Retinol-Binding Protein 4 and Peroxisome Proliferator-Activated Receptor-γ in Steatotic Liver Transplantation

Aralí Casillas-Ramírez, Izabel Alfany-Fernández, Marta Massip-Salcedo, M. Emília Juan, Joana M. Planas, Anna Serafín, Mercè Pallàs, Antoni Rimola, Juan Rodés, and Carmen Peralta

Institut d’Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain (A.C.-R., I.A.-F., M.M.-S., J.R., C.P.); Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, Barcelona, Spain (M.M.-S., A.R., J.R., C.P.); Departament de Fisiologia and Institut de Recerca en Nutrició i Seguritat Alimentària (M.E.J., J.M.P.) and Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Institut de Biomedicina (M.P.), Universitat de Barcelona, Barcelona, Spain; Platform of Laboratory Animal Applied Research, Parc Científic de Barcelona, Barcelona, Spain (A.S.); Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas, Barcelona, Spain (M.P.); and Liver Unit, Hospital Clinic, Barcelona, Spain (A.R.)

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ABSTRACT
Numerous steatotic livers are discarded for transplantation because of their poor tolerance of ischemia-reperfusion (I/R). The injurious effects of retinol-binding protein 4 (RBP4) in various pathologies are well documented. RBP4 levels are reduced by peroxisome proliferator-activated receptor-γ (PPARγ) agonists. Strategies aimed at increasing PPARγ protect steatotic livers under warm ischemia. Ischemic preconditioning (PC) based on brief periods of I/R protects steatotic liver grafts against I/R injury, but the responsible mechanism is poorly understood. We examined the roles of RBP4 and PPARγ in I/R injury associated with steatotic liver transplantation and the benefits of PC in such situations. We report that RBP4 and PPARγ expression levels in nonsteatotic livers were similar to those found in the sham group. However, reduced RBP4 and increased PPARγ levels were observed in steatotic livers. Treatment with either RBP4 or a PPARγ antagonist was effective only in steatotic livers. PC, which increased RBP4 levels, and RBP4 treatment both reduced PPARγ levels and hepatic injury in steatotic livers. When PPARγ was activated, neither RBP4 treatment nor PC (despite RBP4 induction) protected steatotic livers. In conclusion, steatotic liver grafts are more predisposed to down-regulate RBP4 and overexpress PPARγ. RBP4 treatment and PC, through RBP4 induction, reduced PPARγ levels in steatotic liver grafts, thus protecting them from the PPARγ detrimental effects.

Introduction
Numerous steatotic livers are discarded for transplantation because of their poor tolerance to ischemia-reperfusion (I/R), exacerbating the critical shortage of donor livers (D’Alessandro et al., 1991; Ploeg et al., 1993). Therefore, minimizing the adverse effects of I/R in steatotic liver transplantation is an urgent need. New insights into the mechanisms of steatotic liver graft failure could result in new strategies to protect steatotic liver grafts against I/R injury associated with transplantation.

Retinol-binding protein 4 (RBP4) is an adipokine synthesized by the liver, whose known function is to transport retinol in the circulation. However, the role of RBP4 in the liver is largely unknown (Blaner, 1989; Graham et al., 2006; Wagnerberger et al., 2006). Since the discovery of RBP4 in 1992, diverse studies have demonstrated that RBP4 levels are elevated in diabetes, obesity, cardiovascular diseases, and inflammation (Yang et al., 2005; Cho et al., 2006; Gra-
the possibility that lowering RBP4 levels might protect steatotic liver grafts against I/R injury associated with transplantation has not been evaluated to date.

Peroxisome proliferator-activated receptor-γ (PPARγ) agonists reduce I/R injury in steatotic livers under warm hepatic ischemia (Casillas-Ramírez et al., 2008). The role of PPARγ in steatotic liver transplantation has yet to be identified. There is a relationship between PPARγ and RBP4, whereby PPARγ agonists reduce RBP4 mRNA in adipose tissue of mice and serum RBP4 in diabetic subjects (Yang et al., 2005; Teranishi et al., 2007; Lin et al., 2008). This study evaluates the role of RBP4 and PPARγ in steatotic liver transplantation. In addition, we compare the effects of pharmacological treatments that modulate RBP4 and PPARγ in steatotic liver transplantation with those obtained after applying ischemic preconditioning (PC). PC is an endogenous protective mechanism by which brief periods of vascular occlusion confer protection against subsequent sustained hepatic I/R (Casillas-Ramírez et al., 2006; Massip-Salceld et al., 2007). To date, despite intense research efforts, PC is the only surgical strategy that has been successfully applied in patients with steatotic livers undergoing warm ischemia (Clavien et al., 2003). Previous experimental studies of our group have shown the effectiveness of PC in reducing I/R injury in steatotic liver transplantation (Carrasco-Chaumel et al., 2005), but whether PC is appropriate for steatotic liver transplantation in clinical practice remains to be clarified. Although the mechanisms by which PC protects steatotic liver grafts are unknown, liver protection depends on AMP-activated protein kinase (AMPK) activation (Carrasco-Chaumel et al., 2005). Until now, no data had been reported on the capacity of PC to modify RBP4 levels in livers undergoing I/R. However, this possibility should not be ruled out. We have suggested that PC exerts its effect on PPARγ in steatotic livers under warm hepatic ischemia (Casillas-Ramírez et al., 2008) and PPARγ agonists reduce RBP4 levels in various pathologies (Yang et al., 2005; Teranishi et al., 2007; Lin et al., 2008). Only a full appraisal of the underlying protective mechanisms of PC can lead to both new pharmacological strategies to effectively protect steatotic liver grafts and new applications of PC in clinical practice of steatotic liver transplantation.

