Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver

HARTMUT JAESCHKE AND ANWAR FARHOOD
Center for Experimental Therapeutics, Department of Medicine,
and Department of Pathology, Baylor College of Medicine, Houston, Texas 77030

JAESCHKE, HARTMUT, AND ANWAR FARHOOD. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am. J. Physiol. 260 (Gastrointest. Liver Physiol. 23): G355-G362, 1991.—The hypothesis that Kupffer cells and infiltrating neutrophils generate reactive oxygen in the hepatic sinusoids and may contribute to ischemia-reperfusion injury in the liver was investigated in a model of partial no-flow ischemia and reperfusion in male Fischer rats in vivo. During the reperfusion period of 60 min, plasma concentrations of glutathione disulfide (GSSG; index of oxidant stress) increased from 1.62 ± 0.20 μM glutathione (GSH) equivalents to maximal values of 11.82 ± 1.45 (45 min ischemia), 24.19 ± 2.25 (60 min ischemia), and 70.20 ± 7.8 (120 min ischemia). The basal tissue GSSG content in the postischemic lobes (0.19 ± 0.02 nmol GSH eq/mg protein) increased by 50–100%. Although the number of neutrophils in liver and lung increased by 3- to 10-fold during reperfusion, there was no positive correlation between the number of neutrophils and the GSSG concentrations measured in plasma or tissue. However, activation of Kupffer cells with high doses of retinol or with Propionibacterium acnes significantly enhanced plasma GSSG levels, while inactivation of Kupffer cells with methyl palmitate or gadolinium chloride significantly attenuated the increase of plasma GSSG. Inactivation of Kupffer cells protected the liver significantly against ischemia-reperfusion injury. It is concluded that Kupffer cells are the predominant source of reactive oxygen formed during the initial reperfusion period and that Kupffer cell activity (including reactive oxygen formation) contributes to reperfusion injury in the liver in vivo.

THE ROLE of reactive oxygen in the pathogenesis of ischemia-reperfusion injury is under intensive investigation but is still poorly understood. In analogy to the pioneering work of Granger et al. (8) in cat intestine, the effects of the xanthine oxidase inhibitor allopurinol and the detoxifying enzymes superoxide dismutase and catalase had been tested in various animal models of hepatic ischemia and reperfusion injury. These compounds proved to be moderately effective as protective agents in most studies (1, 3, 16), although in a recent report (22) no protective effect was observed. Besides this indirect and not unequivocal pharmacological evidence for the involvement of reactive oxygen in hepatic ischemia-reperfusion injury, the direct quantification of the intracellular formation of reactive oxygen in isolated livers revealed that only small amounts of reactive oxygen were formed during the reoxygenation period after hepatic ischemia (17, 21). On the other hand, there is evidence for an intact glutathione peroxidase system during reperfusion (17, 18), and consequently the postischemic liver is still able to tolerate a considerable chemically induced intracellular oxidant stress without further cell damage (17). Experiments with hepatic hypoxia demonstrated that a severe hypoxic injury of the liver is prerequisite for a significant intracellular formation of reactive oxygen by mitochondria and xanthine oxidase during reoxygenation (14, 18); i.e., reactive oxygen in this model is generated as a consequence of hypoxic cell injury rather than being the cause of the damage (11, 14, 18).

