Peroxisome proliferator-activated receptor-α regulates postischemic liver injury

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Okaya, Tomohisa, and Alex B. Lentsch. Peroxisome proliferator-activated receptor-α regulates postischemic liver injury. Am J Physiol Gastrointest Liver Physiol 286: G606–G612, 2004—Peroxisome proliferator-activated receptor-α (PPARα) is a transcription factor that in some in vitro systems has been linked with downregulation of proinflammatory mediators, thus implicating a potential role for PPARα in the regulation of inflammatory processes. Hepatic ischemia-reperfusion injury is characterized by an intense acute inflammatory response that is dependent on a number of proinflammatory mediators. PPARα is abundantly expressed in hepatic parenchymal cells but not in Kupffer cells. This study examined whether PPARα is involved in regulation of the hepatic inflammatory response to ischemia-reperfusion. Mice nullizygous for PPARα had significantly greater liver injury than did their wild-type counterparts. Consistent with these findings, C57BL/6 mice treated with the PPARα agonist, Wy-14643, had significantly less liver injury than mice receiving vehicle. PPARα-knockout mice also had greatly augmented liver neutrophil accumulation and modest increases in activation of the transcription factors NF-κB and activator protein-1. However, these effects were not associated with increased expression of proinflammatory cytokines or chemokines. In addition, PPARα-knockout mice expressed far less inducible nitric oxide synthase in liver than did wild-type mice after ischemia-reperfusion. Finally, treatment of cultured murine hepatocytes with Wy-14643, a specific agonist of PPARα, protected cells against oxidant-induced injury. The data suggest that PPARα is an important regulator of the hepatic inflammatory response to ischemia-reperfusion in a manner that is dependent of proinflammatory cytokines.

reperfusion injury; neutrophils; inflammation

INTERRUPTION OF HEPATIC INFLOW is a common procedure during trauma surgery, liver transplantation, and resectional surgery. However, the resulting period of hepatic ischemia and subsequent reperfusion can lead to liver injury and dysfunction through the initiation of a biphasic inflammatory response. The acute phase of this response is characterized by activation of Kupffer cells and their subsequent production and release of reactive oxygen species leading to an oxidative shift in the hepatic redox state (13–15). This causes mild injury to the hepatic parenchyma, but the oxidative state is thought to activate redox-sensitive transcription factors, such as NF-κB and activator protein (AP-1), that control the expression of proinflammatory mediators, such as IL-12 and TNF-α (5, 18, 22, 27, 31). The production of these mediators leads to a second phase of liver injury by inducing the expression of secondary mediators, including neutrophil-attracting CXC chemokines and endothelial cell adhesion molecules that mediate the adhesion and transmigration of neutrophils from the vascular space into the hepatic parenchyma (3, 4, 21). Accumulated neutrophils release oxidants and proteases that directly injure hepatocytes and vascular endothelial cells and may also obstruct hepatic sinusoids resulting in hepatic hypoperfusion (17).

Injury to hepatocytes leading to liver dysfunction is the endpoint of this response, yet most of the attempts to limit this injury have targeted proinflammatory mediators. Much less attention has been directed toward the endogenous mechanisms by which hepatocytes try to protect themselves from injury during this response. Hepatocellular injury during both the initial and later phases of ischemia-reperfusion injury is caused in large part by reactive oxygen species (13–15). Peroxisomes, which are subcellular organelles within the hepatocyte, contain a battery of antioxidant enzymes and may help protect hepatocytes from oxidative damage. Proliferation of peroxisomes in hepatocytes is induced, at least in part, by activation of the transcription factor peroxisome proliferator-activated receptor-α (PPARα) (2, 6). PPARα is a member of the nuclear receptor superfamily and within the liver is known to be related to the endogenous mechanisms that lead to hepatocarcinogenesis (28).

In the present studies, we employed PPARα-deficient (PPARα−/−) mice to examine the role of this transcription factor in the regulation of hepatic ischemia-reperfusion injury. Here we show that PPARα−/− mice have significantly more postischemic injury than wild-type mice. There were no differences in proinflammatory mediator production between PPARα−/− and wild-type mice. However, we found that livers from PPARα−/− mice expressed less inducible nitric oxide (NO) synthase (iNOS) than wild-type mice. In addition, treatment of cultured murine hepatocytes with Wy-14643, a specific agonist of PPARα, protected cells against oxidant-induced injury. These findings suggest that PPARα is an important regulator of postischemic liver injury and implicates a hepatocyte-specific therapeutic target for the treatment of inflammatory liver injury.