Materials and Methods

Experimental Animals

The present study was performed using homozygous (obese, Ob) and heterozygous (Lean, Ln) Zucker rats (Iffa-Credo, L’Abresle, France) aged 10 to 11 weeks. Ob rats showed moderate macrovesicular and microvesicular fatty infiltration in hepatocytes, whereas Ln rats showed no evidence of steatosis (Carrasco-Chaumel et al., 2005). Analysis of triglyceride content and fatty droplet accumulation in hepatocytes, whereas Ln and Ob animals (six in each group) were subjected to transverse laparotomy, and silk ligatures were applied in the right suprarenal vein, diaphragmatic vein, and hepatic artery (Carrasco-Chaumel et al., 2005).

Group 2 (TR, 12 transplantations, n = 24 rats) was divided into two subgroups. In subgroup 2.1 (six transplantations, n = 12 rats), steatotic livers from donor rats (Ob Zucker rats, n = 6 rats) were flushed with University of Wisconsin (UW) solution and removed from donor rats. Steatotic livers were then preserved in ice-cold UW solution for 6 h (ice-cold ischemia) (Carrasco-Chaumel et al., 2005) and implanted into recipient rats (Ln Zucker rats, n = 6 rats) according to the Kamada cuff technique without hepatic artery reconstruction (Kamada and Calne, 1979). In subgroup 2.2 (six transplantations, n = 12 rats), nonsteatotic livers from donor rats (Ln Zucker rats, n = 6 rats) were flushed with UW solution and removed from donor rats. Nonsteatotic livers were then preserved in ice-cold UW solution for 6 h (ice-cold ischemia) (Carrasco-Chaumel et al., 2005) and implanted into recipient rats (Ln Zucker rats, n = 6 rats) according to the Kamada cuff technique without hepatic artery reconstruction (Kamada and Calne, 1979).

Group 3 (PC+TR, 12 transplantations, n = 24 rats) was the same as group 2 but with PC (induced by 5 min of ischemia followed by 10 min of reperfusion) before livers were flushed and preserved in UW solution for 6 h (Carrasco-Chaumel et al., 2005).

Protocol 2. This protocol involved dose-response studies of RBP4 and PPARγ antagonist on hepatic injury in steatotic liver transplantation (groups 4 and 5).

Protocol 3. Hepatic injury and RBP4 and PPARγ levels after the pharmacological modulation of RBP4 and PPARγ (groups 6–12) were evaluated.

Protocol 4. AMPK involvement in PC-induced effects on RBP4 and PPARγ in steatotic liver transplantation (groups 13 and 14) was investigated.

Protocol 5. The changes in RBP4 and PPARγ levels in steatotic liver grafts during cold ischemia before the implantation of steatotic liver grafts in the recipient (groups 15–18) were investigated.

All of the drugs used were administered in the donor rats. The doses and pretreatment times used for the different drugs are shown in Table 2. Control experiments were performed using the vehicles of the drugs used in this study.

For analytical determinations, plasma and liver samples from protocols 1 to 4 were collected 4 h after transplantation from the recipient. Liver samples from donor rats were collected after 6 h of

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Drugs

Recombinant RBP4 and anti-RBP4 antibodies were purchased from AdipoGen Inc. (Seoul, Korea). GW9662 (2-chloro-5-nitro-N-phenylbenzamide) and rosiglitazone (RS)-5-[4-[2-(methylpyridin-2-yl)amino]ethoxy]benzylthiazolidine-2,4-dione were purchased from Alexis Biochemicals (Lausen, Switzerland). Aminomidazole-4-carboxamide ribonucleoside (AICAR) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and adenosine 9-β-D-arabinofuranoside was purchased from Sigma-Aldrich (St. Louis, MO).

Experimental Design

The experimental design of the present study is summarized in Table 1. The experimental protocols are the following:

Protocol 1. RBP4 and PPARγ levels in nonsteatotic and steatotic liver transplantation were examined. In group 1 (sham; n = 12 rats), Ln and Ob animals (six in each group) were subjected to transverse laparotomy, and silk ligatures were applied in the right suprarenal vein, diaphragmatic vein, and hepatic artery (Carrasco-Chaumel et al., 2005).

