Alterations in Glutathione Peroxidase Activity following Reperfusion Injury to Rat Liver

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Prevention of cellular damage after warm ischemia is of major importance in liver transplantation. In this study, we determined the extent to which lipid peroxides contribute to the pathogenesis of hepatic cell damage induced by transient warm ischemia with subsequent reperfusion. In addition, the function and immunohistochemical features of glutathione peroxidase, a potent physiological lipid peroxide scavenger of the liver, was assessed.

Reperfusion following 15 or 30 minutes of warm ischemia resulted in a significant elevation in serum and liver lipid peroxidase (LPO) levels. In addition, necrosis of the hepatic perportal area accompanied with remarkable rises in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were observed. In contrast, 30 min of ischemia without reperfusion caused minimal hepatocellular damage. The adverse changes after ischemia/reperfusion were minimized by pretreatment with superoxide dismutase (SOD). These results indicate that increased lipid peroxidation by production of radicals after reperfusion caused the liver cell damage.

After ischemia/reperfusion, liver glutathione peroxidase (GSH-PO) activity was significantly decreased and its location altered in the damaged liver. These findings suggest that GSH-PO contributes significantly to the protection against hepatic reperfusion injuries.

(Key words; liver, reperfusion injury, glutathione peroxidase, lipid peroxide, free radical)

INTRODUCTION
The results of liver transplantation have markedly improved during the last few years owing to standardized surgical techniques and more successful immunosuppressive regimens including cyclosporine(4,13). The current problem in liver transplantation is the shortage of acceptable donors. So, the need to prolong viable ischemic time and possibly to increase the potential donor population is urgent. Liver transplants, even from living donors, have recently been performed in Japan. Therefore, close attention should be focused on the mechanism of injury during hepatic ischemia and methods to improve liver preservation.

The concept that significant tissue damage, caused by oxygen-derived free radicals, may actually occur during the reperfusion phase rather than during the period of ischemia has been advanced (3). Experimental evidence suggests that in several models of ischemia/reper-

fusion injury, the enzyme xanthine oxidase is the source of damaging amounts of oxygen radicals (7). Oxygen radicals occurring in cell membranes are known to initiate lipid peroxidation reactions, which damage the phospholipid layer of the membranes through the peroxidation of structurally important polyunsaturated fatty acids (14). The purposes of this study were to determine the extent to which lipid peroxides are a contributing factor in the pathogenesis of hepatic cell damage from transient warm ischemia with subsequent reperfusion, and to assess the function and immunohistochemical features of glutathione peroxidase, a potent physiological lipid peroxide scavenger of the affected liver.

MATERIALS AND METHODS
Male Wistar rats, 8 weeks old and weighing 250-300 gm, were used. A warm ischemia model was developed as follows: after 16 hours of fasting, laparotomy was carried out.
under general anesthesia with intraperitoneal pentobarbital sodium injection (50mg/ml; 40mg/kg body weight). After systemic heparinization (400IU/kg body weight), liver ischemia was induced in the animals by cross-clamping of the portal vein, hepatic artery, and bile duct at the liver hilum for 15 or 30 minutes, followed by either zero or 60 minutes of reperfusion (declamping). Blood samples were obtained by heart puncture to determine serum glutathione peroxidase (GSH-PO), lipid peroxides (LPO), serum aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels. Livers were flushed with 30 ml of warm (37°C) 0.01M phosphate buffer solution (PBS) (PH 7.2) for 3 minutes (10ml/min.) via an aortic cannula using a peristaltic pump. Approximately 0.5gm of liver tissue, obtained from the left lobe of the liver, was frozen at −20°C for later assay of liver GSH-PO activity and liver LPO. Frozen sections of the liver tissue were made after 5 minutes of irrigation with periodate lysine paraformaldehyde (PLP) solution via the aortic cannula, to facilitate quick immobilization of GSH-PO which is a very soluble cytosolic protein. As described in Table-1, the animals were divided into 6 groups. Animals in the “S” group (n=6) served as controls and received only a simple laparotomy. Livers of group “A” rats (n=6) were subjected to 15 minutes of warm ischemia without reperfusion, and livers from group “B” rats (n=6) were subjected to 15 min. of warm ischemia with subsequent reflow of blood for 60 min. In group “C” animals (n=8), the warm ischemic time was prolonged to 30 min.; in group “D” (n=14), 60 min. of subsequent reperfusion was added to the 30 min of warm ischemia. Group “E” rats (n=6) were pretreated with copper (Cu), zinc (Zn)-superoxide dismutase (EC.1.15.11., SOD activity 4,490U/mg, Nippon Kayaku Co. Tokyo, Japan. Lot No. 10176) 5 minutes prior to receiving an intravenous injection of 70,000 U/kg body weight of SOD and after 50 min of continuous intravenous infusion of the drug (50,000 U/kg body weight).

