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Rebamipide ameliorates hepatic dysfunction induced by ischemia/reperfusion in rats

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Abstract

The relationship between lipid peroxidation and alterations in hepatic secretory function and microsomal function during hepatic ischemia/reperfusion was studied. Rats pretreated with free radical scavengers were subjected to 60 min of hepatic ischemia and to 1 and 5 h of reperfusion thereafter. Serum aminotransferase level and microsomal lipid peroxidation were markedly increased by ischemia/reperfusion. These increases were significantly attenuated by rebamipide, α-tocopherol or allopurinol. Bile flow and cholate output were markedly decreased by ischemia/reperfusion and free radical scavengers, especially rebamipide, restored their secretion. NADPH-cytochrome P_{450} reductase activity and cytochrome P_{450} content were decreased by ischemia/reperfusion. Rebamipide prevented the decrease of the NADPH-cytochrome P_{450} reductase activity but had little effect on the cytochrome P_{450} content. Aminopyrine *N*-demethylase activity was decreased and aniline *p*-hydroxylase was increased by ischemia/reperfusion, which were prevented by α-tocopherol and allopurinol, but not by rebamipide. Our findings suggest that ischemia/reperfusion diminishes hepatic secretory function and microsomal function by increasing lipid peroxidation, and rebamipide significantly ameliorates these changes through its free radical scavenging activity.

Keywords: Rebamipide; Hepatic secretion; Drug metabolism; Ischemia/reperfusion

1. Introduction

Ischemia/reperfusion injury of the liver is involved in the pathogenesis of shock, and it can occur after liver transplantation and hepatic surgery for trauma or cancer. Although hepatic cellular injury may result directly from ischemia or hypoperfusion of the liver, substantial evidence suggests that a major portion of the tissue injury occurs on reperfusion (Parks and Granger, 1986).

Growing evidence indicates that reactive oxygen species play a major role in producing the microvascular and parenchymal cell damage associated with reperfusion of ischemic tissues (Granger et al., 1986; Drugas et al., 1991). When reperfusion supplies large quantities of oxygen to ischemic tissues, the abundant supply of oxygen constitutes the missing substrate for the reaction, catalyzed by xanthine oxidase, that converts hypoxanthine to xanthine and uric acid. The oxidation of hypoxanthine and xanthine produces cytotoxic oxygen metabolites, superoxide, hydroxyl radical, and hydrogen peroxide.

The attack by free radicals of biological membranes may lead to the oxidative destruction of the polyunsaturated fatty acids of the membrane through lipid peroxidation, which results in a loss of membrane integrity, causing edema and ultimately cytolysis (Mead, 1979).

The liver microsomal membrane constitutes an enormous source of free radicals. Cytochrome P_{450} and NADPH-dependent cytochrome P_{450} reductase are involved in lipid peroxidation (Bast, 1986). However, a direct association of hepatic lipid peroxidation in vivo after ischemia/reperfusion injury and changes in the activities of cytochrome P_{450} isozymes and hepatic secretion has not been established.

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Recently rebamipide ((±)-2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic acid) has been introduced as an antiulcer agent (Yamasaki et al., 1989). It inhibits the production of superoxide by neutrophils, scavenges the hydroxy radical and inhibits lipid peroxidation(Yoshikawa et al., 1993).

The purpose of this study was to determine whether specific abnormalities exist in hepatic secretion and microsomal function associated with ischemia/reperfusion injury in which lipid peroxidation occurs. We investigated this relationship by using rebamipide and compared it to α-tocopherol, a lipophilic free radical scavenger, and allopurinol, a xanthine oxidase inhibitor.

2. Materials and methods

2.1. Hepatic ischemic procedure

Male Sprague-Dawley rats, 200–250 g, were fasted 24 h before the experiment and allowed to drink tap water ad libitum. The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and the abdomen was opened by midline incision. The left branches of the portal vein and hepatic artery were clamped to induce complete ischemia of the median and left hepatic lobes while the right lobes remained perfused to prevent intestinal congestion. At the end of 60 min of ischemia, the clip around the left branch of the portal vein was removed, and the branch to the right lobes was ligated. This resulted in all portal and hepatic arterial flow, except for the very small amount to the caudate lobe, being directed through the previously ischemic lobes. At 1 h and 5 h of reperfusion, PE-50 tubing was inserted into the bile duct for collection of bile, and then blood was taken from the abdominal aorta. The left and the median lobes of the liver were removed and used for the experiment. The left lobes were partially cut and weighed (wet weight) and dried for 48 h at 80°C (dry weight) to calculate the ratio of wet/dry liver weight. Sham-operated rats were prepared in a similar manner except that the clip was not placed in the left and the median lobes.

