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ORIGINAL ARTICLE

Retinol Binding Protein 4 and Retinol in Steatotic and Nonsteatotic Rat Livers in the Setting of Partial Hepatectomy Under Ischemia/Reperfusion

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Steatotic livers show increased hepatic damage and impaired regeneration after partial hepatectomy (PH) under ischemia/reperfusion (I/R), which is commonly applied in clinical practice to reduce bleeding. The known function of retinol-binding protein 4 (RBP4) is to transport retinol in the circulation. We examined whether modulating RBP4 and/or retinol could protect steatotic and nonsteatotic livers in the setting of PH under I/R. Steatotic and nonsteatotic livers from Zucker rats were subjected to PH (70%) with 60 minutes of ischemia. RBP4 and retinol levels were measured and altered pharmacologically, and their effects on hepatic damage and regeneration were studied after reperfusion. Decreased RBP4 levels were observed in both liver types, whereas retinol levels were reduced only in steatotic livers. RBP4 administration exacerbated the negative consequences of liver surgery with respect to damage and liver regeneration in both liver types. RBP4 affected the mobilization of retinol from steatotic livers, and this revealed actions of RBP4 independent of simple retinol transport. The injurious effects of RBP4 were not due to changes in retinol levels. Treatment with retinol was effective only for steatotic livers. Indeed, retinol increased hepatic injury and impaired liver regeneration in nonsteatotic livers. In steatotic livers, retinol reduced damage and improved regeneration after surgery. These benefits of retinol were associated with a reduced accumulation of hepatocellular fat. Thus, strategies based on modulating RBP4 could be ineffective and possibly even harmful in both liver types in the setting of PH under I/R. In terms of clinical applications, a retinol pretreatment might open new avenues for liver surgery that specifically benefit the steatotic liver. ***Liver Transpl 18:1198-1208, 2012.*** © **2012 AASLD.**

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In clinical situations, partial hepatectomy (PH) under ischemia/reperfusion (I/R) is usually performed to control bleeding during parenchymal dissection.[1] Hepatic steatosis, a major risk factor for liver surgery, is associated with an increased complication index and increased postoperative mortality after major liver

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BrdU, bromodeoxyuridine; HGF, hepatocyte growth factor; I/R, ischemia/reperfusion; Ln, lean; mRNA, messenger RNA; Ob, obese; PH, partial hepatectomy; RBP4, retinol-binding protein 4; TGF-β, transforming growth factor β; TTR, transthyretin; $I/R_{(RT)}$, ischemia/reperfusion at different reperfusion times.

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resection.[1] In comparison with nonsteatotic livers, steatotic livers show impaired regenerative responses and reduced tolerance to hepatic damage.[2]

Retinol-binding protein 4 (RBP4) is an adipokine synthesized by the liver; its known function is to transport retinol in the circulation.[3] The mobilization of liver vitamin A, which is stored predominantly as retinyl esters, requires the hydrolysis of retinyl esters to free retinol.[4,5] The retinol-RBP4 complex is secreted into the circulation, in which it binds transthyretin (TTR). The association of RBP4 with TTR stabilizes the complex in the circulation. Upon the delivery of retinol to target cells, RBP4 loses its affinity for TTR and is then eliminated through the kidneys.[4,5] It should be considered that RBP4 is not merely a transport protein for retinol. Indeed, RBP4 directly exerts injurious effects in several pathologies, including diabetes and cardiovascular diseases.[3,6,7] However, the administration of RBP4 has been reported to be beneficial against I/R damage in steatotic liver transplantation.[8]

In the present study, we examined whether the modulation of RBP4 could protect steatotic and/or nonsteatotic livers against damage and regenerative liver failure after PH under I/R. Because of the central importance of RBP4 in the homeostatic regulation of retinol,[9] we evaluated whether changes in RBP4 levels induced by PH under I/R could affect the circulating and tissue levels of retinol. Indeed, the accumulation of retinol in the liver during inflammation and the low plasma retinol levels observed in different pathologies have been attributed to a decrease in hepatic RBP4 synthesis.[10,11] We also investigated the role of retinol in nonsteatotic and steatotic livers in the setting of PH under I/R, which is commonly applied in clinical practice to reduce blood loss. To the best of our knowledge, only 1 experimental study of hepatic I/R (notably focused on nonsteatotic livers without hepatectomy) has reported that a derivative of retinol (all-trans retinoic acid) protects against I/R damage.[12] Some studies of PH without vascular occlusion that have focused on nonsteatotic livers have reported apparently controversial effects of retinol or its derivatives on hepatic regeneration.[13,14] A greater understanding of the role of retinol in the setting of PH under I/R could contribute to the development of new pharmacological strategies for hepatic resections.

MATERIALS AND METHODS

Experimental Animals

Male, homozygous, obese (Ob) Zucker rats (400-450 g) and male, heterozygous, lean (Ln) Zucker rats (350-400 g; Iffa Credo, France) that were 14 to 16 weeks old were used in these experiments. The Ob Zucker rats showed severe macrovesicular and microvesicular fatty infiltration in hepatocytes (60%-70% steatosis), whereas the Ln Zucker rats showed no evidence of steatosis. This study complied with European Union regulations on animal experiments (directive 86/609/EEC).

Surgical Procedure

The experiments in this study employed a rat model of PH (70%) with 60 minutes of ischemia, as previously described.[15] Briefly, after anesthesia with isoflurane and resection of the left hepatic lobe, a microvascular clamp was placed for 60 minutes across the portal triad supplying the median lobe. Congestion of the bowel was prevented during the clamping period through the preservation of the portal flow through the right and caudate lobes. At the end of ischemia, the right lobe and caudate lobes were resected, and reperfusion of the median lobe was achieved by the release of the clamp.