Group 2 (TR, 12 transplantations, n = 24 rats) was divided into two subgroups. In subgroup 2.1 (six transplantations, n = 12 rats), steatotic livers from donor rats (Ob Zucker rats, n = 6 rats) were flushed with University of Wisconsin (UW) solution and removed from donor rats. Steatotic livers were then preserved in ice-cold UW solution for 6 h (ice-cold ischemia) (Carrasco-Chaumel et al., 2005) and implanted into recipient rats (Ln Zucker rats, n = 6 rats) according to the Kamada cuff technique without hepatic artery reconstruction (Kamada and Calne, 1979). In subgroup 2.2 (six transplantations, n = 12 rats), nonsteatotic livers from donor rats (Ln Zucker rats, n = 6 rats) were flushed with UW solution and removed from donor rats. Nonsteatotic livers were then preserved in ice-cold UW solution for 6 h (ice-cold ischemia) (Carrasco-Chaumel et al., 2005) and implanted into recipient rats (Ln Zucker rats, n = 6 rats) according to the Kamada cuff technique without hepatic artery reconstruction (Kamada and Calne, 1979).

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Protocol 4. AMPK involvement in PC-induced effects on RBP4 and PPARγ in steatotic liver transplantation (groups 13 and 14) was investigated.

Protocol 5. The changes in RBP4 and PPARγ levels in steatotic liver grafts during cold ischemia before the implantation of steatotic liver grafts in the recipient (groups 15–18) were investigated.

The conditions of this study (including the times of cold ischemia, reperfusion, and PC period) were established on the basis of the results of previous studies (Carrasco-Chaumel et al., 2005). A cold ischemic period of 6 h is long enough to induce liver damage after transplantation in liver grafts and allow high survival 4 h after transplantation. In addition, the PC period used in the present study (5 min of ischemia followed by 10 min of reperfusion) is effective against hepatic I/R injury in both liver types (Carrasco-Chaumel et al., 2005). Thus, these experimental conditions were appropriate for evaluating RBP4 and PPARγ levels in both liver types and their effects on hepatic I/R injury associated with transplantation.

All of the drugs used were administered in the donor rats. The doses and pretreatment times used for the different drugs are shown in Table 2. Control experiments were performed using the vehicles of the drugs used in this study.

For analytical determinations, plasma and liver samples from protocols 1 to 4 were collected 4 h after transplantation from the recipient. Liver samples from donor rats were collected after 6 h of
TABLE 1
Experimental design of the current study

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Drug Administration Protocol</th>
<th>Dose and Pretreatment Time</th>
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<tbody>
<tr>
<td>1. Sham (n = 12, 6 Ln and 6 Ob)</td>
<td>Dissection of hepatic ilium vessels</td>
<td>25, 50, 75, 100, 125, 150, 175, or 200 μg/kg i.v., in donor rat 30 min before surgical procedure</td>
</tr>
<tr>
<td>2. TR (12 transplantations), divided into two groups</td>
<td>Livers preserved in UW solution and subsequently transplanted</td>
<td>250, 500, 750, 1000, 1250, or 1500 μg/kg i.p., in donor rat 1 h before surgical procedure</td>
</tr>
<tr>
<td>2.1 TR (6 transplantations), graft Ob, 6 h ice-cold ischemia</td>
<td>Recipient Ln, 4 h reperfusion</td>
<td>300 μg/kg i.v., in donor rat 30 min before surgical procedure</td>
</tr>
<tr>
<td>2.2 TR (6 transplantations), graft Ln, 6 h ice-cold ischemia</td>
<td>Recipient Ln, 4 h reperfusion</td>
<td>1000 μg/kg i.p., in donor rat 1 h before surgical procedure</td>
</tr>
<tr>
<td>3. PC + TR (12 transplantations)</td>
<td>Same as group, but with PC</td>
<td>Steatotic livers from Ob animals, preserved in UW solution</td>
</tr>
<tr>
<td>Protocol 2. Dose-response studies of RBP4 and PPARγ antagonist on hepatic injury in steatotic liver transplantation</td>
<td>Same as group 2.1, but treated with different doses of RBP4</td>
<td>Same as group 14, but with PC</td>
</tr>
<tr>
<td>4. TR + RBP4 (6 transplantations for each dose evaluated)</td>
<td>Same as group 2.1, but treated with different doses of RBP4 and PPARγ antagonist</td>
<td>Same as group 14, but treated with AMPK activator</td>
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<tr>
<td>5. TR + PPARγ antagonist (6 transplantations for each dose evaluated)</td>
<td>Same as group 2.1, but treated with RBP4 and PPARγ antagonist</td>
<td>Same as group 14, but with PC and treated with AMPK inhibitor</td>
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TABLE 2
Drug administration protocol of the current study

