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# The Role of Oxygen Free Radicals and Phospholipase $A_2$ in Ischemia-reperfusion Injury to the Liver

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The focus of this study was to investigate the influences of enzymatic scavengers of active oxygen metabolites and phospholipase $A_2$ inhibitor on hepatic secretory and microsomal function during hepatic ischemia/reperfusion. Rats were pretreated with free radical scavengers such as superoxide dismutase (SOD), catalase, deferoxamine and phospholipase $A_2$ inhibitor such as quinacrine and then subjected to 60 min. no-flow hepatic ischemia *in vivo*. After 1, 5 hr of reperfusion, bile was collected, blood was obtained from the abdominal aorta, and liver microsomes were isolated. Serum aminotransferase (ALT) level was increased at 1 hr and peaked at 5 hr. The increase in ALT was significantly attenuated by SOD plus catalase, deferoxamine and quinacrine especially at 5 hr of reperfusion. The wet weight-to-dry weight ratio of the liver was significantly increased by ischemia/reperfusion. SOD and catalase treatment minimized the increase in this ratio. Hepatic lipid peroxidation was elevated by ischemia/reperfusion, and this elevation was inhibited by free radical scavengers and quinacrine. Bile flow and cholate output, but not bilirubin output, were markedly decreased by ischemia/reperfusion and quinacrine restored the secretion. Cytochrome $P_{450}$ content was decreased by ischemia/reperfusion and restored by free radical scavengers and quinacrine to the level of that of the sham operated group. Aminopyrine N-demethylase activity was decreased and aniline *p*-hydroxylase was increased by ischemia/reperfusion. The changes in the activities of the two enzymes were prevented by free radical scavengers and quinacrine. Our findings suggest that ischemia/reperfusion diminishes hepatic secretory functions as well as microsomal drug metabolizing systems by increasing lipid peroxidation, and in addition to free radicals, other factors such as phospholipase $A_2$ are involved in pathogenes of hepatic dysfunction after ischemia/reperfusion.

**Key words :** Oxygen free radicals, Phospholipase $A_2$, Hepatic function, Ischemia / reperfusion

## 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 oxygen-derived free radicals 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 the 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.

Oxygen-derived free radicals produced in the tissue during reperfusion but precise mechanisms by which these free radicals might injure the liver have not been substantiated. The attack by free radicals on biological membranes may lead to the oxidative destruction of the polyunsaturated fatty acids of the membrane through lipid peroxidation, which results in loss of membrane integrity, causing edema and cytolysis at the end (Mead, 1976). It has previously been shown that increased phospholipase $A_2$ activity is detected during such peroxidic decomposition of mitochondrial and erythrocyte membrane lipids, and that the removal of peroxidation products originating in membranes is phospholipase $A_2$-dependent, which in turn decompose to yield malondialdehyde (Sevanian

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*et al.*, 1983).

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, direct association of hepatic lipid peroxidation *in vivo* after ischemia/reperfusion injury and changes in activities of cytochrome $P_{450}$ isozymes, as well as hepatic secretion has not been established.

The purpose of this study was to investigate the role of free radical generation and phospholipase $A_2$ activation in hepatic secretion and microsomal function associated with ischemia/reperfusion injury in which lipid peroxidation occurs. To this end, we used superoxide dismutase (SOD), catalase, deferoxamine, free radical scavengers, and quinacrine, a phospholipase $A_2$ inhibitor.

## MATERIALS AND METHODS

### Hepatic ischemic procedure

Male Sprague-Dawley rats, 200-250 g, were fasted 24 hr before the experiment and allowed to drink tap water *ad libitum*. Rats were anesthetized with pentobarbital sodium (50 mg/kg), and abdomen was opened by midline incision. The left part of the portal vein and hepatic artery were clamped to induce ischemia. 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 lobe of livers. At 1 hr and 5 hr 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 wt.) and dried for 48 hr at 80°C (dry wt.) 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.

### Administration of drugs

Superoxide dismutase (Sigma Chemical Co. From bovine liver suspension) and catalase (Sigma Chemical Co. From bovine liver suspension), dissolved in saline, were intravenously injected 5 minutes before ischemia and reperfusion (5,000 units/kg b.wt., 3,000 units/kg b.wt., respectively). Deferoxamine (Sigma Chemical Co.), dissolved in saline, was injected (60 mg/kg b.wt. i.v.) in the same manner as SOD and catalase. Quinacrine (Sigma Chemical Co.) in saline (10 mg/kg b.wt., i.v.) was injected 30 minutes before ischemia and reperfusion. Sham-operated and ischemia/reperfusion control rats were given saline as the vehicle.