The lack of evidence in isolated blood-free perfused livers for an intracellular reactive oxygen formation sufficient to cause direct cell damage through thiol oxidation and lipid peroxidation directed our interest to the in vivo situation, where enhanced plasma levels of glutathione disulfide (GSSG) indicated a significant oxidant stress (12, 16, 21). However, no increase of biliary GSSG was observed in these animals despite a partial recovery of bile flow and anion secretion (12). Furthermore, animals pretreated with the glutathione reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitroso-urea (BCNU) and subject to hepatic ischemia and reperfusion did not show any significant change of plasma, bile, and tissue concentrations of GSSG or enhanced liver cell damage compared with postischemic controls (12). The oxidation of glutathione (GSH) and thus the release of reactive oxygen most likely takes place extracellularly in the hepatic sinusoids (12). Neutrophils [polymorphonuclear leukocytes (PMNs)] and resident macrophages of the liver (Kupffer cells) are both known to generate and release reactive oxygen when stimulated and are potential candidates for an extracellular oxidant stress. We therefore quantified the postischemic accumulation of PMNs in the liver and tried to correlate this with the increase of plasma GSSG levels. Further studies to determine whether manipulation of Kupffer cell activity can affect plasma GSSG levels and hepatic ischemia-reperfusion injury were also carried out. Our results are consistent with the view that Kupffer cells are the main source of reactive oxygen formed during the initial reoxygenation period after hepatic ischemia in vivo and that Kupffer cell activity contributes to hepatic reperfusion injury.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (250–290 g) were purchased from Harlan Sprague Dawley (Houston, TX) and...
Groups of animals were pretreated as follows. 1) Heat-inactivated lyophilized Propionibacterium acnes (28 mg/kg body wt; a gift from Dr. B. D. Barridge, Northwestern State University, Natchitoches, LA) were suspended in saline and injected intravenously 6 days before the experiments (4, 7). 2) Retinol (250,000 IU·kg⁻¹·day⁻¹) was dissolved in corn oil and administered orally for 7 days; the last dose was given 24 h before the experiments (29). 3) Methyl palmitate (2 × 1 g/kg) was suspended in a solution of 0.2% Tween 20 and 5% dextrose and injected intravenously 24 and 20 h before the experiment (24). 4) Gadolinium chloride (5 mg/kg) was dissolved in saline and injected intravenously 24 h before the experiment (10). Because no difference was found between untreated and vehicle-treated rats, all these animals were included in the control groups.

Hepatic ischemia-reperfusion experiments. After anesthetizing the animal with pentobarbital sodium (50 mg/kg ip), the carotid artery was cannulated with PE-50 tubing. The body temperature was monitored and maintained at 37.0 ± 0.3°C by a heating lamp. The blood vessels supplying the median and left lateral hepatic lobes were occluded with an arterial clamp for 45, 60, or 120 min. Reflow was initiated by removal of the clamp. Blood samples of 500 μl were collected before ischemia, at the end of the ischemic period, and three times during reperfusion. The volume of the drawn blood was replaced by saline. An aliquot (200 μl) of blood was mixed immediately with 10 mM N-ethylmaleimide (NEM) in 100 mM potassium phosphate buffer (pH 6.5) for determination of oxidized glutathione (i.e., GSSG). The remaining blood was centrifuged for 1 min, an aliquot of the plasma was pipetted into sulfosalicylic acid (SSA) to avoid spontaneous oxidation of GSH. Plasma concentrations of GSSG did not change significantly during ischemia, during ischemia, and during reperfusion.

Oxidation of GSH in vivo. After anesthetizing the animals with pentobarbital, a carotid artery was cannulated with PE-50 tubing. A midline incision was made and the arteries and veins of both kidneys were ligated with 3-0 silk. The animals received an intravenous injection (penile vein) of GSH in saline (36 μmol/kg). Blood samples of 400 μl were collected before GSH administration and at various time points (2–30 min) afterwards. The blood was processed as described above for the determination of plasma GSII and GSSG. A second group of animals was subjected to hepatic ischemia for 60 min followed by 30 min of reperfusion. At that time the blood vessels of both kidneys were ligated and the animals received the bolus injection of GSH as described above.

Methods. Total soluble glutathione (GSH and GSSG) was measured in bile, plasma, and acidic homogenate from freeze-clamped livers as described in detail (15). For determination of GSSG, a volume of the acidified bile, blood, or liver homogenate was mixed immediately after collection or preparation with 10 mM NEM in 100 mM potassium phosphate buffer (pH 6.5). To separate GSSG from NEM and NEM-GSH adducts, an aliquot of the solution was passed through a Sep-Pak C₁₈ cartridge (Waters, Framingham, MA) followed by 1 ml of buffer. GSSG was determined in the combined eluates as described (15). ALT activity in plasma was determined with Sigma test kit DG159-UV and was expressed in international units per liter. Formalin-fixed portions of the liver were paraffin embedded and 5-μm thick sections were cut. PMNs were stained employing the naphthol AS-D chloroacetate esterase technique (13). Naphthol AS-D chloroacetate was used as a substrate and new fuchsin was used as a coupler. PMNs were identified by positive staining and morphology and were counted in 50 high-power fields using a Nikon Labophot microscope. All chemicals and drugs were purchased from Sigma (St. Louis, MO) or Fisher (Houston, TX).