MATERIALS AND METHODS

Model of hepatic ischemia-reperfusion injury. Wild-type and PPARα−/− mice on a 129 background and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male mice 8–10 wk of age were used in all experiments. This project was approved by the University of Cincinnati Animal Care and Use Committee.

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Committee and conforms to the National Institutes of Health guidelines. Partial hepatic ischemia was induced as described previously (21). Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg ip). A midline laparotomy was performed, and an atraumatic clip was used to interrupt blood supply to the left lateral and median lobes of the liver. After 90 min of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham control mice underwent the same protocol without vascular occlusion. For experiments using the PPARα agonist WY-14463 (Biomol, Plymouth Meeting, PA), C57BL/6 mice were injected intravenously with 10 mg/kg WY-14463 or vehicle 1 h before ischemia. Mice were killed after the indicated periods of reperfusion, and blood and liver samples were taken for analysis.

Western blot. Frozen liver samples were homogenized in lysis buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.6% Nonident-P-40, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin) on ice. Homogenates were sonicated and centrifuged at 5,000 rpm to remove cellular debris. Protein concentration was determined by bicinchoninic acid assay with trichloroacetic acid precipitation using BSA as a reference standard (Pierce). Liver protein (100 μg) was electrophoresed in a denaturing 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with TBS (40 mM Tris, pH 7.6, 300 mM NaCl) containing 5% nonfat dry milk for 1 h at room temperature. Membranes were then incubated in a 1:500 dilution of rabbit polyclonal anti-mouse peroxisome membrane protein-70 (PMP70; Affinity Bioreagents, Golden, CO) or 1:250 dilution of rabbit polyclonal anti-mouse iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS with 0.1% Tween 20 (TBST). After three washes in TBST, membranes were incubated in a 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology). Immunoreactive proteins were detected by enhanced chemiluminescence.

Blood and tissue analysis. Blood was obtained by cardiac puncture at the time of death for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made by using a diagnostic kit (Sigma, St. Louis, MO). Serum samples were also analyzed for TNF-α and macrophage inflammatory protein-2 (MIP-2) by sandwich ELISA according to manufacturer’s instructions (R&D Systems, Minneapolis, MN). Analysis of nitrite/nitrate in liver lysates and serum were performed by using a diagnostic kit from Oxis International (Portland, OR) according to manufacturer’s instructions.

MPO assay. Liver MPO content was assessed by methods similar to those of Schierwagen et al. (29). Liver tissue (100 mg) was homogenized in 2 ml of buffer A (3.4 mM KH₂PO₄, 16 mM Na₂HPO₄, pH 7.4). After centrifugation for 20 min at 10,000 g, the pellet was resuspended in 10 volumes of buffer B (43.2 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 10 mM EDTA, 0.5% hexadecyltrimethylammonium, pH 6.0) and sonicated for 10 s. After being heated for 2 h at 60°C, the supernatant was reacted with 3,3’,5,5’-tetramethyldibenzyline (Sigma), and optical density was determined at 655 nm.

EMSA. Nuclear extracts of liver tissue were prepared by the method of Derryckere and Gannon (10) and analyzed by EMSA. Briefly, double-stranded NF-κB or AP-1 consensus oligonucleotides (Promega, Madison, WI) were end labeled with double-stranded NF-κB or AP-1 consensus oligonucleotides (Promega) and analyzed by EMSA. Briefly, 50,000 counts/min, Cherenkov counting) of oligonucleotide were incubated at room temperature for 30 min. Reaction volumes were held constant to 15 μl. Reaction products were separated in a native 4% polyacrylamide gel and analyzed by autoradiography.

Hepatocyte culture and cytotoxicity assay. Mouse hepatocytes (AML-12; American Type Culture Collection, Manassas, VA) were cultured in a 1:1 mixture of DMEM and Ham’s F-12 media, supplemented with 10% fetal bovine serum, 1% insulin-transferrin-selenium G, 1% penicillin-streptomycin, and 40 ng/ml dexamethasone. Then 1 × 10⁵ cells/well were seeded in 96-well microplates. Twenty-four hours later, cells were treated with either media or WY-14463 in media (5–100 μM; BioMol, Plymouth Meeting, PA). One hour later, cells were stimulated with 1 mM H₂O₂ (final concentration). Twelve hours later, cytotoxicity was analyzed by using the CytoTox-ONE homogeneous membrane integrity assay (Promega) according to the manufacturer’s instructions. This assay was a sensitive, modified lactate dehydrogenase assay.

Statistical analysis. All data are expressed as means ± SE. Data were analyzed with a one-way ANOVA with subsequent Student-Newman-Keuls test. Differences were considered significant when P < 0.05.

