

Are Angiotensin II Receptor Antagonists Useful Strategies in Steatotic and Nonsteatotic Livers in Conditions of Partial Hepatectomy under Ischemia-Reperfusion?

Fernando S. Ramalho, Izabel Alfany-Fernandez, Araní Casillas-Ramirez, Marta Massip-Salcedo, Anna Serafin, Antoni Rimola, Vicente Arroyo, Juan Rodés, Joan Roselló-Catafau, and Carmen Peralta

Experimental Hepatic Ischemia-Reperfusion Unit, Institut d'Investigacions Biomèdiques de Barcelona-Consejo Superior de Investigaciones Científicas, Barcelona, Spain (F.S.R., J.R.-C.); Unitat de Transplantament de Fetge i Viabilitat de l'Empelt, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Consejo Superior de Investigaciones Científicas, Barcelona, Spain (I.A.-F., A.C.-R., M.M.-S., J.R.-C., C.P.); Centro de Investigaciones Biomédicas Esther Koplowitz, Centro de Investigación Biomédica en Red-Enfermedades Hepáticas y Digestivas, Instituto de Salud Carlos III, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain (I.A.-F., A.C.-R., M.M.-S., A.R., V.A., J.R., J.R.-C., C.P.); Centre de Biotecnologia Animal i Teràpia Genica, Universitat Autònoma de Barcelona, Barcelona, Spain (A.S.); and Liver Unit, Hospital Clinic Universitari, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain (A.R., V.A., J.R.)

Received October 23, 2008; accepted December 29, 2008

ABSTRACT

We examined whether angiotensin (Ang) II receptor antagonists could be considered a therapeutic strategy in steatotic and nonsteatotic livers in conditions of partial hepatectomy under ischemia-reperfusion (I/R), which is commonly applied in clinical practice to reduce blood loss. We report that Ang II type I receptor (AT1R) antagonist, but not Ang II type II receptor (AT2R) antagonist, increased regeneration in nonsteatotic livers. In the presence of steatosis, both AT1R and AT2R antagonists increased liver regeneration. This effect was stronger when the two were combined. Neither of the Ang II receptor antagonists protected nonsteatotic livers against damage. Only the AT1R antagonist, through nitric oxide inhibition, reduced damage in steatotic livers. The combination of the AT1R and AT2R antagonists in steatotic livers conferred a similar degree of protection to AT1R antagonist

alone. Herein, we show that p38 mitogen-activated protein kinase (p38) was a key mechanism in the regeneration induced by the Ang II receptor antagonists in both liver types because when this signaling pathway was inhibited, the beneficial effects of the Ang II receptor antagonists on liver regeneration disappeared, regardless of hepatocyte growth factor or transforming growth factor β -hepatic levels. In conclusion, in conditions of partial hepatectomy under I/R, the AT1R antagonist for nonsteatotic livers and the AT1R and AT2R antagonists for steatotic livers improved regeneration in the remnant liver through p38 activation. In addition, the combination of the AT1R and AT2R antagonists in steatotic livers led to stronger liver regeneration than either antagonists used separately and also provided the same protection against damage as that afforded by AT1R antagonist alone.

In clinical situations, partial hepatectomy under ischemia-reperfusion (I/R) is usually performed to control bleeding

This work was supported by the Ministerio de Educación y Ciencia (Madrid, Spain) [Grant SAF 2005-00385]; the Ministerio de Sanidad y Consumo (Madrid, Spain) [Grant PIO60021]; and the Generalitat de Catalunya (Barcelona, Spain) [Grant 2005 SGR/00781].

F.S.R. and I.A.-F. contributed equally to this study.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.108.147835.

during parenchymal dissection (Dixon et al., 2005). Hepatic steatosis, a major risk factor for liver surgery, has been associated with increased complication index and postoperative mortality after major liver resection (McCormack et al., 2007). Steatotic livers show impaired regenerative response and reduced tolerance to hepatic injury compared with nonsteatotic ones in conditions of partial hepatectomy under I/R (Behrns et al., 1998; Veteläinen et al., 2007). A further increase in the prevalence of steatosis in hepatic surgery is to

ABBREVIATIONS: I/R, ischemia-reperfusion; Ang, angiotensin; p38, p38 mitogen-activated protein kinase; NO, nitric oxide; Ob, obese; Ln, lean; PH, partial hepatectomy; AT1R, Ang II type I receptor; losartan, 2 butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl) benzyl] imidazole-5-methanol monopotassium salt; AT2R, Ang II type II receptor; PD123319, S-(+)-1-[(4-(dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo(4,5-c)pyridine-6-carboxylic acid; spermine NONOate, N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosodiazino)-1,2-ethylenediamine; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; PCR, polymerase chain reaction; ACE, angiotensin-converting enzyme; cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; ALT, alanine aminotransferase; HGF, hepatocyte growth factor; TGF, transforming growth factor; MDA, malondialdehyde; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen.

TABLE 1

Experimental design of the current study

The table shows the experimental groups, treatments, and measurements of the current study. Measurement: liver, Ang II levels; gene expression of angiotensinogen, ACE, AT1R, and AT2R; histology, PCNA, and mitotic index; protein expression of cNOS, iNOS, and β -actin and nitrite/nitrate levels; MDA and nitrotyrosine levels; protein expression of total and phosphorylated p38; HGF and TGF- β levels. Plasma: transaminase levels.

Groups	Interventions
1. Sham ($n = 12$, 6 Ln and 6 Ob)	Hepatic helium vessels of Ln and Ob animals were dissected.
2. PH + I/R ($n = 12$, 6 Ln and 6 Ob)	Ln and Ob animals were subjected to partial hepatectomy under I/R.
3. AT1R antagonist ($n = 12$, 6 Ln and 6 Ob)	As in group 2 but treated with an AT1R antagonist.
4. AT2R antagonist ($n = 12$, 6 Ln and 6 Ob)	As in group 2 but treated with an AT2R antagonist.
5. AT1R antagonist + AT2R antagonist ($n = 6$ Ob)	As in group 2, but treated with an AT1R antagonist and an AT2R antagonist.
6. AT1R antagonist + NO ($n = 6$ Ob)	As in group 2 but treated with an AT1R antagonist and a NO donor.
7. AT1R antagonist + p38 inhibitor ($n = 6$ Ln)	As in group 2, but treated with an AT1R antagonist and a p38 inhibitor.
8. AT1R antagonist + AT2R antagonist + p38 inhibitor ($n = 6$ Ob)	As in group 2, but treated with an AT1R antagonist, an AT2R and a p38 inhibitor.

be expected (Nocito et al., 2006). These observations highlight the need to develop protective strategies for steatotic livers in conditions of partial hepatectomy under I/R.

A number of studies suggest that a local renin-angiotensin system is present in the liver that exerts blood pressure-independent actions, including effects on hepatocellular proliferation and damage (Ramalho et al., 2002; Guo et al., 2004; Yayama et al., 2007; Casillas-Ramirez et al., 2008). Two studies on partial hepatectomy without I/R gave opposite results on the effect of angiotensin (Ang) II receptor antagonists on liver regeneration (Ramalho et al., 2002; Yayama et al., 2007). Other studies on I/R without partial hepatectomy reported beneficial effects of Ang II receptor antagonists on hepatic damage (Guo et al., 2004; Casillas-Ramirez et al., 2008). The effect of Ang II receptor antagonists on liver regeneration and damage in conditions of partial hepatectomy under I/R has not been examined. It should be considered that the effectiveness of Ang II receptor antagonists on hepatic regeneration and damage could be different depending on the surgical conditions evaluated. It has been reported that gadolinium chloride treatment protected against hepatic damage in conditions of I/R without hepatectomy and improved liver regeneration after partial hepatectomy without I/R (Rai et al., 1996; Suzuki et al., 1997). However, the same drug had injurious effects on hepatic damage and impaired liver regeneration in conditions of partial hepatectomy under I/R (Watanabe et al., 2000).

In light of these considerations, we examined whether Ang II receptor antagonists could be considered as a therapeutic strategy in steatotic and nonsteatotic livers in conditions of partial hepatectomy under I/R. In an attempt to explain the effects of Ang II receptor antagonists on regeneration and damage in both liver types under conditions of partial hepatectomy under I/R, we focused on p38 mitogen-activated protein kinase (p38) and nitric oxide (NO). This approach was based on: 1) numerous studies

indicating that changes in p38 activity and NO generation are one of the earliest events in the hepatic regenerative response (Hortelano et al., 1995; Spector et al., 1997; Carnovale et al., 2000; Hsu et al., 2006), 2) the involvement of NO in hepatic damage in conditions of partial hepatectomy under I/R (Kukita et al., 2005), and 3) studies in the heart indicating that Ang II blockers could modulate p38 and NO (Wei et al., 2000; Kajihara et al., 2005).