Statistics. All data are expressed as means ± SE. Comparisons of data sets were performed with paired or unpaired Student’s t test, analysis of variance, and Dunnett’s test for multiple comparisons to control means.

RESULTS

We examined the effects of the total interruption of the blood flow to the median and the left lateral hepatic lobes (no flow ischemia) for various time periods with subsequent reperfusion for 1 h. The fraction of the liver for which the vasculature was occluded represents 70.7 ± 0.7% (n = 5) of the total liver mass. As shown in Fig. 1, a significant increase of plasma aminotransferase activities was seen only during reflow; the severity of the damage correlated with the length of the ischemic period. Plasma concentrations of GSSG did not change signifi-

![FIG. 1. Plasma alanine aminotransferase (ALT) activities during hepatic ischemia and reperfusion. Vessels to the median and left lateral lobes were occluded for 45, 60, or 120 min, respectively. Reperfusion was initiated by removal of the vascular clamp. Blood samples were taken before ischemia (Pre-I), at the end of the ischemic period (I), and at 3 time points during reperfusion. Data are expressed as means ± SE; n = 4–8 animals. If no SE is shown, SE is smaller than symbol. *P < 0.05 (ischemic groups vs. sham-operated controls).](http://ajpgi.physiology.org/)

Downloaded from http://ajpgi.physiology.org/ by 10.220.22.246 on June 23, 2017
KUPFFER CELL-INDUCED REACTIVE OXYGEN FORMATION

G357

significantly during ischemia but increased dramatically during reflow (Fig. 2). Plasma GSH levels were significantly reduced during ischemia but increased also during reperfusion. After 2 h of ischemia, the highest concentrations were measured for both compounds between 15 and 30 min of reflow. Shorter periods of hepatic ischemia caused a consistent increase of plasma GSSG during the total 1-h reflow period (Fig. 2). Significantly higher plasma GSSG concentrations were detected as early as 5 min after initiating reflow (data not shown).

Although both GSSG and GSH concentrations were elevated during reperfusion, GSH values increased by 3- to 6-fold while GSSG concentrations were elevated by 9- to 27-fold compared with the preischemic values. Thus the percentage of GSSG of the total plasma glutathione increased from 20 to 25% before and during ischemia to values between 40 and 66% during reperfusion. As has been extensively discussed, there is strong evidence that the vast majority of the plasma GSSG measured under these conditions was oxidized extracellularly (12). To address the question whether the spontaneous oxidation in blood could be responsible for the observed effects, high concentrations of GSH were injected intravenously into nephrectomized rats. Figure 3 shows the time dependency of the GSSG formation in blood in vivo. The oxidation state of plasma glutathione dropped initially after injection of a GSH solution, but recovered to an equilibrium of 24–27% within 10–15 min. In contrast, injection of GSH into animals during the reperfusion period after hepatic ischemia resulted in an accelerated oxidation of the vascular GSH with ~50% higher plasma GSSG levels and an oxidation state of 40–42% (Fig. 3). These data suggest that the high postischemic plasma levels of GSSG are not due to the spontaneous oxidation of GSH in plasma but reflect an extracellular oxidant stress.