The LPO levels in the liver homogenates and sera were assayed by a calorimetric reaction with thiobarbituric acid and were measured as malondialdehyde (MDA)(10). GSH-PO activities in the liver homogenates and sera were determined by Awasthi’s method (2). In addition to hematoxylin and eosin (H&E) staining, enzyme immunohistochemical staining was performed to localize liver GSH-PO (17). The results were presented as means ± standard deviation. Student’s t test was used for statistical analysis, and p values less than 0.05 were considered significant.

RESULTS

Biochemical changes in lipid peroxides and glutathione peroxidase in serum and hepatic tissues

Figure.1 shows the effect of ischemia-reperfusion on serum LPO levels. Reperfusion following 15 and 30 min of warm ischemia resulted in a significant elevation of serum LPO levels (group A to B, and C to D. p<0.02). Pretreatment with SOD seemed to attenuate the rise produced by reperfusion, although not significantly. Figure.2 shows the LPO levels of liver homogenates in the 6 groups of animals. In the warm ischemic preparations of groups A and C, the liver LPO was the same as that of the controls (group S). The LPO of the liver homogenates increased significantly with reperfusion, as observed in groups C and D (p<0.01). A rise in liver LPO after reperfusion was not observed in group E, where the animals were pretreated with SOD. Serum GSH-PO activities increased significantly after either ischemia or reperfusion (p<0.01), as shown in Fig.3. The activity in group D was significantly

<table>
<thead>
<tr>
<th>Table 1 Experimental groups</th>
<th>Number of animals</th>
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<tr>
<td>Groups</td>
<td></td>
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<tr>
<td>S: controls : laparotomy only</td>
<td>6</td>
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<tr>
<td>A: ischemia 15 min</td>
<td>6</td>
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<tr>
<td>B: ischemia 15 min, reperfusion 60 min</td>
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<tr>
<td>C: ischemia 30 min</td>
<td>8</td>
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<td>D: ischemia 30 min, reperfusion 60 min</td>
<td>14</td>
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<tr>
<td>E: pretreatment with SOD ; ischemia 30 min, reperfusion 60 min</td>
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**Fig. 1** Effects of ischemia and subsequent reperfusion on serum lipid peroxide levels measured by the amount of thio-barbituric acid-reactive substance (malon aldehyde).

**Fig. 2** Effects of ischemia and subsequent reperfusion on lipid peroxide levels in liver homogenates.
Fig. 3 Effects of ischemia and subsequent reperfusion on serum GSH-PO activity.

Fig. 4 Effects of ischemia and subsequent reperfusion on GSH-PO activity in liver homogenates.
higher than in group C (p<0.05), indicating that serum GSH-PO activity also increased with reperfusion after prolonged warm ischemia. Pretreatment with SOD did not have a significant effect on serum GSH-PO activity (group D versus group E). Contrary to the serum GSH-PO, liver GSH-PO activity decreased significantly in group A (15min. warm ischemia) vs. group B with subsequent reperfusion (p<0.05), and in group C (30 min. ischemia) vs. group D with reflow of blood (p<0.05). No significant difference in GSH-PO activity was found between groups D and E, indicating that pretreatment with SOD did not prevent the decrease in liver GSH-PO activity after reperfusion. A remarkable rise in serum AST and ALT was observed in groups B and D, indicating hepatic cell damage after reperfusion (shown in Fig.5).

Histopathological changes in rat livers subjected to ischemia and ischemia/reperfusion
Sections of livers, H&E stained, are shown in Figs. 6, 7, 8a, 8b, and 9. No significant change in lobular structure was seen in group A (15 min. of ischemia). Some increase in the number of minute vacuoles, possibly due to fatty metamorphosis (Fig.6, arrows), was recognized mainly in the mid zone to periportal area of the liver. When ischemia was prolonged to 30 min (Group C), fatty metamorphosis increased slightly, but the general histological appearance was largely unchanged from group A rat livers. In group B rats (ischemia plus reperfusion) fatty metamorphosis became far more conspicuous, appearing in almost the entire lobule (Fig.7). In group D (prolonged ischemia and subsequent reperfusion) (Fig.8a,b), many hepatocytes in the vicinity of the portal area underwent necrosis manifested by disappearance of nuclei (Fig.8b, arrows), pyknosis (Fig.8b, arrow heads) and fine granularization of the cytoplasm (Fig. 8b, arrows and arrow heads). Although the “TUNEL” method, electron microscopy, and biochemical procedures such as “ladder formation” to detect “apoptosis” were not performed, those cells with pyknotic nuclei may well be “apoptotic cells”. Hepatocytes near the central vein did not exhibit such severe damage, only moderate fatty metamorphosis (Fig.8a). These necrotic changes were not seen in groups A (Fig. 6) or