### 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 9,000 x g for 60 min. The supernatant was collected and centrifuged at 105,000 x 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.

### Analytical procedures

Serum aminotransferase (ALT) was determined by standard spectrophotometric procedures using Sigma kit #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 AM 301-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. Cytochrome $P_{450}$ content was calculated by using molar extinction coefficient of 104 $mM^{-1}cm^{-1}$ at the absorbance difference between 450 and 500 nm in differential spectrophotometer (Omura and Sato, 1964). Aminopyrine N-demethylase and aniline *p*-hydroxylase activity were 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.

### Statistics

All data were expressed as means$\pm$S.E.M. Overall significance was tested by two-way ANOVA, and the significance level was set at $p<0.05$.

## RESULTS

### Serum ALT activity

Serum level of ALT in sham-operated rats was 55.0 $\pm$ 5.0 IU/L which was similar to that of normal rats, and increased to 330.0$\pm$ 32.1 IU/L at 5 hr of reperfusion. In the ischemia/reperfusion control group, serum ALT increased to 1425.6$\pm$ 170.7 IU/L at 1 hr and 5269.4$\pm$ 575.2 IU/L at 5 hr of reperfusion. Deferoxamine and quinacrine, not SOD-catalase combi-

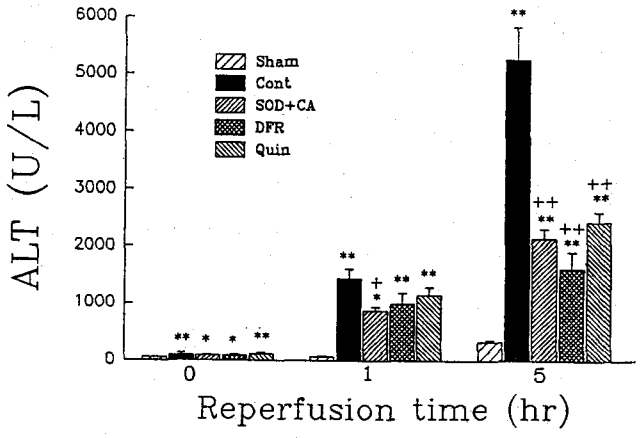


**Fig. 1.** Effect of free radical scavengers and quinacrine on the serum aminotransferase (ALT) activity in ischemia/reperfusion of rat liver. Each bar is expressed as mean±S.E.M. *p<0.05, **p<0.01 Difference from sham operation. ⁺P<0.05, ⁺⁺P<0.01 Difference from ischemia/reperfusion control. Cont; control, SOD+CA; SOD-catalase combination, DFR; deferoxamine, Quin; Quinacrine.

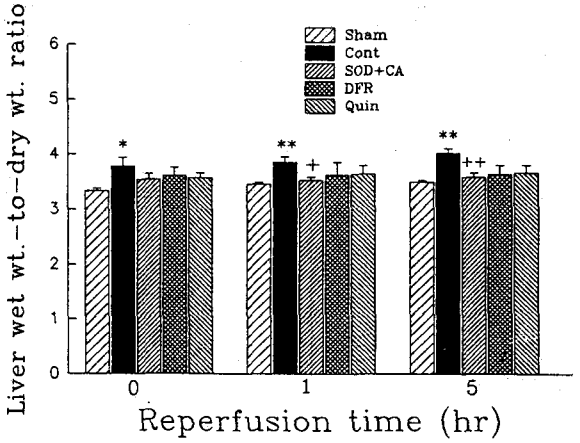


**Fig. 2.** Effect of free radical scavengers and quinacrine on the wet weight-to-dry weight ratio of liver in ischemia/reperfusion of rat liver. Each bar is expressed as mean±S.E.M. *p<0.05, **p<0.01 Difference from sham operation. ⁺P<0.05, ⁺⁺P<0.01 Difference from ischemia/reperfusion control. Cont; control, SOD+CA; SOD-catalase combination, DFR; deferoxamine, Quin; Quinacrine.

nation treatment, had little effect on the increase in ALT activity at 1 hr of reperfusion; however, the increased level of ALT at 5 hr of reperfusion in the ischemia/reperfusion control group was markedly suppressed by combination of SOD and catalase, deferoxamine and quinacrine (Fig. 1).

