Oxidative Stress in the Ischemic and Non-Ischemic Parts of the Rat Liver after Two-Thirds Ischemia/Reperfusion

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Rat liver was subjected to two-thirds warm ischemia for 45 min and reperfusion (I/R) to evaluate the resulting oxidative stress. The plasma alanine aminotransferase and aspartate aminotransferase activities were significantly higher than those in the sham group 1.5–24 h after I/R, showing extensive liver cell death. The level of oxidative stress was compared between the ischemic and non-ischemic regions based on the change in antioxidative vitamins C and E. The vitamin C level was significantly decreased during I/R in both the ischemic and non-ischemic regions 0, 1.5, 3, 6, 12, and 24 h after the start of reperfusion, showing enhanced oxidative stress even in the non-ischemic lobules. This decrease of vitamin C in the ischemic region was significantly higher than that in the non-ischemic lobules, while the vitamin E content was decreased only in the ischemic lobes, demonstrating higher oxidative stress in the ischemic region than that in the non-ischemic region. Early transient activation of cytoprotective extracellular signal-related kinase (ERK) was apparent in both the ischemic and non-ischemic lobules, reflecting oxidative stress in both regions. Early transient activation of c-Jun NH2-terminal kinase (JNK) was only apparent in the ischemic region, corresponding to extensive oxidative stress and liver cell death. These results demonstrate that significant oxidative stress was induced, but that JNK leading to cell death was not activated in the non-ischemic part of the liver.

Key words: ascorbic acid; ischemia; mitogen-activated protein kinase (MAPK); oxidative stress; reperfusion

Tissue damage caused by ischemia-reperfusion (I/R) has received strong attention in a wide field of bioscience and medicine. Hepatic I/R injury is an important process leading to systemic and hepatic damage after circulatory shock, hepatic surgery, or transplantation, and has been the subject of extensive studies.1–3 It is well established that I/R injury is associated with oxidative stress due to excessive inflammatory responses.1–3 Many biochemical events such as lipid peroxidation by reactive oxygen species, modification of proteins by aldehydes (products of lipid peroxidation), and exposure to reactive nitrogen species have been assumed to lead to the death of liver cells.1–3

Although strenuous efforts have been made to finding a biomarker for oxidative stress, no reliable biomarker that quantitatively reflects tissue oxidative stress has yet been established. We have demonstrated in a series of studies4–9 that the liver concentration of vitamin C and the activation of MAPKs (mitogen-activated protein kinases) were both very sensitive indices of oxidative stress by using experimental hepatitis caused by chemicals as the model system. We apply these results in the present study to evaluate the oxidative stress due to I/R injury, especially focusing on the oxidative stress in the non-ischemic lobules as a direct effect of liver I/R.

Materials and Methods

Animal manipulation. This study was approved by the Animal Care Committee of Nara Women’s University. Eight-week-old male rats (SLC Wistar strain) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). The animals were housed in a room at 24 ± 2 °C with a 12h/12h light-dark cycle and were fed ad libitum on commercial laboratory feed (MF, Oriental Yeast Co., Osaka, Japan) and water.

The rats were anesthetized by ether inhalation. We induced two-thirds hepatic ischemia by clamping with a microvascular clip the portal vein, hepatic artery, and bile duct supplying the median and left lobes of the liver, while circulation was maintained through the right and caudate lobes. The clamp was removed to restore blood flow (reperfusion) at the end of the warm ischemic period (45 min). Liver and plasma samples were taken just after the reperfusion (removal of the clamp) and these samples were designated as the 0h samples. Just after the start of the reperfusion the abdomen was closed in two layers with silk (3.0) and then rats were kept in individual cages for the designated periods. Other anesthetized rats underwent a laparotomy for 45 min without a period of hepatic ischemia as a sham operation.

The rats were anesthetized with diethyl ether at the indicated time points and then killed by collecting the blood from the inferior vena cava by using a syringe containing sodium heparin as an anticoagulant. The liver was removed after perfusing with ice-cooled saline through the portal vein. The excised tissue was homogenized in 5 volumes of phosphate-buffered saline (10 mM, pH 7.4) while ice cooling. All determinations were made in duplicate experiments with 4–6 animals in each group.

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Abbreviations: ALT, alanine aminotransferase (EC 2.6.1.2); AST, aspartate aminotransferase (EC 2.6.1.1); ERK, extracellular signal-related kinase; I/R, ischemia/reperfusion; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α
Analytical methods. Vitamin C was determined by a specific and sensitive method involving chemical conversion and HPLC. The concentration of α-tocopherol was determined by HPLC. The conditions for HPLC and fluorescence detection (Shimadzu RF-535, Kyoto, Japan) were as reported previously.

