Generation of hypochlorite-modified proteins by neutrophils during ischemia-reperfusion injury in rat liver: attenuation by ischemic preconditioning

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Hasegawa, Tadashi, Ernst Malle, Anwar Farhood, and Hartmut Jaeschke. Generation of hypochlorite-modified proteins by neutrophils during ischemia-reperfusion injury in rat liver: attenuation by ischemic preconditioning. Am J Physiol Gastrointest Liver Physiol 289: G760–G767, 2005. First published June 30, 2005; doi:10.1152/ajpgi.00141.2005.—Although it is well documented that neutrophils are critical for the delayed phase of hepatic ischemia-reperfusion injury, there is no direct evidence for a specific neutrophil-derived oxidant stress in vivo. Therefore, we used a model of 60 min of partial hepatic ischemia and 0–24 h of reperfusion to investigate neutrophil accumulation and to analyze biomarkers for a general oxidant stress [glutathione disulfide (GSSG) and malondialdehyde (MDA)] and for a neutrophil-specific oxidant stress [hypochlorite (HOCl)-modified epitopes] in rats. Plasma alanine transaminase activities and histology showed progressively increasing liver injury during reperfusion, when hepatic GSSG and soluble MDA levels were elevated. At that time, few neutrophils were present in sinusoids. However, the number of hepatocytes positively stained for HOCl-modified epitopes increased from 6 to 24 h of reperfusion, which correlated with the bulk of hepatic neutrophil accumulation and extravasation into the parenchyma. Consistent with a higher oxidant stress at later times, hepatic GSSG and protein-bound MDA levels further increased. Treatment with the NADPH oxidase inhibitor diphenyleneiodonium chloride attenuated postischemic oxidant stress (GSSG, protein-bound MDA, and hepatocytes positively stained for HOCl-modified epitopes) and liver injury at 24 h of reperfusion. Ischemic preconditioning suppressed all oxidant stress biomarkers, liver injury, and extravasation of neutrophils. In conclusion, extravasated neutrophils generate HOCl, which diffuses into hepatocytes and causes oxidative modifications of intracellular proteins during the neutrophil-mediated reperfusion injury phase. Ischemic preconditioning is an effective intervention for reduction of the overall inflammatory response and, in particular, for limitation of the cytotoxic activity of neutrophils during the later reperfusion period.

hepatic oxidant stress biomarkers reactive oxygen species hypochlorous acid myeloperoxidase hydrogen peroxide chloride system malondialdehyde lipid peroxidation

AN EXCESSIVE INFLAMMATORY response is a critical component of hepatic ischemia-reperfusion injury (24, 42). The initial phase of inflammation is characterized by activation of Kupffer cells (3, 27, 28, 66), which generate reactive oxygen species (24, 25, 27, 66) and aggravate the early injury (27). In addition, polymorphonuclear leukocytes (neutrophils) accumulate in the postischemic liver and actively contribute to hepatocyte injury in the later phase (30). However, the specific mechanisms involved in killing neutrophil-mediated hepatocytes remain controversial (34). Experiments in which neutrophils and hepatocytes were cultured together consistently suggested that neutrophils kill hepatocytes not by reactive oxygen but by protease-associated processes (14, 18, 23, 50, 59). In support of this hypothesis, protease inhibitors were shown to attenuate hepatic ischemia-reperfusion injury in vivo (38, 44). On the other hand, substantial evidence has accumulated that reactive oxygen species derived from neutrophils may be involved in the pathogenesis. First, neutrophils isolated from the postischemic liver at >5 h of reperfusion spontaneously generated superoxide anion radicals (26). These data suggested that liver neutrophils were fully activated at that time. Second, blocking the Mac-1 (CD11b/CD18) receptor, which is critical for adherence-dependent oxidant stress of neutrophils (53, 65), attenuated the spontaneous superoxide anion radical formation of isolated neutrophils and reduced reperfusion injury (29). To reconcile the differences between experiments performed in cell culture and those carried out in vivo, it was suggested that neutrophil-derived reactive oxygen species are important for the inactivation of antiproteases in vivo, rather than directly responsible for killing liver cells (34, 68). However, more recently, we showed that a neutrophil-derived intracellular oxidant stress could cause hepatocellular injury during endotoxemia (21, 32) and obstructive cholestasis (20, 22). This increased insight into the mechanism of neutrophil-induced tissue injury was possible through the use of antibodies directed against chlorotyrosine (20–22) or against hypochlorite (HOCl)-modified epitopes (17, 46, 47), which represent specific footprints of the potent neutrophil-derived oxidant stress (HOCl, 11, 45), generated only from H2O2 and Cl– by myeloperoxidase (MPO). Therefore, the aim of the present investigation was to provide direct evidence for a specific neutrophil-induced oxidant stress in the postischemic liver in vivo and to assess the temporary relation between oxidant formation and extravasation of neutrophils into the parenchyma.