### 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 hrs. 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 hr of reperfusion, and 4.03±0.08 at 5 hr of reperfusion. The data indicate that ischemia/reperfusion induces hepatic edema. The hepatic edema induced by ischemia/reperfusion was significantly reduced by SOD-catalase combination (Fig. 2).

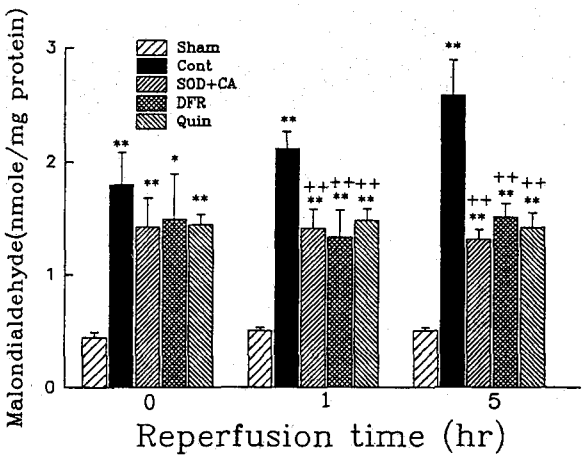


**Fig. 3.** Effect of free radical scavengers and quinacrine on the lipid peroxidation in ischemia/reperfusion of rat liver. Each bar is expressed as mean±S.E.M. * P<0.05, ** P<0.01 Difference from sham operation. ⁺⁺P<0.01 Difference from ischemia/reperfusion control. Cont; control, SOD+CA; SOD-catalase combination, DFR; deferoxamine, Quin; Quinacrine.

### Lipid peroxidation

The data on formation of malondialdehyde (MDA), the product of lipid peroxidation, are presented in Figure 3. In sham-operated rats, the level of MDA in the liver remained constant at approximately 0.5 nmole/mg protein throughout the experiment. However, as with ALT and the wet/dry weight ratio, the level of MDA markedly increased to 1.80±0.28 after ischemia, and 2.11±0.15 at 1 hr, and 2.58±0.31 nmole/mg protein at 5 hr of reperfusion in the ischemia/reperfusion control rats, respectively. Combination of SOD and catalase, deferoxamine and quinacrine treatment prevented the elevations of MDA at 1 hr and 5 hr of reperfusion (Fig. 3).

### Biliary secretion

Bile flow of sham-operated rats was 0.23±0.03 and 0.20±0.01 ml/hr/100g body weight at 1 hr and 5 hr of reperfusion, respectively. The flow was reduced by ischemia/reperfusion to 0.12±0.02 and 0.08±0.03 ml/hr/100 g, at 1 hr and 5 hr of reperfusion, respectively. Quinacrine treatment increased bile secretion at 5 hr of reperfusion compared to that of the ischemia/reperfusion control group. Total bilirubin secretion in all experimental groups was not signifi-

**Table I.** Effect of free radical scavengers and quinacrine on the biliary secretion in ischemia/reperfusion of rat liver

| Reperfusion | Bile Flow[1] | | Cholate[2] | | Bilirubin[2] | |
|---|---|---|---|---|---|---|
| | 1-hour | 5-hour | 1-hour | 5-hour | 1-hour | 5-hour |
| 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** | 0.08±0.03** | 0.61±0.14** | 0.23±0.09** | 1.25±0.36 | 1.27±0.35 |
| SOD-catalase | 0.07±0.02** | 0.08±0.00** | 0.94±0.19* | 0.89±0.14$^{++}$ | 1.14±0.11 | 1.51±0.20 |
| Deferoxamine | 0.16±0.02* | 0.10±0.01** | 1.64±0.15*$^{++}$ | 0.49±0.18* | 1.52±0.11 | 1.31±0.35 |
| Quinacrine | 0.12±0.01** | 0.14±0.03 | 0.73±0.15* | 0.88±0.17$^{++}$ | 1.52±0.27 | 1.08±0.29 |