2.2. Administration of drugs

α-Tocopherol, dissolved in soybean oil, was injected for 3 days (30 mg/kg per day, i.p.). Allopurinol, dissolved in saline, was administered p.o. for 2 days and a third dose 50 mg/kg was given 2 h before the experiment. Rebamipide in 0.5% carboxymethylcellulose (30 mg/kg per day, p.o.) was given twice a day for 1 week. Experimental groups were divided into sham-operated and ischemia/reperfusion groups. In the ischemia/reperfusion groups, rats were pretreated with free radical scavengers such as rebamipide, α-tocopherol, or allopurinol. Sham-operated and ischemia/reperfusion control rats were given soybean oil, saline, or carboxymethylcellulose alone as the vehicle. There was little difference in any parameters between each vehicle treatment, thus the data from all vehicle-treated rats were pooled and simplified to one sham-operated group and one ischemia/reperfusion control group.

2.3. Isolation of hepatic microsomal fraction

The excised liver was sliced and homogenized with a teflon pestle homogenizer with 4 volumes of 0.15 M KCl for 1 g of liver and centrifuged at $9000 \times g$ for 60 min. The supernatant was collected and centrifuged at $105\,000 \times g$ for 60 min. Microsomal precipitates were resuspended with 4 volumes of 0.1 M phosphate buffer, pH 7.4, for 1 g of the liver microsome and stored at −70°C until assayed. All procedures were performed at 2°C.

2.4. Analytical procedures

Serum alanine aminotransferase was determined by standard spectrophotometric procedures using Sigma kit No. 59-UV (Sigma Chemical Co., St. Louis, MO), and bile cholate was determined by the method of Irvin et al. (1944). Bilirubin was spectrophotometrically measured by using a AM301-K kit (Nipponshaji, Tokyo, Japan). Lipid peroxide was assayed by the thiobarbituric acid method of Masugi and Nagamura (1976), and 1,1,3,3-tetraethoxypropane was used as the standard. NADPH-cytochrome P_{450} reductase was determined by using the absorbance difference per 1 min at 550 nm for 3–4 min and 19.1 mM^{-1} cm^{-1} as the molar extinction coefficient (Mazel, 1972). Cytochrome P_{450} content was calculated by using the molar extinction coefficient of 104 mM^{-1} cm^{-1} and the absorbance difference between 450 and 500 nm measured with a differential spectrophotometer (Omura and Sato, 1964). Aminopyrine *N*-demethylase and aniline *p*-hydroxylase activity was determined by measuring the formation of formaldehyde (Schenkman et al., 1967) and *p*-aminophenol (Mieyal and Blumer, 1976), respectively. Protein content was assayed by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

2.5. Statistics

All data were expressed as means ± S.E.M. Overall significance was tested by two-way analysis of variance (ANOVA), and the significance level was set at $P < 0.05$.

3. Results

3.1. Serum aminotransferase activity

The serum level of alanine aminotransferase in sham-operated rats was 55 ± 5 U/l, which was similar to that of normal rats, and increased to 330 ± 32 U/l at 5 h of reperfusion. In the ischemia/reperfusion control group, serum aminotransferase increased to 1425 ± 170 U/l at 1 h and 5269 ± 575 IU/l at 5 h of reperfusion. α-Tocopherol, allopurinol, and rebamipide treatment had little effect on the increase in aminotransferase activity at 1 h of reperfusion; however, the increased level of aminotransferase at 5 h of reperfusion in the ischemia/reperfusion control group was markedly suppressed by α-tocopherol and rebamipide (Fig. 1).

3.2. Wet weight-to-dry weight ratio of liver

The wet weight-to-dry weight ratio of the livers in sham-operated rats was fairly constant (3.33 ± 0.05–3.50 ± 0.03) for 5 h. However, the ratio was significantly increased in the ischemia/reperfusion control group, i.e., 3.78 ± 0.16 after ischemia, 3.86 ± 0.10 at 1 h of reperfusion, and 4.03 ± 0.08 at 5 h of reperfusion. The data indicate that ischemia/reperfusion induces hepatic edema. The hepatic edema induced by ischemia/reperfusion was attenuated by free radical scavengers and among them rebamipide significantly reduced the ratio to 3.41 ± 0.23 at 5 h of reperfusion (Fig. 2).