Ischemic preconditioning (IP), a brief period of ischemic pretreatment followed by reperfusion, is a promising intervention for limitation of subsequent ischemia-reperfusion injury (4, 24, 37, 61, 62). Several studies showed that IP reduced postischemic oxidant stress and neutrophil accumulation in normal (6) and fatty livers (64). The effect of IP on the postischemic oxidant stress was evaluated by quantitating the lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (6, 64). Although MDA and HNE are reliable indicators of the degree of lipid peroxidation, they are
not specific for neutrophils. Similarly, neutrophil accumulation in posts ischemic livers undergoing IP was estimated with the MPO assay (6, 64). Although MPO activity is a good overall indicator of neutrophil content in the tissue, hemorrhage may affect the data by pseudo-MPO activity of hemoglobin (56). In addition, hepatic MPO activity measurements do not give information regarding the localization of neutrophils in the liver. Therefore, a second aim of this study was to evaluate the effect of IP on hepatic neutrophil accumulation and localization and on neutrophil-specific oxidant stress during reperfusion.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (250–300 g body wt; Harlan, Indianapolis, IN) were allowed free access to food and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Arizona and followed the guidelines of the National Research Council for the care and use of laboratory animals in research.

Experimental protocols. Under anesthesia with pentobarbital sodium solution (50 mg/kg ip), a laparotomy was performed, and the blood supply to the median and left hepatic lobes was occluded with an atrumatic vascular clamp for 60 min. Reperfusion was initiated by removal of the clamp. Body temperature was monitored and maintained at 37 ± 0.3°C with a heating lamp. After reperfusion was initiated, 1 ml of physiological saline was injected intraperitoneally, the abdominal incision was closed with 4-0 silk and wound clips, and the animals were allowed to recover. Blood was drawn and the liver was excised before ischemia, at the end of ischemia, and at 1, 6, 12, and 24 h of reperfusion. Plasma was used for determination of alanine transaminase (ALT) activity. A part of the excised liver was fixed in phosphate-buffered 10% formalin solution and embedded in paraffin for histological evaluations, and the remaining tissue was snap frozen in liquid nitrogen for determination of glutathione and MDA levels. In some animals, diphenylethionidion chloride (DPI, 1 mg/kg) (35), a potent inhibitor of NADPH oxidase (51), or its vehicle 5% glucose (4 ml/kg) was injected subcutaneously 24 h and 1 h before ischemia and 12 h after reperfusion.

Ischemic preconditioning. In a series of preliminary experiments, we tested different durations of ischemia to assess the optimal protection by preconditioning. We found the best protection when Sprague-Dawley rats were subjected to 5 min of ischemia and 15 min of reperfusion followed by 45 min of ischemia. In previous studies, we used 45 and 60 min of ischemia and found that the posts ischemic inflammatory response is qualitatively the same. Thus both ischemia intervals can be used to demonstrate the reduced inflammatory response and reduced neutrophil-induced oxidant stress during preconditioning. In a control group without IP, animals underwent a sham operation. Plasma and livers were collected 24 h after reperfusion following 45 min of ischemia.

Quantitative estimation of liver injury and hepatic neutrophil accumulation. Plasma ALT activity was measured with a commercially available test kit (Biotron Diagnostics, Hemet, CA). Hematoxylin-eosin-stained liver sections were used for histological determination. Plasma ALT activity was measured with a commercially available kit (INTERCHIM MDA-586, Oxis Research). Hepatic MPO activity measurements do not give information regarding the localization of neutrophils in the liver. Therefore, a second aim of this study was to evaluate the effect of IP on hepatic neutrophil accumulation and localization and on neutrophil-specific oxidant stress during reperfusion.