Experimental Design

Protocol 1

The effects of RBP4 and retinol on the parameters of liver regeneration and damage 24 hours after reperfusion were examined:

1. Sham group (Ln and Ob rats). The hepatic hilar vessels of the animals were dissected.
2. PH+I/R group (Ln and Ob rats). The animals underwent PH (70%) with 60 minutes of ischemia.[15]
3. PH+I/R+RBP4 group (Ln and Ob rats). The animals were treated as the animals in group 2 were, but they were also treated with RBP4 (5 μg/kg intravenously) before the surgical procedure.[8]
4. PH+I/R+retinol group (Ln and Ob rats). The animals were treated as the animals in group 2 were, but they were also treated with retinol (10 mg/kg intraperitoneally) before the surgical procedure.[16]
5. PH+I/R+RBP4+retinol group (Ob rats). The animals were treated as the animals in group 2 were, but they were also treated with RBP4 (5 μ/kg intravenously) and retinol (10 mg/kg intraperitoneally) before the surgical procedure.[8,16]

RBP4 and retinol levels, hepatic damage (transaminases and damage scores), liver regeneration parameters [percentages of Ki-67–positive hepatocytes and levels of hepatocyte growth factor (HGF) and transforming growth factor β (TGF-β)], retinyl ester and TTR levels, and RBP4/retinol ratios were determined for the groups corresponding to protocol 1 24 hours after reperfusion.

Protocol 2

This protocol involved the reperfusion time–dependent effects of retinol on hepatic damage, proliferative activity, and the degree of steatosis. To establish a relationship between the effects of retinol and proliferative activity in steatotic and nonsteatotic livers, we subjected animals to interventions similar to those used for groups 2 and 4 (protocol 1), but the samples were obtained 12, 24, and 48 hours after reperfusion.

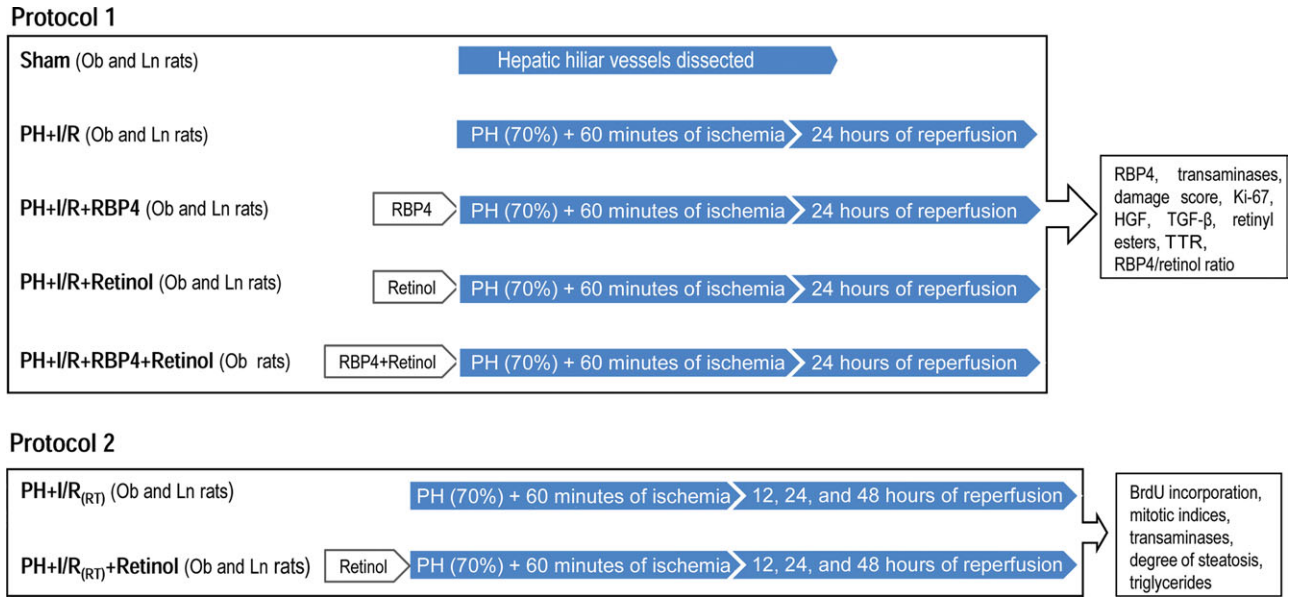

Figure 1. Flow chart of the interventions and measurements corresponding to protocols 1 and 2: (1) effects of RBP4 and retinol on the parameters of liver regeneration and damage 24 hours after reperfusion in the PH+I/R, PH+I/R+RBP4, PH+I/R+retinol, and PH+I/R+RBP4+retinol groups and (2) reperfusion time–dependent effects of retinol on hepatic damage, proliferative activity, and the degree of steatosis throughout reperfusion (12-48 hours) in the PH+I/$R_{(RT)}$ and PH+I/$R_{(RT)}$+retinol groups.

Bromodeoxyuridine (BrdU) was administered intraperitoneally at 50 mg/kg 1 hour before the animals were sacrificed at the indicated times, and they were processed to determine the incorporation of BrdU and mitotic indices.[17] Under these conditions, hepatic damage (transaminases and damage scores) and the degree of steatosis were also evaluated.

The interventions and measurements used in protocols 1 and 2 are shown in Fig. 1. The doses and pretreatment times used for RBP4 and retinol were selected on the basis of previous studies[8,16] and preliminary studies by our group. Control experiments were performed with the corresponding vehicle for each drug (saline and dimethyl sulfoxide for RBP4 and retinol, respectively).

Biochemical Determinations

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), HGF (a potent mitogen), total and active TGF-β (considered the main inhibitor of hepatic proliferation), RBP4, and triglycerides were measured as described elsewhere.[8,15,18]

Analytical Determination of Retinol and Retinyl Esters

Retinol and retinyl esters were measured with a reversed-phase high-performance chromatography system (Waters, Eschborn, Germany).[19] Vitamin A was extracted from the plasma and liver and was separated on a C18 column (Repro-Sil 70, Alltech Grom, Rottenburg-Hailfingen, Germany). Retinol and retinyl esters were quantified by the measurement of the absorption at 325 nm with a photodiode array detector (model 996, Waters). The detection limits for retinol and retinyl palmitate were 2.0 and 2.4 ng, respectively; the coefficient of variation between runs was 4%, and the recovery rate was greater than 95%.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Quantitative real-time reverse-transcription polymerase chain reaction analyses were performed with Assays-on-Demand TaqMan probes (Rn01451317_g1 for RBP4 and Rn00667869_m1 for β-actin, Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.[8]

Western Blotting

Western blotting for TTR was performed as described elsewhere.[20] Anti-TTR antibodies were acquired from DakoCytomation (Hamburg, Germany). Immunoreactive protein bands were visualized with chemiluminescence reagents and were quantified densitometrically with Quantity One software.