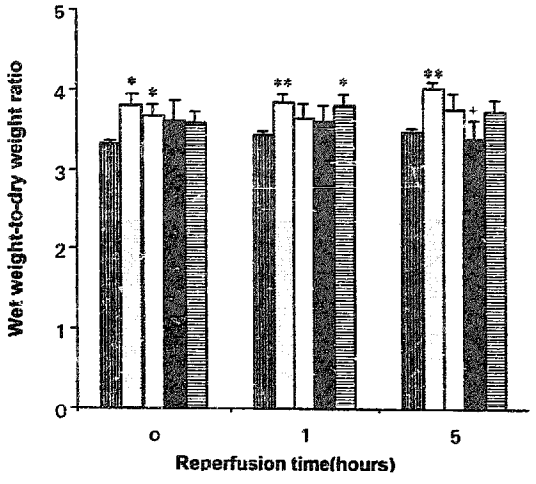

Fig. 2. Effect of free radical scavengers on the wet weight-to-dry weight ratio of liver after ischemia/reperfusion of rat liver. Each bar is expressed as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, difference from sham operation. ‾ $P < 0.05$, difference from ischemia/reperfusion control. For explanation of columns see Fig. 1.

3.3. Lipid peroxidation

The data on the formation of malondialdehyde, the product of lipid peroxidation, are presented in Fig. 3. In sham-operated rats, the level of malondialdehyde in the liver remained constant at approximately 0.5 nmol/mg protein throughout the experiment. However, as with aminotransferase and the wet/dry weight ratio, the level of malondialdehyde markedly increased to 1.80 ± 0.28 after ischemia, and 2.11 ± 0.15 at 1 h, and 2.58 ± 0.31 nmol/mg protein at 5 h of reperfusion in the ischemia/reperfusion control rats, respectively. α-Tocopherol, allopurinol and rebamipide treatment partly prevented the elevations of malondialdehyde at 1 h and 5 h of reperfusion (Fig. 3).

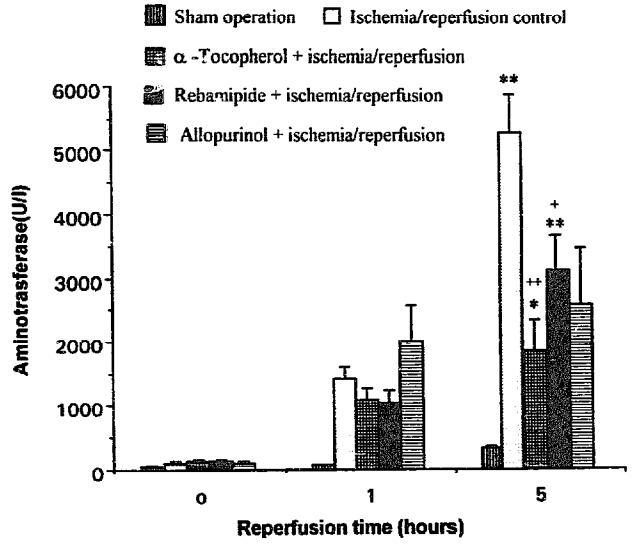

Fig. 1. Effect of free radical scavengers on serum alanine aminotransferase activity after ischemia/reperfusion of rat liver. Each bar is expressed as mean ± S.E.M. ** $P < 0.01$, difference from sham operation. + $P < 0.05$, ++ $P < 0.01$, difference from ischemia/reperfusion control.

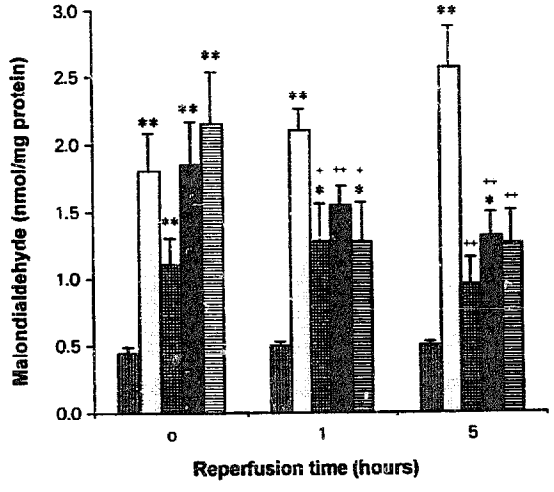

Fig. 3. Effect of free radical scavengers on lipid peroxidation after ischemia/reperfusion of rat liver. Each bar is expressed as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, difference from sham operation. + $P < 0.05$, ++ $P < 0.01$, difference from ischemia/reperfusion control. For explanation of columns see Fig. 1.