Liver injury. Progressive liver injury was observed during 24 h of reperfusion after 60 min of ischemia as indicated by a pronounced increase in plasma ALT activity (Fig. 1A). As a further cumulative indicator of hepatoctye injury, necrosis was estimated in histological sections of the liver (Fig. 1B). Patchy areas of coagulative necrosis appeared 6 h after reperfusion. Thereafter, necrotic areas were progressively enlarged until 24 h of reperfusion. In control rats, plasma ALT levels were low (28 ± 8 U/l), and no necrosis could be seen in any of the liver sections. Consistent with previous results (19), no relevant increase in the number of apoptotic hepatocytes was observed during reperfusion (data not shown).

RESULTS

Liver injury. Progressive liver injury was observed during 24 h of reperfusion after 60 min of ischemia as indicated by a pronounced increase in plasma ALT activity (Fig. 1A). As a further cumulative indicator of hepatoctye injury, necrosis was estimated in histological sections of the liver (Fig. 1B). Patchy areas of coagulative necrosis appeared 6 h after reperfusion. Thereafter, necrotic areas were progressively enlarged until 24 h of reperfusion. In control rats, plasma ALT levels were low (28 ± 8 U/l), and no necrosis could be seen in any of the liver sections. Consistent with previous results (19), no relevant increase in the number of apoptotic hepatocytes was observed during reperfusion (data not shown).

Postischemic oxidant stress. Hepatic GSSG levels and the GSSG-to-GSH + GSSG ratio were measured as indicators of intracellular oxidant stress in the liver (27, 28). GSSG levels and the GSSG-to-GSH + GSSG ratio declined during ischemia but significantly increased during 24 h of reperfusion (Fig. 2). The total glutathione content showed only a temporary decline at 6 h of reperfusion (Fig. 2). On the basis of these parameters, the intracellular oxidant stress during reperfusion appeared to be more severe later in reperfusion. To confirm the postischemic oxidant stress, we measured hepatic MDA (Fig. 3). The total tissue MDA content significantly increased at 1 h and...
reached a plateau at 12 h of reperfusion. Free MDA peaked at 1 h of reperfusion and then decreased to the control level after 24 h (Fig. 3). Only low levels of protein-bound MDA were present in control livers. However, protein-bound MDA was significantly increased between 6 and 24 h of reperfusion. The value at 24 h of reperfusion was 495 ± 42% of baseline (Fig. 3).

Number of neutrophils in the liver. Neutrophils started to accumulate in sinusoids at 1 h of reperfusion (Fig. 4), when only negligible necrotic hepatocytes could be identified in histological sections (Fig. 1). Thereafter, the number of neutrophils increased mainly around and within necrotic areas at 6 h and reached a high plateau level at 12 h of reperfusion (Fig. 4). Extravasation of neutrophils into the hepatic parenchyma was observed at 6 h and then increased until 24 h of reperfusion (Fig. 4). Extravasated neutrophils were seen exclusively around and within necrotic areas.

Immunohistochemical detection of HOCl-modified epitopes. To assess the presence of a specific neutrophil-mediated oxidant stress, liver sections were stained for HOCl-modified proteins. No significant staining was seen before ischemia (Fig. 5A), at the end of 60 min of ischemia (not shown), or at 1 h of reperfusion (Fig. 5B), indicating that hepatocytes (cells devoid of MPO) showed no immunoreactivity for HOCl-modified epitopes. Some viable hepatocytes around the necrotic area were selectively stained at 6 h of reperfusion. Some necrotic hepatocytes in coagulative necrotic areas, which were identified by cell swelling, karyorhexis, and karyolysis, also showed positivity for HOCl-modified proteins at this time (Fig. 5, C and D). An increased number of hepatocytes with pronounced immunoreactivity for HOCl-modified epitopes was observed around necrotic areas at 12 h (data not shown) and 24 h of reperfusion. Many necrotic hepatocytes showing positive staining for HOCl-modified epitopes could be observed in enlarged necrotic areas (Fig. 5E). The distribution of viable and necrotic HOCl-positive hepatocytes colocalized with extravasated neutrophils. Omission of the primary antibody (data not shown) or preabsorption with HOCl-modified (Fig. 5F), but not native, low-density lipoprotein (data not shown) prevented antibody binding, indicating that the staining was specific for HOCl-modified epitopes generated in vivo by the MPO-H2O2-Cl−
system. Next, hepatocytes positively stained for HOCl-modified epitopes were counted in 20 high-power fields and expressed as percentage of the total number of hepatocytes in the corresponding areas (Fig. 6). A few HOCl-positive hepatocytes were observed at 6 h of reperfusion, and the number dramatically increased until 24 h of reperfusion. The appearance of HOCl-positive hepatocytes during reperfusion (Fig. 6) correlated well with the extravasation of neutrophils (Fig. 4).

