Specific Role of Interleukin-1 in Hepatic Neutrophil Recruitment after Ischemia/Reperfusion

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Hepatic ischemia/reperfusion injury is caused primarily by the products of neutrophils recruited into the liver after reperfusion. The mediators responsible for the development of this inflammatory response are thought to be tumor necrosis factor- α and interleukin (IL)-1. Although there is abundant evidence to support a role for tumor necrosis factor- α , much less is known about the function of IL-1 in this injury. In the present studies, we investigated whether IL-1 was a critical mediator for the induction of liver inflammation after ischemia/reperfusion. Wild-type and IL-1 receptor I-knockout (IL-1RI^{-/-}) mice were exposed to 90 minutes of partial hepatic ischemia and up to 24 hours of reperfusion. In wild-type mice, IL-1 β expression was maximal after ischemia and 8 hours of reperfusion. At the same time, both wild-type and IL-1RI^{-/-} mice had severe liver injury as assessed by serum alanine aminotransferase levels and hepatic histopathology. However, IL-1RI^{-/-} mice had significantly less neutrophil accumulation in liver tissues as measured by liver myeloperoxidase content and histology. The reduction in hepatic neutrophil recruitment in IL-1RI^{-/-} mice was associated with decreased activation of the transcription factor, nuclear factor- κ B, and reduced expression of the CXC chemokine, macrophage inflammatory protein-2. These data suggest that IL-1 functions to augment neutrophil accumulation, but does not play an essential role in this response. (*Am J Pathol* 2002, 161:1797–1803)

Ischemia/reperfusion injury is a complication of liver resectional surgery, transplantation, and trauma.^{1–3} The hepatic inflammatory response to ischemia and reperfusion is comprised of two distinct phases. In the acute phase, the ischemic insult induces oxidant stress within the liver resulting in Kupffer cell activation and oxidant-mediated injury to hepatocytes.^{4,5} The activation of Kupffer cells results in their production of the early response cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-1.^{6,7} These potent proinflammatory cyto-

kines are generally thought to have similar overlapping functions. Similar to other acute inflammatory responses, a central role of TNF- α has been demonstrated in hepatic ischemia/reperfusion injury.⁸ Numerous studies have demonstrated that TNF- α is the primary intercellular stimulus for the production of CXC chemokines and up-regulation of vascular cell adhesion molecules.^{8–10} The effects of TNF- α culminate in the accumulation of activated neutrophils in the hepatic parenchyma. These events comprise the later phase of ischemia/reperfusion injury, which is represented by neutrophil-dependent injury to hepatocytes.^{11,12} Although the primary mode of hepatocyte death after liver ischemia/reperfusion is necrosis,¹³ there is data suggesting that TNF- α may also directly induce hepatocyte apoptosis.¹⁴ Based on this large body of data it is clear that TNF- α has multiple roles and is the dominant soluble mediator for promotion of the inflammatory response to hepatic ischemia/reperfusion injury. Surprisingly, there is a paucity of data on the role of IL-1 in this response.

IL-1 is also produced by activated Kupffer cells after ischemia/reperfusion but little is known about its function in this response. In a rat model of lethal liver ischemia/reperfusion, administration of IL-1 receptor antagonist reduced TNF- α production and mortality.¹⁵ However, the manner in which IL-1 contributes to the inflammatory response in this setting has not been fully investigated. IL-1 (both α and β isoforms) mediate their functions through interactions with the IL-1 receptor type I (IL-1RI).¹⁶ There also exists a type II IL-1 receptor, but this receptor does not signal and therefore is thought to either sequester or present IL-1.¹⁶ In the current study, we used mice lacking IL-1RI to assess the function of IL-1 in hepatic ischemia/reperfusion injury. Our data suggest that IL-1 plays an important role in the induction of CXC chemokines and subsequent neutrophil recruitment, but does not affect expression of TNF- α or the extent of hepatocellular injury.

Supported by the National Institutes of Health (grants DK56029 and HL072552) and the Swiss National Science Foundation (grant 3200-054955.98).