![Fig. 5 Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) after ischemia and reperfusion.](image-url)
Fig. 6  Section of a group "A" (Table 1) rat liver (H&E). Fatty droplets scattered in the cytoplasm of some periportal located hepatocytes (mild fatty metamorphosis). \( \times 200. \)

Fig. 7  Section of group "B" rat liver (H&E). Hepatocytes exhibiting larger fat droplets; widely distributed throughout the hepatic lobules (moderate to severe fatty metamorphosis). Some necrotic cells are seen in the periphery of the lobule. \( \times 200. \)
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Fig. 8 Section of group “D” rat liver (H&E)
[a] Necrotic change recognized only in the periportal region. ×200.
[b] Some hepatocytes show typical necrotic changes (karyolysis; arrows); others exhibit highly pyknotic nuclei. These cells might represent “apoptosis” (arrow heads). ×400.
Fig. 9 Section of group “E” rat liver (H&E). Necrotic cells very rare; the few apoptotic cells are scattered. ×200

Fig. 10 Immunohistochemical localization of glutathione peroxidase (GSH-PO) in control rat liver (group “S”). GSH-PO is stained intensely in peripheral zone hepatocytes in the hepatic lobules. Central zone hepatocytes stain poory or not at all. C-central vein, P-portal area, ×100.
Fig. 11 GSH-PO staining of group “D” rat liver. (a) GSH-PO staining generally less intense, but karyolytic cells in the heavily damaged portal area are deeply stained. × 200
(b) Necrotic cells (arrows) are intensely stained. The increased intensity is finely granular, rather than the usual homogeneous staining seen in the controls. × 400
C although they were consistently seen in group B (Fig. 7) and to a lesser extent in group D rats (Fig. 8a,b). In the liver of group E animals, pretreated with SOD, such necrotic changes were rarely present. Instead, moderate fatty metamorphosis was commonly seen (Fig.9).

Changes in immunohistochemical localization of GSH-PO in livers undergoing ischemia and ischemia/reperfusion

Figure.10 shows the localization of GSH-PO in the liver of control rats (group S). The zonal distribution of GSH-PO staining is obvious. The peripheries of hepatic lobules are more intensely stained for GSH-PO, in clear contrast to the poor staining in the central zones. In group B livers, immunostaining of GSH-PO was slightly decreased, especially in the cells in the periphery of the lobule, plus a concomitant increase in fatty changes. GSH-PO weakly positive cells appeared around the central vein occasionally. An increase in staining of GSH-PO in the periphery of the lobules of group D livers was seen (Fig. 11a, b). This increased staining coincided with the necrotic cells appearing in the peripheral zones (Fig. 11b, arrows). When serial sections, taken from the same tissue block used for Fig.11a and 11b, were reacted with normal rabbit serum and subsequently with anti-rabbit IgG Goat antibody (the second antibody in the immunoperoxidase method), only those necrotic cells were positively stained. This findings may indicate that the positive staining could be due to artifactual deposits of rabbit IgG in the cytoplasm of the necrotic cells. Figure. 12 shows the localization of GSH-PO in the liver of group E rats (SOD pretreatment). Necrotic changes in the periportal regions, seen in group D livers, was not evident. Although the cells in the periportal regions were definitely stained for GSH-PO, the intensity of the staining was somewhat weaker than that seen in normal rat liver hepatocytes.

DISCUSSION

Since the cells of the rat undergo aerobic energy metabolism, a cessation in blood flow and its concomitant decrease in oxygen supply would cause cell damage and even death. This is more prominent in organs with an active metabolism. The liver is especially susceptible to low oxygen levels, and it is known to develop irreversible cell damage after relatively short periods of ischemia (11,12). In our preliminary experiments, ischemic periods of only 5 or 10 minutes failed to result in any signifi-
cant histological and biochemical changes. When the ischemic time was prolonged to 15 minutes or longer, distinct changes occurred. Incidentally, the period of 15 minutes coincides with the period of cessation of blood flow during Pringle’s maneuver in hepatectomy.