Histology, Red Oil Staining, and Immunohistochemistry

To appraise the severity of hepatic injury, we graded hematoxylin and eosin–stained sections with a point-counting method on an ordinal scale: (0) minimal or no evidence of injury; (1) mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; (2) moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and a loss of intercellular borders; (3) severe necrosis with

disintegration of hepatic cords, hemorrhaging, and neutrophil infiltration; and (4) very severe necrosis with disintegration of hepatic cords, hemorrhaging, and neutrophil infiltration.[8,21] Liver steatosis was evaluated via red oil staining on frozen specimens, and the percentage of steatosis was calculated by image analysis according to the standard procedure.[18] For liver regeneration, liver samples were immunostained with a rabbit monoclonal antibody against Ki-67 (clone SP6, Abcam, Cambridge, MA), developed with diaminobenzidine, and counterstained with hematoxylin.[22] The percentages of proliferating hepatocytes were also estimated through the quantification of hepatocytes that incorporated BrdU. BrdU-positive cells were detected with a mouse anti-BrdU antibody (GE Healthcare, United States).[17] The mitotic index was determined in hematoxylin and eosin–stained liver sections.[15] At least 30 high-power fields were counted.

Statistics

Data are expressed as means and standard deviations, and they were compared statistically via a 1-way analysis of variance and then a post hoc Student-Newman-Keuls test; a P value < 0.05 was considered significant. The Spearman correlation coefficient was used to investigate correlations between the effects of retinol therapy and the degree of steatosis; P values < 0.05 were considered statistically significant.

RESULTS

Effect of RBP4 24 Hours After Reperfusion

RBP4 Levels in Steatotic and Nonsteatotic Livers

The RBP4 messenger RNA (mRNA) and protein levels in nonsteatotic and steatotic livers from the PH+I/R group were lower than the levels in livers from the sham group (Fig. 2). The plasma RBP4 levels showed a pattern similar to that described for hepatic RPB4 levels (data not shown).

Hepatic Damage and Regeneration

The administration of RBP4 exacerbated hepatic damage in steatotic and nonsteatotic livers from the PH+I/R+RBP4 group and increased damage scores and transaminase levels in comparison with the PH+I/R group (Fig. 3A). The number of Ki-67–positive hepatocytes in both liver types was lower for the PH+I/R+RBP4 group versus the PH+I/R group. This decrease in proliferative cells was associated with low HGF levels and high levels of active TGF-β (Fig. 3A); the total hepatic TGF-β levels were similar for all groups (data not shown). We confirmed that the administration of RBP4 at the used dose raised RBP4 levels in both liver types with respect to the levels in the sham group. The RBP4 (pmol/g of tissue) protein levels in steatotic livers from the sham, PH+I/R, and PH+I/R+RBP4 groups were 242.17 ± 19.55, 82.41 ± 3.91, and 239.18 ± 20.62, respectively ($P < 0.05$ for the PH+I/R+RBP4 group versus the PH+I/R group; P = not significant for the PH+I/R+RBP4 group versus the sham group).

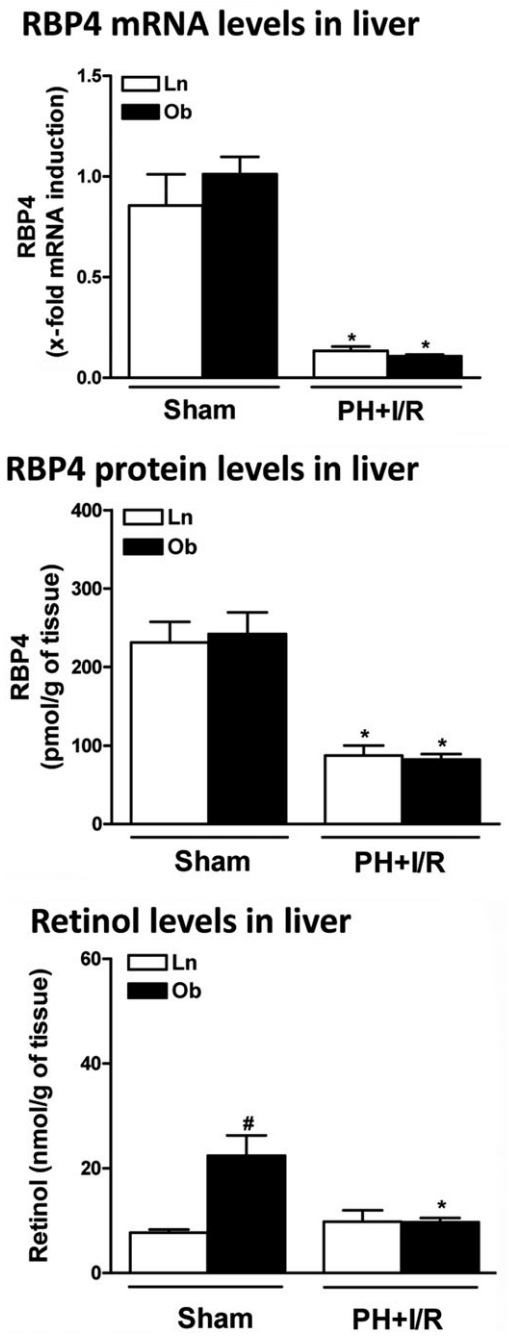

Figure 2. RBP4 mRNA and protein levels and retinol levels in both liver types 24 hours after reperfusion. Six Ln animals and 6 Ob animals from each group were included for each measurement. The retinol levels in steatotic livers from the sham group were higher than the levels in nonsteatotic livers. The polymerase chain reaction fluorescent signals for RBP4 were normalized to the signals obtained from an endogenous reference (β-actin). The β-actin–normalized RBP4 mRNA levels were calculated with respect to the levels of the sham control group with the $2^{-\Delta\Delta C_t}$ method. *$P < 0.05$ versus sham animals; #$P < 0.05$.