Accepted for publication August 6, 2002.

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Materials and Methods

Model of Hepatic Ischemia/Reperfusion Injury

Wild-type and IL-1RI-deficient (IL-1RI^{-/-}) mice on a C57BL/6 background were obtained from the Jackson Laboratories (Bar Harbor, ME). Male mice 10 to 12 weeks of age were used in all experiments. This project was approved by the University of Cincinnati Animal Care and Use Committee and conforms to the National Institutes of Health guidelines. Partial hepatic ischemia was induced as described previously.¹⁷ Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.m.). A mid-line 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 minutes of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham control mice underwent the same protocol without vascular occlusion. Mice were sacrificed after the indicated periods of reperfusion and blood and liver samples were taken for analysis.

Blood and Tissue Analysis

Blood was obtained by cardiac puncture at the time of sacrifice for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnostic kit (Sigma Chemical Co., St. Louis, MO). Serum samples were also analyzed for IL-1 β , TNF- α , and macrophage inflammatory protein-2 (MIP-2) by sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Ischemic lobes (or corresponding lobes in the sham group) were excised for tissue analysis. Liver edema was determined by organ wet-to-dry weight ratios. Liver sections were stained with hematoxylin and eosin for histological examination.

Ribonuclease Protection Assay and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA from lung and liver were extracted with Trizol Reagent (Life Technologies, Inc., Rockville, MD). Ribonuclease protection assays were performed using Ribo-Quant kits purchased from Pharmingen (San Diego, CA) as described by the manufacturer. A customized DNA template was used to analyze the expression of the IL-1 β as well as the housekeeping gene, GAPDH. The probes were hybridized in excess to 10 μ g of total RNA for 16 hours at 56°C, after which free probe and other single-stranded RNA were digested with RNAses. Double-stranded (protected) RNA was purified and resolved using the QuickPoint Gel system (Novex, San Diego, CA). For RT-PCR analysis of MIP-2 mRNA, 1 μ g of RNA was reverse-transcribed to cDNA using random hexamers. cDNA products were co-amplified by PCR (30 cycles; 95°C for 60 seconds, 59°C for 90 seconds, and 72°C for 10 seconds). Primers for MIP-2 (205-bp product) and β -actin (245-bp product) have been described else-

where.¹⁷ PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide, and photographed.

Electrophoretic Mobility Shift Assay

Nuclear extracts of liver tissue were prepared by the method of Deryckere and Gannon¹⁸ and analyzed by electrophoretic mobility shift assay. Briefly, double-stranded nuclear factor (NF)- κ B or AP-1 consensus oligonucleotides (Promega, Madison, WI) were end-labeled with γ [³²P] ATP (3000 Ci/mmol at 10 mCi/ml; Amersham, Arlington Heights, IL). Binding reactions containing equal amounts of nuclear protein extract (20 μ g) and 35 fmol (~50,000 cpm, Cherenkov counting) of oligonucleotide were incubated at room temperature for 30 minutes. Reaction volumes were held constant to 15 μ l. Reaction products were separated in a native 4% polyacrylamide gel and analyzed by autoradiography.

Myeloperoxidase (MPO) Assay

Liver MPO content was assessed by methods similar to Schierwagen and colleagues.¹⁹ Liver tissue (100 mg) was homogenized in 2 ml of buffer A (3.4 mmol/L KH₂HPO₄, 16 mmol/L Na₂HPO₄, pH 7.4). After centrifugation for 20 minutes at 10,000 $\times$ g, the pellet was resuspended in 10 vol of buffer B (43.2 mmol/L KH₂HPO₄, 6.5 mmol/L Na₂HPO₄, 10 mmol/L ethylenediaminetetraacetic acid, 0.5% hexadecyltrimethylammonium, pH 6.0) and sonicated for 10 seconds. After heating for 2 hours at 60°C, the supernatant was reacted with 3,3',3,5'-tetramethylbenzidine (Sigma Chemical Co.) and optical density determined at 655 nm.