3.4. Biliary secretion

The bile flow of sham-operated rats was 0.23 ± 0.03 and 0.20 ± 0.01 ml/h per 100 g body weight at 1 h and 5 h of reperfusion, respectively. The flow was reduced by ischemia/reperfusion to 0.12 ± 0.02 and 0.08 ± 0.03 ml/h per 100 g, at 1 h and 5 h reperfusion, respectively. α-Tocopherol and rebamipide treatment increased bile secretion at 5 h of reperfusion compared to that of the ischemia/reperfusion control group. Total bilirubin secretion in all experimental groups was not significantly different. Cholate output, which is bile acid-dependent secretion, was significantly reduced by ischemia/reperfusion. The decreases were markedly restored by rebamipide treatment in the 1-h and 5-h reperfusion groups, and by α-tocopherol in the 1-h reperfusion group (Table 1).

3.5. Cytochrome P_{450} content and NADPH-cytochrome P_{450} reductase activity

The hepatic microsomal cytochrome P_{450} content in the sham-operated group was 0.76 ± 0.03 and 0.74 ± 0.07 nmol/mg protein at 1 h and 5 h of reperfusion, respectively. Ischemia/reperfusion did not affect the cytochrome P_{450} content after 1 h of reperfusion, but markedly reduced it to 0.33 ± 0.08 nmol/mg protein after 5 h of reperfusion. Pretreatment with α-tocopherol and allopurinol significantly attenuated the decrease in cytochrome P_{450} content after 5 h of reper-

Table 1
Effect of free radical scavengers on biliary secretion during ischemia/reperfusion of rat liver

	Bile Flow [1]		Cholate [2]		Bilirubin [2]	
Reperfusion	1 h	5 h	1 h	5 h	1 h	5 h
Sham operation	0.23 ± 0.03	0.20 ± 0.01	1.23 ± 0.07	1.00 ± 0.06	1.49 ± 0.18	1.39 ± 0.19
Ischemia/reperfusion						
Control	0.12 ± 0.02 [b]	0.08 ± 0.03 [b]	0.62 ± 0.14 [b]	0.23 ± 0.99 [b]	1.25 ± 0.36	1.27 ± 0.35
α-Tocopherol	0.13 ± 0.02 [b]	0.13 ± 0.03 [b]	1.74 ± 0.26 [c]	0.32 ± 0.07 [b]	2.07 ± 0.51	1.70 ± 0.25
Allopurinol	0.11 ± 0.02 [b]	0.12 ± 0.03	0.66 ± 0.13 [b]	0.51 ± 0.11 [b]	1.38 ± 0.41	1.79 ± 0.44
Rebamipide	0.13 ± 0.02 [b]	0.20 ± 0.03 [c]	1.49 ± 0.37 [c]	0.64 ± 0.12 [ac]	1.07 ± 0.19	1.59 ± 0.35

Each value is mean ± S.E.M. from seven rats. [a] $P < 0.05$, [b] $P < 0.01$; difference from sham operation. [c] $P < 0.05$; difference from ischemia/reperfusion control. [1] ml/h per 100 g body weight. [2] mg/h per 100 g body weight.

Table 2
Effect of free radical scavengers on cytochrone P_{450} and NADPH cytochrome P_{450} reductase during ischemia/reperfusion of rat liver

	Cytochrome P_{450} [1]			NADPH cytochrome P_{450} reductase [2]		
Reperfusion	0 h	1 h	5 h	0 h	1 h	5 h
Sham operation	0.80 ± 0.03	0.76 ± 0.03	0.74 ± 0.07	128.4 ± 10.4	128.6 ± 10.4	137.0 ± 10.2
Ischemia/reperfusion						
Control	0.70 ± 0.15	0.65 ± 0.21	0.33 ± 0.08 [a]	58.4 ± 17.0 [a]	57.1 ± 9.72 [a]	67.6 ± 11.1 [a]
α-Tocopherol	0.71 ± 0.22	0.83 ± 0.31	0.89 ± 0.38	84.6 ± 11.1	148.0 ± 35.0 [b]	106.9 ± 11.6 [b]
Allopurinol	1.47 ± 1.35	0.98 ± 0.62	1.05 ± 0.63	100.0 ± 42.2	107.2 ± 48.0	82.5 ± 93.3
Rebamipide	1.01 ± 0.37	0.84 ± 0.32	0.26 ± 0.09 [a]	91.2 ± 36.5	159.9 ± 24.0 [c]	166.7 ± 23.4