Blood was centrifuged at 8,400 rpm for 5 min at 4 °C to separate the plasma. The activities of plasma aspartate aminotransferase (AST: EC 2.6.1.1) and alanine aminotransferase (ALT: EC 2.6.1.2) were determined by using diagnostic kits (GOT and GPT-UV Test Wako, Wako Pure Chemicals Co., Osaka, Japan) and are expressed as Karmen units.

Western blot analysis of JNK, ERK1/2, and p38 MAPK. Liver tissue was removed and frozen at −83 °C until needed for use, the determination being made as previously described. Homogenization was conducted basically as reported. The extraction buffer contained 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM EDTA, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM of sodium orthovanadate, 1 μg/ml of pepstatin A, 2 μg/ml of leupeptin, and 1 mM of phenylmethylsulfonyl fluoride (PMSF). Each sample was homogenized in five volumes of the extraction buffer on ice. All debris and nuclei were removed by centrifugation at 8,400 rpm at 4 °C for 10 min, and the supernatant obtained was used for the Western blot analysis. The protein concentration was determined according to the method of Lowry et al. using BSA as the standard. One hundred micrograms of protein was electrophoresed on 10% SDS–PAGE gel and then transferred to a BioTrace NT membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA).

Phosphorylated p38 MAPK, JNK, and ERK1/2 were detected according to the instruction manual provided by Cell Signaling Technology (Beverly, MA, USA), respectively using a PhosphoPlus p38 MAP Kinase (Thr180/Tyr182), PhosphoPlus SAPK/JNK (Thr183/Tyr185), and a PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) antibody kits purchased from Cell Signaling Technology (Beverly, MA, USA), respectively using a PhosphoPlus antibody kit purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Chemiluminescence was recorded with an AE-6972 cooled CCD camera system (ATTO Co., Tokyo, Japan) and analyzed with ATTO CS Analyzer Ver. 2.0 densitograph software.

Statistical analyses. Each data value is expressed as the mean ± SE. The statistical analysis was carried out with Statcel (Excel 2000). Differences between the group means were considered significant at \( p < 0.05 \) by using Fisher’s protected least significant difference test (PLSD), following the detection of an effect by one-way ANOVA.

Results

Changes in plasma ALT, AST, vitamin C, α-tocopherol (vitamin E) and hepatic vitamins C and E

Two-thirds hepatic ischemia and reperfusion was performed by stopping the blood flow through the left and median lobes (ischemic) for 45 min, while maintaining circulation to the rest of the tissue (non-ischemic) and subsequent perfusion. Values were determined 0, 1.5, 3, 6, 12, and 24 h after starting reperfusion, all data being shown in Table 1.

The plasma ALT and AST values were significantly higher than those in the sham group as early as 1.5 h after I/R. Plasma ALT and AST respectively reached maximal values 6 and 12 h after the start of reperfusion. The plasma vitamin C level 0, 1.5, 3, and 6 h after reperfusion was significantly higher than that in the sham group, while the plasma vitamin E level (only the α-isomer was detected among the tocopherols) was significantly lower 1.5, 3, and 6 h after reperfusion than that of the sham group.

The concentration of vitamin C in the ischemic lobes was significantly lower than that of the sham group at all times and lower than that in the non-ischemic lobes, except 24 h after reperfusion, showing enhanced oxidative stress during ischemia and after reperfusion. The vitamin C level in the non-ischemic region was also significantly lower than that of the sham group 0 and 1.5 h after reperfusion, was restored 3 h after reperfusion, and lower again 6, 12, and 24 h after reperfusion.

The concentration of α-tocopherol in the non-ischemic lobes remained unchanged by I/R, while that in the ischemic region was lower 0, 1.5, 3, 6, and 12 h after reperfusion than that of the sham group, showing stronger oxidative stress in the ischemic lobes than in the non-ischemic region.

Change to MAPKs in the liver

Significant activation of JNK2 (54 kDa) was only observed in the ischemic region 1.5 and 3 h after reperfusion (Figs. 1 and 2).