**Effect of the NADPH oxidase inhibitor DPI.** To provide further support for the presence of neutrophil-induced oxidant stress, animals were treated with DPI, an inhibitor of NADPH oxidase, the enzyme generating superoxide anion radicals from molecular oxygen. We used 1 mg/kg DPI as previously described by Kono et al. (35) in experiments with rats. Treatment with DPI significantly attenuated liver injury (ALT, histological evaluation) after 60 min of ischemia and 24 h of reperfusion (Table 1). DPI treatment did not significantly affect hepatic GSSG but significantly reduced the GSSG-to-GSH + GSSG ratio by 31%, protein-bound MDA by 47%, and the number of hepatocytes positively stained for HOCl-modified epitopes by 50% (Table 1). Furthermore, DPI treatment attenuated accumulation of neutrophils in the liver by 54% and the number of extravasated neutrophils by 62% and reduced the areas of necrosis.

**Effect of IP on neutrophil-induced oxidant stress and neutrophil extravasation.** In preliminary experiments, the optimal duration of ischemia for IP was assessed. We found the best protection with 5 min of ischemia and 15 min of reperfusion before 45 min of ischemia. Under these conditions, IP significantly reduced the increase in plasma ALT activities and the area of necrosis (Table 2). IP attenuated hepatic GSSG by 40%, the GSSG-to-GSH + GSSG ratio by 44%, protein-bound MDA by 34%, and the number of hepatocytes (positively stained for HOCl-modified epitopes) by 78%. In addition, IP reduced accumulating neutrophils in the liver by 45% and extravasated neutrophils by 62%.