Materials and Methods

Experimental Animals. Homozygous [obese (Ob)] and heterozygous [lean (Ln)] Zucker rats (Iffa Credo, L'Arbresle, France), 16 to 18 weeks old, were used in the experiments. Ob Zucker rats showed severe macrovesicular and microvesicular fatty infiltration in hepatocytes (60–70% steatosis). Ln Zucker rats showed no evidence of steatosis. This study complied with European Union regulations (Directive 86/609 EEC) on animal experiments.

Surgical Procedure. To achieve the aims of the present study, we used a well characterized model of partial hepatectomy under vascular occlusion, as described below (Selzner et al., 1999). Although I/R and partial hepatectomy are likely to affect one on another (Clavien et al., 2003), examination of this issue was not the purpose of the present study. Therefore, comparisons between experimental models of partial hepatectomy without vascular occlusion and experimental models of partial hepatectomy under vascular occlusion were not performed. In the present study, a rat model of partial (70%) hepatectomy (PH) under 60 min of ischemia was examined (Selzner et al., 1999). A vascular occlusion of 60 min is currently used in liver surgery (Huguet et al., 1992; Clavien et al., 2003). After anesthesia with isoflurane and resection of left hepatic lobe, a microvascular clamp was placed for 60 min across the portal triad supplying the median lobe (30%). Congestion of the bowel was avoided during the clamping period by preserving the portal flow through the right and caudate lobes. At the end of ischemia time, the right lobe and caudate lobes were resected, and reperfusion of the median lobe was achieved by releasing the clamp.

Experimental Design. The animals were divided at random into 12 experimental groups (Tables 1 and 2). The experimental treatments

TABLE 2

Experimental design of the current study

This table shows the drug administration protocol of the current study.

Drug Administration Protocol		
Drug		Dose and Pretreatment Times
AT1R antagonist	Losartan	5 mg/kg orally, 24 and 1.5 h before the surgical procedure
AT2R antagonist	PD123319	30 mg/kg i.v., 5 min before the surgical procedure
NO	Spermine NONOate	10 mg/kg i.v., 5 min before the surgical procedure
p38 inhibitor	SB203580	1 mg/kg i.p., 24 h before the surgical procedure

and the measurements are listed in Table 1. The following drugs were administered: the Ang II type I receptor (AT1R) antagonist losartan (Ramalho et al., 2002; Guo et al., 2004), the Ang II type II receptor (AT2R) antagonist PD123319 (Gohlke et al., 1998; Casillas-Ramirez et al., 2008), the NO donor spermine NONOate (Peralta et al., 2001; Serafin et al., 2002), and the p38 inhibitor SB203580 (Zhao et al., 2001; Massip-Salcedo et al., 2006). The dose and the pretreatment times of the different drugs used in the present study were selected on the basis of previous dose-response studies (Gohlke et al., 1998; Peralta et al., 2001; Zhao et al., 2001; Ramalho et al., 2002; Serafin et al., 2002; Guo et al., 2004; Massip-Salcedo et al., 2006; Casillas-Ramirez et al., 2008)

and preliminary studies from our group. Control experiments were performed with the vehicles used for the different drugs: saline for losartan and PD123319, phosphate-buffered saline for spermine NONOate, and dimethylsulfoxide for SB203580. Under our conditions, the vehicles used did not modify the postsurgical outcomes. These drugs did not significantly affect systolic blood pressure, which was measured by a noninvasive tail-cuff method (Pressure Meter LE5001; Panlab, S.L., Barcelona, Spain). Plasma and liver samples corresponding to all experimental groups were collected at 24 h of reperfusion.

Reverse Transcription and Real-Time PCR. Quantitative real-time PCR analysis was performed using the Assays-on-Demand Taq-

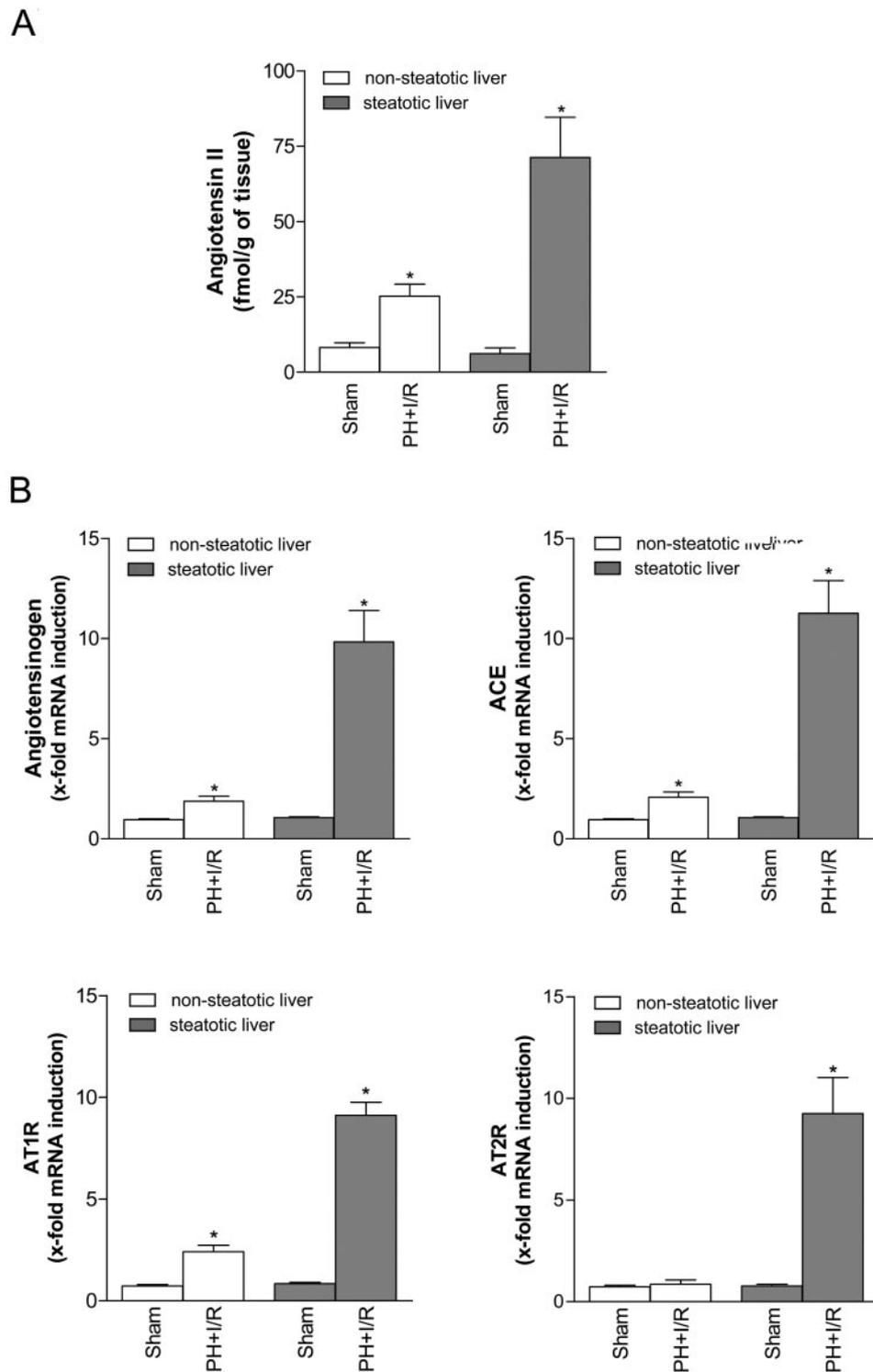


Fig. 1. The renin-angiotensin system in partial hepatectomy under I/R. Angiotensin II levels (A) and angiotensinogen, ACE, AT1R, and AT2R mRNA expression (B) were measured in both liver types. PCR fluorescent signals for angiotensinogen, ACE, AT1R, and AT2R were standardized to PCR fluorescent signals obtained from an endogenous reference (β -actin). Comparative and relative quantifications of angiotensinogen, ACE, AT1R, and AT2R gene products normalized to β -actin and control Sham group were calculated by the $2^{-\Delta\Delta C_t}$ method. *, $P < 0.05$ versus Sham; +, $P < 0.05$ versus PH + I/R.

