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Effects of Hepatic Ischemia-Reperfusion Injury on the Hepatic Mixed Function Oxidase System in Rats

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SUMMARY

Hepatic ischemia induced *in vivo* by ligation of the left hepatic lobe of rats for up to 2 hr had no effect on cytochrome P-450, cytochrome *c* reductase, or lobe histology; however, cytochrome b_5 increased with ischemia duration. Ethylmorphine demethylation decreased 35% after 2 hr of ischemia. Reperfusion of tissue previously made ischemic for up to 2 hr was associated with appreciable necrosis as well as decreases in cytochrome P-450, cytochrome b_5, cytochrome *c* reductase, and ethylmorphine demethylation. Serum alanine transaminase and aspartate transaminase concentrations were increased by reperfusion of previously ischemic tissue. Reperfusion of the previously ischemic lobe for 18 hr was associated with a greater loss of cytochromes P-450 and b_5, cytochrome *c* reductase, and ethylmorphine demethylation than reperfusion for 1 hr. The total decrease in cytochrome P-450 and b_5 content was equal to the decrease in total microsomal heme content, although cytochrome P-450 decreased more than cytochrome b_5. Ethoxyresorufin deethylation by hepatic microsomes from 3-methylcholanthrene-treated rats was decreased by ischemia-reperfusion; however, pentoxyresorufin dealkylation by hepatic microsomes from phenobarbital-treated rats was not, suggesting specific cytochrome P-450 isozyme loss. *In vitro* NADPH-dependent lipid peroxidation in hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats resulted in a selective decrease of ethoxyresorufin but not pentoxyresorufin dealkylation, similar to that observed in livers subjected to ischemia-reperfusion *in vivo*. These data suggest that cytochrome P-450, ethylmorphine demethylation, and ethoxyresorufin deethylation are more susceptible to ischemia-reperfusion injury than cytochrome b_5 or pentoxyresorufin dealkylation.

The tissue pathology observed in organs that have been allowed to reperfuse after a period of ischemia is very complex and the etiology is not understood. There are alterations of cellular energy charge (1, 2), pH (3), glutathione status (1, 3), and calcium homeostasis (4), as well as phospholipase activation (5, 6) and lipid peroxidation (7, 8). The presence of toxic oxygen species that lead to peroxidative damage has been directly and indirectly shown in several tissues subjected to ischemia followed by reperfusion (9, 10). It is known that cytochrome P-450 is susceptible to peroxidative damage (11, 12). Chemical agents that are known to stimulate lipid peroxidation result in cytochrome P-450 degradation (13, 14) and the purified cytochrome P-450 is destroyed in the presence of various lipid peroxides (15, 16). It is, therefore, of interest to determine whether ischemia-reperfusion injury, which may be mediated by lipid peroxidation, can alter cytochrome P-450 levels and associated activities *in vivo*. In this communication, we report the effects of hepatic ischemia followed by reperfusion on hepatic cytochromes P-450 and b_5, cytochrome *c* reductase, ethylmorphine, ethoxyresorufin and pentoxyresorufin dealkylation, and serum levels of ALT and AST in rats.

Materials and Methods

Chemicals. Isocitric dehydrogenase (type IV), DL-sodium isocitrate (type I), NADP, NADPH (type X), cytochrome *c* (type VI), 3-methylcholanthrene, and ethoxyresorufin were obtained from Sigma Chemical Co., pentoxyresorufin was obtained from Pierce Chemical Co., and resorufin was obtained from Aldrich Chemical Co. All other chemicals and biochemicals employed were of the highest purity available.

Animal treatments. Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250–300 g, were kept in a controlled environment with a 12-hr light-dark cycle and had free access to food and water until the morning of the study. All rats, including controls, were anesthetized with ketamine (7.5 mg/100 g) and xylazine (1.6 mg/100 g), which provided approximately 15 min of anesthesia. In order to induce hepatic ischemia, laparotomy was performed on experimental animals and the blood supply to the left lobe of the liver was interrupted by placement of a Silastic noose around the left lobe at the level of the porta. The noose was tightened and secured with a bulldog clip, which was then exteriorized, and the wound was closed with stainless steel wound clips. After a predetermined interval, the noose was loosened by release of the bulldog clip, which required no anesthesia or wound opening, and the lobe was allowed to reperfuse with blood for either 1 or 18 hr. Where indicated, rats were administered phenobarbital sodium (60 mg/kg/day, intraperitoneally) in saline for 4 days before hepatic

ABBREVIATIONS: ALT, alanine transaminase; AST, aspartate transaminase; PMN, polymorphonuclear leukocytes.

