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COMPARISON OF THE SUSCEPTIBILITY OF HEPATOCYTES FROM POSTNATAL AND ADULT MICE TO HEPATOTOXINS

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Abstract—Age-related changes of susceptibility to hepatotoxicity induced by four hepatotoxic compounds were investigated using an isolated mouse hepatocyte model. Hepatocytes isolated from 2-week-old mice and adult mice (8–10 weeks old) were exposed to different concentrations (including toxic concentrations) of paracetamol, furosemide, iodoacetic acid and *t*-butylhydroperoxide for incubation times up to 24 hr. Cell damage was assessed by leakage of lactate dehydrogenase. Analysis of variance indicated that the hepatocytes from the 2-week-old mice were less susceptible to the toxic effects of all four hepatotoxins. The activities of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase were determined in both hepatocytes and whole liver from the two age groups. While catalase was significantly greater in adults, glutathione peroxidase, glutathione reductase and superoxide dismutase were all higher in the 2-week-old mice. Since these three enzymes are involved with protection against oxidative stress, it is likely that the higher activity in hepatocytes from 2-week-old mice is responsible for the reduced susceptibility to damage induced by the four hepatotoxins.

Some compounds are more toxic to the liver during the postnatal period than they are in adults. For example, chloramphenicol is more hepatotoxic to neonates because of the low activity of the glucuronidation pathway in the very young [1]. However, a number of other chemicals appear to be less toxic during the postnatal period. Rats of 7 and 11 days old are less susceptible to the hepatotoxic effects of paracetamol, bromobenzene and tannic acid when compared to young adults [2, 3], while 10-day-old mice show less liver damage than adults when exposed to paracetamol [4].

This resistance could be due to an immaturity of the enzyme systems responsible for activating chemicals to reactive toxic metabolites. However, it has been shown that at certain periods during postnatal development hepatocytes from both rats and mice have greater capacity to activate compounds, such as paracetamol, than do adults [3, 5, 6]. Also, the toxicity of tannic acid is not dependent on metabolic activation as it is a directly-acting hepatotoxin.

One possibility is that these postnatal animals have superior cellular defence mechanisms to deal with the damaging events initiated by toxic agents in the liver. Glutathione peroxidase (GSH-peroxidase),† glutathione reductase (GSSG-reductase), superoxide dismutase (SOD) and catalase have been implicated in protection against some forms of chemical-induced injury. This study examined if there is an age-related difference in the susceptibility to hepatotoxins and whether this correlated with activity of protective enzyme systems. Because factors such as rate of absorption, distribution, metabolism and excretion have the potential to affect susceptibility to hepatotoxic agents, the present study examined susceptibility to toxic agents in isolated hepatocytes. This hepatocyte model has been shown to be a useful *in vitro* system for examining age-related toxic phenomena in the liver [7]. Hepatocytes from 2-week old and adult mice were exposed to four hepatotoxic compounds. These were: paracetamol and furosemide, which are metabolised by the microsomal mixed function oxidase enzymes to toxic reactive metabolites [8, 9]; iodoacetic acid, which is a directly-acting toxin [10]; and *t*-butylhydroperoxide (t-BOOH), which initiates an oxidative stress in hepatocytes [11].

MATERIALS AND METHODS

Chemicals. RPMI 1640 culture media was obtained from Gibco (Grand Island, NY). Collagenase type II enzyme was purchased from Worthington Biomedical Corp., U.S.A. Iodoacetic acid was from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were obtained from Sigma (St Louis, MO).

Hepatocyte preparation. Hepatocytes were isolated from 2-week-old and adult (8–10 week old) Swiss mice (Animal Resources Centre, Murdoch, WA) by collagenase perfusion of the liver, as described previously [7]. The cells were suspended into RPMI 1640 culture media containing 20 mM HEPES, 100 I.U./ml penicillin, and 100 μg/ml streptomycin, pH 7.4. Following isolation, the viability of hepatocytes from adult and 2-week-old mice were 90 ± 4% and 92 ± 5% respectively (Trypan Blue

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† Abbreviations used: GSH, reduced glutathione; GSSG, glutathione disulphide; SOD, superoxide dismutase; t-BOOH, *t*-butylhydroperoxide; LDH, lactate dehydrogenase.

exclusion). Three-millilitre aliquots of cell suspension (12 mg wet weight cells/ml) were added onto collagen-coated culture dishes (55 mm dia.) prepared as described previously [12]. These were then placed in an incubator (Forma Scientific, Model 3164) and maintained at 37° in a humidified 95% air:5% CO_2 atmosphere. Hepatocytes were allowed to equilibrate in the incubator for 2 hr, after which they were washed with 2 × 3 ml phosphate buffered saline, pH 7.4, to remove non-viable cells. Viable cells adhered to the collagen and washing removed dead cells [7]. Microscopic examination of hepatocytes from both age groups showed > 99% were parenchymal cells. Hepatocytes were then incubated for up to 24 hr in the presence or absence of various concentrations of either paracetamol, furosemide, iodoacetic acid or t-BOOH in RPMI 1640 medium.