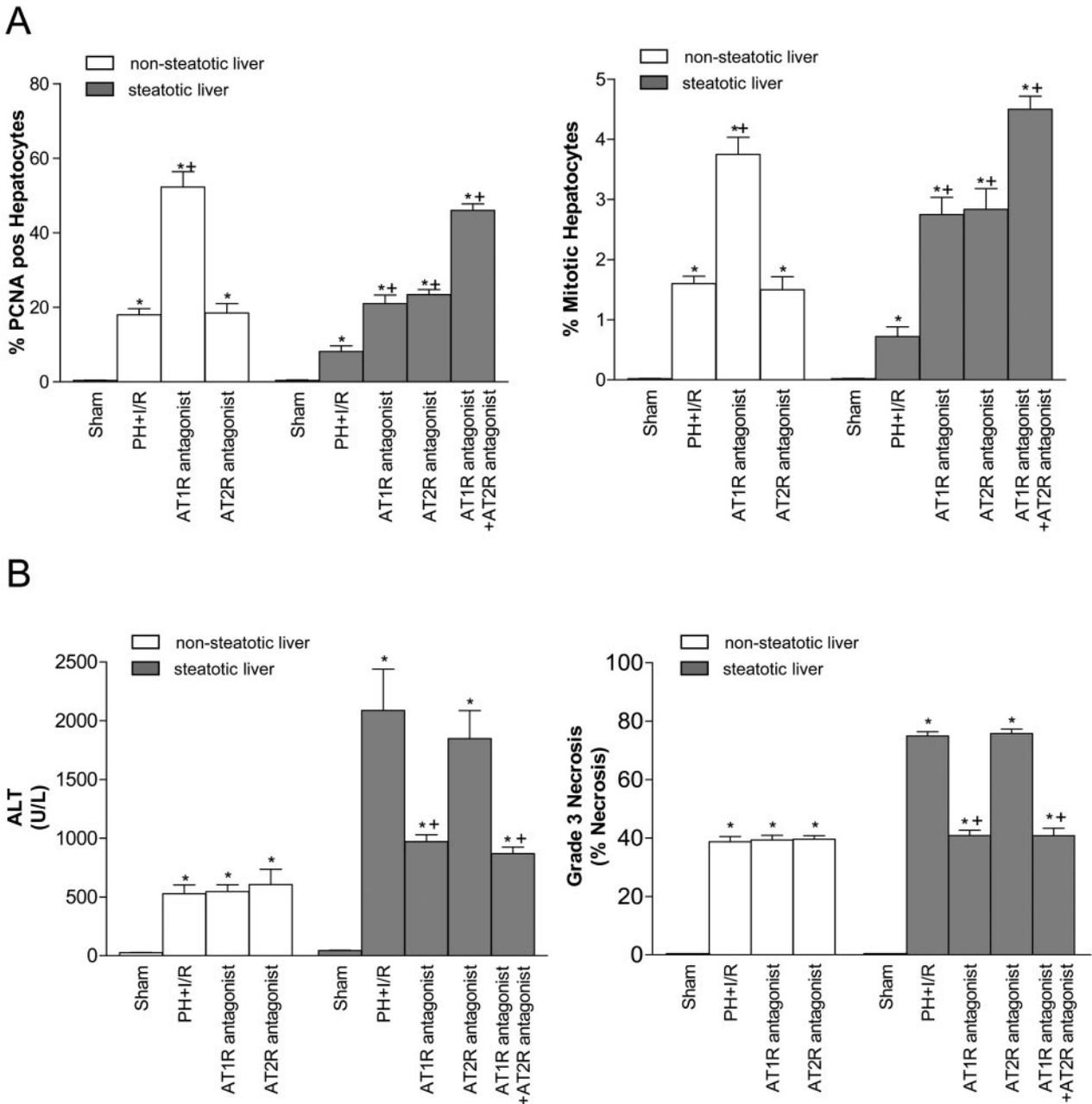


Fig. 2. Effect of Ang II receptor antagonists on hepatic regeneration and damage. A, percentage PCNA-positive hepatocytes and percentage mitotic hepatocytes were analyzed in both liver types. B, ALT levels and grade 3 necrosis were analyzed in plasma and both liver types, respectively. *, $P < 0.05$ versus Sham; +, $P < 0.05$ versus PH + I/R.

Man probes [Rn00593114_m1 for angiotensinogen, Rn00561094_m1 for angiotensin-converting enzyme (ACE), Rn00578456_m1 for AT1R, Rn00560677_s1 for AT2R, and Rn00667869_m1 for β -actin] (Applied Biosystems, Foster City, CA). The TaqMan gene expression assay was performed according to the manufacturer's protocol (Applied Biosystems).

Western Blotting of NOS and p38-MAPK. This was done as described elsewhere (Carrasco-Chaumeil et al., 2005; Massip-Salcedo et al., 2006), using the following antibodies: constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS) (Transduction Laboratories, Lexington, KY), total p38 and phosphorylated p38 (Cell Signaling Technology Inc., Danvers, MA), and β -actin (Sigma-Aldrich, St. Louis, MO). The bands were visualized using an enhanced chemiluminescence kit (Bio-Rad, Hercules, CA). The values were obtained by densitometric scanning and the Quantity One software program. The scanning values for cNOS and iNOS were

divided by the scanning values for β -actin and those for phosphorylated p38 by the total p38.

Biochemical Determinations. Alanine aminotransferase (ALT), hepatocyte growth factor (HGF), total and active transforming growth factor (TGF) β , Ang II, malondialdehyde (MDA), nitrite and nitrate, and nitrotyrosine levels (as an index of peroxynitrite) were measured as described elsewhere (Carrasco-Chaumeil et al., 2005; Franco-Gou et al., 2006; Casillas-Ramirez et al., 2008).

Assessment of Hepatocyte Proliferation. Hepatic proliferation was assessed in liver biopsies by measurement of mitotic index in H&E-stained sections and S phase cell percentage in sections stained for proliferating cell nuclear antigen (PCNA) (Selzner et al., 1999; Franco-Gou et al., 2006). Data are expressed as the percentage of mitotic hepatocytes and PCNA-stained hepatocytes per total number of hepatocytes in 30 high-power fields.

Histology and Red-Oil Staining. H&E-stained sections were evaluated by a point-counting method using an ordinal scale (Serafin et al., 2002). Steatosis in liver was evaluated by red oil staining on frozen specimens, according to standard procedures.

Statistics. Data are expressed as means \pm S.E. and were compared statistically by variance analysis, followed by the Student-Newman-Keuls test. $P < 0.05$ was considered significant.

Results

Ang II levels were higher in steatotic and nonsteatotic livers of the PH + I/R group than in the Sham group (Fig. 1A). Ang II levels were markedly higher in steatotic livers. This is consistent with the finding that the expression of angiotensinogen (Ang II precursor) and ACE was higher in steatotic than in nonsteatotic livers (Fig. 1B). The expression of AT1R increased in both liver types from the PH + I/R group with respect to the Sham group (Fig. 1B). AT1R expression was higher in steatotic livers. The expression of AT2R increased only in steatotic livers from the PH + I/R group, compared with the results of the Sham group.

Effects of Ang II Receptor Antagonists on Hepatic Regeneration and Damage. The AT1R antagonist, but not the AT2R antagonist, increased the number of PCNA-positive hepatocytes and the mitotic index in nonsteatotic livers

compared with the PH + I/R group (Fig. 2A), indicating that Ang II impaired liver regeneration through AT1R in this type of liver. In the presence of steatosis, both the AT1R and AT2R antagonists increased liver regeneration compared with the PH + I/R group, indicating that Ang II impaired steatotic liver regeneration through both Ang II receptors (Fig. 2A). Given these results, we evaluated the effect of the combination of both Ang II receptor antagonists on regeneration in steatotic liver. The increase in the parameters of hepatic proliferation was stronger in the AT1R antagonist + AT2R antagonist group than that caused by either antagonist separately (Fig. 2A). Photomicrographs of PCNA from the main groups in both liver types are shown in Fig. 3. Neither of the Ang II receptor antagonists protected nonsteatotic livers against damage because ALT levels and grade 3 necrosis in the AT1R antagonist and AT2R antagonist groups were similar to those of the PH + I/R group (Fig. 2B). Thus, in our conditions, Ang II is not the mechanism responsible for inducing damage in nonsteatotic livers. Only the AT1R antagonist reduced damage in steatotic livers compared with the PH + I/R group, indicating that Ang II, through AT1R, damaged the remnant steatotic liver. Thus, the AT1R antagonist + AT2R antagonist conferred a similar degree of protection to the AT1R antagonist alone (Fig. 2B). The histological