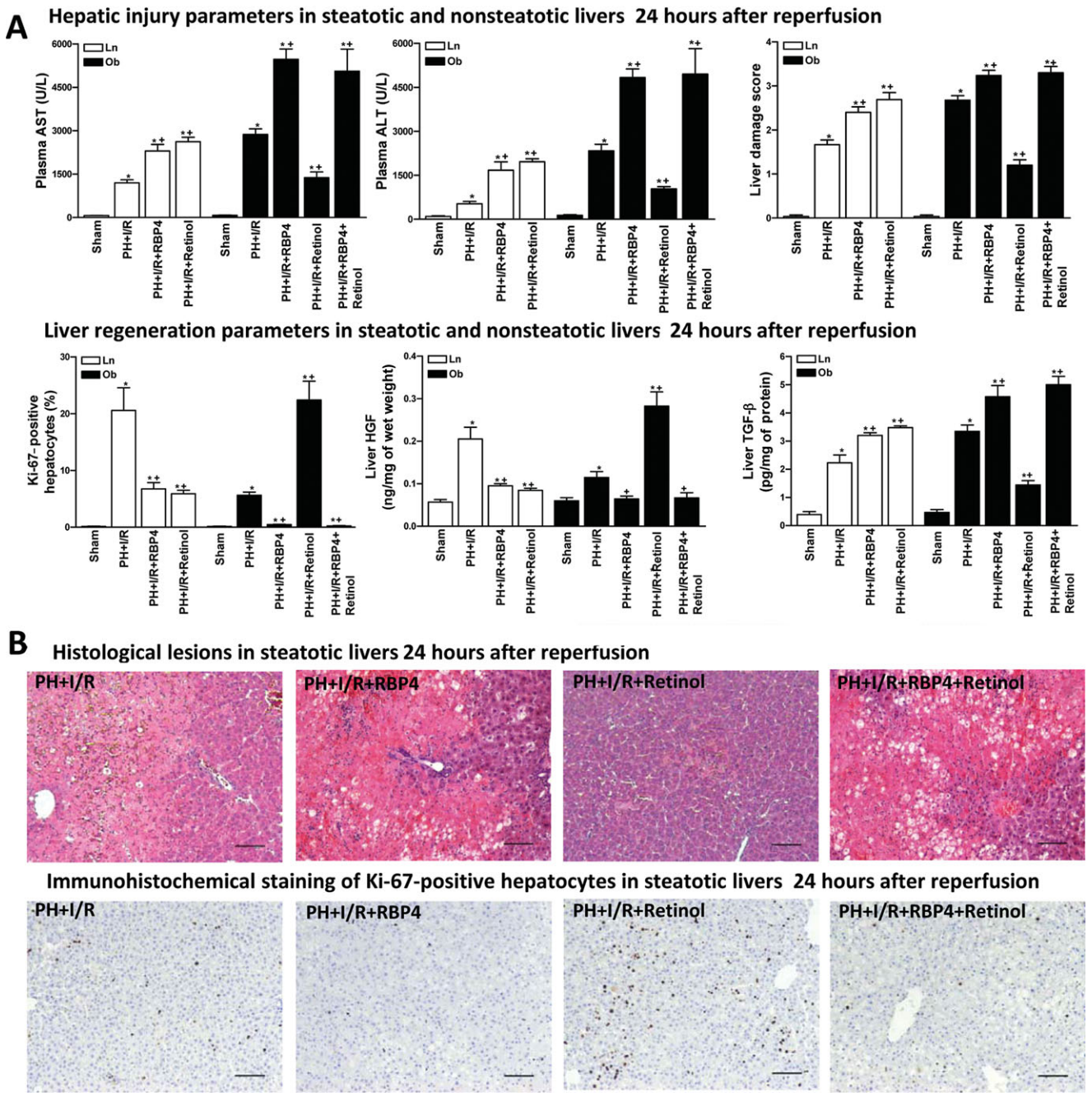

Figure 3. (A) Effects of RBP4 and retinol (separately or in combination) on hepatic injury (plasma AST and ALT levels and liver damage scores) and liver regeneration parameters (percentages of Ki-67–positive hepatocytes and plasma HGF and TGF-β levels) 24 hours after reperfusion. Six Ln animals and 6 Ob animals from each group were included for each measurement. $^{*}P < 0.05$ versus sham animals; $^{+}P < 0.05$ versus PH+I/R animals. (B) Representative photographs of hematoxylin and eosin staining 24 hours after reperfusion show extensive areas of coagulative necrosis in the PH+I/R, PH+I/R+RBP4, and PH+I/R+RBP4+retinol groups and small areas of coagulative necrosis in the retinol group (scale bar = 1000 μm). Representative photographs of immunohistochemical staining of Ki-67–positive hepatocytes in steatotic livers 24 hours after reperfusion show that the number of Ki-67–positive hepatocytes was lower in the PH+I/R, RBP4, and RBP4+retinol groups versus the retinol group (scale bar = 1000 μm).

Effect of Retinol 24 Hours After Reperfusion

Retinol Levels in Steatotic and Nonsteatotic Livers

In nonsteatotic livers, the retinol levels for the PH+I/R group were similar to those for the sham group. The retinol levels in steatotic livers from the sham group were significantly higher than the levels in nonsteatotic livers (Fig. 2), and this indicated that the presence of fatty infiltration in and of itself (without any surgical intervention) induced changes in retinol metabolism, as previously suggested.[23] The retinol

levels in steatotic livers from the PH+I/R group were reduced in comparison with the sham group.

Hepatic Damage and Regeneration

The effects of retinol were dependent on the type of liver. The administration of retinol negatively affected hepatic damage and liver regeneration in nonsteatotic livers from the PH+I/R+retinol group because the parameters of hepatic injury were higher than those for the PH+I/R group. This was associated with reduced percentages of Ki-67–positive hepatocytes, reduced HGF levels, and high TGF-β levels in comparison with the PH+I/R group (Fig. 3A). The administration of retinol to Ob animals in the PH+I/R+retinol group reduced hepatic damage; this was indicated by the reduction in transaminases and damage scores in comparison with the PH+I/R group. Retinol administration also increased the percentage of Ki-67–positive hepatocytes in steatotic livers in comparison with the PH+I/R group. This improvement was associated with high HGF levels and low TGF-β levels (Fig. 3A).

Effects of RBP4 and Retinol in Combination 24 Hours After Reperfusion

Taken together, the results presented up to this point show that neither the administration of RBP4 nor the administration of retinol protects nonsteatotic livers in the setting of PH under I/R. Because of our demonstration of the differential effects of RBP4 and retinol on steatotic livers and data indicating that changes in RBP4 induce changes in retinol levels,[10,11] we evaluated whether the injurious effects of RBP4 with respect to damage and regeneration in steatotic livers could be explained by changes in retinol levels.