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ischemia or 3-methylcholanthrene (60 mg/kg, intraperitoneally) in corn oil once 2 days before hepatic ischemia.

Assays. Microsomes were prepared by perfusion of the liver with ice-cold 1.15% KCl and homogenization of the lobe in ice-cold 0.25 M sucrose with a Tissuemizer (Tekmar). The homogenate was centrifuged for 20 min at 9,000 × *g* at 5°. The supernatant fraction was centrifuged for 60 min at 105,000 × *g* to obtain the microsomal pellet, which was then resuspended in ice-cold 1.15% KCl and centrifuged for an additional 30 min at 105,000 × *g*. The microsomal pellet was resuspended in ice-cold 100 mM Tris buffer, pH 7.4, before biochemical evaluation. For NADPH-dependent microsomal lipid peroxidation experiments, the final washed microsomal pellet was resuspended in ice-cold 100 mM phosphate buffer, pH 7.4. Microsomal lipid peroxidation was induced in 3-ml incubation mixtures containing 2–4 mg of microsomal protein, 11 nmol of sodium isocitrate, 1 nmol of NADP, 15 nmol of $MgCl_2$, and 2 units of isocitric dehydrogenase in 100 mM phosphate buffer, pH 7.4. Microsomal cytochromes P-450 and b_5 were measured by the method of Omura and Sato (17) and heme was assayed by the hemochromogen method of Paul *et al.* (18). Ethoxyresorufin *O*-deethylation and pentoxyresorufin *O*-dealkylation were assayed by the method of Lubet *et al.* (19), using excitation and emission wavelengths of 569 and 596 nm, respectively. Ethylmorphine *N*-demethylation was quantitated as described previously (20), except that microsomal suspensions were incubated in 100 mM Tris buffer, pH 7.4, for 20 min. Cytochrome *c* reductase activity was quantitated by a modification of the method of Phillips and Langdon (21), in 1-ml incubation mixtures containing 0.1 mM NADPH, 1 mg of cytochrome *c*, and 10 μl of microsomal suspension in 300 mM phosphate buffer, pH 7.3, containing 0.1 mM EDTA. The rate of cytochrome *c* reduction was calculated from the absorbance change at 550 nm, using an extinction coefficient of 21 $mM^{-1}\ cm^{-1}$. Malondialdehyde was assayed by the method of Buege and Aust (22) and microsomal protein was assayed by the method of Bradford (23), using human serum albumin as the standard. Serum ALT and AST were assayed with a Monarch 761 Automated Centrifugal Analyzer (Instrumentation Laboratories) and are reported as IU/liter. Liver samples for histological evaluation were obtained immediately after animal sacrifice. Samples were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin and eosin.

Statistics. Bartlett's test ($p < 0.05$) was used to test for homogeneity of variances. When variances were not homogeneous, a natural logarithm transformation was used to transform the data before a one-way (Tables 1–6) or two-way (Table 7) analysis of variance was used ($p < 0.05$). A Student's *t* test, Dunnett's *t* test, or Duncan's multiple range test was used to test for significance of differences at the $p < 0.05$ level.

Results

Rats were subjected to hepatic ischemia for 0.5 to 2 hr, and the effects on microsomal enzymes and associated activities are shown in Table 1. Cytochrome P-450 and cytochrome *c* reductase were not significantly altered by up to 2 hr of hepatic ischemia; however, ethylmorphine demethylation was decreased 35% after 2 hr of ischemia. The specific content of cytochrome b_5 increased in a time-dependent manner, such that 2 hr after the onset of ischemia a 60% increase in cytochrome b_5 specific content was observed. Release of the cytosolic enzymes ALT and AST into the serum was minimal.

Reperfusion of previously ischemic liver tissue resulted in appreciable changes in microsomal enzyme content and cytosolic enzyme release (Table 2). Cytochrome P-450, cytochrome *c* reductase, and ethylmorphine demethylation decreased 41, 32, and 42%, respectively, subsequent to 2 hr of ischemia followed by 18 hr of reperfusion. The majority of the loss of cytochrome P-450, cytochrome *c* reductase, and ethylmorphine demethylation was associated with the first 0.5 hr of ischemia. Cytochrome b_5 levels also decreased upon reperfusion of previously ischemic tissue; however, the decrease was less after 1 and 2 hr of ischemia than after 0.5 hr of ischemia. Serum ALT and AST levels were significantly elevated subsequent to 1 hr of ischemia followed by 18 hr of reperfusion and were 13 and 30 times control levels after 2 hr of ischemia followed by 18 hr of reperfusion.