No significant increase in phosphorylated p38 MAPK was apparent at any time (data not shown). Phosphorylated ERK2 (42 kDa) significantly increased transiently 1.5 h after reperfusion in both the ischemic and non-ischemic regions (Figs. 1 and 2), while phosphorylated ERK1 (44 kDa) was not significantly changed in either region after I/R (data not shown). The protein concentrations of ERK, JNK, and p38 MAPK were not affected by I/R at any time point (Fig. 1 for ERK and JNK).

Discussion

It is very important to accurately evaluate oxidative stress. Antioxidants react with oxidants and therefore are assumed to be decreased by the generated oxidants. Among the physiological antioxidants, glutathione (GSH) and its oxidized state (GSSG) have been used in the study of liver I/R. Although there are some differences in the degree of change of GSH among previous studies, it has been established that total GSH decreased and GSSG increased during I/R.14–20) GSH contributes to the recycling of vitamin C, and a decreased level of GSH will affect the content of vitamin C; this has been shown to be sensitive to decrease by the oxidative stress resulting from hepatitis induced by such drugs as carbon tetrachloride,5 thiocetamide,4 and galactosamine.5 We followed the change in antioxidative vitamins in the present study, namely water-soluble vitamin C and vitamin E, a hydrophobic antioxidant during I/R injury to the liver.

The vitamin C content in the ischemic region was decreased to less than half the level of the sham group and remained at a low level until 24 h after reperfusion. This result demonstrates highly enhanced oxidative stress occurring in the aqueous region of ischemic lobes. The significant decrease of vitamin C in the non-ischemic region demonstrates that oxidative stress was caused by I/R in these lobes neighboring the ischemic liver region.

The vitamin E content in the ischemic lobes was significantly lower than that in the sham group at all times, except at 24 h. This result indicates that oxidative stress was also enhanced in the membrane (hydrophobic) region of the ischemic liver. In contrast, the vitamin E content in the non-ischemic lobes was not affected by I/R, suggesting that oxidative stress did not fully extend to the membrane region of the non-ischemic lobes.
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The levels of vitamins C and E in the ischemic region were significantly lower immediately after reperfusion, thus only resulting from ischemia, although it has generally been assumed that oxidative stress was caused when the oxygen supply and blood flow were restored after reperfusion.

The plasma vitamin C concentration increased 0, 1.5, 3, and 6 h after reperfusion. This may be ascribed to the release of this vitamin from the necrotized liver, which contained a 40-fold higher concentration of vitamin C (about 2,000 versus 50 μM) than that in the plasma. In contrast, the plasma α-tocopherol level was significantly decreased 1.5, 3, and 6 h after reperfusion. A simultaneous decrease of vitamin E in the plasma and liver has also been observed with hepatitis caused by thioacetamide and D-galactosamine. An unknown mechanism to transferring vitamin E from the plasma to tissue during oxidative stress may operate.

The plasma ALT and AST activities were significantly higher than those in the sham group as early as 1.5 h after I/R. The ALT and AST levels increased during the prolonged reperfusion period, respectively reaching maximal values 6 and 12 h after reperfusion. These values were still high 24 h after reperfusion when the oxidative stress in the membrane region was ameliorated, as shown by the vitamin E level, although the vitamin C level in the ischemic and non-ischemic lobes was still at a similar low level.

Damage to tissues remote from the liver such as the brain and kidney is well known in hepatic failure. Oxidative stress in the non-ischemic lobes caused by I/R may be mainly due to inflammatory cytokines, especially tumor necrosis factor-α (TNF-α) which is released by Kupffer cells in I/R liver[12,23] and results in cell death of the hepatocytes involving oxidative stress and JNK activation[23–25].

The activation of MAPK is linked with cell death in the rat liver via oxidative stress[7–9]. The three major subclasses of the MAPK family are JNK, ERK1/2, and p38 MAPK. Phosphorylated JNK in the ischemic lobes was significantly higher 1.5 and 3 h after reperfusion when the oxidative stress was also significantly higher, as was evident by the liver vitamin C level (Table 1), and liver necrosis was active based on the plasma AST and ALT levels (Table 1). The activation of JNK in I/R injury to the rat liver has been well documented[12,26–28].