Each value is mean±S.E.M. from 7 rats

*p<0.05 **p<0.01; Difference from sham operation, $^{++}$p<0.01; Difference from ischemia/reperfusion control, [1]: ml/hr/100 g body weight, [2]: mg/hr/100 g body weight

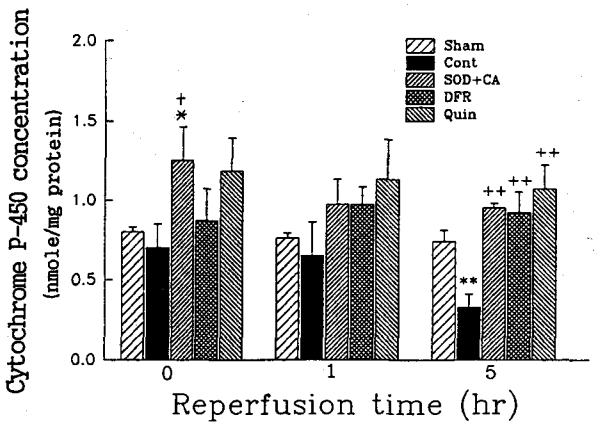


**Fig. 4.** Effect of free radical scavengers and quinacrine on the microsomal cytochrome $P_{450}$ concentration in ischemia/reperfusion of rat liver. Each bar is expressed as mean±S.E.M. *P<0.05, **P<0.01 Difference from sham operation. $^{+}$P<0.05, $^{++}$P<0.01 Difference from ischemia/reperfusion control. Cont; control, SOD+CA; SOD-catalase combination, DFR; deferoxamine, Quin; Quinacrine.

cantly different. Cholate output which is bile acid-dependent secretion was significantly reduced by ischemia/reperfusion. The decreases were markedly restored by deferoxamine treatment in 1 hr reperfusion group, and by SOD-catalase combination and quinacrine in 5 hr reperfusion group (Table I).

### Cytochrome $P_{450}$ content

Hepatic microsomal cytochrome $P_{450}$ content in the sham-operated group was 0.76±0.03~0.74±0.07 nmole/mg protein. Ischemia/reperfusion did not affect the cytochrome $P_{450}$ content after 1 hr of reperfusion, but markedly reduced to 0.33±0.08 nmole/mg protein after 5 hr of reperfusion. SOD-catalase combination, deferoxamine and quinacrine significantly attenuated the decrease in cytochrome $P_{450}$ content after 5 hr of reperfusion (Fig. 4).

### Drug metabolizing enzyme activity

Aminopyrine N-demethylase activity in the sham-operated group was 21.41±1.37 nmole HCHO/mg protein/10 min and constant throughout the whole experiment, but ischemia/reperfusion markedly reduced enzyme activity to 6.61±1.27~5.36±1.03 nmole HCHO/mg protein/10 min following reperfusion. The decrease were markedly increased by all experimental drug treatment at 1 hr of reperfusion. Furthermore, SOD-catalase combination and quinacrine treatment, but not deferoxamine, increased aminopyrine N-demethylase activity at 5 hr of reperfusion (Table II). Aniline *p*-hydroxylase activity was not significantly different among all experimental groups after ischemia and 5 hr of reperfusion, but after 1 hr of reperfusion,

**Table II.** Effect of free radical scavengers and quinacrine on the drug metabolizing enzyme activity in ischemia/reperfusion

| Reperfusion | Aminopyrine N-demethylase[1] | | | Aniline-*p*-hydroxylase[2] | | |
|---|---|---|---|---|---|---|
| | 0-hour | 1-hour | 5-hour | 0-hour | 1-hour | 5-hour |
| 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** | 5.36±1.03** | 6.91±0.72 | 13.87±1.82** | 7.76±1.39 |
| SOD-catalase | 15.67±4.08 | 16.73±2.62**$^{++}$ | 22.81±3.55$^{++}$ | 6.80±0.98 | 9.42±0.53**$^{+}$ | 9.95±0.64** |
| Deferoxamine | 19.70±3.10 | 11.83±2.03**$^{+}$ | 12.20±5.61 | 8.63±0.83 | 8.78±1.90 | 8.15±1.91 |
| Quinacrine | 24.04±2.81 | 16.12±1.58**$^{++}$ | 15.57±2.13*$^{++}$ | 9.76±2.67 | 9.78±1.19* | 9.21±2.73 |

Each value is mean±S.E.M. from 7 rats

*p<0.05 **p<0.01; Difference from sham operation, $^{++}$p<0.05 $^{++}$p<0.01; Difference from ischemia/reperfusion control, [1]: nmol HCHO/mg protein/10 min [2]: nmol PAP/mg protein/15 min

aniline p-hydroxylase activity in the ischemia/reperfusion control group was significantly increased. The increases in microsomal aniline *p*-hydroxylase activity were prevented by SOD-catalase combination (Table II).

