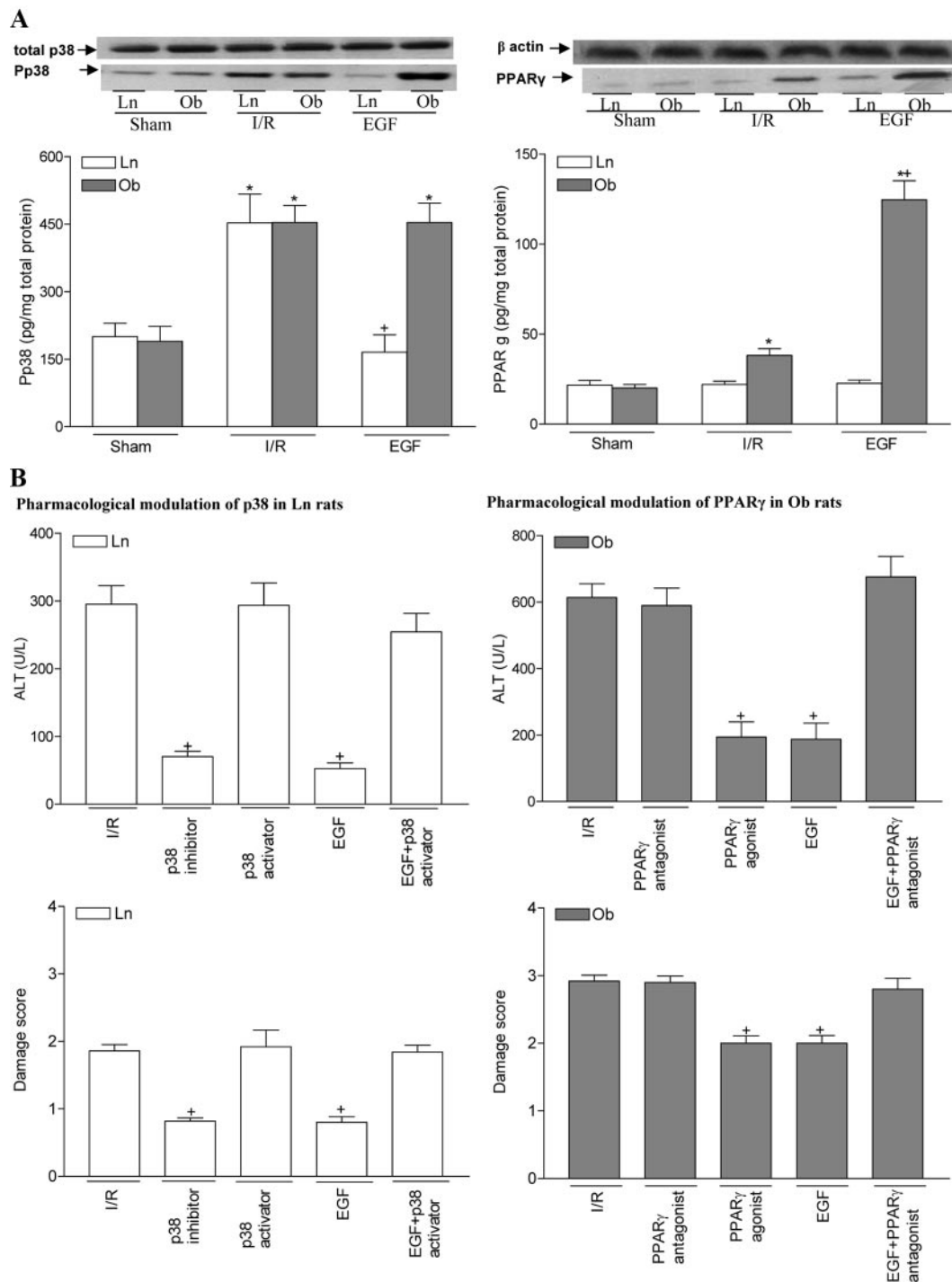
from a decrease in the rate of synthesis and an increase in the rate of removal from the blood (28, 37). The correlation between hepatic and circulating IGF-I indicates that the reduction in circulating IGF-I levels observed in Ob animals subjected to hepatic I/R could mainly be due to hepatic IGF-I synthesis reduction. Nevertheless, an increase in the rate of removal from the blood should not be ruled out.

The causes of the decrease in IGF-I synthesis observed in steatotic livers undergoing I/R were not explored in this study. However, a failure in the GH signaling pathway in steatotic livers under I/R conditions should not be ruled out. GH is released from the anterior pituitary gland, binds to its receptors in the liver, and thus, in turn synthesizes IGF-I (38). A decrease in GH secretion and alterations in the number of GH receptors have been observed in liver disorders caused by alcohol consumption, thermal injury, or Laron's syndrome. This leads to GH resistance in the liver, with a corresponding reduction in hepatic IGF-I synthesis (28, 39, 40).

We show here that in contrast to Ln animals, IGF-I administration at the dose of 100  $\mu\text{g}/\text{kg}$  in Ob animals was not associated with either an increase in hepatic IGF-I protein levels or

protection against damage. Thus, a higher dose of IGF-I had to be administered in Ob animals to protect steatotic livers effectively. This could be explained, at least partially, by the impairment of IGF-I synthesis induced by I/R in steatotic livers.