Hepatic Damage and Regeneration

The combined administration of RBP4 and retinol resulted in injury and regeneration parameters for steatotic livers from the PH+I/R+RBP4+retinol group that were similar to those for steatotic livers from the PH+I/R+RBP4 group (Figs. 3A). The histological analysis revealed severe, extensive, and confluent areas of coagulative necrosis with neutrophil infiltration in steatotic livers from the PH+I/R, PH+I/R+RBP4, and PH+I/R+RBP4+retinol groups (Fig. 3B). The liver damage scores for the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups were significantly higher than those recorded for the PH+I/R group. Both the number and extent of necrotic areas in steatotic livers were reduced by retinol. In comparison with the PH+I/R group, the PH+I/R+retinol group also exhibited an increased number of Ki-67–positive hepatocytes in steatotic livers. The number of Ki-67–positive hepatocytes in the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups was lower than the number in the PH+I/R group (Fig. 3A,B). Thus, the administration of RBP4, separately or in combination with retinol, negatively affected hepatic damage and regeneration.

Retinyl Esters and Retinol in Liver and Plasma

Vitamin A is stored in the liver as retinyl esters, and they must be hydrolyzed into retinol before vitamin A can be mobilized into the circulation.[4] In line with this, retinyl ester and retinol levels in steatotic livers were reduced for the PH+I/R group versus the sham group (Fig. 4A). This decrease was associated with high plasma retinol levels (Fig. 4B). As previously suggested on the basis of studies in cultured cells,[24] RBP4 could affect the storage and mobilization of retinol in steatotic livers. In fact, we found that retinyl ester levels (but not retinol levels) were increased in steatotic livers (Fig. 4A) and circulating retinol levels were reduced (Fig. 4B) for the PH+I/R+RBP4 group versus the PH+I/R group or the sham group. As expected, retinol administration increased both retinyl ester and retinol levels in steatotic livers from the PH+I/R+retinol and PH+I/R+RBP4+retinol groups versus the PH+I/R group and increased retinyl ester levels (but not retinol levels) in plasma (Fig. 4A,B). Our results confirmed that RBP4 administration led to higher RBP4 levels in steatotic livers from the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups versus the PH+I/R group. For instance, the hepatic RBP4 levels for the PH+I/R+RBP4+retinol and PH+I/R groups were 239.6 ± 21.65 and 82.41 ± 3.91 respectively ($P < 0.05$). The hepatic RBP4 (pmol/g of tissue) levels for the PH+I/R+retinol group (83.45 ± 4.56) were similar to the levels for the PH+I/R group (82.41 ± 3.91, $P =$ not significant).

Transport of Retinol in Plasma

Reduced plasma RBP4 levels were observed for the PH+I/R group versus the sham group (Fig. 4C). The plasma RBP4 levels for the PH+I/R+RBP4 and PH+I/R+RBP4+retinol groups were lower than those for the PH+I/R group. The plasma RBP4 levels for the PH+I/R+retinol group were similar to those for the PH+I/R group. The plasma TTR levels were reduced for the PH+I/R group versus the sham group, whereas the TTR levels for the PH+I/R+RBP4, PH+I/R+retinol, and PH+I/R+RBP4+retinol groups were similar to those for the sham group. It has been reported that retinol is normally found in plasma in a 1:1 molar ratio with RBP4.[25] In the sham group, the RBP4/retinol ratio was approximately 2; in the PH+I/R and PH+I/R+RBP4 groups, the RBP4/retinol ratio was greater than 1; and in the PH+I/R+retinol and PH+I/R+RBP4+retinol groups, the RBP4/retinol ratio was less than 1 (Fig. 4C).

Reperfusion Time–Dependent Effect of Retinol on Hepatic Damage, Proliferative Activity, and Degree of Steatosis

Hepatic damage and proliferative activity in steatotic and nonsteatotic livers were measured throughout reperfusion (12, 24, and 48 hours; Fig. 5). In Ln animals, the parameters of hepatic injury were higher for the partial hepatectomy under ischemia-reperfusion