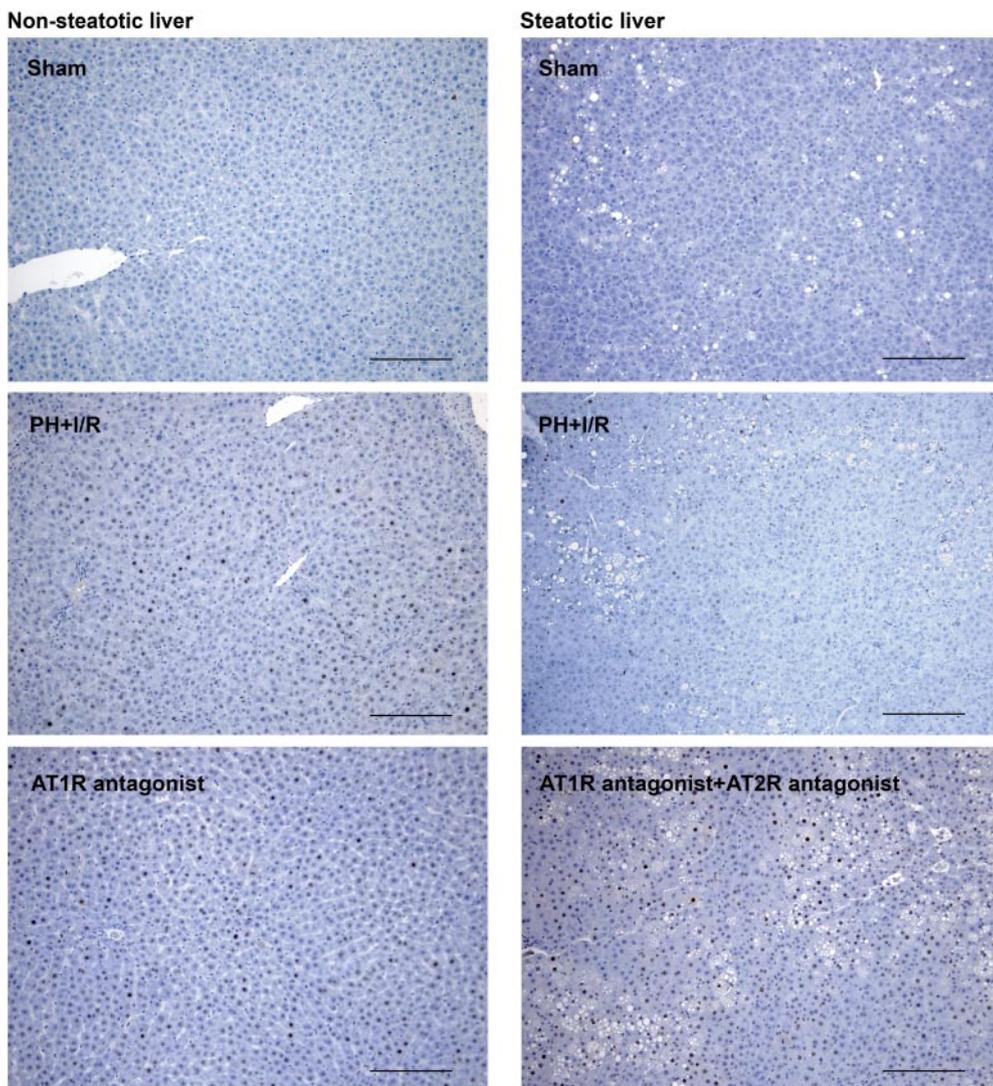


Fig. 3. Immunohistochemical staining of PCNA-positive hepatocytes. In nonsteatotic livers, Sham showed no PCNA-positive hepatocytes, and PH + I/R showed lower positive cell numbers than AT1R antagonist. In steatotic livers, Sham showed no PCNA-positive hepatocytes, and PH + I/R showed lower positive cell numbers than AT1R antagonist + AT2R antagonist (bar, 200 μ m).

study of nonsteatotic livers from the PH + I/R group showed extensive and multifocal areas of coagulative necrosis and neutrophil infiltration, randomly distributed throughout the parenchyma (Fig. 4). The AT1R antagonist led to histological lesions in nonsteatotic livers that were similar to those of the PH + I/R group. The histological study of steatotic livers from the PH + I/R group showed severe, very extensive, and confluent areas of coagulative necrosis with neutrophil infiltration (Fig. 4). The AT1R antagonist + AT2R antagonist reduced the extent and number of necrotic areas in steatotic livers. Preliminary studies from our group demonstrated that the dose and the pretreatment times of Ang II receptor antagonists used in the present study were the most effective for protecting against damage and for enhancing liver regeneration in both liver types in conditions of partial hepatectomy under I/R.

Protective Mechanisms of Ang II Receptor Antagonist on Hepatic Regeneration and Damage. Given that NO and p38 are involved in the hepatic regenerative response (Hortelano et al., 1995; Spector et al., 1997; Carnovale et al., 2000; Hsu et al., 2006), we evaluated whether the beneficial effects of Ang II receptor antagonists on liver regeneration (AT1R antagonist for nonsteatotic livers and both

Ang II receptor antagonists for steatotic ones) could be explained by changes in NO and p38. Our results indicated that NO was not responsible for the beneficial effects of Ang II receptor antagonists on regeneration in either type of liver. In fact, the AT1R antagonist for nonsteatotic livers resulted in NO generation (cNOS, iNOS, and nitrate and nitrite levels) similar to the PH + I/R group (Fig. 5A). In the presence of steatosis, AT1R antagonist reduced NO generation (cNOS, iNOS, and nitrate and nitrite levels) compared with the PH + I/R group (Fig. 5A), but this did not affect liver regeneration. In fact, as shown in Fig. 5B, AT1R antagonist and AT1R antagonist + NO had similar effects on liver regeneration in steatotic livers. Because AT2R antagonist did not modify NO generation in steatotic livers compared with the PH + I/R group (Fig. 5A), the reduction in NO generation induced by the AT1R antagonist + AT2R antagonist was due to AT1R antagonist alone. Next, we examined the relevance of NO inhibition induced by AT1R antagonist in steatotic livers. Given the injurious effects of NO in the remnant liver in conditions of partial hepatectomy under I/R previously reported (Kukita et al., 2005), we hypothesized that in our conditions, NO inhibition induced by AT1R antagonist could explain the beneficial effects of this strategy on damage in