Samples of control liver, liver subjected to 1 hr of ischemia, and liver subjected to 1 hr of ischemia followed by 18 hr of reperfusion were evaluated microscopically. Liver from control animals was histologically normal (Fig. 1). Centrilobular congestion was evident in liver subjected to 1 hr of ischemia only (Fig. 2); however, the hepatic architecture remained intact. In contrast, livers that had been allowed to reperfuse for 18 hr subsequent to 1 hr of ischemia (Fig. 3) contained multifocal areas of coagulation necrosis. Sinusoidal spaces were dilated and the necrotic portions of the tissue were infiltrated with PMN. Enlarged vacuolated hepatocytes were occasionally present in less severely affected areas in which overt necrosis was not present.

The integrity of the mixed function oxidase system was also dependent upon the duration of reperfusion subsequent to a constant interval of ischemia (Table 3). Cytochrome P-450 levels decreased 10% subsequent to 1 hr of ischemia followed by 1 hr of reperfusion and decreased 30% after 1 hr of ischemia followed by 18 hr of reperfusion. Cytochrome b_5 levels decreased 15% subsequent to 1 hr of ischemia followed by 1 hr of reperfusion and 25% after 1 hr of ischemia followed by 18 hr of reperfusion. Cytochrome *c* reductase and ethylmorphine demethylation activity were not significantly altered by 1 hr of ischemia followed by 1 hr of reperfusion, yet both were decreased about 30% subsequent to 1 hr of ischemia followed by 18 hr of reperfusion. Serum ALT and AST were significantly elevated subsequent to 1 hr of ischemia followed by either 1 or 18 hr of reperfusion.

Total microsomal heme content was equal to the sum of the cytochrome P-450 and cytochrome b_5 contents in control mi-

TABLE 1

Effects of ischemia duration on hepatic microsomal enzymes and enzyme release

Hepatic ischemia was maintained for 0.5 to 2 hr. Animals were sacrificed immediately after the ischemic interval (no reperfusion period). Values are the mean ± standard error ($n = 3$).

	Cytochrome P-450	Cytochrome b_5	Cytochrome *c* reductase	Ethylmorphine demethylation	ALT	AST
	nmol of cytochrome/mg of protein	*nmol of cytochrome/mg of protein*	*μmol of cytochrome c reduced/min/mg of protein*	nmol of formaldehyde/min/mg of protein	IU/liter	IU/liter
Control	1.00 ± 0.04	0.56 ± 0.02	7.6 ± 0.3	6.1 ± 0.3	31 ± 1	146 ± 7
0.5-hr ischemia	0.96 ± 0.03	0.65 ± 0.03	7.2 ± 0.6	5.7 ± 0.5	38 ± 2	149 ± 19
1.0-hr ischemia	1.02 ± 0.09	0.75 ± 0.07*	7.5 ± 0.7	6.0 ± 0.6	41 ± 2	139 ± 10
2.0-hr ischemia	0.90 ± 0.08	0.89 ± 0.02*	6.7 ± 0.3	3.9 ± 0.4*	95 ± 38*	247 ± 58

* Significantly different from control group (Dunnett's *t* test, $p < 0.05$).

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TABLE 2

Effects of ischemia-reperfusion on hepatic microsomal enzymes and enzyme release

Hepatic ischemia was maintained for 0.5–2.0 hr and animals were sacrificed after 18 hr of reperfusion. Values are the mean ± standard error (n = 6).

	Cytochrome P-450	Cytochrome b_5	Cytochrome c reductase	Ethylmorphine demethylation	ALT	AST
	nmol of cytochrome/mg of protein	nmol of cytochrome/mg of protein	μmol of cytochrome c reduced/min/mg of protein	nmol of formaldehyde/min/mg of protein	IU/liter	IU/liter
Control	1.11 ± 0.06	0.66 ± 0.02	11.0 ± 0.8	9.0 ± 0.9	45 ± 3	157 ± 19
Ischemia time						
0.5 hr	0.73 ± 0.02*	0.52 ± 0.01*	8.3 ± 0.5*	6.5 ± 0.4*	110 ± 16	257 ± 21
1.0 hr	0.72 ± 0.04*	0.54 ± 0.03*	7.9 ± 0.2*	6.3 ± 0.6*	928 ± 440*	1291 ± 665*
2.0 hr	0.65 ± 0.06*	0.55 ± 0.04*	7.5 ± 0.5*	5.2 ± 0.4*	1366 ± 393*	1973 ± 776*

* Significantly different from control group (Dunnett's t test, $p < 0.05$).