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Data were analyzed with a one-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

Results

Expression of IL-1 β during Hepatic Ischemia/Reperfusion Injury

Previous investigations of IL-1 production during liver ischemia/reperfusion have focused on IL-1 α .^{6,7} Because IL-1 β is also a primary proinflammatory cytokine, we assessed whether this mediator was expressed in response to hepatic ischemia and reperfusion. Figure 1 shows the expression of mRNA for IL-1 β in wild-type mice during a time course of liver ischemia/reperfusion as determined by RNase protection assay. There was no increase in IL-1 β mRNA expression at the end of the ischemic insult (0 hours of reperfusion), but within 1 hour of reperfusion expression was increased. IL-1 β mRNA expression continued to increase and peaked at 8 hours of reperfusion

Figure 1. Hepatic expression of IL-1 β mRNA during ischemia/reperfusion injury. RNA extracts from wild-type mice were analyzed for IL-1 β and GAPDH expression by RNase protection assay. Autoradiographs were digitized and IL-1 β mRNA expression was expressed as percent GAPDH mRNA expression.

and was substantially reduced after 16 hours of reperfusion. To determine whether increases in mRNA expression were associated with increases in protein expression, we assessed the level of IL-1 β protein in serum samples from wild-type mice throughout a time course of ischemia/reperfusion injury. IL-1 β protein expression increased significantly after 1 hour of reperfusion (Figure 2). IL-1 β protein levels continued to increase and were maximal after 8 hours of reperfusion. After 24 hours of reperfusion, IL-1 β protein levels were similar to that observed after 1 hour of reperfusion, but remained significantly greater than sham controls (Figure 2).

Production of TNF- α and Liver Injury Are Unaffected in Mice Lacking IL-1RI

Because we found that IL-1 β expression was greatly increased after liver ischemia/reperfusion (Figures 1 and 2) and because others have reported similar finding with IL-1 α ,^{6,7} we next assessed the function of IL-1 in this model of injury. Both IL-1 α and - β elicit their effects by interactions with the IL-1 receptor type I (IL-1RI).¹⁶ Therefore, we used mice lacking the IL-1RI to determine the role of IL-1 in the hepatic inflammatory response to ischemia and reperfusion. We first sought to determine whether IL-1 functioned to augment the production of TNF- α in this response. Toward this end, we measured

Figure 2. Serum levels of IL-1 β after hepatic ischemia/reperfusion. Serum samples from wild-type mice were analyzed by enzyme-linked immunosorbent assay. Data are expressed as mean $\pm$ SEM with $n = 5$ to 10 per group. *, $P < 0.05$ compared to the sham group.

serum levels of TNF- α in wild-type and IL-1RI^{-/-} mice (Figure 3). There were no differences in serum TNF- α levels in sham-operated mice or in mice after ischemia and 8 or 24 hours of reperfusion. We next assessed the extent of liver injury induced by ischemia/reperfusion in wild-type and IL-1RI^{-/-} mice. Serum levels of ALT were similar in wild-type and IL-1RI^{-/-} mice at all time points investigated (Figure 4). Similarly, liver wet-to-dry weight ratios were identical after 8 hours of reperfusion (wild-type, 4.66 ± 0.12 ; IL-1RI^{-/-}, 4.65 ± 0.25). Collectively, these data suggest that IL-1 does not affect the hepatic production of TNF- α or the extent of hepatocellular injury induced by ischemia and reperfusion.

Figure 3. Serum levels of TNF- α in wild-type and IL-1RI^{-/-} mice after hepatic ischemia and 8 or 24 hours of reperfusion. Serum samples were analyzed by enzyme-linked immunosorbent assay. Data are expressed as mean $\pm$ SEM with $n = 8$ to 10 per group.

Figure 4. Liver injury in wild-type and IL-1RI^{-/-} mice after hepatic ischemia/reperfusion. Hepatocellular injury was assessed by measuring serum ALT levels. Data are expressed as mean $\pm$ SEM with $n = 8$ to 10 per group.