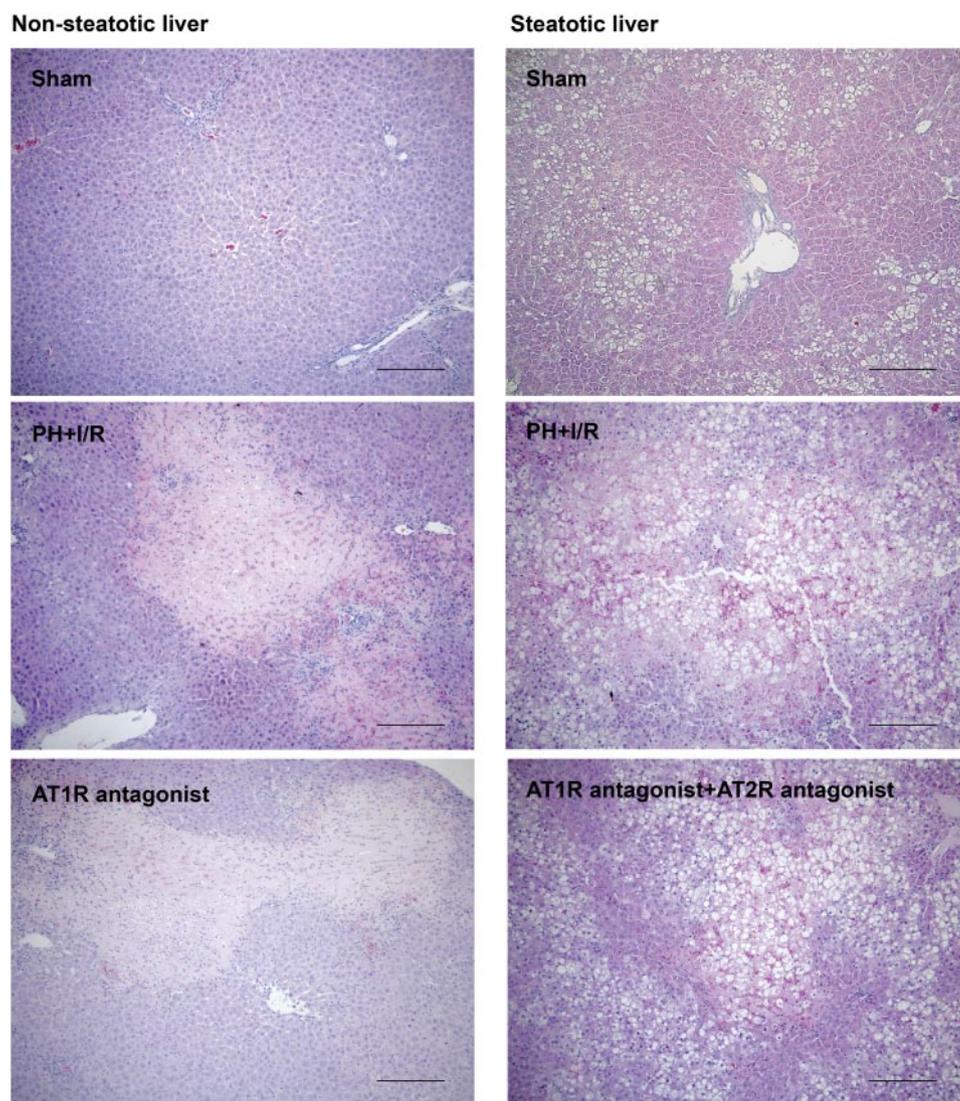


Fig. 4. Histological lesions. In nonsteatotic livers, Sham showed no hepatic lesions, PH + I/R showed extensive areas of coagulative hepatic necrosis with neutrophil infiltration, and AT1R antagonist showed hepatic lesions similar to PH + I/R. In steatotic livers, Sham showed no hepatic lesions, PH + I/R showed very extensive and confluent areas of coagulative necrosis with neutrophil infiltration, and AT1R antagonist + AT2R antagonist showed extensive areas of coagulative hepatic necrosis with neutrophil infiltration (H&E; bar, 200 μ m).

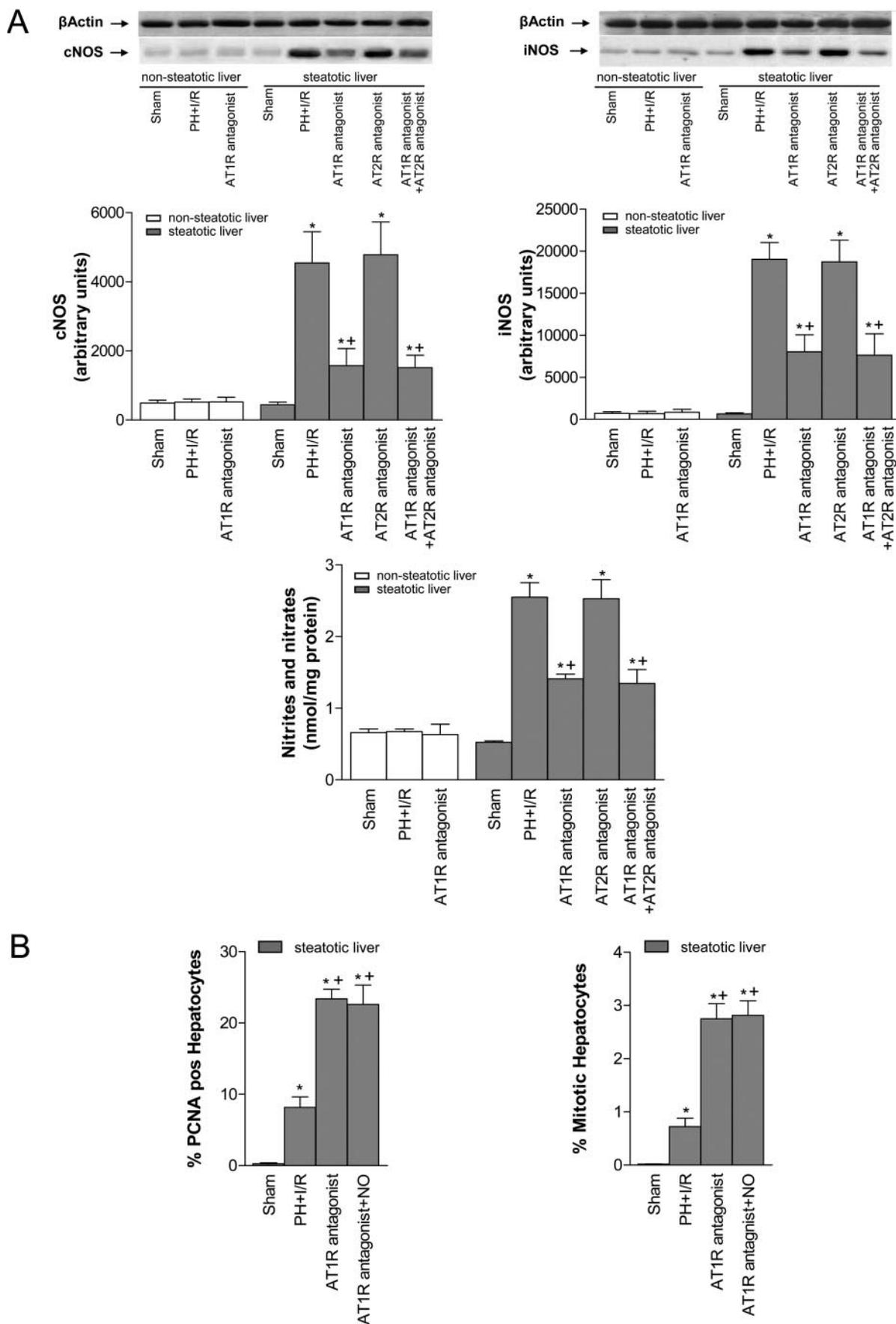


Fig. 5. Role of NO in the beneficial effects of Ang II receptor antagonists on liver regeneration. A, protein levels of cNOS and iNOS and nitrite and nitrate levels were measured in both liver types. At the top of densitometric analysis, representative Western blot of cNOS and iNOS with β -actin as loading control are shown. B, percentage PCNA-positive hepatocytes and percentage mitotic hepatocytes were analyzed in steatotic livers. *, $P < 0.05$ versus Sham; +, $P < 0.05$ versus PH + I/R.

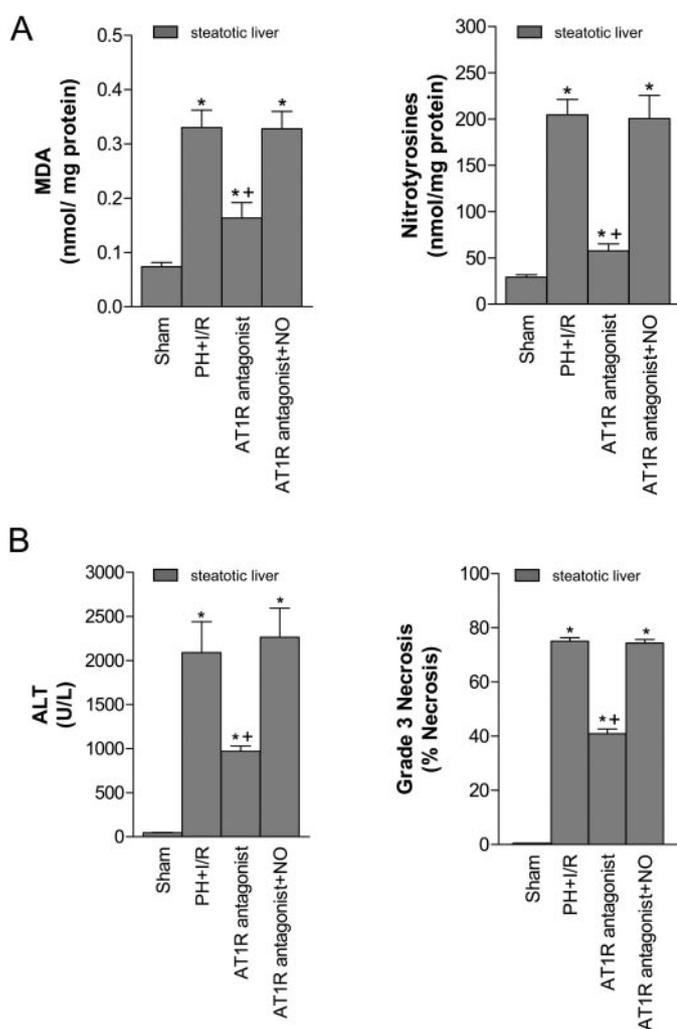


Fig. 6. Role of NO in the beneficial effects of Ang II receptor antagonists on hepatic damage. A, MDA and nitrotyrosine levels were measured in steatotic livers. B, ALT and grade 3 necrosis were analyzed in plasma and steatotic livers, respectively. *, $P < 0.05$ versus Sham; +, $P < 0.05$ versus PH + I/R.

steatotic livers. Herein, we showed that the reduction in NO generation induced by AT1R antagonist in steatotic livers (Fig. 5A) was associated with lower MDA and nitrotyrosine levels (Fig. 6A), which was consistent with the reduction in peroxynitrite levels in steatotic livers. Conversely, AT1R antagonist + NO abolished the beneficial effects of AT1R antagonist on oxidative stress (Fig. 6A) and hepatic injury (Fig. 6B).