Each value is mean ± S.E.M. from seven rats. [a] $P < 0.01$; difference from sham operation. [b] $P < 0.05$, [c] $P < 0.01$; difference from ischemia/reperfusion control. [1] nmol/mg protein. [2] nmol/mg protein per min.

Table 3
Effects of free radical scavengers on drug-metabolizing enzyme activity during ischemia/reperfusion of rat liver

	Aminopyrine *N*-demethylase [1]			Aniline-*p*-hydroxylase [2]		
Reperfusion	0 h	1 h	5 h	0 h	1 h	5 h
Sham operation	21.41 ± 1.37	21.52 ± 0.51	21.63 ± 0.51	7.30 ± 0.53	6.40 ± 0.42	5.50 ± 0.52
Ischemia/reperfusion						
Control	21.24 ± 1.59	6.61 ± 1.27 [a]	5.36 ± 1.03 [a]	6.91 ± 0.72	13.87 ± 1.82 [a]	7.76 ± 1.39
α-Tocopherol	21.51 ± 2.26	16.16 ± 2.76 [b]	8.74 ± 0.90 [ab]	7.33 ± 1.22	7.73 ± 2.15 [b]	7.23 ± 1.27
Allopurinol	23.59 ± 8.85	12.87 ± 2.02 [ac]	5.21 ± 4.21 [a]	9.62 ± 4.58	6.16 ± 0.65 [c]	6.72 ± 1.98
Rebamipide	24.94 ± 3.99	6.29 ± 0.99 [a]	4.96 ± 1.07 [a]	6.87 ± 2.02	13.29 ± 1.88	7.06 ± 2.18

Each value is mean ± S.E.M. from seven rats. [a] $P < 0.01$; difference from sham operation. [b] $P < 0.05$, [c] $P < 0.01$; difference from ischemia/reperfusion control. [1] nmol HCHO/mg protein per 10 min. [2] nmol *p*-aminophenol/mg protein per 15 min.

fusion. Rebamipide did not affect the ischemia/reperfusion-induced changes of cytochrome P_{450}.

NADPH cytochrome P_{450} reductase activity in sham-operated rats was 128.6 ± 10.4 and 137.0 ± 10.2 nmol/mg protein per min and those in ischemia/reperfusion groups were 57.1 ± 9.72 and 67.6 ± 11.1 nmol/mg protein/min at 1 h and 5 h of reperfusion, respectively. The ischemia/reperfusion-induced decrease in NADPH cytochrome P_{450} reductase activity was prevented by rebamipide and α-tocopherol (Table 2).

3.6. Drug-metabolizing enzyme activity

Aminopyrine *N*-demethylase activity in the sham-operated group was 21.41 ± 1.37 nmol HCHO/mg protein per 10 min and constant throughout the whole experiment, but ischemia/reperfusion markedly reduced enzyme activity to 6.61 ± 1.27 and 5.36 ± 1.03 nmol HCHO/mg protein per 10 min following 1 and 5 h of reperfusion. At 1 h and 5 h of reperfusion, α-tocopherol and allopurinol treatment, but not rebamipide, increased aminopyrine *N*-demethylase activity (Table 3). Aniline *p*-hydroxylase activity was not significantly different among all experimental groups after ischemia and 5 h of reperfusion, but after 1 h of reperfusion, aniline *p*-hydroxylase activity in the ischemia/reperfusion control group was significantly increased. The increases in microsomal aniline *p*-hydroxylase activity were prevented by α-tocopherol and allopurinol, but not by rebamipide (Table 3).