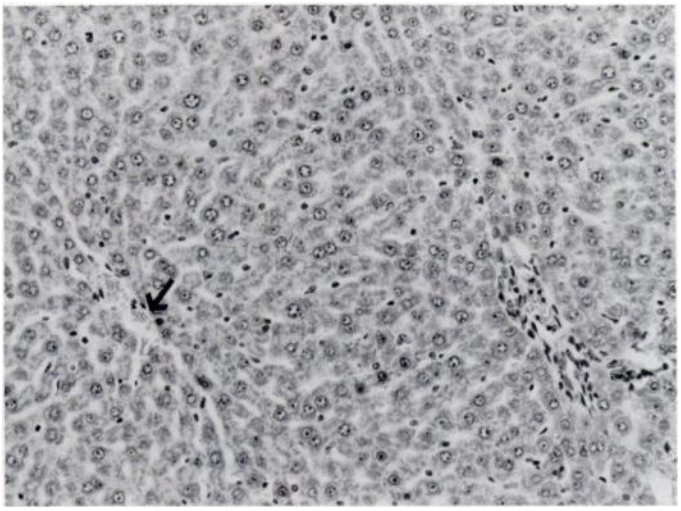

Fig. 1. Photomicrograph of liver from a control animal, illustrating normal hepatic architecture. *Arrow*, central vein. Hematoxylin and eosin, 250×.

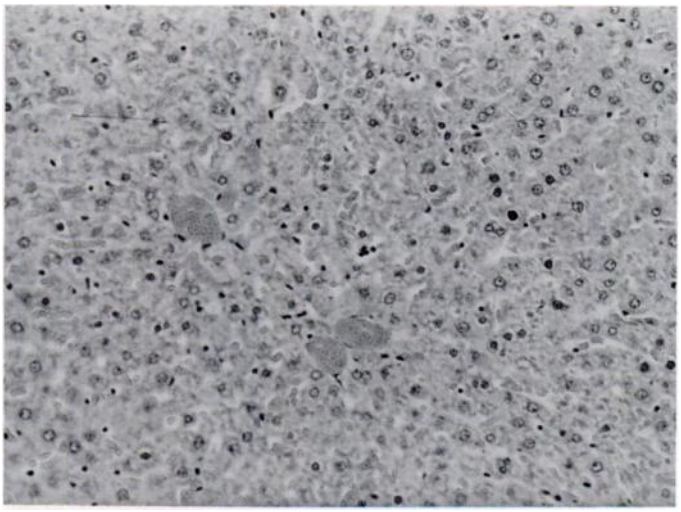

Fig. 2. Photomicrograph of a liver lobe subjected to 1 hr of ischemia, showing red blood cells within a central vein and sinusoidal spaces but otherwise normal hepatic morphology. Hematoxylin and eosin, 250×.

crosomes (Table 4). Subsequent to 1 hr of ischemia followed by 18 hr of reperfusion, cytochromes P-450 and b_5 were decreased 30 and 14%, respectively. Under this condition, microsomal heme content decreased 21% but remained equivalent to the sum of the cytochrome P-450 and cytochrome b_5 contents.

Phenobarbital pretreatment of rats increased cytochrome P-450 and cytochrome c reductase activity 88 and 21%, respectively; however, no significant effect was observed on cytochrome b_5, ethylmorphine demethylation, or serum ALT and AST (Table 5). In phenobarbital-pretreated rats, induction of hepatic ischemia for 1 hr followed by reperfusion for 18 hr resulted in a significant loss of cytochrome P-450 but not cytochrome b_5 or cytochrome c reductase activity. A significant elevation of serum ALT and AST was caused by this procedure. 3-Methylcholanthrene pretreatment of rats significantly increased cytochrome P-450 and b_5 contents by 60 and 25%, respectively, whereas ethylmorphine demethylation was decreased and cytochrome c reductase activity was unaltered. In 3-methylcholanthrene-pretreated animals, induction of hepatic ischemia for 1 hr followed by reperfusion for 18 hr caused no significant effect on cytochrome P-450 or b_5 contents, cytochrome c reductase activity or ethylmorphine demethylation; however, serum ALT and AST were significantly elevated.