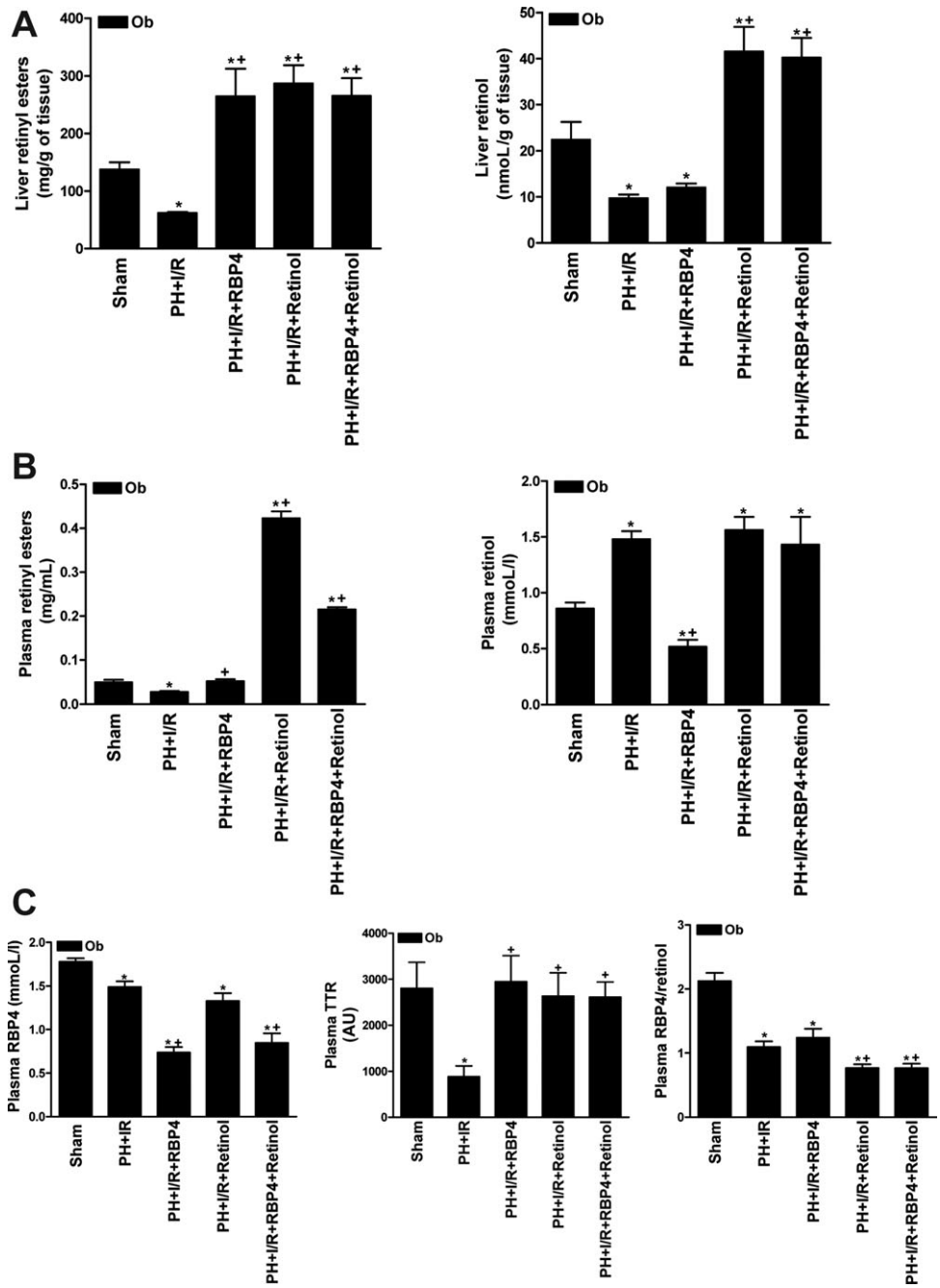

Figure 4. Effects of RBP4 and retinol (separately or in combination) on retinyl esters and retinol in (A) steatotic livers and (B) plasma and (C) RBP4 and TTR levels and RBP4/retinol ratios in plasma 24 hours after reperfusion. Six Ob animals from each group were included for each measurement. $^{*}P < 0.05$ versus sham animals; $^{+}P < 0.05$ versus PH+I/R animals.

(PH+I/R) at different reperfusion times (RT) treated with retinol (PH+I/$R_{(RT)}$+retinol) group versus the partial hepatectomy under ischemia-reperfusion (PH+I/R) at different reperfusion times (RT) (PH+I/$R_{(RT)}$) group at each time point (Fig. 5A). In Ln animals, the administration of retinol impaired the mitotic index and hepatocellular BrdU incorporation after reperfusion. For instance, the percentages for BrdU-positive hepatocytes were 9.3% ± 1.09% and 19.5% ± 1.67% in the PH+I/$R_{(RT)}$+retinol and PH+I/$R_{(RT)}$ groups, respectively, 48 hours after reperfusion. In Ob animals, the hepatic damage parameters were lower for the PH+I/$R_{(RT)}$+retinol group versus the PH+I/$R_{(RT)}$ group at each time (Fig. 5B). Our results revealed an improvement of the regenerative process in steatotic livers from the PH+I/$R_{(RT)}$+retinol group versus the PH+I/$R_{(RT)}$ group. Thus, 48 hours after reperfusion, the percentage of BrdU-positive hepatocytes was 17.3% ± 0.99% for the PH+I/$R_{(RT)}$+retinol group, whereas only 6.5% ± 0.76% of BrdU-labeled hepatocytes were detected at that reperfusion time in the PH+I/$R_{(RT)}$ group (Fig. 5B). Our results suggest a relationship between the effects of retinol therapy and the degree of steatosis in steatotic livers. Forty-eight hours after reperfusion, increased hepatocellular fatty infiltration and triglyceride levels were observed in the PH+I/$R_{(RT)}$ group versus the sham group (Fig. 5C,D). This is in line with a previous report of hepatic lipid