RESULTS

Peroxisome proliferation occurs after ischemia-reperfusion. Because PPARα activation is linked to the proliferation of peroxisomes, we sought to determine whether peroxisome proliferation was induced by hepatic ischemia-reperfusion and whether this process was dependent on PPARα. Western blot analysis of the PMP70 was performed on liver protein extracts from sham-operated mice and mice undergoing ischemia and 8 h of reperfusion. Figure 1 shows that abundant PMP70 protein was present in sham-operated wild-type mice but that sham-operated PPARα−/− mice had far less expression. After ischemia-reperfusion, there was a significant increase in PMP70 expression in wild-type mice and PPARα−/− mice, but wild-type mice appeared to have more PMP70 than PPARα−/−, suggesting that PPARα was required for full induction of peroxisome proliferation.

PPARα regulates neutrophil accumulation and hepatocellular injury. To determine whether PPARα was involved in the regulation of hepatic ischemia-reperfusion injury, we assessed
the response of wild-type and PPARα−/− mice to ischemia-reperfusion. After ischemia and 4 h of reperfusion, there were no differences between wild-type and PPARα−/− mice in the extent of hepatocellular injury, as measured by serum levels of ALT (wild-type, 5,909 ± 502 IU/l; PPARα−/−, 5,916 ± 394 IU/l). However, after ischemia and 8 h of reperfusion, hepatocellular injury was significantly increased in PPARα−/− mice (Fig. 2A). Because much of the injury to hepatocytes at this time point is thought to be due to reactive oxygen species and proteases released from recruited neutrophils, we then examined whether PPARα deficiency caused any change in the hepatic recruitment of neutrophils. After ischemia and 4 h of reperfusion, there was no difference in liver content of MPO, a surrogate marker of neutrophil accumulation (wild-type, 2.30 ± 0.18 U/g; PPARα−/−, 3.43 ± 0.69 U/g). In contrast, after ischemia and 8 h of reperfusion, neutrophil accumulation was significantly increased in PPARα−/− mice compared with wild-type mice (Fig. 2B).

We then conducted experiments with the specific PPARα agonist WY-14643. Treatment of C57BL/6 mice with 10 mg/kg iv WY-14643 1 h before ischemia resulted in a modest but significant reduction in hepatocellular injury (Fig. 3A). A similar reduction, yet not statistically significant, was observed when liver content of MPO was measured (Fig. 3B). To determine whether WY-14643 reduced hepatocellular injury directly through PPARα activation, we treated wild-type and PPARα−/− mice with 10 mg/kg WY-14643 and assessed the extent of liver injury after ischemia and 8 h of reperfusion. In wild-type mice, WY-14643 significantly reduced hepatocellular injury (Fig. 4). However, in PPARα−/− mice, no effect of WY-14643 was observed. These studies suggest that a single treatment with WY-14643 confers protective effects against ischemia-reperfusion injury through activation of PPARα.

PPARα does not regulate hepatic production of proinflammatory cytokines and chemokines. Recruitment of neutrophils to the liver after ischemia-reperfusion has been shown to be controlled in large part by the expression of proinflammatory cytokines such as TNF-α (5) and CXC chemokines such as MIP-2 (21). The gene expression of these mediators is under the control of the transcription factors NF-κB and AP-1 (18, 19).
27), and there is evidence that PPARα may negatively regulate NF-κB and AP-1 in some cell types (7, 8, 9). Therefore, we sought to determine whether PPARα deficiency altered the hepatic inflammatory response to ischemia-reperfusion by altering transcription factor activation and production of inflammatory mediators. No differences in NF-κB or AP-1 activation were detected between sham-operated wild-type and PPARα−/− mice (Fig. 5). Ischemia and 8 h of reperfusion resulted in a marked increase in both NF-κB and AP-1 activation in wild-type mice. In livers from PPARα−/− mice there was a slight but consistent increase in the activation of both NF-κB and AP-1 (Fig. 5). We then assessed whether these apparent differences corresponded with increased production of the proinflammatory mediators TNF-α and MIP-2. Surprisingly, no differences were detected in the serum levels of TNF-α and MIP-2 in wild-type and PPARα−/− mice (Fig. 6). Consistent with these findings, wild-type mice receiving 10 mg/kg WY-14643 showed no significant changes in serum levels of TNF-α compared with untreated mice (Table 1) despite having significantly reduced hepatocellular injury (Fig. 3A).