Reduced Hepatic Neutrophil Accumulation in IL-1RI^{-/-} Mice

To confirm the ALT and liver wet-to-dry weight ratio data, we assessed the histopathology of livers from wild-type and IL-1RI^{-/-} mice (Figure 5). Hepatic architecture was normal in wild-type mice and IL-1RI^{-/-} mice undergoing sham surgery (Figure 5, A and B). Extensive areas of hepatocyte necrosis were present in both wild-type and IL-1RI^{-/-} mice after ischemia and 8 or 24 hours of reperfusion (Figure 5; C to F), consistent with ALT and liver wet-to-dry weight ratio data (Figure 4). However, we observed consistent differences between wild-type mice and IL-1RI^{-/-} mice in terms of the neutrophilic infiltrates. After ischemia and 8 hours of reperfusion, wild-type mice exhibited a pattern of neutrophil accumulation both around central veins as well as diffuse accumulation in sinusoids (Figure 5C). In contrast, IL-1RI^{-/-} mice appeared to have fewer neutrophil infiltrates that were associated almost exclusively around central veins (Figure 5D). After ischemia and 24 hours of reperfusion, this pattern was still present in IL-1RI^{-/-} mice, although not as uniform as observed 8 hours after reperfusion (Figure 5, E and F). This led us to quantitatively measure the amount of liver neutrophil accumulation by determining the hepatic content of MPO. Livers from wild-type and IL-1RI^{-/-} mice undergoing sham surgery had very low levels of MPO (Figure 6). After ischemia and 4 hours of reperfusion, liver MPO levels began to increase in wild-type mice. After 8 hours of reperfusion, liver MPO content was greatly increased in wild-type mice. At this time point, livers from IL-1RI^{-/-} mice undergoing ischemia/reperfusion also had elevated MPO contents compared to sham controls, but these levels were 60% lower than those in wild-type mice ($P = 0.015$). After 24 hours of reperfusion, IL-1RI^{-/-} mice had less liver MPO, but this was not statistically different from wild-type mice. These

data suggest that IL-1 has a significant role in the hepatic recruitment of neutrophils after ischemia and reperfusion.

Reduced MIP-2 Expression and NF- κ B Activation in IL-1RI^{-/-} Mice

To further explore the mechanism by which fewer neutrophils were recruited to the livers of IL-1RI^{-/-} mice, we measured the expression of MIP-2, a CXC chemokine that has been shown to be critical for the accumulation of neutrophils in this model.¹⁷ Liver RNA extracts from wild-type and IL-1RI^{-/-} mice were analyzed by RT-PCR. As shown in Figure 7A, there was no MIP-2 mRNA expressed in liver extracts from wild-type or IL-1RI^{-/-} sham mice. After ischemia and 1 hour of reperfusion, MIP-2 mRNA expression was induced to a similar level in both wild-type and IL-1RI^{-/-} mice. Interestingly, after ischemia and 8 hours of reperfusion, MIP-2 mRNA expression in wild-type mice was greatly increased, whereas in IL-1RI^{-/-} mice it was only slightly greater than after 1 hour of reperfusion (Figure 7A). Thus, MIP-2 mRNA expression was decreased in IL-1RI^{-/-} mice compared to wild-type mice after ischemia and 8 hours of reperfusion. To determine whether there was any difference in the production of MIP-2 protein, we analyzed serum samples by enzyme-linked immunosorbent assay. In sham mice, MIP-2 levels were nearly undetectable (Figure 7B). After ischemia and 4 hours of reperfusion, MIP-2 levels were lower in IL-1RI^{-/-} mice, but this difference was not statistically significant. After 8 hours of reperfusion, wild-type mice had a marked increase in serum MIP-2, whereas IL-1RI^{-/-} mice had far lower serum levels of MIP-2 (Figure 7B). After 24 hours of reperfusion, serum levels of MIP-2 were similar in wild-type and IL-1RI^{-/-} mice.