Because phagocytizing PMNs produce reactive oxygen species and have been shown to accumulate in postischemic tissue in the heart (19) and intestine (9), the extent of PMN infiltration was determined in this model of hepatic ischemia and reperfusion. As shown in Table 1, reperfusion after hepatic no-flow ischemia for various time periods was associated with a significant recruitment of PMNs into the liver. This accumulation occurred not only in the postischemic but also in the nonischemic tissue. Although the number of PMNs in the nonischemic liver lobes correlated with the length of the ischemic period, the maximal number of PMNs was found in the postischemic lobes during reperfusion after

![Figure 2](https://api.png素材.com/figure2.png)

**Fig. 2.** Plasma concentrations of GSH (A) and GSSG (B) during hepatic ischemia and reperfusion (all values are given as GSH equivalents). Vessels to the median and left lateral lobes were occluded for 45, 60, or 120 min, respectively. Reperfusion was initiated by removal of the vascular clamp. Blood samples were taken before ischemia (Pre-I), at the end of the ischemic period (I), and at 3 time points during reperfusion. Data are expressed as means ± SE; n = 4–8 animals. If no SE is shown, SE is smaller than symbol. *P < 0.05 (ischemic groups vs. sham operated controls).

![Figure 3](https://api.png素材.com/figure3.png)

**Fig. 3.** Plasma concentrations of total glutathione (GSSG + GSH) (A) and GSSG (B) after iv injection of 36.2 ± 0.8 μmol/kg GSH into nephrectomized animals. All values are given as GSH equivalents. An untreated control group is compared with a group of rats subjected to 60 min of hepatic ischemia and 30 min of reflow before GSH injection. Data are expressed as means ± SE; n = 3 animals. *P < 0.05.
PMNs plugging capillaries and/or tissue swelling due to accumulated metabolites. Hepatic wet-to-dry weight ratios (controls, 3.54 ± 0.09) increased significantly only in the postischemic lobes and postischemic liver lobes content in lung during reperfusion after hepatic ischemia.

**Table 1. Number of neutrophils in nonischemic and postischemic liver lobes**

<table>
<thead>
<tr>
<th>n</th>
<th>PMNs per 50 HPF</th>
<th>I</th>
<th>NI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>6</td>
<td>14 ± 3</td>
<td></td>
</tr>
<tr>
<td>120 min I</td>
<td>4</td>
<td>4 ± 2*</td>
<td></td>
</tr>
<tr>
<td>120 min I/60 min R</td>
<td>8</td>
<td>56 ± 12†</td>
<td></td>
</tr>
<tr>
<td>60 min I/60 min R</td>
<td>6</td>
<td>101 ± 19†</td>
<td></td>
</tr>
<tr>
<td>40 min I/60 min R</td>
<td>4</td>
<td>61 ± 13†</td>
<td></td>
</tr>
</tbody>
</table>

Number of PMNs counted in 50 high-power fields (HPF) are given as means ± SE; n, no. of animals. Left lateral and median lobes of the liver were ischemic for 45, 60, or 120 min, respectively. One group of animals was killed after 120 min of ischemia. In 3 groups of animals, blood flow was restored for 60 min (I/60 min R). Controls were killed 120 min after cannulation of the carotid artery. Nonischemic lobes (NI) are compared with postischemic lobes (I). † P < 0.05 (I vs. NI). * P < 0.05 (controls vs. I/R).

**Table 2. GSH and GSSG content of nonischemic and postischemic liver lobes**

<table>
<thead>
<tr>
<th>Glutathione</th>
<th>GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>I</td>
</tr>
<tr>
<td>45 min I/60 min R</td>
<td>29 ± 2.7</td>
</tr>
<tr>
<td>60 min I/60 min R</td>
<td>27.1 ± 1.6*</td>
</tr>
<tr>
<td>120 min I/60 min R</td>
<td>12.7 ± 1.3*</td>
</tr>
</tbody>
</table>

Total glutathione (GSH and GSSG) and GSSG values are given as mmol GSH eq/mg protein; n = 6–8 animals. Left lateral and median lobes of the liver were ischemic for 45, 60, or 120 min, respectively, followed by a reperfusion period of 60 min. Tissue glutathione content of the nonischemic lobes (NI) are compared with the postischemic lobes (I). Control animals were killed 120 min after cannulation of the carotid artery. * P < 0.05 (I vs. NI).