iNOS expression is decreased in PPARα−/− mice. Because iNOS expression has been shown to be protective in models of hepatic ischemia-reperfusion (11) and because it has recently been shown that iNOS is associated with the membranes of peroxisomes (30), we investigated whether the expression of this potentially hepatoprotective enzyme was altered in PPARα−/− mice. Western blot analysis of liver protein extracts demonstrated that there was no detectable difference in iNOS expression between sham-operated wild-type and PPARα−/− mice (Fig. 7). However, after ischemia and 8 h of reperfusion, there was a marked increase in the expression of iNOS in wild-type mice but no increase in PPARα−/− mice (Fig. 7). Additionally, in both wild-type and PPARα−/− mice undergoing ischemia-reperfusion, a smaller, faster, migrating form of iNOS was detected. A similar band was also detected in a positive control obtained from activated RAW cells (Fig. 7) and may represent a degradation product found in activated cells.

To determine whether expression of endothelial NOS (eNOS) was altered in PPARα−/− mice, we performed a Western blot analysis of liver protein extracts for eNOS. In contrast to iNOS, we found similar expression of eNOS protein in all groups. There were no differences in eNOS expression between wild-type and PPARα−/− sham-operated mice, nor was there any induction of eNOS after ischemia-reperfusion in either of these mice (data not shown).

To examine whether the reduced iNOS protein expression observed in PPARα−/− mice was accompanied by reduced appearance of the NO metabolites nitrite and nitrate, we examined nitrite/nitrate levels in liver lysates and serum. We found that nitrate/nitrate levels were significantly increased by ischemia-reperfusion in both wild-type and PPARα−/− mice in liver lysates but not in serum (Table 2). There was no differ-

![Fig. 5. Hepatic activation of NF-κB and activator protein-1 (AP-1) after I/R in WT (+/+ ) and PPARα−/− (−/− ) mice. Liver nuclear extracts from sham-operated mice and mice undergoing ischemia and 8 h of reperfusion were analyzed by electrophoretic mobility shift assay.](image)

![Fig. 6. Production of TNF-α (A) and macrophage inflammatory protein-2 (MIP-2) (B) after hepatic I/R in WT and PPARα−/− mice. Serum samples from sham-operated mice and mice undergoing ischemia and 8 h of reperfusion were analyzed by ELISA. Values represent means ± SE with n = 5 mice per group.](image)

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<th>Table 1. Effect of WY-14643 on serum TNF-α levels</th>
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<td>Serum TNF-α, pg/ml</td>
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*P < 0.05, compared with sham group.
ence between nitrite/nitrate levels in liver lysates from wild-type and PPARα−/− mice undergoing ischemia-reperfusion.

**PPARα activation protects cultured hepatocytes from H2O2-induced injury.** Because we found that PPARα−/− mice exhibited enhanced hepatic ischemia-reperfusion injury and because the hepatocellular injury observed after ischemia-reperfusion is largely mediated by oxidant-induced cellular stress, we then sought to determine whether activation of PPARα in hepatocytes was directly protective against oxidant-induced injury. With the use of an in vitro model of H2O2-induced hepatocyte injury that we (19) have previously described, cultured murine hepatocytes (AML-12) were treated with WY-14643 dose-dependently inhibited H2O2-induced cell death. These studies demonstrate that PPARα activation within hepatocytes directly protects against oxidant-induced cell injury.

**DISCUSSION**

The findings presented herein demonstrate that PPARα, a transcription factor that within the liver is expressed preferentially in hepatocytes, regulates inflammatory injury induced by ischemia-reperfusion. A unique aspect of this regulation was that it was independent of the expression of the proinflammatory cytokine TNF-α and the CXC chemokine MIP-2. We and others have demonstrated essential roles for these two mediators in the propagation of hepatic inflammation after ischemia and reperfusion (5, 21). TNF-α drives this inflammatory response by upregulating vascular cell adhesion molecules and by inducing the expression of MIP-2 and other neutrophil-attracting CXC chemokines (26). Mice nullizygous for PPARα had significantly more injury to hepatocytes than did their wild-type counterparts, despite similar production of TNF-α and MIP-2. These findings can be explained by the fact that PPARα is not expressed in Kupffer cells, which is the primary site of production of TNF-α and MIP-2 in this model. We did, however, observe an increase in the DNA binding of NF-κB and AP-1 in liver nuclear extracts. NF-κB and AP-1 are both involved in the transcriptional regulation of genes for TNF-α and MIP-2 (18, 27). Unfortunately, the use of nuclear extracts from whole liver does not provide information on specific liver cell types. In fact, these experiments are likely to be more representative of hepatocytes, because these are by far the most predominant cell type in the liver. Given that PPARα is found only in hepatocytes, the increased activation of NF-κB and AP-1 observed probably represents the hepatocyte milieu and therefore would not be expected to have a significant impact on overall hepatic production of TNF-α and MIP-2 after ischemia-reperfusion.