**DISCUSSION**

**Neutrophil-induced oxidant stress and reperfusion injury.** The aims of the present study were to test the presence and the potential pathophysiological role of a neutrophil-specific oxidant stress during hepatic ischemia-reperfusion with or without IP. Consistent with our earlier report (27), we found a significant number of neutrophils in hepatic sinusoids after 1 h of reperfusion. Neutrophils continued to accumulate in the liver up to 12 h of reperfusion. On the other hand, neutrophil extravasation into the parenchyma started at −6 h of reperfusion and then progressed rapidly. The earliest evidence for a specific neutrophil-mediated oxidant stress (hepatocytes positively stained for HOCl-modified epitopes) was detected at 6 h of reperfusion. The number of HOCl-positive hepatocytes increased substantially between 6 and 12 h and even further at 24 h of reperfusion. This close temporal correlation between extravasation of neutrophils and a neutrophil-derived oxidant stress in vivo suggests that neutrophils in sinusoids are only partially activated, and the full cytotoxic potential is only reached after extravasation into the parenchyma. These conclusions are supported by analysis of neutrophils isolated from the postischemic liver. At 1 h of reperfusion, these isolated neutrophils were primed for enhanced reactive oxygen formation but did not produce superoxide anion radicals spontaneously (25). On the other hand, hepatic neutrophils isolated at 5 or 24 h of reperfusion generated superoxide anion radicals without stimulation, suggesting a full activation at this time (26). Our data in a model of hepatic ischemia-reperfusion injury are consistent with findings in other experimental models of neutrophil-mediated liver damage, such as galactosamine/endotoxin shock or bile duct ligation (obstructive cholestasis). In these models, neutrophil extravasation is a prerequisite for neutrophil-derived oxidant stress and neutrophil-mediated liver injury (7, 20–22). It is generally assumed that the final activation of neutrophils occurs during the extravasation process and adherence to the target cells (39, 52, 65). The activation may be related to engagement of adhesion molecules (39) and exposure to chemotactic agents (43, 55). Consistent with this hypothesis, the neutrophil-derived oxidant stress and injury by neutrophils are eliminated when the extravasation of neutrophils is prevented (12, 13, 20, 22, 31, 40) or the formation of reactive oxygen is inhibited (21, 29). Thus the neutrophil-mediated injury is critically dependent on extravasation into the parenchyma and induction of a neutrophil-induced oxidant stress.
H₂O₂, HOCl, and proteases. The presence of HOCl-modified epitopes is a specific footprint for the MPO-H₂O₂-Cl⁻/H⁰ system of activated neutrophils, where MPO makes up as much as 5% of the total cell protein content. Using a specific antibody (cross-reacting with HOCl-modified epitopes, but not with epitopes generated via lipid peroxidation) (45), we could stain hepatocytes colocalized with extravasated neutrophils. The finding that the staining was intracellular indicates that HOCl or a derivative (48) diffused inside hepatocytes and reacted with intracellular proteins. Similar findings were reported using an antichlorotyrosine antibody in other models of neutrophil-induced liver injury (20–22). In addition, HOCl can cause formation of chloramines, which are potent oxidants and cytotoxic agents (1). However, during the oxidative burst, neutrophils generate superoxide anion radicals via NADPH oxidase. Superoxide anion radicals dismutate to oxygen and H₂O₂, which can be used to generate HOCl by neutrophil-derived MPO or can directly diffuse into hepatocytes. Evidence for the intracellular presence of H₂O₂ is the increase in intracellular GSSG levels (Fig. 2) or the enhanced biliary efflux of GSSG (2). Sources of H₂O₂ other than neutrophils, e.g., mitochondria and Kupffer cells, may contribute to the

Table 1. Effect of DPI on ischemia-reperfusion liver injury

<table>
<thead>
<tr>
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<th>Vehicle</th>
<th>DPI</th>
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<tbody>
<tr>
<td>ALT, IU/l</td>
<td>4,904±273</td>
<td>2,519±538*</td>
</tr>
<tr>
<td>Necrosis, %</td>
<td>77.5±2.5</td>
<td>52.5±8.5*</td>
</tr>
<tr>
<td>GSSG, μmol/g liver</td>
<td>0.187±0.039</td>
<td>0.216±0.057</td>
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<tr>
<td>GSSG/GSH+GSSG, %</td>
<td>5.83±0.54</td>
<td>4.18±0.06*</td>
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<tr>
<td>Protein-bound MDA, nmol/mg protein</td>
<td>0.486±0.042</td>
<td>0.259±0.042*</td>
</tr>
<tr>
<td>Total neutrophils, cells/20 HPFs</td>
<td>623±43</td>
<td>289±68*</td>
</tr>
<tr>
<td>Extravasated neutrophils, cells/20 HPFs</td>
<td>149±11</td>
<td>56±13*</td>
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<tr>
<td>Extravasated neutrophils, %</td>
<td>23.9±0.4</td>
<td>19.6±1.9</td>
</tr>
<tr>
<td>HOCl-positive hepatocytes, %</td>
<td>14.5±1.2</td>
<td>7.2±1.2*</td>
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Values are mean ± SE of 4 animals in each group. Effect of diphenyleneiodonium chloride (DPI) on liver injury, sequestration of neutrophils, and oxidant stress markers was measured 24 h after reperfusion-following 60 min of ischemia. DPI (1 mg/kg) or its vehicle 5% glucose (4 ml/kg) was injected subcutaneously 24 h and 1 h before ischemia and 12 h after reperfusion. ALT, alanine transaminase; GSSG, glutathione disulfide; GSH, glutathione; MDA, malondialdehyde; HPFs, high-power fields; HOCl, hypochlorite. *P < 0.05 vs. vehicle.
The importance of H2O2 for a neutrophil-mediated injury was ramine formation, were at least partially responsible for the stress (32). Thus H2O2, HOCl, and HOCl-derived chloramines oxidase gene knockout mice to the neutrophil-induced oxidant stress underwent a sham operation before prolonged ischemia. In the control group, animals underwent a sham operation before prolonged ischemia. In the IP group, animals were subjected to 5 min of ischemia and 15 min of reperfusion before prolonged ischemia. In the control group, animals underwent a sham operation before prolonged ischemia. *P < 0.05 vs control.