Biochemical analysis of LPO and GSH-PO in serum and liver
Ischemia alone of 15 or 30 min did not induce significant changes in LPO and GSH-PO either in serum or liver, whereas ischemia followed by 60 min of reperfusion elicited distinct changes. The marked increase of serum LPO and GSH-PO due to ischemia/reperfusion may be related to severe damage to hepatocytes which allowed the release of intracytoplasmic substances into the blood stream. This view is supported by the fact that the serum AST and ALT were also markedly increased by the ischemia/reperfusion procedure. The LPO level in livers exposed to ischemia/reperfusion exhibited a significant rise, but GSH-PO activity in the liver was greatly decreased. The decrease in GSH-PO, a known potent LPO scavenger, may account for the increase in LPO. The significance of the changes in LPO and GSH-PO becomes more apparent after taking into account changes in morphology, fatty metamorphosis, and GSH-PO localization as described below.

Histological and immunohistochemical observations on GSH-PO
Morphologically, after ischemia of 15 minutes, only a mild fatty metamorphosis was observed. Following 60 min of reperfusion, however, more dramatic changes in the peripheral zones of the lobules occurred. The more pronounced fatty metamorphosis induced in the peripheral zone was presumably due to the fact that GSH-PO, which is an effective lipid peroxide scavenger, is concentrated in the peripheral zones (17) and greatly decreased after ischemia/reperfusion. Since the oxygen tension in the perportal zone is higher than in the central zone, oxygen radicals and lipid peroxidation tend to develop more readily in the former. Consequently, factors acting in the protective mechanism against lipid peroxidation, especially GSH-PO, would be distributed in the perportal zones in higher concentration, because the enhancement of LPO can stimulate GSH-PO production (8,16). As McCord (7) has suggested, we assume that ischemia caused an increase in perportal O2 and the resultant increase in lipid peroxidation in the same region led to the development of fatty metamorphosis (16). After ischemia without reperfusion, the above changes are still reversible because of the preserved activity of GSH-PO, which will protect against lipid peroxidation. When an oxygen supply is reestablished by reperfusion, the amount of O2 is apparently increased and extreme enhancement of lipid peroxidation probably occurs, provoking irreversible hepatocyte injury (predominantly necrosis). A decrease in the amount of GSH-PO in “ischemia/reperfusion” rat livers may have accelerated this cell injury. In fact, in our experiments, pretreatment with SOD ameliorated these changes and thereby supports our explanation. It has also been reported that the survival rate of rats after 90 minutes of ischemia/reperfusion was clearly increased by pretreatment with α-tocopherol (6) (Vitamine E), a known naturally occurring antioxidant and radical scavenger. Superoxide dismutase (SOD) is known to prevent lipid peroxidation by deoxidization of superoxide radicals. In cardiac ischemia/reperfusion experiments, the administration of SOD alone (1) or with catalase (5), greatly reduced necrotic damage to the cardiac muscles. Some investigators, however, have reported that SOD is unable to prevent cell injury (9,15).

The decrease in GSH-PO in the livers of group D can be explained by a decrease in GSH-PO synthesis, related to an overwhelming occurrence of radicals (such as O2• and ·OH) and subsequent lipid peroxidation. These changes (decreased GSH-PO and increased fatty metamorphosis) in the perportal zone are similar to those induced by starvation and administration of protein synthesis inhibitors such as ethionine which are known to markedly inhibit GSH-PO synthesis (16). In a normal state, the central zones of the hepatic lobules, which show much lower oxygen tension and are less susceptible to ischemia than the periphery, exhibit less intense GSH-PO immunostaining. However, “ischemia/reperfusion” resulted in a moderate increase in immunostainability of hepatocytes in the cen-
The increased oxygenation induced by "reperfusion" may have brought about enhancement of oxygen radicals and subsequent lipid peroxidation to stimulate GSH-PO synthesis. As a matter of fact, the increased lipid peroxidation induced in rat hepatocytes and peritoneal macrophages was proved to enhance GSH-PO production (8,16).

The ischemic stimulation given to the rat liver failed to cause any serious hepatic damage, but the reperfusion which followed the ischemia induced severe damage to hepatocytes, especially to those located in the peripheral zones of the hepatic lobules. Since damage was effectively prevented by SOD pretreatment, it is relevant to consider that the damage may have been due to the increased occurrence of oxygen radicals and subsequent lipid peroxidation.

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