<table>
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<th>Protocol</th>
<th>Drug</th>
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<tr>
<td>2</td>
<td>RBP4</td>
<td>Recombinant RBP4</td>
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<tr>
<td></td>
<td>PPARγ antagonist</td>
<td>GW9662</td>
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<tr>
<td>2</td>
<td>3</td>
<td>25, 50, 75, 100, 125, 150, 175, or 200 μg/kg i.v., in donor rat 30 min before surgical procedure</td>
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<tr>
<td></td>
<td></td>
<td>250, 500, 750, 1000, 1250, or 1500 μg/kg i.p., in donor rat 1 h before surgical procedure</td>
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<tr>
<td>3</td>
<td>RBP4</td>
<td>Recombinant RBP4</td>
</tr>
<tr>
<td></td>
<td>Anti-RBP4</td>
<td>Anti-RBP4 antibody</td>
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<tr>
<td></td>
<td>PPARγ antagonist</td>
<td>GW9662</td>
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<td></td>
<td>PPARγ agonist</td>
<td>Rosiglitazone</td>
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<td>4 and 5</td>
<td>AMPK activator</td>
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<td></td>
<td>AMPK inhibitor</td>
<td>Adenine 9-β-d-arabinofuranoside.</td>
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<tr>
<td>2</td>
<td>3</td>
<td>150 μg/kg i.v., in donor rat 30 min before surgical procedure</td>
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<td></td>
<td></td>
<td>1000 μg/kg i.p., in donor rat 1 h before surgical procedure</td>
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<td>4 and 5</td>
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<td>3000 μg/kg i.v., in donor rat 30 min before surgical procedure</td>
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<td>3000 μg/kg i.p., in donor rat 1 h before surgical procedure</td>
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<td>2</td>
<td>3</td>
<td>100 mg/kg i.v., in donor rat 5 min before surgical procedure</td>
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<td></td>
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<td>100 μg/kg/min i.v. in donor rat 10 min before surgical procedure</td>
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ice-cold ischemia (protocol 5); thus, in these rats steatotic liver grafts were not subjected to transplantation.

Reverse Transcription and Real-Time Polymerase Chain Reaction
Quantitative real-time PCR analysis was performed using the Assays-on-Demand TaqMan probes (Rn01451317_g1 for RBP4, Rn00440945_m1 for PPARγ, and Rn00667869_m1 for β-actin; Applied Biosystems, Foster City, CA) following the manufacturer’s protocol.

Biochemical Determinations
Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and triglyceride levels were measured following previously described protocols (Carrasco-Chaumel et al., 2005; Man et al., 2006). RBP4 and PPARγ were measured using enzyme-linked immunosorbent assay kits (Adipogen Inc., Seoul, Korea, and Antibodies-online GmbH, Aache, Germany, respectively) according to the manufacturer’s instructions.

Histology
To appraise the severity of hepatic injury, hematoxylin and eosin-stained sections were evaluated by a point-counting method on an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmatic vacuolization and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmatic hydropsynephiasis, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (Serafin et al., 2004). To appraise the hepatic fatty droplet content, red-oil staining on frozen specimens was evaluated according to standard procedures (Carrasco-Chaumel et al., 2005).

Statistics
Data are expressed as means ± S.E. and were compared statistically via one-way analysis of variance followed by a post hoc Student-Newman-Keuls test. A P value <0.05 was considered significant. The dose responses of RBP4 and PPARγ antagonist were analyzed using Prism version 4 (GraphPad Software Inc., San Diego, CA). To derive median effective dose (ED50), a nonlinear approximation model of the least-square method based on a competition curve using one component was calculated. Data were evaluated by one-way analysis of variance and Bonferroni’s post-test.
Results

RBP4 and PPARγ Levels in Nonsteatotic and Steatotic Liver Transplantation. RBP4 mRNA and protein levels in nonsteatotic liver grafts of the TR and PC+TR groups were similar to those found in the sham group (Fig. 1, A and B). In steatotic liver grafts, a reduction in RBP4 mRNA and protein levels was observed in the TR group compared with the results found in the sham group (Fig. 1, A and B). In the PC+TR group, RBP4 mRNA and protein levels were increased in steatotic liver grafts compared with the TR group.

PPARγ mRNA and protein levels in nonsteatotic liver grafts of the TR and PC+TR groups were similar to those found in the sham group (Fig. 1, C and D). In the presence of steatosis, PPARγ mRNA and protein levels in the TR group were markedly higher than in the sham group. In the PC+TR group, PPARγ mRNA and protein levels were reduced in steatotic liver grafts compared with the TR group (Fig. 1, C and D).

Dose-Response Effect of RBP4 and PPARγ Antagonist on Hepatic Injury in Steatotic Liver Transplantation. We evaluated the relevance of changes in RBP4 and PPARγ levels observed in steatotic liver grafts undergoing transplantation on hepatic injury. For this, we administered RBP4 at doses of 25, 50, 75, 100, 125, 150, and 200 μg/kg in donor rats 30 min before the surgical procedure, and the effects on hepatic injury were determined 4 h after transplantation in recipients (Fig. 2, A–C). The most effective dose of RBP4 in reducing the parameters of hepatic injury in steatotic liver grafts was 150 μg/kg, because higher doses were not associated with lower hepatic damage. The pretreatment time of RBP4 used in the present study (30 min before the surgical procedure) was selected on the basis of previous studies from our group. Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed using longer pretreatment times (60 or 120 min before the surgical procedure; data not shown).