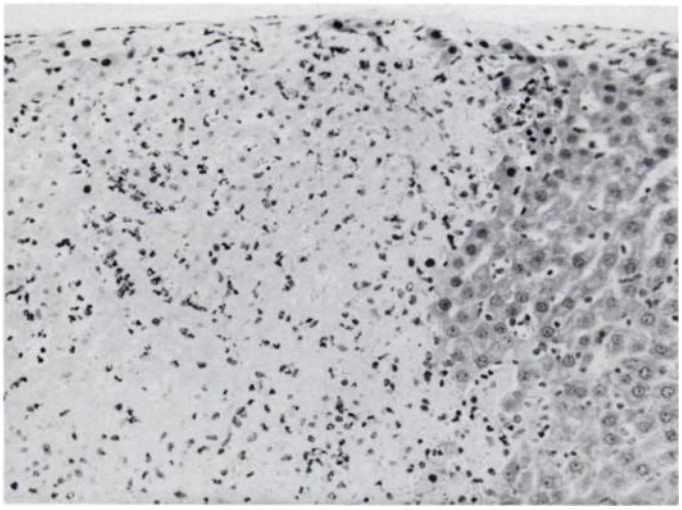

Fig. 3. Photomicrograph of a liver lobe subjected to 1 hr of ischemia followed by 18 hr of reperfusion. A focal subcapsular area of coagulation necrosis containing neutrophils and necrotic cellular debris is evident. Hematoxylin and eosin, 250×.

The effects of ischemia followed by reperfusion on hepatic microsomal pentoxyresorufin and ethoxyresorufin dealkylation were also investigated in phenobarbital- and 3-methylcholanthrene-pretreated rats (Table 6). Phenobarbital pretreatment caused a 14-fold increase in pentoxyresorufin dealkylation but no significant increase in ethoxyresorufin deethylation. In contrast, 3-methylcholanthrene pretreatment caused a 30-fold increase in ethoxyresorufin deethylation and no significant increase in pentoxyresorufin dealkylation. Pentoxyresorufin dealkylation in microsomes from phenobarbital-pretreated rats was not significantly altered by 1 hr of ischemia followed by 18

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TABLE 3

Effects of reperfusion duration on hepatic microsomal enzymes and enzyme release

Hepatic ischemia was maintained for 1 hr and animals were sacrificed after 1 or 18 hr of reperfusion. Values are the mean ± standard error (n = 4).

	Cytochrome P-450	Cytochrome b_5	Cytochrome c reductase	Ethylmorphine demethylation	ALT	AST
	nmol of cytochrome/mg of protein	nmol of cytochrome/mg of protein	μmol of cytochrome c reduced/min/mg of protein	nmol of formaldehyde/min/mg of protein	IU/liter	IU/liter
Control	1.10 ± 0.03	0.67 ± 0.02	12.1 ± 0.4	6.8 ± 0.6	35 ± 2	131 ± 9
Reperfusion time						
1 hr	0.98 ± 0.02[a]	0.56 ± 0.01[a]	10.4 ± 0.7	6.4 ± 0.1	233 ± 8[a]	381 ± 25[a]
18 hr	0.77 ± 0.02[a,b]	0.51 ± 0.02[a]	8.6 ± 0.6[a]	4.6 ± 0.3[a,b]	297 ± 22[a,b]	464 ± 21[a,b]

[a] Significantly different from control group (Duncan's test, $p < 0.05$).
[b] Significantly different from 1-hr reperfusion group (Duncan's test, $p < 0.05$).

TABLE 4

Effects of ischemia-reperfusion on hepatic microsomal hemoproteins and heme content

Hepatic ischemia was maintained for 1 hr and animals were sacrificed after 18 hr of reperfusion. Values are the mean ± standard error (n = 4).

	Cytochrome P-450	Cytochrome b_5	Heme
	nmol of cytochrome/mg of protein	nmol of cytochrome/mg of protein	nmol of heme/mg of protein
Control	0.99 ± 0.07	0.62 ± 0.04	1.53 ± 0.08
Ischemia-reperfusion	0.68 ± 0.03[a]	0.54 ± 0.02[a]	1.21 ± 0.03[a]

[a] Significantly different from control group (Student's t test, $p < 0.05$).

hr of reperfusion. Similarly, 1 hr of ischemia followed by 18 hr of reperfusion had no significant effect on the low pentoxyresorufin dealkylation activity in hepatic microsomes from 3-methylcholanthrene-treated rats. The low level of ethoxyresorufin deethylation in microsomes from phenobarbital-pretreated rats was also not significantly affected by 1 hr of ischemia followed by 18 hr of reperfusion. In contrast, ethoxyresorufin deethylation in microsomes from 3-methylcholanthrene-pretreated rats decreased 50% subsequent to 1 hr of ischemia followed by 18 hr of reperfusion. Furthermore, the reduced cytochrome P-450 carbon monoxide binding spectrum maximum shifted from 448 nm in microsomes from 3-methylcholanthrene-pretreated rats to 449 nm or higher in microsomes from 3-methylcholanthrene-pretreated rats subjected to 1 hr of ischemia followed by 18 hr of reperfusion (data not shown).