Assays. At various times after exposure to the toxins, aliquots of cell free supernatant were measured for leakage of lactate dehydrogenase (LDH) (EC 1.1.1.27). This activity was expressed as a percentage of total LDH activity, determined after treating the cells with Triton X-100 (0.01% final concentration) as described previously [12]. None of the hepatotoxins decreased total LDH activity, compared to controls, during their incubation period. Total LDH activity did not decrease during the 24 hr incubation period. Paracetamol metabolites were measured by HPLC analysis [5] in aliquots (0.1 ml) of cell free supernatant from culture plates containing hepatocytes incubated in 1.0 mM paracetamol.

Enzyme assays. Enzyme activities were measured in the cytosolic fractions of whole liver. Mice were sacrificed by cervical dislocation and organs were excised, weighed and placed in ice cold phosphate buffered saline. The organs were then homogenised (in the appropriate reaction buffer) using an Ultra-

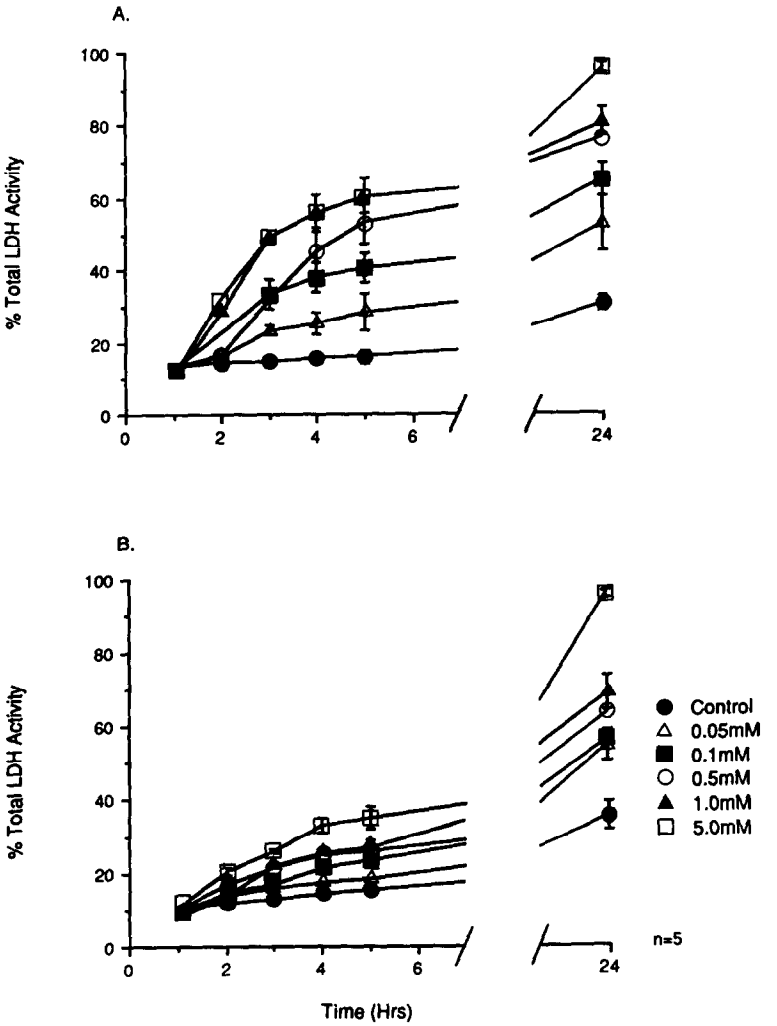

Fig. 1. The effect of paracetamol on the time course of LDH leakage from hepatocytes isolated from adult (A), and from 2-week-old mice (B). The LDH leakage from cells is expressed as activity in the cell-free supernatant as a percentage of the total activity. Hepatocytes from both age groups were exposed to paracetamol at concentrations of 0.0–5.0 mM, over a 24-hr incubation period. Individual data points represent the mean ± SE of 5 experiments.