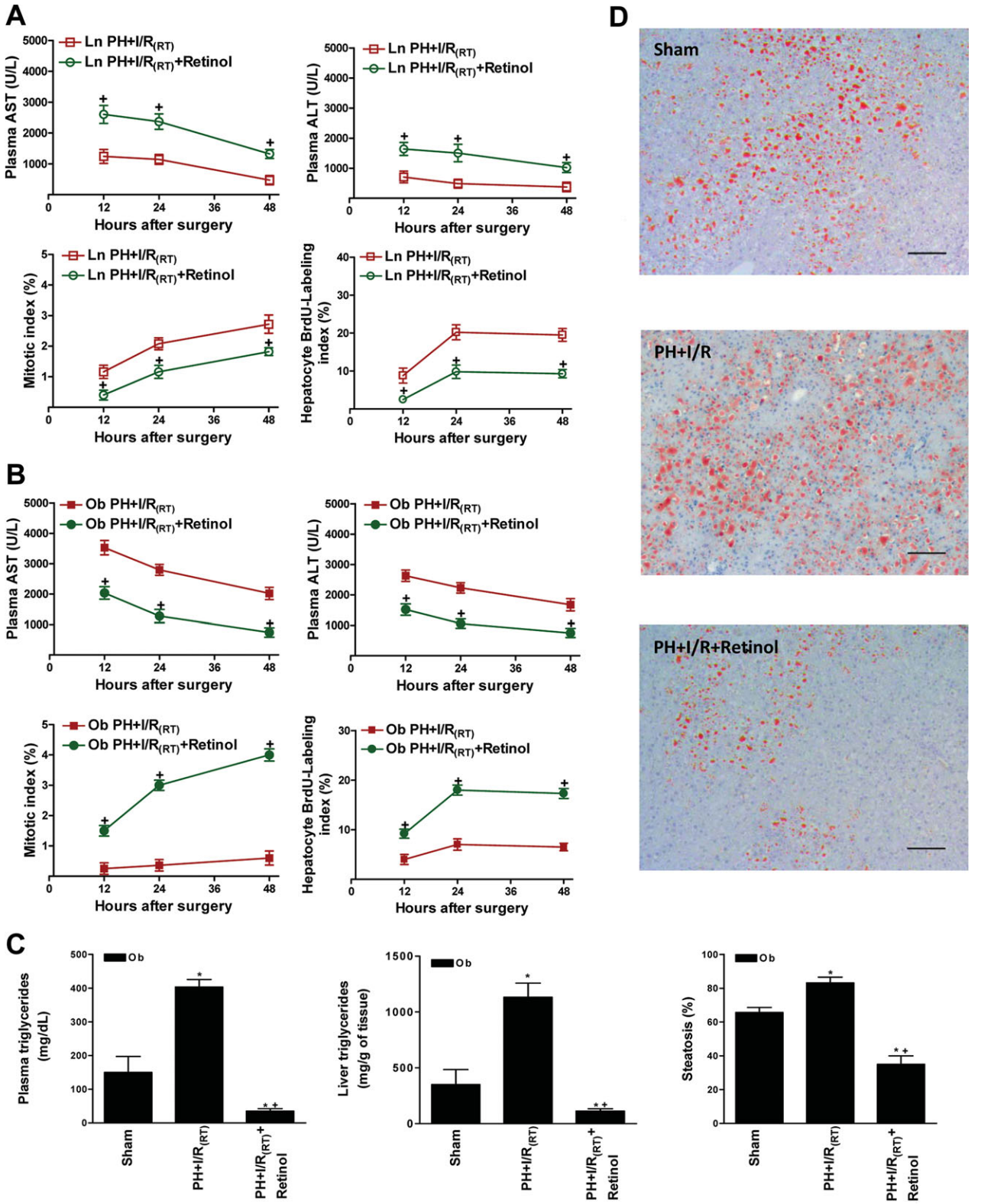

Figure 5. Effects of retinol on hepatic injury (plasma AST and ALT levels) and liver regeneration (percentages of BrdU-positive hepatocytes and mitotic indices) throughout reperfusion (12, 24, and 48 hours) in (A) Ln animals and (B) Ob animals. Eighteen Ln animals and 18 Ob animals (6 Ln and 6 Ob animals for each reperfusion time) were included for each measurement. (C) Triglyceride levels and steatosis percentages and (D) representative photographs of red oil staining 48 hours after reperfusion are shown. Steatotic livers from the retinol group showed reduced fat globules (red) in hepatocytes in comparison with steatotic livers from the sham and PH+I/R groups (scale bar = 1000 μm). Six Ob animals from each group were included for each measurement. *$P < 0.05$ versus sham animals; $^{+}P < 0.05$ versus PH+I/R animals.

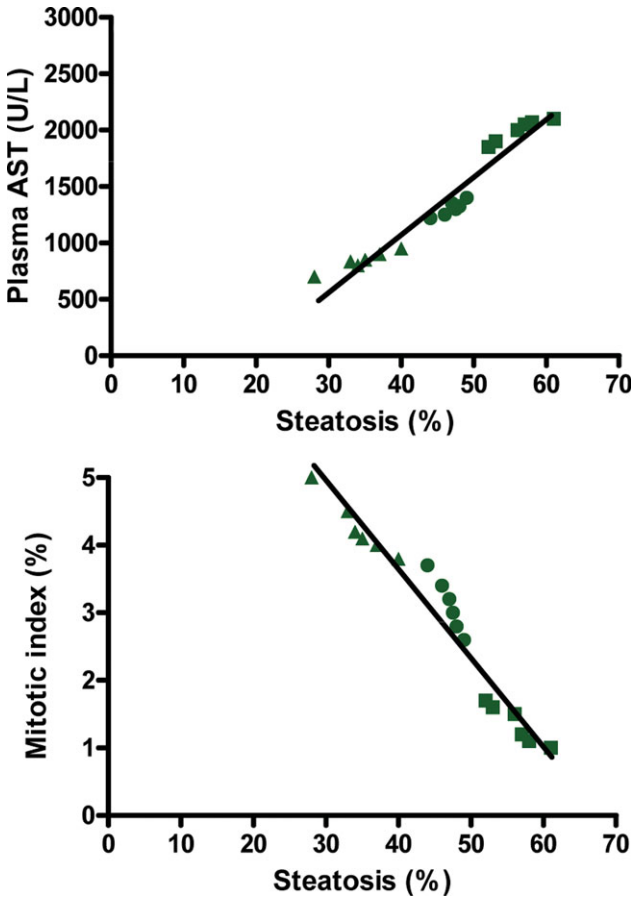

Figure 6. Relationship between the degree of steatosis and (A) the plasma AST level ($r = 0.9677$, $P < 0.05$) and (B) the mitotic index ($r = -0.9724$, $P < 0.05$): (■) Ob PH+I/$R_{(RT)}$+retinol animals 12 hours after reperfusion, (●) Ob PH+I/$R_{(RT)}$+retinol animals 24 hours after reperfusion, and (▲) Ob PH+I/$R_{(RT)}$+retinol animals 48 hours after reperfusion.

accumulation during regeneration after PH.[26] The treatment with retinol decreased the accumulation of fatty droplets and triglycerides in steatotic livers from the PH+I/$R_{(RT)}$+retinol group versus the PH+I/$R_{(RT)}$ group. The steatosis percentages were 35.0% ± 1.78% and 83.13% ± 3.40% for the PH+I/$R_{(RT)}$+retinol and PH+I/$R_{(RT)}$ groups, respectively, 48 hours after reperfusion. Figure 6 shows the relationship between the effects of retinol therapy and the degree of steatosis 12, 24, and 48 hours after reperfusion. A significant correlation between the degree of fat infiltration and the plasma AST level ($r = 0.9677$, $P < 0.05$) and an inverse correlation between the degree of fat infiltration and the mitotic index ($r = -0.9724$, $P < 0.05$) were observed. Treatment with retinol, therefore, attenuated hepatic damage and improved hepatocellular proliferation during liver regeneration. This was associated with a reduced accumulation of hepatocellular fat.

DISCUSSION

The decreased RBP4 levels observed in the circulation after PH under I/R might reflect decreased hepatic synthesis of RBP4 due to a loss of functional hepatic tissue. Because the liver is the main site of RBP4 synthesis,[8,27] hepatocyte damage decreases circulating RBP4 levels. Moreover, the synthesis of negative acute-phase proteins such as RBP4 might be reduced in the liver during inflammation or hepatic resection as part of a compensatory response to the sudden increase in the synthesis of positive acute-phase proteins.[11] The effects of RBP4 could depend on the surgical conditions. Indeed, in contrast to steatotic liver transplantation,[8] RBP4 administration worsens liver damage and regenerative failure in the setting of PH under I/R.