The effect of NADPH-dependent lipid peroxidation upon pentoxyresorufin and ethoxyresorufin dealkylation in hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats was also evaluated (Table 7). Formation of the lipid peroxidation product malondialdehyde was stimulated in microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats by 1 hr of incubation with NADPH; however, peroxidation was greatest in the latter. Furthermore, a significant decrease in cytochrome P-450 was observed after incubation with NADPH in microsomes obtained from control and phenobarbital- and 3-methylcholanthrene-pretreated rats. Pentoxyresorufin dealkylation in hepatic microsomes from control and phenobarbital- or 3-methylcholanthrene-induced microsomes was not decreased by incubation with NADPH, even though phenobarbital increased pentoxyresorufin dealkylation 44-fold. In contrast, ethoxyresorufin dealkylation was significantly decreased by incubation with NADPH in microsomes from control (45%) and phenobarbital- (54%) and 3-methylcholanthrene-treated (30%) rats.

Discussion

A number of hypotheses have been put forth to attempt to explain the organ pathology associated with reperfusion of ischemic tissue. The peroxidation hypothesis (24) suggests that, during the ischemia-reperfusion event, intracellular mechanisms are altered whereby reactive oxygen species are produced (hydrogen peroxide, superoxide, hydroxyl radical, etc.), which lead to peroxidation of intracellular molecules critical to cell homeostasis. Several of these reactive oxygen species have been detected directly and indirectly in organ systems undergoing ischemia followed by reperfusion (9, 10). Lipid peroxidation has also been observed in organs subjected to ischemia-reperfusion injury (8, 9). The cytochrome P-450 mixed function oxidase system, which is ubiquitous in mammalian tissues, is susceptible to lipid peroxidation damage induced by chemicals

TABLE 5

Effects of hepatic ischemia-reperfusion on microsomal enzymes and serum enzyme release in phenobarbital- and 3-methylcholanthrene-treated rats

Animals were administered phenobarbital at 60 mg/kg/day for 4 days or 3-methylcholanthrene at 60 mg/kg/day once 2 days before ischemia. Hepatic ischemia was maintained for 1 hr and animals were sacrificed after 18 hr of reperfusion. Values are the mean ± standard error (n = 4–12).

	Cytochrome P-450	Cytochrome b_5	Cytochrome c reductase	Ethylmorphine demethylation	ALT	AST
	nmol of cytochrome/mg of protein	nmol of cytochrome/mg of protein	μmol of cytochrome c reduced/min/mg of protein	nmol of formaldehyde/min/mg of protein	IU/liter	IU/liter
Control	1.03 ± 0.04	0.63 ± 0.02	11.5 ± 0.6	7.7 ± 0.2	46 ± 2	168 ± 10
Phenobarbital	1.94 ± 0.13[a]	0.65 ± 0.03	13.9 ± 0.5[a]	8.1 ± 0.6	43 ± 4	150 ± 7
Phenobarbital + ischemia-reperfusion	1.48 ± 0.09[a,b]	0.58 ± 0.01	12.5 ± 0.4	8.2 ± 0.4	671 ± 125[a,b]	721 ± 102[a,b]
3-Methycholanthrene	1.65 ± 0.38[a]	0.79 ± 0.08[a]	11.8 ± 0.5	5.2 ± 0.5[a]	41 ± 2	165 ± 13
3-Methylcholanthrene + ischemia-reperfusion	1.38 ± 0.24	0.81 ± 0.04[a]	10.1 ± 1.0	4.2 ± 0.5[a]	389 ± 47[a,b]	664 ± 44[a,b]

[a] Significantly different from control group (Duncan's test, $p < 0.05$).
[b] Significantly different from respective phenobarbital or 3-methylcholanthrene group (Duncan's test, $p < 0.05$).

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TABLE 6

Effects of hepatic ischemia-reperfusion on microsomal pentoxyresorufin and ethoxyresorufin dealkylation in phenobarbital- and 3-methylcholanthrene-treated rats

Animals were administered phenobarbital sodium (60 mg/kg/day, intraperitoneally) in saline for 4 days before ischemia or 3-methylcholanthrene (60 mg/kg, intraperitoneally) in corn oil once 2 days before ischemia. Hepatic ischemia was maintained for 1 hr and animals were sacrificed after 18 hr of reperfusion. Values are the mean ± standard error (n = 8–12).