Our results indicated the key role of p38 in the beneficial effects of Ang II receptor antagonists on regeneration in both liver types. All treatments that improved liver regeneration in either type of liver (AT1R antagonist for nonsteatotic livers and both Ang II receptor antagonists for steatotic ones) raised phosphorylated p38 levels compared with the PH + I/R group (Fig. 7A). Similarly to the parameters of liver regeneration, the increase in phosphorylated p38 activity in steatotic livers was stronger in AT1R antagonist + AT2R antagonist group than that obtained by either antagonist separately (Fig. 7A). Total p38 was unchanged in all groups (Fig. 7A). In nonsteatotic livers, AT1R antagonist + p38 inhibitor resulted in parameters of liver regeneration similar to those of the PH + I/R group (Fig. 7B). Thus, the benefits of

AT1R antagonist on liver regeneration in nonsteatotic livers were abolished when p38 was inhibited. The key role of p38 in the beneficial effects of Ang II receptor antagonists on regeneration was also evidenced in steatotic livers. For instance, AT1R antagonist + AT2R antagonist + p38 inhibitor resulted in parameters of liver regeneration in steatotic livers similar to those of the PH + I/R group (Fig. 7B).

In addition to p38, liver regeneration may be controlled by growth factors. HGF is a potent mitogen, whereas TGF β has been considered as the main inhibitor of hepatocyte proliferation (Fausto et al., 1995). Our results indicated that, in nonsteatotic livers, the AT1R antagonist (which improved liver regeneration) resulted in HGF and active TGF β levels that were similar to those of PH + I/R group (Fig. 8). In the presence of steatosis, both Ang II receptor antagonists, separately or in combination (which improved liver regeneration), increased HGF and reduced active TGF β levels with respect to the PH + I/R group (Fig. 8). Total hepatic TGF β levels were similar in all groups (data not shown). Our results indicated that, when p38 was inhibited, the beneficial effects of AT1R and AT2R antagonists on liver regeneration in steatotic livers disappeared despite the presence of high HGF levels and reduced TGF β . Thus, AT1R antagonist + AT2R antagonist + p38 inhibitor resulted in parameters of liver regeneration in steatotic livers that were similar to those of the PH + I/R group (Fig. 7B) and higher HGF and lower TGF β levels compared with the PH + I/R group (Fig. 8). In summary, p38 is a key mechanism in the liver regeneration induced by the Ang II receptor antagonists in both liver types because when this signaling pathway was inhibited, the beneficial effects of the Ang II receptor antagonists on liver regeneration disappeared, regardless of HGF or TGF β hepatic levels.

Discussion

Herein, we show that in conditions of partial hepatectomy under I/R, the AT1R antagonist for nonsteatotic livers and the AT1R and AT2R antagonists for steatotic ones improved regeneration in the remnant liver. The combination of AT1R and AT2R antagonists in steatotic livers showed stronger liver regeneration than either antagonist used separately and also provided the same protection against damage as that afforded by AT1R antagonist alone. These results could be of clinical interest in liver surgery.

The loss of protection of Ang II receptor antagonists against damage in our conditions (only AT1R antagonist protected steatotic liver against damage) compared with the study of I/R without hepatectomy (in which both Ang II receptor antagonists reduced damage in both liver types) (Casillas-Ramirez et al., 2008) could not be explained by differences in the dose or frequency of drug administration but, rather, by the different surgical conditions. In the model of I/R without hepatectomy (Casillas-Ramirez et al., 2008), the blood supply to the left and median liver lobes (70% hepatic mass) was interrupted, and the other hepatic lobes remained intact. However, in the conditions evaluated herein, only blood supply to the remnant liver (30% hepatic mass) was interrupted and the other hepatic lobes were excised. Compared with the study of I/R without hepatectomy (Casillas-Ramirez et al., 2008), in our conditions, there are two main differences, the percentage of hepatic mass that is

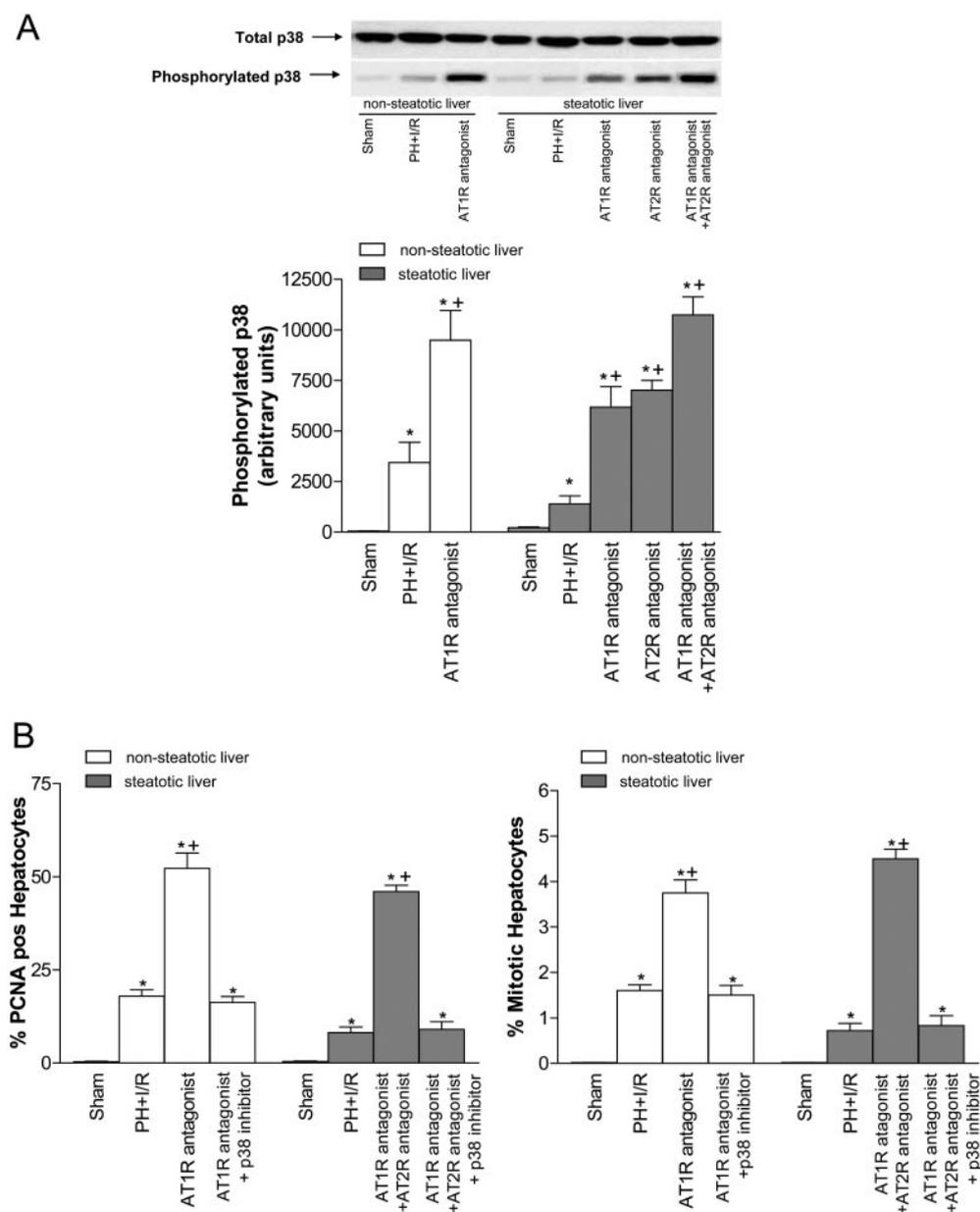


Fig. 7. Role of p38 in the beneficial effects of Ang II receptor antagonists on liver regeneration. A, phosphorylated p38 protein levels were measured in both liver types. At the top of the densitometric analysis of phosphorylated p38, representative Western blot of phosphorylated p38 and total p38 as loading control are shown. B, percentage PCNA-positive hepatocytes and percentage mitotic hepatocytes were analyzed in both liver types. *, $P < 0.05$ versus Sham; +, $P < 0.05$ versus PH + I/R.

deprived of blood supply and hepatic resection. It is well known that the mechanisms of hepatic damage are different depending on the percentage of hepatic mass that is deprived of blood supply (Chaudry et al., 1982; Hayashi et al., 1986; Massip-Salcedo et al., 2007). In addition, the inherent mechanisms of hepatic damage derived from the massive removal of hepatic mass should be considered (Kamiyama et al., 1976; Kurokawa et al., 1991; Watanabe et al., 2000). This may explain, at least partially, why the same drug, such as an Ang II receptor antagonist, may show differential effect on hepatic injury depending on surgical conditions.