60 min of ischemia. A major problem of postischemic tissue is the no-reflow phenomenon, which may be caused by PMNs plugging capillaries and/or tissue swelling due to accumulated metabolites. Hepatic wet-to-dry weight ratios (controls, 3.54 ± 0.09) increased significantly only in the postischemic lobes and only after ischemic periods of 60 min (4.38 ± 0.09) and 120 min (4.98 ± 0.11), not after 45 min (3.87 ± 0.11). To determine whether PMNs, regardless of their site of accumulation in the liver, produce reactive oxygen species, tissue GSSG levels were measured at the end of the reperfusion period in both ischemic and nonischemic lobes. As shown in Table 2, a moderate increase of the tissue GSSG content was only observed in the postischemic lobes. The total glutathione content in the liver did not change during ischemia (data not shown), but there was a selective loss of glutathione from the postischemic lobes during the reflow period (Table 2). The extent of the decline of tissue glutathione correlated with the length of the ischemic period and the plasma glutathione concentrations (Fig. 2).

The significant accumulation of PMNs in nonischemic liver lobes suggests that an upregulation of adhesion molecules on the PMN surface may take place during reperfusion, thus increasing the tendency of PMNs to adhere to vascular endothelium. To test whether PMNs also accumulate in capillary beds of other organs, PMNs were determined in lung tissue. The data in Table 3 indicate a five- to sixfold increase in the number of PMNs in the lung after hepatic ischemia and reperfusion. As in the nonischemic hepatic lobes, there was no change of the GSH or GSSG content in the lung.

**Table 3. Neutrophil accumulation and tissue GSSG content in lung during reperfusion after hepatic ischemia**

<table>
<thead>
<tr>
<th>PMNs per 50 HPF</th>
<th>Glutathione, μmol/g</th>
<th>GSSG, nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>138 ± 43</td>
<td>1.92 ± 0.10</td>
</tr>
<tr>
<td>60 min 1/60 min R</td>
<td>778 ± 224*</td>
<td>2.18 ± 0.14</td>
</tr>
<tr>
<td>45 min 1/60 min R</td>
<td>644 ± 134*</td>
<td>2.08 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 animals/experimental group. Number of PMNs counted in 50 high-power fields (HPF). Total glutathione (GSH and GSSG) and GSSG are given in GSH eq/g lung wet wt. Left lateral and median lobes of the liver were ischemic for 45 or 60 min, respectively, followed by a reperfusion period of 60 min. Control animals were killed 120 min after cannulation of the carotid artery. At the end of the experiment, a sample of lung tissue was fixed in Formalin. Another sample was freeze-clamped and stored in liquid nitrogen.
In the present study, Kupffer cells were implicated in the induction of reactive oxygen formation during hepatic ischemia and reperfusion. Animals were untreated or pretreated with methyl palmitate (MP, 2 x 1 g/kg iv 24 and 20 h before ischemia). Vessels to the median and left lateral hepatic lobes were occluded for 60 min. Reperfusion was initiated by removal of the vascular clamp. Blood samples were taken before ischemia (t = 0), at the end of the ischemic period (60 min), and at 3 time points during reperfusion. Data are expressed as means ± SE; n = 6 animals. If no SE is shown, SE is smaller than symbol. *p < 0.05 (methyl palmitate vs. controls).

Increase of plasma GSSG levels were obtained with retinol treatment and the lowest increase was measured in gadolinium chloride-treated animals. On the other hand, the highest GSH levels were found in gadolinium chloride-treated rats and the lowest increase was seen in controls. Furthermore, controls and methyl palmitate-treated rats showed a similar enhancement of plasma GSH concentrations, but the relative increase of plasma GSSG levels were greatly attenuated with methyl palmitate (Fig. 6). These data strongly support plasma GSSG as an index of a postischemic oxidant stress and do not suggest that the posts ischemic increase of GSSG levels is due to the increased release of GSH. None of the compounds used to pretreat the animals caused any PMN infiltration in the liver before the ischemia experiment. The number of PMNs in the postischemic liver lobes was identical or attenuated in animals pretreated with P. acnes (65 ± 21), gadolinium chloride (43 ± 16), retinol (17 ± 8; *P < 0.05), or methyl palmitate (13 ± 6; *P < 0.05) compared with controls (Table 1).