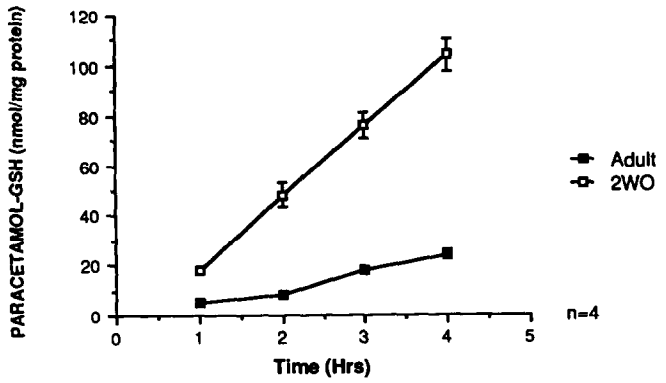

Fig. 2. Paracetamol-GSH metabolite released into the medium during incubation of isolated hepatocytes from 2-week-old (2WO) and adult mice, exposed to 1.0 mM paracetamol over a 4 hr period. Individual points represent the mean ± SE of 4 experiments.

Turrax homogeniser (Janke & Kunkel). Homogenates were then centrifuged at 100,000 *g* for 10 min at 4° in a Beckman TL-100 Ultracentrifuge. Enzyme activities were also measured in isolated hepatocytes. Cells were removed from culture dishes using a rubber policeman and resuspended in the appropriate reaction buffer. The resulting cell suspensions were sonicated for 20 sec (Branson Sonifier Cell Disruptor B15, Branson, U.S.A.), then centrifuged as for whole liver. Aliquots of cytosolic fraction were then assayed for enzyme activity. Protein was determined using the method of Hartree [13]. Catalase (EC 1.11.16) [14], SOD (EC 1.15.1.1) [15] and GSH-peroxidase (EC 1.11.1.9) [16] were measured using established procedures. GSSG-reductase (EC 1.6.4.2) was measured by a method adapted from Paglia and Valentine [16]. A 50 μl aliquot of sample was added to 2.95 ml reaction mixture containing 8.4 mM NADPH, 0.15 M oxidised glutathione (GSSG) and 1.125 M NaN_3 in 0.1 M Tris buffer (pH 7.0). Consumption of NADPH was then followed spectrophotometrically at 340 nm over 2 min at 25°.

Statistical analysis. Three-way analysis of variance was performed using the computer statistical package GENSTAT (Rothamsted Experimental Station, U.K.). If a significant variance ratio was indicated, individual group differences were investigated using a multiple range test (either Duncan's multiple range test or Dunnett's test) as previously described [17].

RESULTS

Hepatocytes from adult and 2-week-old mice were exposed to paracetamol over a concentration range of 0.0–5.0 mM for up to 24 hr (Fig. 1). The viability of control (non-exposed) cells from both age groups were similar during this incubation period ($F = 2.0$, $P > 0.05$). Three-way analysis of variance indicated significant effects of paracetamol concentration ($F = 88$, $P < 0.01$), exposure time ($F = 376$, $P < 0.01$) and age ($F = 201$, $P < 0.01$) on LDH leakage from hepatocytes from 2-week-old and adult mice. Onset of damage was faster in hepatocytes from adult mice. At concentrations of 0.1 mM and greater, paracetamol produced significant damage after 3 hr exposure when compared to control (Dunnett's test, $P < 0.05$), whereas no significant damage had occurred at any concentration in hepatocytes from 2-week-old mice at this time (Dunnett's test, $P > 0.05$). Damage did not occur in hepatocytes from the postnatal mice until after 4 hr of exposure, and then only to 5.0 mM paracetamol (Dunnett's test, $P < 0.05$).

Hepatocytes from the 2-week-old mice were capable of activating paracetamol to its reactive metabolite, as evidenced by their ability to form the paracetamol–GSH conjugate. The hepatocytes from the younger mice produced more of this conjugate than did adults (Fig. 2).

Furosemide-induced damage was studied over a concentration range of 0.0–1.0 mM for up to 24 hr in hepatocytes from both age groups (Fig. 3). Furosemide produced both concentration ($F = 136$, $P < 0.01$) and time dependent changes ($F = 657$, $P < 0.01$) on LDH leakage. Analysis of variance also demonstrated that hepatocytes from 2-week-old mice were less susceptible to hepatotoxicity induced by furosemide than were hepatocytes from adults ($F = 257$, $P < 0.01$). As with paracetamol, the onset of damage in hepatocytes from adults occurred after only 2 hr of exposure to furosemide, whereas in hepatocytes from 2-week-old mice onset of damage did not occur until 3 hr of exposure (Dunnett's test, $P < 0.05$).