We administered a PPARγ antagonist at doses of 250, 500, 750, 1500, and 2000 μg/kg in donor rats 1 h before the surgical procedure, and the effects on hepatic injury were determined 4 h after transplantation in recipients (Fig. 2, D–F). The PPARγ antagonist protected steatotic liver grafts against damage in a dose-dependent manner. The ED50 values for AST, ALT, and damage score were 503.0 ± 5.53, 315.0 ± 1.03, and 224.9 ± 2.07 μg/kg, respectively (Fig. 2, D–F). The most effective dose of PPARγ antagonist in protecting steatotic livers against damage was 1000 μg/kg. Higher doses were unnecessary because they were not associated with lower hepatic damage. The pretreatment time of the PPARγ antagonist (1 h before the surgical procedure) was selected on the basis of previous studies (Si-varajah et al., 2005; Casillas-Ramírez et al., 2008) and preliminary studies from our group. Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed at longer pretreatment times (2 or 3 h before the surgical procedure; data not shown).

Hepatic Injury and RBP4 and PPARγ Levels after the Pharmacological Modulation of RBP4 and PPARγ. As shown in Fig. 3, A and B, RBP4 treatment at the selected dose, 150 μg/kg (TR+RBP4 group), reduced transaminase levels in steatotic liver grafts with respect to those recorded
in the TR group. Nonsteatotic liver grafts were not protected against hepatic injury in the TR+RBP4 group. Indeed, transaminase levels and damage score values in nonsteatotic liver grafts of the TR+RBP4 group were similar to those of the TR group (AST: 2135 ± 232 and 2075 ± 329 U/l for the TR+RBP4 and TR groups, respectively; ALT: 1549 ± 129 and 1540 ± 205 U/l for the TR+RBP4 and TR groups, respectively; damage score values: 1.80 ± 0.20 and 1.80 ± 0.12 for the TR+RBP4 and TR groups, respectively). We evaluated whether the increase in RBP4 induced by PC+TR protects steatotic liver grafts. We attempted to identify the dose of anti-RBP4 antibody that reduced RBP4 levels to those of the TR group. Among the doses evaluated (1000, 3000, and 5000 μg/kg), the appropriate dose of anti-RBP4 antibody was 3000 μg/kg (data not shown). At this dose, the administration of anti-RBP4 antibody in the PC+TR group (PC+TR+anti-RBP4 group) resulted in RBP4 protein levels similar to those of the TR group (2804 ± 80, 7316 ± 709, and 2863 ± 212 ng/g tissue for the TR, PC+TR, and PC+TR+anti-RBP4 groups, respectively). This effect was associated with transaminase levels similar to those detected in the TR group (Fig. 3, A and B). The damage score values showed a similar pattern to that described for transaminase (Fig. 3C). Thus, the administration of anti-RBP4 antibody to the PC+TR group (PC+TR+anti-RBP4 group) abolished the benefits of PC+TR on hepatic damage. The pretreatment time of anti-RBP4 antibody used in the present study (30 min before the surgical procedure) was selected on the basis of previous studies from our group. Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed at longer pretreatment times (60 or 120 min before the surgical procedure; data not shown).

PPARγ antagonist treatment at the selected dose, 1000 μg/kg (TR+PPARγ antagonist group), resulted in lower biochemical and histological parameters of hepatic injury in steatotic liver grafts than in the TR group (Fig. 3). In nonsteatotic liver grafts, the TR+PPARγ antagonist did not induce changes in hepatic injury. Indeed, transaminase levels and damage score values in nonsteatotic liver grafts of the TR+PPARγ antagonist group were similar to those of the TR group (AST: 2143 ± 192 and 2075 ± 329 U/l for the TR+PPARγ antagonist and TR groups, respectively; ALT:
Inclusion of each measurement. The evaluated doses (1000, 3000, and 5000 μg/kg), the appropriate dose of PPARγ agonist was 3000 μg/kg (data not shown). PPARγ agonist administration at 3000 μg/kg (PC+TR+PPARγ agonist group) abolished the benefits of PC+TR, resulting in transaminase and damage score values in steatotic liver grafts similar to those observed in the TR group (Fig. 3). The pretreatment time of PPARγ agonist used in the present study (1 h before the surgical procedure) was selected on the basis of previous studies from our group and others (Yue et al., 2001; Casillas-Ramírez et al., 2008). Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed at longer pretreatment times (2 or 3 h before the surgical procedure; data not shown).

The histological findings revealed that steatotic liver grafts of the TR group showed extensive and confluent areas of coagulative necrosis with neutrophil infiltration (Fig. 4A) that were reduced in number and extension in the TR+RBP4 group (Fig. 4B). The hepatic lesions observed in steatotic liver grafts of the PC+TR (Fig. 4C) and PC+TR+anti-RBP4 (Fig. 4D) groups were comparable with those observed in the TR+RBP4 (Fig. 4B) and TR (Fig. 4A) groups, respectively. In the TR+PPARγ antagonist group (Fig. 4E), the extent and the number of necrosis areas in steatotic liver grafts were reduced compared with the TR group (Fig. 4A). The hepatic lesions observed in the PC+TR+PPARγ agonist group (Fig. 4F) were similar to those observed in the TR group (Fig. 4A).