Pretreatment with gadolinium chloride (Fig. 7) or methyl palmitate (Fig. 8) was very effective in reducing hepatocellular injury during the reperfusion period; both compounds prevented the increase in plasma ALT activities by 55–72%. Retinol and P. acnes pretreatment did not significantly enhance liver injury during the early reperfusion period (Fig. 7). Histological evaluation of livers subject to 45 min ischemia and 60 min of reperfusion revealed no signs of cell necrosis in any group at this early time point. The only detectable histological change was a moderate centrilobular congestion in the retinol- and P. acnes-treated animals; control animals did not show any histological changes.

**DISCUSSION**

The objective of the present study was to investigate the involvement of PMNs and Kupffer cells in the formation of reactive oxygen species in ischemia-reflow damage in vivo. We used a model of no-flow ischemia to the two main lobes, i.e., the left lateral and the median lobes, representing ~70% of the total liver mass. Advantages of the model were that the splanchnic circulation could be maintained without the need to establish a portocaval shunt and ischemic vs. nonischemic liver lobes could be compared in the same animal. This model has been also used by others in various studies (5, 21) of ischemia-reflow injury in vivo.

**Postischemic accumulation of PMNs in the liver.** Initially myeloperoxidase was used as an index of PMN accumulation in the liver (10) as was reported for the intestine (9). However, the measurement of myeloperoxidase activities in liver tissue proved to be unreliable because of considerable interference by hemoglobin (pseudoperoxidase activity) and inactivation of myeloperoxidase in liver homogenate (25). Thus in this study an esterase was used to stain PMNs in histological sections. Our data indicate a significant accumulation of PMNs not only in the postischemic lobes but also in the nonischemic lobes. This observation is consistent with the view that ischemia-reperfusion promotes upregulation of adhesion molecules on circulating PMNs leading to an increase in their adherence. The good correlation between the length of the ischemic interval and the number of PMNs found in the nonischemic tissue suggests that PMN accumulation in these lobes indeed...
reflects systemic PMN activation. This view is supported by the increased number of PMNs detected in the lung after hepatic ischemia and reperfusion. Furthermore, when isolated and labeled rat PMNs were reinjected into an animal, the majority of these cells accumulated in the liver and lung within 1 h (Jaeschke, unpublished data). The fact that relatively more PMNs accumulated in the postischemic lobes versus the ischemic period indicates that PMNs may not be just passively trapped in the postischemic lobes. However, restriction of the blood flow because of capillary plugging and edema formation in these lobes could further impede PMN infiltration. This could explain the lower PMN counts measured after 2 h of ischemia.

Plasma GSSG levels as index of an extracellular oxidant stress. A major question is whether plasma GSSG reflects generation of reactive oxygen species by PMNs or Kupffer cells. Previous studies (17, 21) with the blood-free perfused liver found only a very small release of GSSG into the perfusate during reperfusion. Studies in vivo demonstrated increased plasma GSSG concentrations during reperfusion but no enhanced biliary GSSG efflux (12, 16, 21). The enzyme glutathione reductase is the rate-limiting step of the glutathione redox cycle and consequently determines the fraction of GSSG that is released vs. recycled. Therefore, pretreatment of rats with the glutathione reductase inhibitor BCNU caused a dramatic increase of GSSG levels in bile, plasma, and liver tissue during a peroxide-induced intracellular oxidant stress (12). On the other hand, BCNU treatment did neither affect GSSG concentrations in plasma or bile nor the tissue GSSG content during reperfusion after hepatic ischemia (12). These data are not compatible with the intracellular formation or even the intracellular detoxification of reactive oxygen during hepatic ischemia and reperfusion. Thus reactive oxygen release and GSSG formation is likely to take place extracellularly.