Differences in susceptibility between 2-week-old and adult mice were also examined with the direct-acting toxin, iodoacetic acid (Fig. 4). Although the age-related difference with iodoacetic acid exposure does not appear as great as with either paracetamol or furosemide, analysis of variance indicated a significant effect of age ($F = 9.0$, $P < 0.05$). Again, hepatocytes from the two week-old mice were less susceptible to damage.

The susceptibility of hepatocytes from 2- and 3-week-old postnatal mice, as well as from adult mice, to an oxidative stress was examined by exposure to t-BOOH at concentrations of between 0 and 1.0 mM over a 3.5 hr exposure period (Fig. 5). As with the other toxins, there were significant effects of t-BOOH concentration ($F = 381$, $P < 0.01$) and

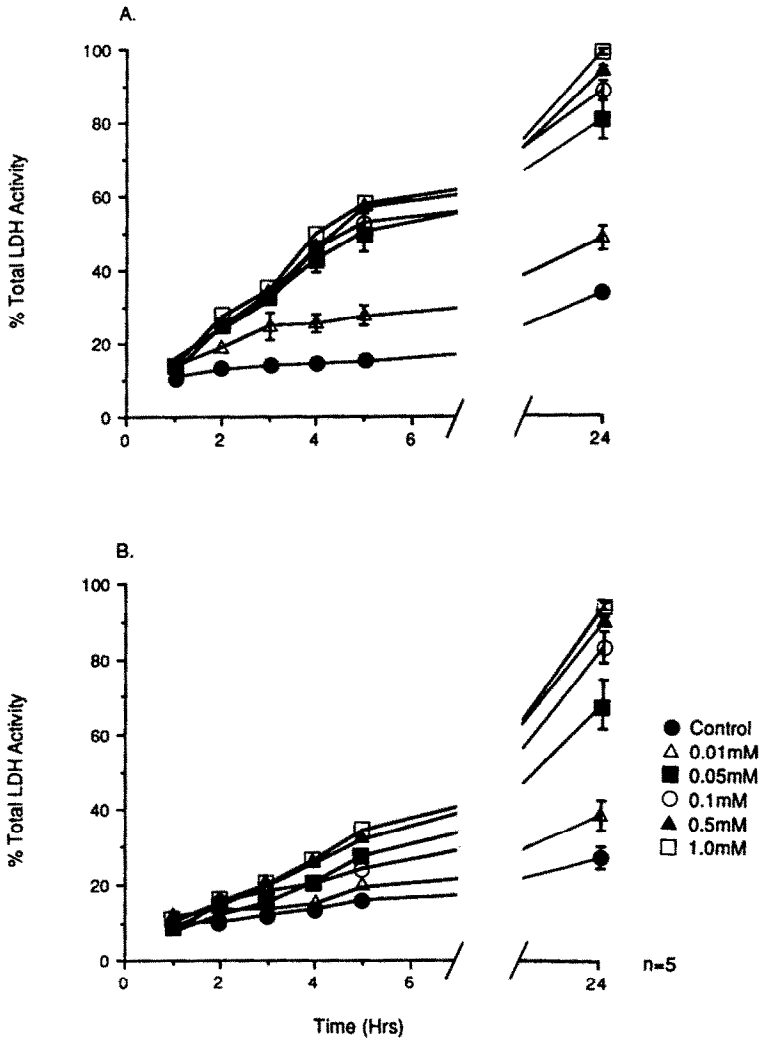

Fig. 3. The effect of furosemide on the time course of LDH leakage from hepatocytes isolated from adult (A), and 2-week-old mice (B). Cells were exposed to concentrations of 0.0–1.0 mM furosemide, over a 24 hr incubation period. Data points represent mean ± SE of 5 experiments.

exposure time ($F = 136$, $P < 0.01$) on LDH leakage. Also, there was a significant effect of age, with hepatocytes from the 2-week-old mice being less susceptible to the damaging effects of t-BOOH ($F = 20$, $P < 0.01$). It was found that hepatocytes from three week-old mice were intermediate in their toxic response to t-BOOH. The LDH leakage was greater than in two week-olds but less than in adults (Duncan's multiple range test, $P < 0.05$).