In our conditions, the NO generation after partial hepatectomy was associated with a marked increase in iNOS and cNOS protein levels. The up-regulation of cNOS expression is surprising, given the fact that this protein is considered relatively constitutive and mostly regulated at the level of cofactor supply (Förstermann et al., 1998). Nevertheless, it has been reported that cNOS is not only regulated at the level of its enzymatic activity but also at the level of the

protein expression and that different factors (including shear stress) induce a marked increase in cNOS expression (Nadaud et al., 1996; Tazi et al., 2002). This could occur in our conditions because it is well known that alteration in the hepatic blood flow in the remnant liver caused by I/R injury and the massive removal of hepatic mass lead to shear stress (Pannen, 2002; Schoen and Lautt, 2002). Clinical and experimental studies revealed the injurious effects of NO on damage in the remnant liver in conditions of partial hepatectomy under I/R, whereas the role of NO in liver regeneration was not assessed (Kukita et al., 2005). Our results indicated that NO is not responsible for the beneficial effects on liver regeneration induced by either Ang II receptor antagonists in either liver type. In nonsteatotic livers, the AT1R antagonist improved liver regeneration but did not modify NO generation. In the presence of steatosis, only the AT1R antagonist reduced NO generation, but NO donor administration did not modify the beneficial effects of AT1R antagonist on regeneration in this type of liver. On the other hand, the AT1R

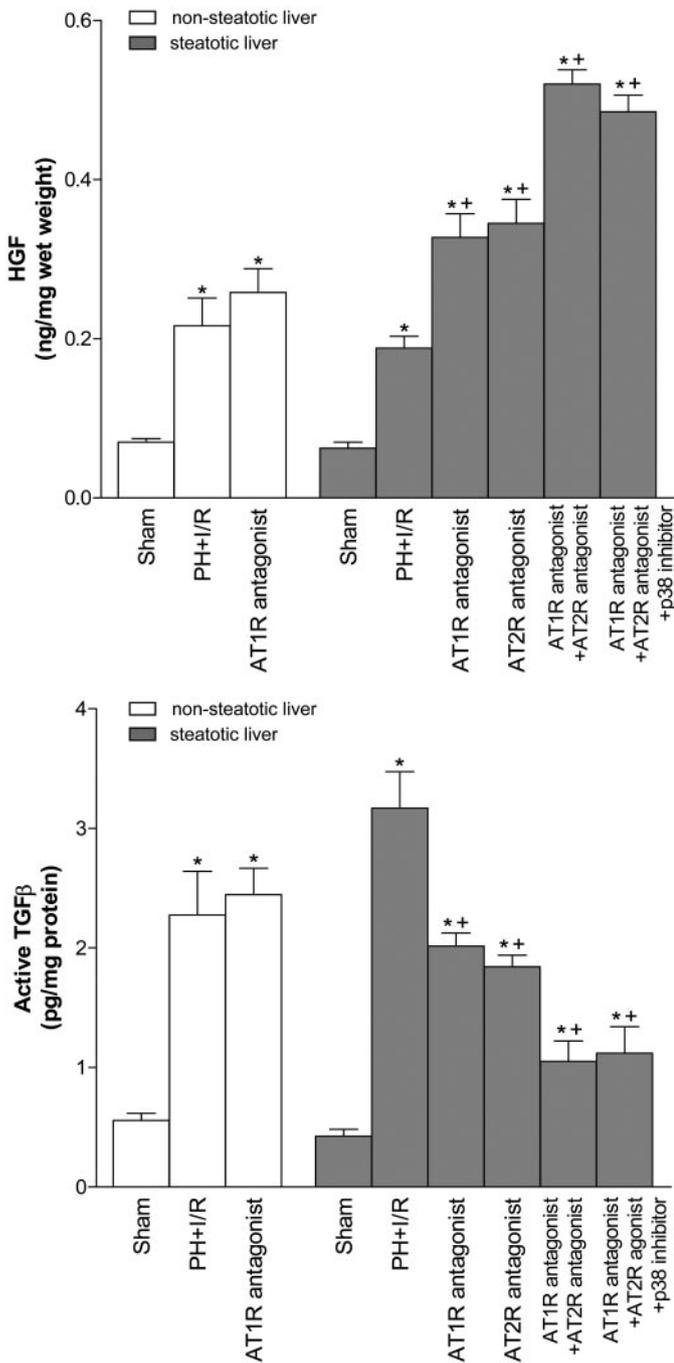


Fig. 8. Involvement of growth factors in the beneficial effects of Ang II receptor antagonists on liver regeneration. HGF and active TGF β levels were measured in both liver types. *, $P < 0.05$ versus Sham; +, $P < 0.05$ versus PH + I/R.

antagonist, through NO inhibition, protected steatotic livers against oxidative stress and damage. This indicated the key role of NO in the beneficial effects of the AT1R antagonist on damage in steatotic livers.

In conditions of partial hepatectomy under I/R, p38 was a key mechanism for the liver regeneration induced by Ang II receptor antagonists in both liver types. Thus, strategies that increased p38 (AT1R antagonist for nonsteatotic livers and both Ang II receptor antagonists for steatotic ones) improved liver regeneration. In addition, when p38 was inhibited, the beneficial effects of Ang II antagonists on liver regeneration

disappeared. In addition to p38, liver regeneration may be controlled by growth factors that promote or inhibit liver regeneration (Fausto et al., 1995). Our results suggest a minor role of HGF and TGF β in the beneficial effects of Ang II receptor antagonists on liver regeneration in nonsteatotic livers in conditions of partial hepatectomy under I/R. In the presence of steatosis, when p38 was inhibited, the beneficial effects of Ang II receptor antagonists on liver regeneration disappeared despite the presence of high HGF levels and reduced TGF β . As a consequence, HGF and TGF β may not play a role in the benefits of these Ang II receptor antagonists on liver regeneration in steatotic livers. An alternative hypothesis is that these growth factors are upstream of p38 and that they are unmodified when p38 is inhibited or activated. Further studies will be required to clarify this.

In summary, in conditions of hepatectomy under I/R, the AT1R antagonist for nonsteatotic livers and AT1R and AT2R antagonists for steatotic ones trigger p38 activation in the remnant liver intended to favor cell growth. In addition, the combination of AT1R and AT2R antagonists in steatotic livers lead to stronger liver regeneration than either antagonist used separately and also provided the same protection against damage as that afforded by the AT1R antagonist alone.

Acknowledgments

We thank Robin Rycroft (Language Advisory Service, University of Barcelona, Barcelona, Spain) for revising the English text and Emma Puig-Oriol and Llorenç Quintó (Epidemiology and Biostatistics Unit, Barcelona University, Barcelona, Spain) for help in the statistical analyses.