Could the spontaneous oxidation of GSH in blood be responsible for the high concentrations of GSSG measured during reperfusion? Our experiments clearly demonstrated that the spontaneous oxidation of GSH in vivo was a relatively slow process and that the GSSG values did not exceed 27% of the total glutathione concentrations in blood even when the half-life of GSH was artificially prolonged (Fig. 3). Thus the observed immediate increase of plasma GSSG levels to 40–70% (Fig. 2) or even 95% in phorone-pretreated animals (12) cannot be explained by the spontaneous oxidation of GSH in blood, but reflects an accelerated oxidation of GSH (oxidant stress) during reperfusion after hepatic ischemia. This conclusion is strongly supported by our findings that an intravenously injected solution of GSH is oxidized faster and more significantly in the vasculature of a postischemic animal than in a control animal (Fig. 3). The half-life of plasma glutathione is <2 min (31), a value that was confirmed in our animals after 2 h ischemia and 60 min reperfusion (Jaeschke, unpublished data). Thus the consistent increase of plasma GSSG during the entire reperfusion period of 60 min can only be maintained by a continuous hepatic release of GSH and a continuous increased oxidation rate, indicative of a long-lasting oxidant stress during reperfusion.

Kupffer cells and PMNs as potential sources of reactive oxygen. Kupffer cells and PMNs can both generate reactive oxygen species. As indicated by the hepatic GSSG content, reactive oxygen formation took place only in the postischemic lobes. Furthermore, plasma GSSG concentrations are strictly dependent on the length of the ischemic period but correlate with the number of PMNs in the tissue only for the shorter ischemic intervals. On the other hand, pretreatment with P. acnes or retinol significantly enhanced plasma GSSG concentrations without increasing the number of PMNs in the liver. The clear separation of plasma GSSG concentrations and tissue GSSG content from the number of PMNs counted in the tissue makes it very unlikely that PMNs are a major source of reactive oxygen formation in the
postischemic liver during this early reperfusion period. Experiments with isolated PMNs in vitro showed that the adherence of PMNs to a surface such as endothelial cells or extracellular matrix proteins promoted a massive long-lasting PMN respiratory burst (24). However, reactive oxygen formation by adhered PMNs was only seen after a chemotactic stimulus, e.g., tumor necrosis factor, and only after a lag-phase of 15–90 min (24). In our experiments in vivo we observed increases of GSSG as early as 5 min after initiating reperfusion, i.e., at a time when PMNs only started to accumulate. Thus these data clearly do not support PMNs as a quantitatively relevant source of reactive oxygen at this time.

Kupffer cells are another potential source of reactive oxygen in the hepatic sinusoids (6). Various stimuli are known to activate Kupffer cells in vivo (galactosamine, retinol); i.e., activated Kupffer cells release considerably more reactive oxygen than control Kupffer cells when challenged with phagocytosable material (27, 29). For example, hepatic macrophages isolated from galactosamine-pretreated rats released 5- to 10-fold more reactive oxygen during stimulation with latex particles or phorbol 12-myristate 13-acetate (27). Retinol potentiated CCl4-induced lipid peroxidation and hepatic injury in rats, an effect that was inhibitable by superoxide dismutase and catalase (29). It was concluded that reactive oxygen released from Kupffer cells was responsible for the aggravation of CCl4 toxicity in vivo. Heat-inactivated bacteria or endotoxin recruit mononuclear cells into the liver (7, 26), increasing the number of macrophages in the liver severalfold. Pilaro and Laskin (26) demonstrated that these phagocytes were activated and release more reactive oxygen when stimulated with 12-0-tetradecanoylphorbol 13-acetate. In our experiments in vivo, retinol and P. acnes pretreatment (Fig. 4) as well as galactosamine pretreatment (unpublished data) significantly enhanced plasma GSSG concentrations during reperfusion, indicating the enhanced release of reactive oxygen. On the other hand, when Kupffer cells are inactivated with two different compounds, i.e., methyl palmitate (2, 29) or gadolinium chloride (10), the postischemic increase of plasma GSSG levels was significantly suppressed (Figs. 4 and 5), a finding that is consistent with a reduced formation of reactive oxygen species during reperfusion. These experiments provide strong evidence that Kupffer cells are the dominant source of reactive oxygen formation in the hepatic sinusoids. At present it is unknown which factor stimulates Kupffer cells to generate reactive oxygen during the reperfusion period. Further studies are necessary to address these problems.