RBP4 and PPARγ levels were investigated after the pharmacological modulation of PPARγ and RBP4. In the TR+PPARγ antagonist group, RBP4 levels in steatotic liver grafts were similar to those of the TR group (Fig. 5, A and B), indicating that PPARγ antagonist treatment did not modify RBP4 levels in steatotic liver grafts. In addition, in the PC+TR+PPARγ agonist group hepatic RBP4 levels were similar to those of the PC+TR group (Fig. 5, A and B). We evaluated the effects of different doses of RBP4 on PPARγ levels in steatotic livers 4 h after transplantation, and the calculated ED₅₀ was 111.5 ± 1.33 μg/kg. The most effective dose of RBP4 in reducing PPARγ levels was 150 μg/kg. Higher doses of RBP4 were not associated with lower PPARγ levels in steatotic liver grafts (Fig. 5C). On the other hand, it should be noted that at the selected dose of RBP4 (150 μg/kg) PPARγ levels and hepatic injury were reduced in steatotic livers in the TR+RBP4 group, but not to levels observed in the sham group (Figs. 3 and 5, D and E). Then, we assessed whether the administration of a PPARγ antagonist in the TR+RBP4 group would inhibit PPARγ levels and hepatic injury in steatotic livers. Our results indicated that in the TR+RBP4+PPARγ antagonist group PPARγ levels were reduced to those seen in the sham group (4.12 ± 0.59 and 4.24 ± 0.86 ng/g tissue in the TR+RBP4+PPARγ antagonist and sham groups, respectively) but resulted in parameters of hepatic injury similar to those of the TR+RBP4 group (AST: 1287 ± 432 and 1188 ± 360 U/l for the TR+RBP4+PPARγ and TR+RBP4 groups, respectively; ALT: 1005 ± 179 and 964 ± 170 U/l for the TR+RBP4+PPARγ and TR+RBP4 groups, respectively; damage score: 1.38 ± 0.24 and 1.35 ± 0.15 for the TR+RBP4+PPARγ and TR+RBP4 groups, respectively).

Our results indicate that RBP4 treatment (at the selected dose, 150 μg/kg) and PC mediation by RBP4 reduced PPARγ in steatotic liver grafts and ameliorated hepatic injury. In the
TR+RBP4 group, PPARγ levels (Fig. 5, D and E) and hepatic injury (Fig. 3) were reduced in steatotic liver grafts compared with the TR group. In the TR+RBP4+PPARγ agonist group, transaminase and damage score values were similar to the TR group (Fig. 3), indicating that the PPARγ agonist abolished the benefits of RBP4 treatment in steatotic liver grafts. During RBP4 treatment, PPARγ levels (Fig. 5, D and E) and hepatic injury (Fig. 3) were reduced in steatotic liver grafts in the PC+TR group compared with the TR group. In the PC+TR+anti-RBP4 group, PPARγ levels and hepatic injury in steatotic liver grafts were similar to those in the TR group, indicating the injurious effects of anti-RBP4 antibody in steatotic liver grafts. In the PC+TR+anti-RBP4+PPARγ antagonist group, hepatic injury was similar to the PC+TR group (Fig. 3), indicating that the PPARγ antagonist prevented the injurious effects of anti-RBP4 antibody in steatotic liver grafts. In the TR+RBP4+PPARγ agonist group, histological lesions were observed in steatotic liver grafts (Fig. 4G) that were similar to those of the TR group (Fig. 4A). In the PC+TR+anti-RBP4+PPARγ antagonist group, histological lesions were observed in steatotic liver grafts (Fig. 4H) that were similar to those of the PC+TR group (Fig. 4C).

Effect of AMPK on RBP4 and PPARγ Levels in Steatotic Liver Transplantation. The beneficial effects of AMPK activators such as AICAR and the involvement of AMPK in the benefits of PC in steatotic liver grafts were previously reported by our group using the same experimental model of liver transplantation described here (Carrasco-Chaumel et al., 2005). From such studies, we selected the doses and pretreatment times for AMPK activator (100 mg/kg, 5 min before the surgical procedure) and AMPK inhibitor (100 μg/kg/min for 10 min, 10 min before the surgical procedure). This dose of AMPK activator protected steatotic liver grafts. The dose of AMPK inhibitor selected inhibited AMPK activity.