Intracellular GSSG formation. However, the fact that the highest levels of GSSG were found during neutrophil-induced oxidant stress suggests that neutrophils, through H2O2 or chloramines formation, were at least partially responsible for the increased intracellular GSSG levels after ischemia-reperfusion. The importance of H2O2 for a neutrophil-mediated injury was documented by the increased susceptibility of glutathione peroxidase gene knockout mice to the neutrophil-induced oxidant stress (32). Thus H2O2, HOCl, and HOCl-derived chloramines may contribute to the oxidant stress-mediated injury process. Consistent with this hypothesis, the NADPH oxidase inhibitor DPI attenuated the number of hepatocytes positively stained for HOCl-modified epitopes and partially protected against ischemia-reperfusion injury. The dose of DPI used in our studies and previously in rats (35) was only partially effective. On the other hand, a higher dose completely eliminated the neutrophil-induced oxidant stress during endotoxemia in mice (21). However, this dose of DPI (2.5 mg/kg body wt sc) proved to be toxic in rats (unpublished observation). The effect of DPI may not be restricted to neutrophils, because Kupffer cells and endothelial cells also contain NADPH oxidase. Because a Kupffer cell-induced oxidant stress is critical for the early injury phase (27, 28), DPI may have affected the initial injury and, thus, reduced neutrophil recruitment and the later neutrophil-induced injury phase indirectly in addition to the direct effect on neutrophil NADPH oxidase. Although we found that very few superoxide anion radicals are released by endothelial cells during reperfusion (25), reduced intracellular superoxide anion radical formation may trap less nitric oxide, which then could be available to attenuate postischemic vasoconstriction (24). Thus the protective effect of DPI may be caused by multiple mechanisms related to inhibition of NADPH oxidase in several cell types.

A consequence of the intracellular oxidant stress during reperfusion is lipid peroxidation. Our data suggest that protein-bound MDA (Fig. 3) and HNE (21, 22) are more sensitive parameters of lipid peroxidation in vivo than the soluble primary products. However, even if the accumulating products are analyzed, lipid peroxidation is quantitatively insufficient to explain the massive cell injury during reperfusion (49). Instead, lipid aldehydes (10) and HOCl-modified (lipid)proteins and reactive chloramines, which are in turn powerful oxidants (1), can act as chemotactic signals (36, 69), which continue to recruit neutrophils into the postischemic liver. On the other hand, the intracellular oxidant stress can trigger mitochondrial dysfunction, leading to loss of mitochondrial membrane potential and cell necrosis (41, 54). Thus neutrophil-derived oxidants, which diffuse into hepatocytes, have two major functions: 1) they are involved in cell killing, and 2) they are responsible for maintaining the inflammatory response. Because of the self-aggravating nature of the posts ischemic inflammatory injury (24), any intervention that reduces cell damage early in the process will reduce neutrophil recruitment into the postischemic liver. Thus the reduced number of neutrophils at 24 h of reperfusion and the reduced neutrophil-induced oxidant stress in DPI-treated animals are caused by a combination of effects, i.e., the direct inhibition of NADPH oxidase in neutrophils and the reduced overall inflammatory response due to an early reduction of Kupffer cell and neutrophil cytotoxicity. Similarly, IP attenuates the initial ischemic injury, which leads to a reduced progression of the inflammatory response, including less hepatic neutrophil recruitment and less neutrophil-mediated oxidant stress. In this case, a direct inhibition of neutrophil NADPH oxidase is not involved in the mechanism.