Because the gene expression of MIP-2 is controlled by the transcription factors, NF- κ B and AP-1,^{20,21} we assessed the activation of these transcriptional regulators in liver nuclear extracts. After ischemia and 1 hour of reperfusion, NF- κ B was activated to a similar degree in wild-type and IL-1RI^{-/-} mice (Figure 8, top). After ischemia and 8 hours of reperfusion, however, NF- κ B activation persisted in wild-type mice but was greatly reduced in IL-1RI^{-/-} mice. There were no apparent differences in AP-1 activation between wild-type and IL-1RI^{-/-} mice at any time point examined (Figure 8, bottom). These data suggest that MIP-2 expression in the liver is decreased in IL-1RI^{-/-} mice in association with reduced activation of NF- κ B.

Discussion

The present study provides new information regarding the role of IL-1 in the liver inflammation occurring as a result of ischemia/reperfusion. Our data suggests that IL-1 contributes to this inflammatory response by augmenting the production of CXC chemokines and subsequent recruitment of neutrophils. It seems, however, that IL-1 is not required for the hepatocellular injury induced by ischemia/reperfusion and thus, it seems that IL-1 plays

Figure 5. Histological analysis of livers from wild-type and IL-1RI^{-/-} mice after hepatic ischemia/reperfusion. Normal liver architecture was observed in sham-operated wild-type (A) and IL-1RI^{-/-} (B) mice. After ischemia and 8 hours of reperfusion, similar degrees of hepatocyte necrosis were observed in wild-type (C) and IL-1RI^{-/-} (D) mice. After ischemia and 24 hours of reperfusion, there were similar degrees of necrosis and areas of normal liver architecture, indicating resolution of liver injury. In wild-type mice, ischemia/reperfusion induced abundant neutrophil accumulation around central veins and more diffusely in sinusoids (C and E). In IL-1RI^{-/-} mice, neutrophils were observed primarily around central veins (D and F). Original magnifications, $\times 100$.

only an accessory role during this inflammatory response.

IL-1 has long been considered to be interchangeable with TNF- α in terms of proinflammatory function. Numerous acute inflammatory responses are known to be dependent on the expression of both TNF- α and IL-1, such that inhibition of either mediator or signaling pathway greatly reduces the development of inflammatory injury. Our study demonstrates that, in the setting of hepatic ischemia/reperfusion injury, TNF- α and IL-1 have distinctly different properties. It is well established that he-

patic ischemia/reperfusion induces the expression of TNF- α , and that TNF- α is essential for the induction and promotion of liver inflammation.⁸⁻¹⁰ Here we show that IL-1 β is also expressed in liver after ischemia/reperfusion, but that mice lacking IL-1RI experience a similar degree of liver injury as wild-type controls. However, IL-1RI^{-/-} mice had greatly reduced expression of the CXC chemokine, MIP-2, and reduced neutrophil accumulation in the liver. Recent studies suggest that IL-1 is more potent than TNF- α in inducing hepatic CXC chemokine production.²² In addition, IL-1 is a potent neutrophil ac-

Figure 6. Liver neutrophil recruitment in wild-type and IL-1RI^{-/-} mice after hepatic ischemia/reperfusion. Liver neutrophil accumulation was assessed by liver content of MPO. Data are expressed as mean $\pm$ SEM with $n = 8$ to 10 per group. *, $P < 0.05$ compared to wild-type mice.

tivator,²³ and thus, the reduced neutrophil accumulation in IL-1RI^{-/-} mice is likely a result of the combined lack of these effects. However, a striking association was observed between reduced neutrophil accumulation and MIP-2 expression. Furthermore, the reduced expression of MIP-2 was linked with a similar reduction in the activation of the transcription factor, NF- κ B, in liver nuclei.

Figure 7. Expression of MIP-2 in wild-type and IL-1RI^{-/-} mice during hepatic ischemia/reperfusion injury. **A:** MIP-2 and β -actin mRNA expression were analyzed by RT-PCR in sham-operated mice and mice undergoing hepatic ischemia and 1 or 8 hours of reperfusion. **B:** Serum levels of MIP-2 were measured in wild-type and IL-1RI^{-/-} mice after hepatic ischemia/reperfusion. Data are expressed as mean $\pm$ SEM with $n = 8$ to 10 per group. *, $P < 0.05$ compared to wild-type mice.