	Cytochrome P-450	Pentoxyresorufin dealkylation	Ethoxyresorufin dealkylation
	nmol of cytochrome P-450/mg of protein	*nmol of resorufin formed/min/nmol of cytochrome P-450*	*nmol of resorufin formed/min/nmol of cytochrome P-450*
Control	0.77 ± 0.02	0.010 ± 0.002	0.025 ± 0.004
Phenobarbital	1.49 ± 0.10[a]	0.140 ± 0.029[a]	0.056 ± 0.006
Phenobarbital + ischemia-reperfusion	1.34 ± 0.23[a]	0.195 ± 0.031[a]	0.080 ± 0.007[a]
3-Methylcholanthrene	1.56 ± 0.05[a]	0.011 ± 0.001	0.754 ± 0.049[a]
3-Methylcholanthrene + ischemia-reperfusion	1.04 ± 0.04[b]	0.006 ± 0.001[a]	0.389 ± 0.064[a,b]

[a] Significantly different from control group (Duncan's test, $p < 0.05$).
[b] Significantly different from respective phenobarbital or 3-methylcholanthrene group (Duncan's test, $p < 0.05$).

TABLE 7

Effects of NADPH-dependent lipid peroxidation on pentoxyresorufin and ethoxyresorufin dealkylation in hepatic microsomes from control and phenobarbital- or 3-methylcholanthrene-treated rats

Animals were administered phenobarbital sodium (60 mg/kg/day, intraperitoneally) for 4 days or 3-methylcholanthrene (60 mg/kg, intraperitoneally) once 2 days before preparation of hepatic microsomes. Microsomes were assayed for various parameters immediately or after 1 hr of incubation with NADPH at 37°. Values are the mean ± standard error (n = 3 or 4).

	Malondialdehyde formed	Cytochrome P-450	Pentoxyresorufin dealkylation	Ethoxyresorufin dealkylation
	nmol of malondialdehyde/mg of protein	*nmol of cytochrome P-450/mg of protein*	*nmol of resorufin formed/min/nmol of cytochrome P-450*	*nmol of resorufin formed/min/nmol of cytochrome P-450*
Control microsomes				
0-hr incubation	0.61 ± 0.03	0.81 ± 0.06	0.007 ± 0.001	0.139 ± 0.009
1-hr incubation	5.72 ± 0.16[a]	0.63 ± 0.04[a]	0.007 ± 0.001	0.077 ± 0.007[a]
Phenobarbital-treated microsomes				
0-hr incubation	0.19 ± 0.03[b]	1.32 ± 0.02[b]	0.309 ± 0.008[c]	0.240 ± 0.006[b]
1-hr incubation	4.92 ± 0.13[a]	1.07 ± 0.03[a]	0.306 ± 0.022[c]	0.110 ± 0.005[a]
3-Methylcholanthrene-treated microsomes				
0-hr incubation	0.40 ± 0.03[b]	1.65 ± 0.02[b]	0.010 ± 0.001[c]	2.700 ± 0.080[b]
1-hr incubation	35.34 ± 0.82[a]	0.86 ± 0.03[a]	0.012 ± 0.001[c]	1.897 ± 0.199[a]

[a] Significantly different from 0-hr incubation within induction group (Duncan's test, $p < 0.05$).
[b] Significantly different from 0-hr incubation in control group (Duncan's test, $p < 0.05$).
[c] Control, phenobarbital, and 3-methylcholanthrene groups significantly different from each other irrespective of time (Duncan's test, $p < 0.05$).

in vivo (13, 14), as well as in microsomes (12) and in highly purified form (15). In view of the success with organ transplantation and recent intense research in ischemia-reperfusion injury, it is of interest to elucidate the effects of ischemia and reperfusion on the cytochrome P-450 mixed function oxidase system and associated enzyme activities.

The data presented herein indicate that there are significant alterations of the hepatic mixed-function oxidase system upon reperfusion of previously ischemic liver tissue that are not characteristic of the ischemic state *per se*. Liver histology, cytochrome P-450 content, cytochrome *c* reductase activity, ethylmorphine demethylation, and serum ALT and AST are not appreciably altered by 1 hr of ischemia as such. However, reperfusion of previously ischemic tissue is associated with significant decreases in microsomal cytochrome P-450 and b_5 contents, cytochrome *c* reductase activity, and ethylmorphine demethylation, as well as significant increases in serum ALT and AST. The decrease in microsomal enzymes and release of cytosolic enzymes into the serum were also related to the duration of reperfusion following ischemia, such that 18 hr of reperfusion resulted in greater effects than 1 hr of reperfusion. Although hepatic morphology was not appreciably altered by 1 hr of ischemia *per se*, except for the presence of some centrilobular congestion, morphology was appreciably altered by reperfusion of previously ischemic tissue, resulting in dilated sinusoids, PMN infiltration and coagulation necrosis. Infiltration by, and stimulation of, PMNs is associated with NADPH oxidase activity, leading to increased oxygen consumption and reactive oxygen species formation (25, 26). Jeffery *et al.* (11) found that destruction of microsomal cytochrome P-450 by linoleic acid hydroperoxide was biphasic, and they suggested that labile and stable forms of cytochrome P-450 existed in microsomes. Additionally, microsomal cytochrome b_5 was not appreciably decreased by linoleic acid hydroperoxide at low concentrations (<50, μM) but was decreased at higher concentrations of the hydroperoxide. It is possible that the 30–40% decrease in microsomal cytochrome P-450 content in control and phenobarbital- and 3-methylcholanthrene-pretreated animals induced by 1 hr of ischemia followed by 18 hr of reperfusion results from the destruction of more labile forms of cytochrome P-450. However, if the destruction of the cytochromes are peroxide mediated in ischemia-reperfusion injury, it appears necessary that locally high peroxide concentrations be present to account for the destruction of the cytochrome b_5 observed.