PPARα−/− mice had augmented hepatocellular injury in association with a marked increase in the amount of neutrophils recruited to the liver. Neutrophils are responsible for much of the hepatocellular injury in this model, as documented by studies (16, 25) in which neutrophil depletion strategies ameliorated liver injury. Neutrophils are recruited from the vascular space through a complex series of events that involves upregulated expression of cellular adhesion molecules on hepatic vascular endothelial cells and increased production of CXC chemokines, both of which are induced by TNF-α (3, 4, 21). Whereas we did not examine the expression of adhesion molecules in this study, we found no differences in the production of TNF-α and MIP-2, suggesting that the enhanced liver recruitment of neutrophils observed in PPARα−/− mice may involve other mechanisms and/or mediators. One alternative mechanism that we examined was the possibility that PPARα may regulate the expression of iNOS and therefore alter the production of NO. iNOS has been shown to localize within the peroxisomal membranes in hepatocytes (30), and our data demonstrate that hepatic ischemia-reperfusion induces peroxisomal proliferation, an effect that is reduced in PPARα−/− mice. NO is known to regulate hepatic neutrophil...

### Table 2. Liver and serum levels of nitrite/nitrate after hepatic I/R

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<th>Nitrite/Nitrate</th>
<th>Liver, μmol/g</th>
<th>Serum, μM</th>
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<tr>
<td>Wild-type sham</td>
<td>14.4 ± 1.0</td>
<td>158 ± 28</td>
</tr>
<tr>
<td>PPARα−/− sham</td>
<td>15.1 ± 0.8</td>
<td>131 ± 19</td>
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<tr>
<td>Wild-type I/R</td>
<td>22.0 ± 1.3*</td>
<td>147 ± 13</td>
</tr>
<tr>
<td>PPARα−/− I/R</td>
<td>23.8 ± 1.9*</td>
<td>142 ± 19</td>
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I/R, ischemia-reperfusion; PPARα, peroxisome proliferator-activated receptor-α. *P < 0.05, compared with sham group.
accumulation after ischemia-reperfusion. Nonspecific blockade of all NOS isoforms results in large increases in the number of neutrophils sequestered in the liver after ischemia-reperfusion and results in increased liver injury (24). It was subsequently shown that this nonspecific inhibition of NOS isoforms resulted in augmented mRNA expression of P-selectin and ICAM-1 in the liver (23). The roles of specific NOS isoforms have been studied recently by different laboratories using gene-targeted mice. These studies have provided consistent evidence that eNOS serves a protective function, because eNOS-deficient mice had increased liver injury (11, 12, 20). However, the role of iNOS is less clear. Conflicting data exist as to whether iNOS is protective or injurious (11, 20). We found that iNOS expression was dramatically reduced in livers from PPARα−/− mice in association with augmented liver injury. However, our analysis of nitrite/nitrate in liver lysates did not detect any differences in NO metabolites between wild-type and PPARα−/− mice. Because iNOS expression and activity in Kupffer cells is much greater, it is possible that Kupffer cell-derived NO metabolites may have masked any differences attributable to hepatocyte production. The precise manner in which PPARα may regulate iNOS expression needs further analysis.

Complementing our studies of PPARα−/− mice were experiments that employed the PPARα-specific agonist WY-14643. These experiments demonstrated that a single dose of WY-14643 was sufficient to reduce liver injury induced by ischemia-reperfusion. Although significant, this reduction in liver injury was not dramatic, and WY-14643 did not significantly suppress the degree of neutrophil accumulation. Higher doses of WY-14643 were no more effective than the 10 mg/kg dose employed in these studies (data not shown). A possible explanation for these findings is that hepatic ischemia-reperfusion may induce PPARα activation to a near-maximal state, such that the administration of PPARα agonists like WY-14643 may have little additive effect. We also found that treatment of cultured hepatocytes with WY-14643 was directly protective against oxidant-induced injury.

In summary, the present study is the first to address the important role of PPARα in the liver inflammatory response to ischemia-reperfusion. Our data suggest that PPARα regulates hepatic neutrophil accumulation and hepatocellular injury. This regulation does not occur through suppression of proinflammatory mediators but is associated with reduced hepatic expression of iNOS. Furthermore, activation of PPARα in cultured hepatocytes was directly protective against oxidant-induced injury. These findings are of particular interest because they demonstrate that a regulatory factor expressed in liver parenchymal cells, but not in Kupffer cells, may have significant impact on the hepatic inflammatory response.

GRANTS

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REFERENCES