Figure 8. Activation of the transcription factors, NF- κ B and AP-1, in wild-type and IL-1RI^{-/-} mice during hepatic ischemia/reperfusion injury. Liver nuclear extracts from sham-operated mice and mice undergoing hepatic ischemia and 1 or 8 hours of reperfusion were subjected to electrophoretic mobility shift assay. Results are representative of duplicate experiments.

NF- κ B is a rapidly activated transcription factor that regulates the expression of a large number of proinflammatory genes, including MIP-2.²⁴ The finding that IL-1RI^{-/-} mice had reduced NF- κ B activation provides evidence that IL-1 is an important stimulus for NF- κ B activation during hepatic ischemia/reperfusion injury. However, activation of NF- κ B by IL-1 appeared to occur near the apex of the inflammatory response. Liver NF- κ B activation was normal in IL-1RI^{-/-} mice after 1 hour of reperfusion, but greatly reduced after 8 hours of reperfusion. This was consistent with the expression of IL-1 β mRNA and protein in the liver, both of which were maximal 8 hours after reperfusion. Thus, unlike TNF- α , which is expressed rapidly after reperfusion, IL-1 expression is delayed. It is well known that TNF- α can stimulate the production of IL-1.^{25,26} Furthermore, based on our current data and previous reports,²⁷ it seems likely that TNF- α is responsible for the initial activation of NF- κ B in the liver. This may lead to the delayed production of IL-1, which itself is a potent activator of NF- κ B. IL-1 may then activate NF- κ B during the later phases of the reperfusion injury.

The activation of NF- κ B at these later times by IL-1 appears to augment the hepatic recruitment of neutrophils by increasing the expression of MIP-2. However, IL-1 is not the primary stimulus for liver neutrophil accumulation. Although IL-1RI^{-/-} mice had reduced neutrophil accumulation 8 hours after reperfusion, these mice still had a significant number of neutrophils sequestered in the hepatic parenchyma. Furthermore, the hepatocellular injury that occurs in this model is predominantly because of oxidants and proteases released by accumulated neutrophils, yet there was no reduction in liver injury in IL-1RI^{-/-} mice. This suggests that despite the reduced neutrophil accumulation in IL-1RI^{-/-} mice, there were still

enough neutrophils present to cause extensive liver injury.

Our findings are similar to those in a murine model of renal ischemia/reperfusion injury. In those studies, mice treated with IL-1Ra or IL-1RI^{-/-} mice had acute tubular necrosis and increases in blood urea nitrogen and serum creatine levels similar to untreated or wild-type mice, respectively.²⁸ Interestingly, mice treated with IL-1Ra or IL-1RI^{-/-} mice had significantly less infiltration of neutrophils in postischemic renal tissues. Furthermore, these mice experienced a marked acceleration in the recovery of renal function. This latter finding is in contrast to our observations of liver injury after 24 hours of reperfusion. At this late time point in wild-type mice, ALT levels were greatly reduced, indicating that the hepatocellular injury was in a phase of resolution. However, similar levels of ALT as well as similar hepatic architecture were found in IL-1RI^{-/-} mice. These findings suggest that in kidney and liver, IL-1 has similar properties related to the recruitment of neutrophils, but that unlike the kidney, IL-1 is not critical for the inflammatory injury to the liver.

In summary, the current study provides important information regarding the role of IL-1 in the inflammatory injury induced by hepatic ischemia/reperfusion. Our data indicate that IL-1 functions to induce NF- κ B activation and expression of CXC chemokines during the later phases of this inflammatory response. These effects augment the recruitment of neutrophils to the hepatic parenchyma, but do not significantly alter the extent of hepatocellular injury. Thus, IL-1 is not a primary mediator of this response and has limited significance in the inflammatory injury to the liver.

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