4. Discussion

Lipid peroxidation is related to pathologic states such as liver necrosis, ischemic brain damage (Bromont et al., 1989), ischemic liver damage (Omar et al., 1989), and ischemic heart disease (Petty et al., 1991) and induces structural and functional injury to the membranes of cell organelles. Reactive oxygen species-induced lipid peroxidation plays an important role in liver damage caused by ischemia/reperfusion (Atalla et al., 1985) and also is involved in drug-induced lipid peroxidation in hepatic injury. In addition, CCl_4 is converted to $\cdot CCl_3$, which causes lipid peroxidation in hepatocytes, and lipid peroxide levels are increased in chronic alcoholic patients. These symptoms are reduced by antioxidants, promethazine and α-tocopherol (Kawase et al., 1989). These findings are in agreement with our data in which free radical scavenger pretreatment prevented any increases in hepatic lipid peroxidation during ischemia/reperfusion.

Lipid peroxidation was also increased after 60 min of ischemia, after 1 h of reperfusion, and reached a peak after 5 h of reperfusion. Interestingly, serum aminotransferase and the wet/dry weight ratio of the liver were increased after an episode of ischemia, followed by a further increase after 1 h and a peak after 5 h of reperfusion. Thus, our data show that a temporal association exists between increased lipid peroxidation and hepatic injury. Moreover, pretreatment with α-tocopherol or allopurinol prevented lipid peroxidation and hepatic injury. The effect of rebamipide on ischemia/reperfusion-induced hepatic injury is also similar to that of α-tocopherol and allopurinol, suggesting that rebamipide inhibits lipid peroxidation by scavenging free radicals.

Bile acid-dependent bile secretion decreased progressively up to 5 h of reperfusion, which is temporally similar to the pattern of the effect on lipid peroxidation. Pretreatment with rebamipide or α-tocopherol increased bile secretion, and this means that failure of bile secretion is a direct result of ischemia/reperfusion-induced lipid peroxidation.

Alterations in the cytochrome P_{450} drug-metabolizing enzyme system during ischemia/reperfusion in the liver are closely related to lipid peroxidation, and pretreatment with α-tocopherol reduces hepatocellular damage (Lee and Clemens, 1992). In the ischemia/reperfusion control group, cytochrome P_{450} was not changed until 1 h of reperfusion, but was significantly decreased at 5 h of reperfusion. This decrease was inhibited by α-tocopherol and allopurinol treatment. Such a decrease in the total content of cytochrome P_{450} would suggest that the overall activity of the cytochrome P_{450}-dependent oxidases would be similarly decreased. It seems likely that the loss of cytochrome P_{450} is a direct result of ischemia/reperfusion-induced lipid peroxidation. Unexpectedly, rebamipide caused a similar reduction in cytochrome P_{450} content to that shown in rats subjected to ischemia/reperfusion alone. However, rebamipide has a somewhat different mechanism of action from that of α-tocopherol or allopurinol in a drug-metabolizing enzyme system. Since ischemia/reperfusion-induced lipid peroxidation affects other cell organelles such as mitochondria, we cannot exclude the possibility of different mechanisms of action for rebamipide in hepatic drug metabolism.

In an in vitro study, it was reported that cytochrome P_{450} is converted to cytochrome P_{420} during lipid peroxidation, and the reduction in aminopyrine *N*-demethylase and 3,4-benzopyrene hydroxylation was parallel to the reduction in cytochrome P_{450} (Hrycay and O'Brien, 1971). However, glucuronyl transferase activity increases in early lipid peroxidation and becomes normal with the process of lipid peroxidation (Hogberg et al., 1973). In the present study, the activity of aminopyrine *N*-demethylase was reduced while aniline *p*-hydroxylase activity was increased during hepatic ischemia/reperfusion. This contrasting phenomenon was alleviated by treatment with α-tocopherol and allopuri-

nol. Even though the mechanisms of these inconsistent alterations in drug-metabolizing systems have not been identified, the individual cytochrome P_{450} isozymes seem to be differentially affected by ischemia/reperfusion injury. In connection with the clinical situation, unexpected alterations in drug metabolism could occur in patients with liver diseases, and thus more careful administration of drugs to these patients is necessary. Rebamipide did not affect the ischemia/reperfusion-induced abnormal alterations in aminopyrine *N*-demethylase and aniline *p*-hydroxylase activities.

In summary, we have demonstrated that abnormalities in hepatic secretion and microsomal drug-metabolizing function associated with lipid peroxidation occur during ischemia/reperfusion in vivo. In addition, our findings suggest that pretreatment with rebamipide reduces the hepatocellular damage caused by ischemia/reperfusion, and this protection is due to a decrease in lipid peroxidation.

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