Retinol is decreased in steatotic livers after PH under I/R and is increased in plasma. This is consistent with the increased secretion of retinol from steatotic livers into the circulation. However, other hypotheses proposed for different liver diseases,[28] including increased catabolism of retinol in the liver and decreased liver uptake of retinyl esters formed in peripheral tissues, should not be discounted. Retinol is normally found in plasma in a 1:1 molar ratio with RBP4 (the RBP4/retinol ratio is approximately 1.0).[29] However, in our study and in agreement with the reports of others,[4] the RBP4/retinol ratio for the sham group of Ob (steatotic) animals was approximately 2, and this indicated that the proportion of RBP4 in plasma was higher than the proportion of retinol. In this context, it should be considered that RBP4 is present as a holoform (retinol-bound) and an apoform (retinol-unbound) because RBP4 may transport and deliver other lipophilic molecules in addition to retinol.[6,30] A question that arises is how the high plasma retinol levels in the PH+I/R group are maintained in light of the low plasma RBP4 and TTR levels. Like RBP4, TTR is a negative acute-phase protein, and the hepatic syntheses of both are also decreased during inflammation.[11] However, because the plasma RBP4/retinol ratio in the PH+I/R group was greater than 1, there was enough RBP4 to bind retinol despite the reduced RBP4 levels. It could be assumed that the reduced plasma TTR levels in the PH+I/R group were also high enough to maintain the retinol-RBP4 complex in plasma, insofar as there is usually a 3- to 5-fold molar excess of circulating TTR with respect to circulating RBP4.[11,27] The lack of a correlation between RBP4 and TTR levels in plasma under our surgical conditions could be explained, at least in part, by the differential clearance of RBP4 and TTR by the kidneys: TTR has a higher molecular weight and is, therefore, retained to a greater extent than RBP4.[27] Moreover, TTR has additional functions (eg, the transporter for T4).[4]

RBP4 exerted injurious effects on steatotic livers with respect to damage and regeneration independently of retinol. Moreover, the changes in retinol metabolism induced by RBP4 could have occurred simply as a result of disease progression and thus do not explain the injurious effects of RBP4. The mechanism behind this is unclear. Retinyl esters seem to be affected by RBP4 administration, and this also

suggests that RBP4 has actions independent of simple retinol transport. However, the RBP4-induced increase in retinyl esters does not explain the injurious effects of RBP4. Indeed, the loss of retinoid (rather than an increase in retinoid levels) contributes to the development of hepatic diseases.[31]

Serum RBP4 levels are elevated in insulin-resistant mice and humans with obesity and type 2 diabetes. The transgenic overexpression of human RBP4 or the injection of recombinant RBP4 into normal mice causes insulin resistance. Conversely, the genetic deletion of RBP4 enhances insulin sensitivity.[6] Elucidating whether RBP4 also has metabolic effects was not an aim of the present study. Nevertheless, because of our results, this seems to not be the case for our experimental model. The Ob Zucker rats used in the present study have a mutated leptin receptor and, as a result, are hyperphagic, Ob, and hyperinsulinemic. They are insulin-resistant, but they have normal blood glucose levels. Ob Zucker rats do not develop diabetes. Ln Zucker rats maintain an Ln phenotype throughout life, and they show normal blood insulin and glucose levels. RBP4 administration did not alter the plasma insulin or glucose levels in the Ob Zucker rats undergoing PH and I/R (data not shown). Under our conditions, there was no relationship between RBP4 levels and insulin resistance. Indeed, Ln and Ob Zucker rats in the sham group showed similar RBP4 levels in the liver and plasma, and only Ob Zucker rats are insulin-resistant. In contrast, Yang et al.[6] have shown that genetically Ob mice and high-fat diet–induced Ob mice with insulin resistance exhibit increased plasma RBP4 in comparison with Ln controls. The differences in plasma RBP4 levels observed in the 2 studies can be explained at least partially by the differences in RBP4 regulation between rats and mice.[32] All these data indicate that under our conditions, RBP4 does not affect insulin resistance.

Further studies, which are not part of the objectives of the present study, are required to answer why the pharmacological modulation of RBP4 exerted damaging effects in the setting of PH under I/R. It is possible that the compensatory changes in the protein synthesis of positive and negative acute-phase proteins, which were necessary to restore protein homeostasis after hepatic resection, were disturbed in the remaining liver when RBP4 was administered. From a clinical perspective, strategies based on modulating RBP4 might not be appropriate for hepatic resection or under surgical conditions (including small-for-size liver transplantation). When we administered RBP4 to compensate for the reduced RBP4 levels induced by liver surgery, we observed more injurious effects with respect to damage and liver regeneration.

In contrast, pretreatment with retinol alone may create new possibilities for therapeutic interventions in the resection of steatotic livers. As expected, retinol administration alone increased vitamin A storage in the liver. The increase in retinyl esters (but not retinol or RBP4) in plasma after retinol administration suggests that retinol is incorporated as retinyl esters in hepatocytes and is secreted together with lipoproteins. Thus, retinol administration could supply vitamin A to target tissues. Data indicate that the RBP4 molecule contains 1 binding site for a single molecule of retinol,[25] so the fact that the RBP4/retinol ratio after retinol administration was less than 1 might suggest that a fraction of retinol is bound to carriers other than RBP4, just as with other pathologies.[33]

The results presented here indicate that retinol reduces damage and improves liver regeneration in an experimental model combining PH and I/R. Unpublished results from our group indicate that retinol therapy for steatotic livers affects both I/R injury and PH. Thus, retinol reduces hepatic damage in Ob rats subjected to partial hepatic ischemia (60 minutes) without hepatectomy. Retinol administration also reduces hepatic damage and improves liver regeneration in Ob rats subjected to 70% resection only (data not shown).

Further studies will be required to explain how retinol reduces lipid accumulation in the regenerating liver after surgery. The effects of retinol on the mobilization of fatty acids from peripheral stores and hepatic lipogenesis, fatty acid oxidation, and triglyceride secretory mechanisms in the liver should be explored as possible answers to this question. Our results show that the benefits of retinol with respect to damage and liver regeneration in steatotic livers are associated with reduced hepatic lipid accumulation. It has been reported that it is crucial to reduce steatosis to prevent the vulnerability of steatotic livers to I/R injury and regenerative failure.[34]

In conclusion, PH under I/R affects hepatic vitamin A metabolism by reducing hepatic RBP4 expression and increasing the mobilization of retinol into the circulation. The results presented here suggest that under surgical conditions requiring liver regeneration, modulating RBP4 levels worsens the outcome and is, therefore, not advised. This study also points to new possibilities for therapeutic interventions based on retinol pretreatment to protect steatotic livers against damage and regenerative failure after liver surgery.

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