Fig. 4. Histological lesions in steatotic liver transplantation. Representative photographs of histological changes in steatotic livers are shown. A, TR, widespread coagulative hepatic necrosis with neutrophil infiltration. B, TR+RBP4, small area of coagulative hepatic necrosis with neutrophil infiltration. C, E, and H, PC+TR (C), TR+PPARγ antagonist (E), and PC+TR+anti-RBP4+PPARγ antagonist (H), hepatic lesions similar to the TR+RBP4 group. D, F, and G, PC+TR+anti-RBP4 (D), PC+TR+PPARγ agonist (F), and TR+RBP4+PPARγ agonist (G), hepatic lesions similar to the TR group. Hematoxylin and eosin staining was used. Bars, 100 μm.
in preconditioned steatotic livers, leading to AMPK and transaminase levels similar to those of the TR group (Carrasco-Chaumel et al., 2005). The results of the present study indicated that in the TR/AMPK activator and PC/TR groups RBP4 mRNA and protein levels increased in steatotic liver grafts compared with the TR group (Fig. 6, A.1 and A.2). This finding was associated with reduced PPARγ mRNA and protein levels (Fig. 6, A.3 and A.4). Conversely, in the PC/AMPK inhibitor group RBP4 mRNA and protein levels in steatotic liver grafts were similar to those of the TR group (Fig. 6, A.1 and A.2). This effect was associated with similar PPARγ mRNA and protein levels in steatotic liver grafts to those of the TR group (Fig. 6, A.3 and A.4). Thus, AMPK activators and PC, through AMPK, increased RBP4 levels in steatotic liver grafts after transplantation. This result was associated with a reduction in PPARγ levels.

RBP4 and PPARγ in Steatotic Liver Grafts after Ice-Cold Ischemia. As shown above, our results reveal a close relationship between RBP4 mRNA and RBP4 protein levels in steatotic liver grafts after transplantation, suggesting that this type of graft by itself generates RBP4 after either AMPK activator treatment or PC induction (TR+AMPK activator and PC+TR groups). In addition to the liver, adipose tissue is able to generate RBP4 that may be taken up by liver from the circulation (Gjøen et al., 1987; Tsutsumi et al., 1992). To confirm that the steatotic liver graft by itself, without the influence of other tissues or plasma constituents, can generate RBP4 after either AMPK activator treatment or PC induction, we measured RBP4 mRNA levels in steatotic liver grafts during ice-cold ischemia (before the implantation of liver grafts in the recipient). Under these conditions, the liver is isolated from the influence of other tissues and plasma constituents. Our results indicated that during ice-cold ischemia increased RBP4 mRNA levels in steatotic liver grafts were observed after either AMPK activator treatment or PC induction (Fig. 6B.1). This finding was also associated with reduced PPARγ mRNA levels (Fig. 6B.2). Conversely, in the PC+ischemia+AMPK inhibitor group, RBP4 mRNA levels in steatotic liver grafts were similar to those of the ischemia group (Fig. 6B.1). This result was associated with similar PPARγ mRNA levels in steatotic liver grafts to those of the ischemia group (Fig. 6B.2).

Discussion
In line with previous data in high-fat diet-induced obese rats (Wu et al., 2009) and Zucker rats (Lanne et al., 2006), our results indicated that the presence of fatty infiltration by itself in the liver (without any surgical intervention) does not induce changes in either RBP4 or PPARγ levels, because no differences in RBP4 or PPARγ levels were observed in steatotic or nonsteatotic livers of the sham group of Zucker rats. These results contrast with reports from the literature indicating reduced RBP4 levels (Mody et al., 2008) and high or low PPARγ levels (Zhao et al., 2004; Inoue et al., 2005) in steatotic compared with nonsteatotic livers. These different results for RBP4 and PPARγ levels could be explained, at least in part, by differences in the level of RBP4 and PPARγ.
regulation between rats and mice (Lanne et al., 2006), the different obesity experimental models evaluated, and the degree of steatosis.

The present study provides evidence for the generation of RBP4 in steatotic and nonsteatotic liver grafts after transplantation. In contrast with nonsteatotic liver grafts, steatotic liver grafts clearly had lower RBP4 mRNA and protein levels after transplantation. This finding is in line with previous data showing decreased RBP4 levels in liver diseases, including cirrhosis, acute hepatitis, and malnutrition (Smith and Goodman, 1971; Smith et al., 1975; McClain et al., 1979) as well as different types of inflammation, induced by either lipopolysaccharide or interleukin-6 (Rosales et al., 1996; Rosales and Ross, 1998; Gieng et al., 2005). To date, RBP4 has been described as an adipokine that exerts injurious effects in several pathologies, including diabetes and cardiovascular diseases (Yang et al., 2005; Cho et al., 2006; Graham et al., 2006; Lee et al., 2007; Yao-Borengasser et al., 2007). Here, we report evidence of the beneficial effects of RBP4 treatment in steatotic liver transplantation.

The protective pathway of PC based on AMPK activation has been demonstrated elsewhere (Carrasco-Chaumel et al., 2005). AMPK mediates some of the effects of hormones such as adiponectin and resistin (Kahn et al., 2005; Kola et al., 2006; Lage et al., 2008). Here, we report, for the first time, that RBP4 could be a downstream effector of AMPK in steat-
totic liver transplantation, and we suggest a new mechanism, namely, the induction of RBP4 generation, that might explain why AMPK activation induced by PC protects steatotic liver grafts against I/R injury.