An ongoing controversy is whether neutrophils kill hepatocytes through oxidant stress or proteases (34). Although all in vitro coculture studies suggest a dominant role for proteases (14, 18, 23, 50, 59), there are a number of limitations to this approach. The most important limitation is the fact that neutrophils do not adhere to and attack healthy control hepatocytes (20), which are generally used in these studies. Thus the activated neutrophils release mainly proteases, which slowly digest the cultured hepatocytes over 15–20 h. In contrast, neutrophils in vivo actively migrate and adhere to the target. This then triggers degranulation and a long-term adherence-dependent oxidant stress (53, 65), which can kill stressed hepatocytes within 1 h (31, 32). Nevertheless, during a neutrophilic hepatitis such as occurs during ischemia-reperfusion, neutrophil-derived proteases also contribute to the overall cell killing (38, 44).

**Neutrophil-induced oxidant stress and IP.** IP is an effective intervention to attenuate reperfusion injury in the liver in experimental models (4, 24, 37, 61, 62) and in humans (8, 9). A number of mechanisms have been discussed, including improvement of energy metabolism, reduced ischemic injury, enhanced antioxidant defense mechanisms, enhanced anti-inflammatory cytokine formation, enhanced regeneration, and reduced posts ischemic oxidant stress (5, 6, 16, 57, 58, 60, 63, 67), all of which can contribute to various degrees to the overall protective effect after IP. An increased resistance to ischemic injury (4, 5) and an enhanced capacity to detoxify reactive oxygen species (60) are important mechanisms for a reduced inflammatory response during reperfusion with less neutrophil accumulation in the liver (6, 16, 63, 67) and less lipid peroxidation (6). Our data confirm the reduced number of neutrophils in the preconditioned liver and an overall reduced oxidant stress and lipid peroxidation. In addition, we also demonstrate a significantly reduced extravasation of neutrophils into the parenchyma and a near elimination of specific neutrophil-induced oxidant stress. These findings suggest that, after IP, not only are fewer neutrophils accumulating and extravasating in the liver, but these neutrophils are much less activated than those in animals subjected only to ischemia-

| Table 2. Effect of IP on ischemia-reperfusion liver injury |
|-------------------|----------------|
|                   | Control       | IP            |
| ALT, IU/l         | 2,461±582     | 1,066±123*    |
| Necrosis, %       | 47.5±4.8      | 27.5±6.3*     |
| GSSG, μmol/g liver| 0.284±0.032   | 0.169±0.016*  |
| GSSG/GSH+GSSG, %  | 3.76±0.38     | 2.10±0.17*    |
| Protein-bound MDA, nmol/mg protein | 0.232±0.021 | 0.153±0.018* |
| Total neutrophils, cells/20 HPF | 330±35      | 183±12*       |
| Extravasated neutrophils, cells/20 HPF | 75±7       | 27±3*         |
| Extravasated neutrophils, % | 22±6.5      | 14.8±0.6*     |
| HOCI-positive hepatocytes, % | 11.6±2.1 | 2.5±0.5*      |

Values are means ± SE of 4 animals in each group. Effect of ischemic preconditioning (IP) on liver injury, sequestration of neutrophils, and oxidant stress markers was measured 24 h after reperfusion following 45 min of ischemia. In the IP group, animals were subjected to 5 min of ischemia and 15 min of reperfusion before prolonged ischemia. In the control group, animals underwent a sham operation before prolonged ischemia. *P < 0.05 vs control.
reperfusion. These conclusions are supported by a recent human study showing reduced CD11b integrin expression and reduced priming of peripheral neutrophils after IP in patients undergoing the Pringle maneuver during hepatic resections (8). In addition, the increase in plasma interleukin-8 levels was substantially reduced in preconditioned patients (8). Thus IP reduces the overall posts ischemic inflammatory response and substantially attenuates neutrophil activation and cytotoxicity in experimental animals and in humans.

In summary, our data provide direct evidence for a specific neutrophil-mediated oxidant stress during reperfusion when a relevant number of neutrophils had extravasated from sinusoids. Neutrophils enzymatically generate superoxide anion radicals through NADPH oxidase and HOCl through the MPO-H$_2$O$_2$-Cl$^-$ system. HOCl diffuses into hepatocytes and causes oxidative modifications and chlorination of tyrosine residues on intracellular proteins during the neutrophil-mediated reperfusion injury phase. IP is an effective intervention for reduction of the overall inflammatory injury response and, in particular, for limitation of the cytotoxic activity of neutrophils later in reperfusion.

GRANTS

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