Because cytochrome P-450 is integrally associated with the lipid of the endoplasmic reticulum, decreased levels of the cytochrome and associated activities subsequent to reperfusion of previously ischemic liver tissue may result from destruction

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of the cytochrome apoprotein, heme moiety, or lipid environment. Cytochromes P-450 and b_5 are the only heme-containing enzymes in the hepatic microsomal fraction. Ischemia-reperfusion injury is believed to be mediated in part by peroxidative reactions, and heme is highly susceptible to peroxidative destruction (27, 28). The data herein show that the total heme content of microsomes is equal to the cytochrome P-450 and b_5 contents. However, cytochrome P-450 and b_5 levels were both decreased by ischemia followed by reperfusion, and the total microsomal heme content was also decreased exactly proportionally to the loss of both of these cytochromes. These data suggest that the loss of cytochromes P-450 and b_5 resulting from ischemia-reperfusion may be mediated by destruction of cytochrome heme rather than an alteration of the apoprotein or the lipid environment.

Phenobarbital and 3-methylcholanthrene induce predominantly two specific isozymes of cytochrome P-450 in rat hepatic microsomes, designated P-450$_{PB\text{-}B}$ (P450IIB1) and P-450$_{\beta NF\text{-}B}$ (P450IA1), respectively (29). Cytochrome P-450 in microsomes from phenobarbital- and 3-methylcholanthrene-pretreated rats is susceptible to ischemia-reperfusion injury. However, the cytochrome P-450 ferrous-CO binding spectrum maximum in microsomes from 3-methylcholanthrene-pretreated rats shifted from 448 nm to 449–450 nm, suggesting a differential destruction of the cytochrome P-450 isozymes that give rise to the composite ferrous-CO binding spectrum. Upon indirect analysis of the P-450$_{PB\text{-}B}$ and P-450$_{\beta NF\text{-}B}$ content with the isozyme-specific substrates pentoxyresorufin and ethoxyresorufin, it was observed that 3-methylcholanthrene-inducible P-450$_{\beta NF\text{-}B}$ activity was decreased more by 1 hr of ischemia followed by 18 hr of reperfusion than was phenobarbital-induced P-450$_{PB\text{-}B}$ activity. If the decrease in cytochrome P-450 level associated with ischemia-reperfusion injury is related to heme destruction, as discussed previously, it is possible that the selective decrease in ethoxyresorufin deethylation due to tissue ischemia followed by reperfusion reflects a selective destruction of the P-450$_{\beta NF\text{-}B}$ isozyme. Because P-450$_{\beta NF\text{-}B}$ exhibits a ferrous-CO wavelength maximum at 446–448 nm rather than at 450 nm, characteristic of uninduced microsomes, the shift of the ferrous-CO wavelength maximum of microsomes from 3-methylcholanthrene-treated rats from 448 nm to 449–450 nm after ischemia and reperfusion is consistent with a selective loss of the P-450$_{\beta NF\text{-}B}$ isozyme. Furthermore, incubation of microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats in the presence of NADPH produces oxidative destruction of cytochrome P-450 and leads to a selective decrease in ethoxyresorufin dealkylation relative to pentoxyresorufin dealkylation, which is analogous to the *in vivo* ischemia-reperfusion results. Recently, Kitada *et al.* (30) have demonstrated, in rat hepatic microsomes peroxidized *in vitro*, that there is a greater decrease in testosterone 6β-hydroxylation than in testosterone 16α-hydroxylation. These results are consistent with the ischemia-reperfusion results presented herein, because ethoxyresorufin dethylation and testosterone 6β-hydroxylation are both mediated by P-450$_{\beta NF\text{-}B}$; whereas pentoxyresorufin dealkylation and testosterone 16α-hydroxylation are mediated by P-450$_{PB\text{-}B}$ (29). Thus, it appears that the destruction of cytochrome P-450 associated with reperfusion of ischemic liver tissue may be mediated by oxidative processes that lead to the selective destruction of cytochrome P-450 isozymes.

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