To date, the liver has been considered the major site of endogenous RBP4 production (Blaner, 1989; Tsutsumi et al., 1992). Although adipose tissue could be another source of RBP4, its contribution seems to be minimal, according to previously reported data indicating that increases in RBP4 levels are not associated with the amount of subcutaneous abdominal fat (Gjøen et al., 1987; Tsutsumi et al., 1992; Janke et al., 2006; Stefan et al., 2007). In the present study, increased hepatic RBP4 levels were detected in steatotic liver grafts after transplantation in the groups treated with AMPK activators and PC. This RBP4 accumulation in the liver is generated by steatotic liver grafts by themselves, without the influence of other tissues or plasma constituents.

In line with this result, a relationship between mRNA and protein RBP4 levels was seen in steatotic liver grafts after transplantation. In addition, the increases in RBP4 levels observed in steatotic liver grafts after transplantation after AMPK activator treatment or PC induction were observed during ice-cold ischemia (before the implantation of liver grafts in the recipient). Taking into account the fact that the liver was isolated from the influence of other organs and plasma constituents under these conditions, it is suggested that steatotic liver grafts alone were responsible for the PC- and AMPK-induced generation of RBP4.

The results presented here indicate that both RBP4 treatment and PC, via RBP4 induction, reduced PPARγ overexpression in steatotic liver grafts, thus protecting against the worsening effects of PPARγ on hepatic injury. In steatotic liver grafts, the pharmacological modulation of RBP4 activity induced changes in PPARγ levels, and the benefits of RBP4 induction by either PC or RBP4 pretreatment on hepatic injury were abolished when PPARγ was activated. Moreover, the injurious effects of anti-RBP4 antibody on steatotic liver grafts were prevented by treatment with PPARγ antagonists. In contrast with previous studies in steatotic livers under warm ischemic conditions, indicating the benefits of PPARγ (Casillas-Ramírez et al., 2008), we report that in the setting of steatotic liver transplantation reduction in PPARγ levels protects steatotic liver grafts. Moreover, in contrast with previous studies indicating that PPARγ agonists reduce RBP4 levels (Yang et al., 2005; Teranishi et al., 2007; Lin et al., 2008), we report that in the setting of steatotic liver transplantation strategies aimed at increasing RBP4 levels (RBP4 treatment and PC induction) reduce PPARγ levels and hepatic injury. On the other hand, our results do not elucidate why levels of PPARγ and RBP4 are increased and reduced, respectively, in steatotic liver grafts, or whether RBP4 is responsible for the reduced PPARγ levels observed in steatotic liver grafts. The data presented herein are insufficient to establish a regulatory relationship between RBP4 and PPARγ in steatotic liver transplantation because other genes may be involved. Further studies (beyond the scope of this work) will be required to answer this question.

It should also be considered that RBP4 treatment, at the most effective dose (150 μg/kg), reduced hepatic injury and PPARγ levels but did not completely inhibit either hepatic injury or PPARγ in steatotic liver grafts. That RBP4 did not prevent hepatic injury cannot be explained by the fact that PPARγ levels were not completely reduced by RBP4. Treatment with a PPARγ antagonist reduced but did not prevent hepatic injury in steatotic liver grafts. In addition, the administration of a PPARγ antagonist in the TR+RBP4 group, at the most effective dose, reduced PPARγ to sham levels but resulted in hepatic injury parameters similar to those of the TR+RBP4 group. Thus, these results suggest that, in addition to RBP4 and PPARγ, other mechanisms are involved in the hepatic I/R injury associated with transplantation in steatotic liver grafts. This idea is not surprising given that numerous mechanisms and mediators are involved in hepatic I/R injury associated with transplantation (Casillas-Ramírez et al., 2006; Massip-Salcedo et al., 2007), which makes it difficult to find a strategy to prevent the hepatic I/R associated with transplantation.

In conclusion, steatotic liver grafts were found to be more vulnerable to the down-regulation of RBP4 and the overexpression of PPARγ. RBP4 treatment and PC (through AMPK induction) reduced PPARγ overexpression, thus protecting steatotic liver grafts against I/R injury associated with transplantation. In terms of clinical application, therapies based on RBP4 treatment and PPARγ antagonists might open new avenues for steatotic liver transplantation and improve the initial conditions of donor livers with low steatosis that are available for transplantation. Such therapies could also increase the use of numerous steatotic livers currently discarded for transplantation, thus reducing the risk of death of those patients on liver transplant waiting lists.

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Authorship Contributions

Participants in research design: Casillas-Ramírez, Alfany-Fernández, and Peralta.

Conducted experiments: Casillas-Ramírez, Alfany-Fernández, Massip-Salcedo, and Serafin.

Performed data analysis: Juan, Planas, Pallás, Rimola, Rodés, and Peralta.

Wrote or contributed to the writing of the manuscript: Casillas-Ramírez, Alfany-Fernández, Massip-Salcedo, Serafin, Rimola, Rodés, and Peralta.

References


Address correspondence to: Dr. Carmen Peralta, Institut d’Investigacions Biomèdiques August Pi i Sunyer, Esther Koplowitz Center, Roselló 149–153, 3rd Floor, Office 3.8, 08036 Barcelona, Spain. E-mail: cperalta@clinic.ub.es