| Table 1. Change in Plasma ALT, and AST, and Vitamin C, and Vitamin E in the Liver and Plasma |
|-------------------------------------------------|-----------------|-----------------|
| AL (Karmen units)                               | Non-ischemic    | Ischemic        |
| 0 h (n = 4)                                     |                 |                 |
| ALT (Karmen units)                              | 66.0 ± 14.9     | 402.9 ± 113.9   |
| AST (Karmen units)                              | 155.6 ± 34.0    | 667.1 ± 184.4   |
| Vitamin C Liver (nmol/g liver)                  | 2084.8 ± 124.7  | 1610.8 ± 91.1*  |
| Plasma (nmol/ml)                                | 55.5 ± 7.6      | 102.5 ± 14.1    |
| 1.5 h (n = 6)                                   |                 |                 |
| Vitamin E Liver (nmol/g liver)                  | 24.9 ± 2.1      | 27.5 ± 2.4      |
| Plasma (nmol/ml)                                | 10.7 ± 0.5      | 10.5 ± 0.5      |
| 3 h (n = 4)                                     |                 |                 |
| Vitamin E Liver (nmol/g liver)                  | 30.5 ± 3.2      | 13.7 ± 2.1*     |
| Plasma (nmol/ml)                                | 7.2 ± 0.6*      | 7.8 ± 0.4*      |
| 6 h (n = 5)                                     |                 |                 |
| Vitamin E Liver (nmol/g liver)                  | 22.7 ± 2.7      | 16.1 ± 1.8*     |
| Plasma (nmol/ml)                                | 10.5 ± 0.3      | 10.5 ± 0.6*     |
| 12 h (n = 5)                                    |                 |                 |
| Vitamin E Liver (nmol/g liver)                  | 1471.7 ± 230.9* | 917.2 ± 137.0*  |
| Plasma (nmol/ml)                                | 96.6 ± 16.4     | 39.0 ± 1.7      |
| 24 h (n = 4)                                    |                 |                 |
| Vitamin E Liver (nmol/g liver)                  | 22.7 ± 2.7      | 26.7 ± 1.5      |
| Plasma (nmol/ml)                                | 10.5 ± 0.3      | 10.5 ± 0.6      |

Rat liver was subjected to 70% ischemia (45 min) and reperfusion for indicated times. Determinations were made for plasma (ALT, AST, vitamin C, and vitamin E), ischemic (median and left lobes), and non-ischemic (right and caudate lobes) regions of the liver as described in the text. The numbers of rats are shown in parentheses. Value is mean ± SE for 4–5 rats. Signals indicate significant differences at p < 0.05 (* vs. sham and † vs. non-ischemic). (ANOVA Fisher’s protected least significant difference test (PLSD)).
JNK activation has been assumed to cause cell death, while the hepato-protective effect of JNK has also been reported. ERK functions cytoprotectively against the apoptosis that is triggered by oxidative stress, TNF-α, growth factor deprivation, and proapoptotic drugs, in contrast to JNK. In addition, hepatocyte resistance to oxidative stress depended on the protein kinase C and ERK-mediated down regulation of JNK signaling. The activation of ERK in I/R injury to the rat liver has been previously reported.

Phospho-ERK2 was transiently elevated 1.5 h after reperfusion in both the ischemic and non-ischemic regions (Fig. 1), while ERK1 was not activated. ERK is particularly sensitive to oxidative stress and is activated by 100 μM hydrogen peroxide, i.e., as low a level as 100 μM of reactive oxygen species, in cultured rat hepatocytes, so it may be difficult to detect a change of 100 μM in 2,000 μM of vitamin C in the liver, even if all hydrogen peroxide caused an equivalent decrease in vitamin C. The activation of ERK has preceded the decrease in liver ascorbate level with the oxidative stress caused by carbon tetrachloride and thioacetamide. The vitamin C level in the present study was significantly decreased in the non-ischemic region, so the notion of ERK activation in this region is reasonable. This may be a cytoprotective reaction of the non-ischemic lobes to the oxidative stress caused by I/R. In contrast, both ERK and JNK in the ischemic region were activated, and the activation of JNK leading to cell death caused by a larger oxidative stress surpassed the cytoprotective action of ERK.

In conclusion, rat liver was subjected to two-thirds ischemia for 45 min and reperfusion. The decrease of vitamin C in the ischemic region was significantly higher than that in the non-ischemic lobules, and the vitamin E content was only lower in the ischemic lobes, demonstrating a higher oxidative stress in the ischemic region than that in the non-ischemic region. Early transient activation of ERK was observed in both the ischemic and non-ischemic lobules, reflecting oxidative stress in both regions, while early transient activation of JNK was only apparent in the ischemic region, corresponding to extensive oxidative stress and liver cell death. These results demonstrate that a significant oxidative stress was induced in the non-ischemic part of the liver, but JNK leading to cell death was not activated.
Acknowledgments

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