IGFBP-3 binds most circulating IGF-I (41), but a smaller proportion of IGF-I is associated with other serum IGFBPs (IGFBP-1, -2, -4, -5, and -6) (42, 43). Surprisingly, this did not appear to occur in the conditions of the present study. According to our results, in plasma of the sham Ob group, total IGF-I levels were about 200 nM, IGFBP-3 levels were about 30 nM, and the percentage of IGF-I bound to IGFBPs was about 50%. Considering that one molecule of IGF-I binds one molecule of IGFBP-3 (44, 45), this indicates that another IGFBP apart from IGFBP-3 must be present at unusually high concentration for 50% of IGF-I to be in complexes. Similarly, in plasma of Ob rats undergoing I/R and treated with IGF-I at the effective dose, total IGF-I levels were about 640 nM, IGFBP-3 levels were about 15 nM, and the percentage of IGF-I bound to IGFBPs was 80%. This indicates that another IGFBP apart from IGFBP-3 must be present at unusually high concentration for 80% of IGF-I to be in complexes. Thus, further research will be needed to identify the IG-



**FIG. 5.** A, Effect of EGF administration on phosphorylated p38 (Pp38) protein levels and PPAR $\gamma$  protein levels in liver. Representative Western blot (top) and densitometric analysis (bottom) are shown. B, Hepatic injury (alanine aminotransferase levels and damage score) after pharmacological modulation of p38 in nonsteatotic livers and PPAR $\gamma$  in steatotic livers. \*,  $P < 0.05$  vs. sham; +,  $P < 0.05$  vs. I/R.

FBPs other than IGFBP-3 that should be considered under warm hepatic I/R conditions in both liver types.

In addition to acting as a binding protein of circulating IGF-I, IGFBP-3 has been reported to have IGF-I-independent actions; IGFBP-3 inhibits cellular proliferation and enhances apoptosis by binding to the cell membrane (46–49). These effects of IGFBP-3 are injurious in conditions of hepatic I/R. Because our results showed that increases in hepatic IGFBP-3 were in parallel

with hepatic protection, it seems unlikely that in the conditions evaluated herein, IGFBP-3 has IGF-independent effects. Further studies will be required to clarify why IGF-I administration increased hepatic IGF-I protein levels but reduced plasma IGFBP-3 levels, considering that IGF-I is one of the main positive regulators of IGFBP-3 levels (50, 51). Nevertheless, on the basis of previous reports in other pathologies, we hypothesize that a decrease in circulating IGFBP-3 could be explained, at least par-

tially, by increased IGFBP-3 rate of clearance or enhanced IGFBP-3 proteolysis (41, 52).

It should be considered that IGF-I and EGF decrease hepatic I/R injury but do not preserve the liver from I/R injury. The latter is a complex process in which several mechanisms participate (53) but not all of which are affected by EGF and IGF-I. The ability of IGF-I and EGF to protect both liver types against I/R injury is of particular interest because numerous strategies that are effective in nonsteatotic livers may not be useful in the presence of steatosis (31, 53). It should be noted that the administration of EGF resulted in hepatic injury parameters similar to those obtained by IGF. However, EGF seems to be the most appropriate therapeutic strategy. Our main argument is that the IGF-I dose that protected against I/R injury is different for steatotic and nonsteatotic livers. This may be an obstacle to its therapeutic use in clinical practice. Moreover, it would appear to be more reasonable to administer the ultimate effector of the cascade, EGF in this case, because its effects are more direct.

Herein we showed that the combined administration of IGF-I and EGF resulted in hepatic injury parameters in both liver types that were similar to those induced by IGF-I and EGF separately. Thus, a combination of IGF-I and EGF seems unnecessary to protect both liver types against I/R injury. Our results showed a potential relationship between IGF-I and EGF in both liver types under warm ischemic conditions. In contrast with the results obtained in different types of cells (12–14), EGF did not enhance *igf1* expression in our conditions. The results reported here indicate that IGF-I induces *egf* expression in both steatotic and nonsteatotic livers under hepatic I/R conditions. Previous studies from our group revealed the key role of both p38 and PPAR $\gamma$  in nonsteatotic and steatotic livers, respectively, under I/R conditions (16, 17), and data reported in the literature have indicated that IGF-I and EGF affect p38 and PPAR $\gamma$  in several different conditions (18–21). In the present study, we hypothesize that the benefits of EGF on hepatic I/R injury could be explained, at least partially, by a reduction in p38 activation in nonsteatotic livers and by overexpression of PPAR $\gamma$  in the presence of steatosis. Thus, EGF protected both liver types, possibly by different mechanisms. This is in line with reports indicating that the multiple intracellular signaling induced by EGF depends on cell type. Moreover, the EGF signaling pathway in the same cell type may result in completely different effects depending on numerous factors including the concentration of growth factor, the number of receptors displayed on the cell surface, and docking and target proteins and their initial activity state (54–56). Taking these observations into account, and the structural and functional differences between hepatocytes with or without fatty infiltration (57–59), it is not surprising that our results show a differential effect of EGF on p38 and PPAR $\gamma$ , depending on the type of liver.

In conclusion, pharmacological strategies based on IGF-I and EGF administration could open new pathways for protecting steatotic livers against I/R, although they do not need to be administered in combination. Moreover, EGF administration could be a more appropriate clinical therapy because EGF protected both liver types at the same dose and its effects are more direct than IGF-I. Finally, EGF protected the liver against I/R

injury by reducing p38 activation in nonsteatotic livers and inducing PPAR $\gamma$  overexpression in steatotic livers.

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