A recent publication (32) demonstrated the release of xanthine oxidase from the liver during the reperfusion period and hypothesized that plasma xanthine oxidase activity could be a relevant source of extracellular reactive oxygen formation. The data in the present paper and in a preceding communication (12) provide evidence for an extracellular oxidant stress. Although the present data suggest Kupffer cells as the main source of reactive oxygen formation, we cannot exclude a certain contribution of other sources such as plasma xanthine oxidase to the postischemic oxidant stress.

Kupffer cell-induced reperfusion injury. Does the Kupffer cell-induced oxidant stress contribute to the hepatic ischemia-reperfusion injury? The protective effect of methyl palmitate and gadolinium chloride seem to support this hypothesis. On the other hand, neither retinol nor P. acnes pretreatment enhanced parenchymal cell injury at this initial reperfusion period despite significantly higher GSSG values in plasma. A possible explanation for these results could be the enhanced detoxification of reactive oxygen as indicated by the higher plasma GSSG values. As was shown previously, only a 90% depletion of the hepatic glutathione content with the subsequent reduction of the hepatic glutathione release significantly aggravated the postischemic injury (12). Therefore, the increased formation of reactive oxygen in the presence of an intact glutathione system must not necessarily enhance the injury. Because Kupffer cells release not only reactive oxygen but also proteolytic enzymes (6), Kupffer cell activity can cause hepatocellular injury through several mechanisms. This hypothesis is supported by the observation that activation of Kupffer cells with methyl palmitate or gadolinium chloride was equally effective in inhibiting phagocytosis (Jaeschke, unpublished data) and postischemic injury (Figs. 7 and 8), but methyl palmitate was more effective in preventing the postischemic oxidant stress (Figs. 5 and 6). Nevertheless, the beneficial effects of extracellular superoxide dismutase and catalase (1, 3) and vitamin E (20), as well as the damaging effect of hepatic glutathione depletion (12), strongly support a contribution of reactive oxygen in the pathogenesis of ischemia-reperfusion injury in the liver. No evidence was found for gross lipid peroxidation in the postischemic liver (23, 28), but a recent preliminary report (30) indicated evidence for lipid peroxidation selectively in the nonparenchymal fraction of the liver. These findings also support our data of a vascular oxidant stress.

In summary, the present study demonstrated a significant vascular oxidant stress during the initial reperfusion period after hepatic no-flow ischemia. Although a considerable number of PMNs accumulated in liver and lung during reperfusion, no correlation was observed between plasma and tissue GSSG concentrations as an index of oxidant stress and the number of PMNs in various tissues. In contrast, plasma GSSG and tissue injury could be manipulated with the activation or suppression of Kupffer cell activity. It is concluded that Kupffer cells are the predominant source of reactive oxygen formation during the initial reperfusion period and that Kupffer cell activity (including reactive oxygen formation) contributes to reperfusion injury in the liver in vivo at that time. Recent investigations of longer reperfusion periods revealed a second, PMN-dependent injury phase in the liver between 6 and 24 h of reperfusion (13). Thus an important aspect of the early reperfusion period with the initial injury and oxidant stress is the activation of PMNs and their accumulation in the postischemic liver tissue, a prerequisite for the later, PMN-dependent injury phase.

The authors thank Brad Black and Michael Fisher for skillful technical assistance.
This work was supported by National Institute of General Medical Sciences Grant GM-42957.

Address for reprint requests: H. Jaeschke, Dept. of Medicine, Baylor College of Medicine, One Baylor Plaza, Room 826E, Houston, TX 77030.

Received 29 May 1990; accepted in final form 16 October 1990.

REFERENCES