## 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.,* 1990) and induces structural and functional injury to membranes of cellular 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 $CCl_3$ which causes lipid peroxidation in hepatocytes, and lipid peroxide levels were increased in chronic alcoholic patients. These symptoms were reduced by antioxidants, promethazine and α-tocopherol (Kawase *et al.,* 1989). Phospholipase $A_2$ ($PLA_2$) is involved in the generation of a variety of bioactive and potentially toxic lipid metabolites, such as arachidonic acid metabolites, lysophospholipids, and platelet-activating factor (Benveniste *et al.,* 1982). Otamiri and Tagesson (1989) found that ischemia and revascularization in the small intestine caused not only accumulation of malondialdehyde in the mucosa, but also increased activity of $PLA_2$ and ratio between lysophosphatidylcholine and phosphatidylcholine. Moreover, the intestinal mucosa were protected against ischemic injury by quinacrine, a $PLA_2$ inhibitor (Otamiri *et al.,* 1987). These findings are in agreement with our data in which free radical scavenger and quinacrine treatments prevented any increases in hepatic lipid peroxidation during ischemia/reperfusion.

Lipid peroxidation was increased after 60 min of ischemia, after 1 hr of reperfusion, and reached a peak after 5 hr of reperfusion. Interestingly, serum ALT and the wet/dry weight ratio of the liver were increased after an episode of ischemia, followed by a further increase after 1 hr and a peak after 5 hr of reperfusion. Thus, our data showed a temporal association exists between increased lipid peroxidation and hepatic injury. Moreover, treatment with SOD, catalase or deferoxamine prevented lipid peroxidation and hepatic injury. The effect of quinacrine on ischemia/reperfusion-induced hepatic injury is also similar to those of free radical scavengers, suggesting that quinacrine inhibits lipid peroxidation by inhibition of phospholipase $A_2$.

Bile acid-dependent bile secretion decreased progressively up to 5 hr of reperfusion, which is temporally similar to the pattern of the effect on lipid peroxidation. Treatment of free radical scavengers or quinacrine 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 is closely related to lipid peroxidation and pretreatment with α-tocopherol reduced hepatocellular damage (Lee and Clemens, 1992). In the ischemia/reperfusion control group, cytochrome $P_{450}$ was not changed until 1 hr of reperfusion, but significantly decreased at 5 hr of reperfusion was inhibited by SOD, catalase, deferoxamine and quinacrine treatment. Such a decrease in 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 loss of cytochrome $P_{450}$ is a direct result of ischemia/reperfusion-induced lipid peroxidation.

In the *in vitro* study, it was reported that cytochrome $P_{450}$ is converted to cytochrome $P_{420}$ during lipid peroxidation, and reduction of aminopyrine N-demethylase and 3,4-benzopyrene hydroxylation were parallel to the reduction in cytochrome $P_{450}$ (Hrycay and O'brien, 1971). However, glucuronyl transferase activity increased in early lipid peroxidation and became normal with the process of lipid peroxidation (Hogberg *et al.,* 1973). In present study, the activity of aminopyrine N-demethylase was reduced while aniline *p*-hydroxylase activity was increased during hepatic ischemia/reperfusion. This contrasting phenomenon is alleviated by treatment with free radical scavengers and quinacrine. 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 addition, it is well established that at least six to nine cytochrome $P_{450}$ isozymes are present in hepatic microsomes of normal rats, although the levels of many are low (Astrom and DePierre, 1986). In the clinical situation, unexpected alterations in drug metabolism could occur in patients with liver diseases, and thus, more careful administration of drugs to patients with liver dysfuntion is necessary.

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.* Our findings suggest that in addition to oxygen free radicals, activation of phospholipase $A_2$ may be regarded as an additional and important mediator of hepatic injury after ischemia and reperfusion.

## ACKNOWLEDGEMENTS

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