References

- Behrns KE, Tsiotos GG, DeSouza NF, Krishna MK, Ludwig J, and Nagorney DM (1998) Hepatic steatosis as a potential risk factor for major hepatic resection. *J Gastrointest Surg* **2**:292–298.
- Carnovale CE, Scapini C, Alvarez ML, Favre C, Monti J, and Carrillo MC (2000) Nitric oxide release and enhancement of lipid peroxidation in regenerating rat liver. *J Hepatol* **32**:798–804.
- Carrasco-Chaumel E, Roselló-Catafau J, Bartrons R, Franco-Gou R, Xaus C, Casillas A, Gelpí E, Rodés J, and Peralta C (2005) Adenosine monophosphate-activated protein kinase and nitric oxide in rat steatotic liver transplantation. *J Hepatol* **43**:997–1006.
- Casillas-Ramirez A, Amine-Zaouali M, Massip-Salcedo M, Padriisa-Altés S, Bintanel-Morcillo M, Ramalho F, Serafin A, Rimola A, Arroyo V, Rodés J, et al. (2008) Inhibition of angiotensin II action protects rat steatotic livers against ischemia-reperfusion injury. *Crit Care Med* **36**:1256–1266.
- Chaudry IH, Clemens MG, Ohkawa M, Schleck S, and Baue AE (1982) Restoration of hepatocellular function and blood flow following hepatic ischemia with ATP-MgCl₂. *Adv Shock Res* **8**:177–186.
- Clavien PA, Selzner M, Rüdiger HA, Graf R, Kadry Z, Rousson V, and Jochum W (2003) A prospective randomized study in 100 consecutive patients undergoing major liver resection with versus without ischemic preconditioning. *Ann Surg* **238**:843–850; discussion 851–852.
- Dixon E, Vollmer CM Jr, Bathe OF, and Sutherland F (2005) Vascular occlusion to decrease blood loss during hepatic resection. *Am J Surg* **190**:75–86.
- Fausto N, Laird AD, and Webber EM (1995) Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. *FASEB J* **9**:1527–1536.
- Förstermann U, Boissel JP, and Kleinert H (1998) Expressional control of the “constitutive” isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J* **12**:773–790.
- Franco-Gou R, Roselló-Catafau J, Casillas-Ramirez A, Massip-Salcedo M, Rimola A, Calvo N, Bartrons R, and Peralta C (2006) How ischaemic preconditioning protects small liver grafts. *J Pathol* **208**:62–73.
- Gohlke P, Pees C, and Unger T (1998) AT2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. *Hypertension* **31**:349–355.
- Guo L, Richardson KS, Tucker LM, Doll MA, Hein DW, and Artele GE (2004) Role of the renin-angiotensin system in hepatic ischemia reperfusion injury in rats. *Hepatology* **40**:583–589.
- Hayashi H, Chaudry IH, Clemens MG, and Baue AE (1986) Hepatic ischemia models for determining the effects of ATP-MgCl₂ treatment. *J Surg Res* **40**:167–175.
- Hortelano S, Dewez B, Genaro AM, Diaz-Guerra MJ, and Bosca L (1995) Nitric oxide is released in regenerating liver after partial hepatectomy. *Hepatology* **21**:776–786.
- Hsu MK, Qiao L, Ho V, Zhang BH, Zhang H, Teoh N, Dent P, and Farrell GC (2006)

- Ethanol reduces p38 kinase activation and cyclin D1 protein expression after partial hepatectomy in rats. *J Hepatol* **44**:375–382.
- Huguet C, Gavelli A, Chieco PA, Bona S, Harb J, Joseph JM, Jobard J, Gramaglia M, and Lasserre M (1992) Liver ischemia for hepatic resection: where is the limit? *Surgery* **111**:251–259.
- Kajihara N, Nishida T, Boku N, Tatewaki H, Eto M, and Morita S (2005) Angiotensin II type 1 receptor antagonist protects ventricular and coronary endothelial function after 24-hour heart preservation. *J Heart Lung Transplant* **24**:2211–2217.
- Kamiyama Y, Ozawa K, and Honjo I (1976) Changes in mitochondrial phosphorylative activity and adenylate energy charge of regenerating rabbit liver. *J Biochem* **80**:875–881.
- Kukita K, Katsuramaki T, Kikuchi H, Meguro M, Nagayama M, Kimura H, Isobe M, and Hirata K (2005) Remnant liver injury after hepatectomy with the Pringle maneuver and its inhibition by an iNOS inhibitor (ONO-1714) in a pig model. *J Surg Res* **125**:78–87.
- Kurokawa T, Nonami T, Sugiyama S, Ozawa T, and Takagi H (1991) Effects of long-acting superoxide dismutase on liver metabolism after major hepatic resection in rats with cirrhosis. *Eur Surg Res* **23**:65–72.
- Massip-Salcedo M, Casillas-Ramirez A, Franco-Gou R, Bartrons R, Ben Mosbah I, Serafin A, Roselló-Catafau J, and Peralta C (2006) Heat shock proteins and mitogen-activated protein kinases in steatotic livers undergoing ischemia-reperfusion: some answers. *Am J Pathol* **168**:1474–1485.
- Massip-Salcedo M, Roselló-Catafau J, Prieto J, Avila MA, and Peralta C (2007) The response of the hepatocyte to ischemia. *Liver Int* **27**:6–16.
- McCormack L, Petrowsky H, Jochum W, Furrer K, and Clavien PA (2007) Hepatic steatosis is a risk factor for postoperative complications after major hepatectomy: a matched case-control study. *Ann Surg* **245**:923–930.
- Nadaud S, Philippe M, Arnal JF, Michel JB, and Soubrier F (1996) Sustained increase in aortic endothelial nitric oxide synthase expression in vivo in a model of chronic high blood flow. *Circ Res* **79**:857–863.
- Nocito A, El-Badry AM, and Clavien PA (2006) When is steatosis too much for transplantation? *J Hepatol* **45**:494–499.
- Pannen BH (2002) New insights into the regulation of hepatic blood flow after ischemia and reperfusion. *Anesth Analg* **94**:1448–1457.
- Peralta C, Rull R, Rimola A, Deulofeu R, Roselló-Catafau J, Gelpi E, and Rodés J (2001) Endogenous nitric oxide and exogenous nitric oxide supplementation in hepatic ischemia-reperfusion injury in the rat. *Transplantation* **71**:529–536.
- Rai RM, Yang SQ, McClain C, Karp CL, Klein AS, and Diehl AM (1996) Kupffer cell depletion by gadolinium chloride enhances liver regeneration after partial hepatectomy in rats. *Am J Physiol* **270**:G909–G918.
- Ramalho FS, Ramalho LN, Castro-e-Silva Júnior O, Zucoloto S, and Corrêa FM (2002) Effect of angiotensin-converting enzyme inhibitors on liver regeneration in rats. *Hepato-gastroenterology* **49**:1347–1351.
- Schoen JM and Lauth WW (2002) Nitric oxide potentiates C-Fos mRNA expression after 2/3 partial hepatectomy. *Proc West Pharmacol Soc* **45**:47–48.
- Selzner M, Camargo CA, and Clavien PA (1999) Ischemia impairs liver regeneration after major tissue loss in rodents: protective effects of interleukin-6. *Hepatology* **30**:469–475.
- Serafin A, Roselló-Catafau J, Prats N, Xaus C, Gelpi E, and Peralta C (2002) Ischemic preconditioning increases the tolerance of Fatty liver to hepatic ischemia-reperfusion injury in the rat. *Am J Pathol* **161**:587–601.
- Spector MS, Auer KL, Jarvis WD, Ishac EJ, Gao B, Kunos G, and Dent P (1997) Differential regulation of the mitogen-activated protein and stress-activated protein kinase cascades by adrenergic agonists in quiescent and regenerating adult rat hepatocytes. *Mol Cell Biol* **17**:3556–3565.
- Suzuki S, Nakamura S, Sakaguchi T, Ochiai H, Konno H, Baba S, and Baba S (1997) Alteration of reticuloendothelial phagocytic function and tumor necrosis factor- α production after total hepatic ischemia. *Transplantation* **64**:821–827.
- Tazi KA, Barrière E, Moreau R, Heller J, Sogni P, Pateron D, Poirel O, and Lebrec D (2002) Role of shear stress in aortic eNOS up-regulation in rats with biliary cirrhosis. *Gastroenterology* **122**:1869–1877.
- Veteläinen R, van Vliet A, Gouma DJ, and van Gulik TM (2007) Steatosis as a risk factor in liver surgery. *Ann Surg* **245**:20–30.
- Watanabe M, Chijiwa K, Kameoka N, Yamaguchi K, Kuroki S, and Tanaka M (2000) Gadolinium pretreatment decreases survival and impairs liver regeneration after partial hepatectomy under ischemia/reperfusion in rats. *Surgery* **127**:456–463.
- Wei C, Cardarelli MG, Downing SW, and McLaughlin JS (2000) The effect of angiotensin II on mitogen-activated protein kinase in human cardiomyocytes. *J Renin Angiotensin Aldosterone Syst* **1**:379–384.
- Yayama K, Sugiyama K, Miyagi R, and Okamoto H (2007) Angiotensin-converting enzyme inhibitor enhances liver regeneration following partial hepatectomy: involvement of bradykinin B2 and angiotensin AT1 receptors. *Biol Pharm Bull* **30**:591–594.
- Zhao TC, Taher MM, Valerie KC, and Kukreja RC (2001) p38 Triggers late preconditioning elicited by anisomycin in heart: involvement of NF- κ B and iNOS. *Circ Res* **89**:915–922.

Address correspondence to: Dr. Joan Roselló-Catafau, Institut d'Investigacions Biomèdiques August Pi i Sunyer-Consejo Superior de Investigaciones Científicas, C/ Roselló 161, 7th floor, 08036 Barcelona, Spain. E-mail: jrccbam@iibb.csic.es