GSH-peroxidase, GSSG-reductase and SOD activities in hepatocytes from two week-old mice were greater than values found in those from adults (Table 1). In contrast, catalase activity was far less in hepatocytes from two week-old mice than in adults (Table 1). These age-related differences in activity were also found in whole liver (Table 1). Values for GSH-peroxidase and GSSG-reductase activities in hepatocytes from 3-week-old mice were intermediate to those seen in 2-week-olds and adults (Table 1). Differences in the activity of these enzymes were not due simply to differences in the protein content of liver cells from the three age groups. The protein contents of 2-week-old, 3-week-old and adult livers were 166 ± 2, 168 ± 6 and 178 ± 1 mg protein/g liver, respectively (N = 4). The corresponding values for isolated liver cells were 151 ± 4, 154 ± 5 and 165 ± 2 mg/g wet weight of cells (N = 4).

DISCUSSION

Hepatocytes from 2-week-old mice are less susceptible to the toxic effects of paracetamol, furosemide, iodoacetic acid and t-BOOH. These four agents exert their toxicity, at least in part, by different mechanisms. Hence, it is likely that the reduced susceptibility is a characteristic of the cells themselves, rather than a property of the interaction between the individual compounds and the cells.

For example, the reduced susceptibility of hepatocytes from the younger mice to paracetamol is not

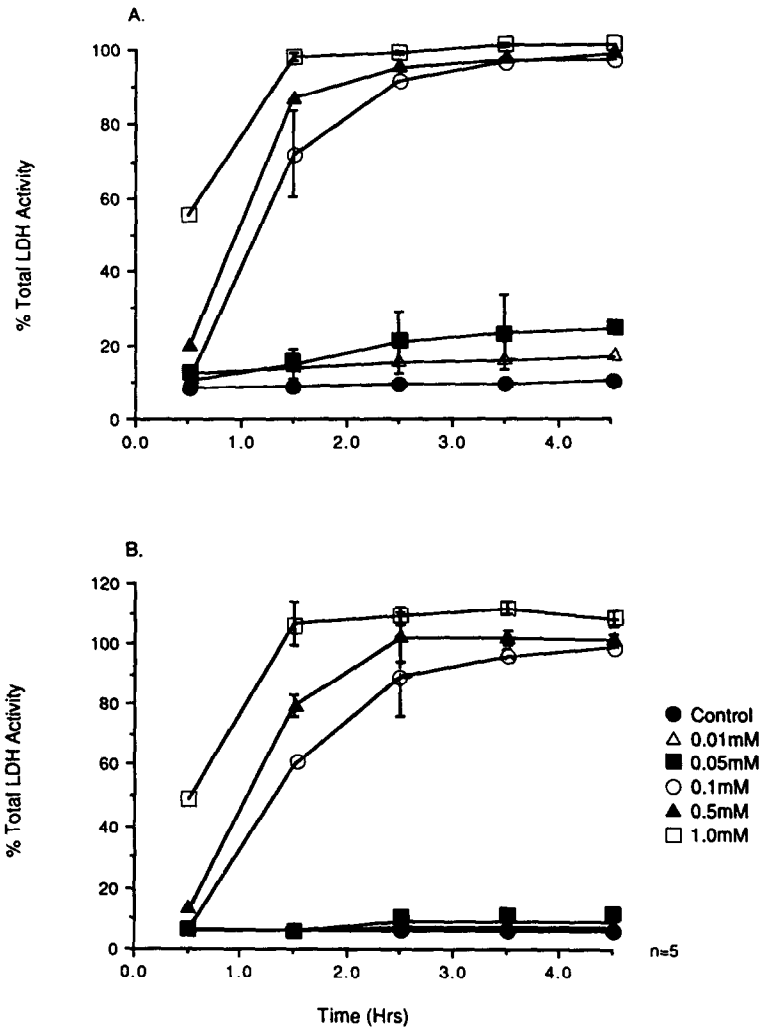

Fig. 4. The effect of iodoacetic acid on the time course of LDH leakage from hepatocytes from adult (A), and 2-week-old mice (B). Hepatocytes were exposed to iodoacetic acid concentrations of 0.0–1.0 mM, over a 4.5 hr incubation period. Individual points represent mean ± SE of 5 experiments.

due to an inability to activate this compound to its reactive metabolite. Indeed, hepatocytes from the 2-week-old mice produced more of the detoxification product of the reactive metabolite (the paracetamol-GSH conjugate) than did those from adults. In a previous study, it was found that the binding of the reactive metabolite of paracetamol to cellular protein was also greater in hepatocytes from postnatal mice compared to adults [5]. Hence, despite producing more toxic metabolite, as evidenced by both increased binding and conjugate formation, the hepatocytes from the younger mice were less susceptible to the toxic effects of paracetamol. This reduced susceptibility is consistent with previous findings that young rats of 11 days old [2, 3] and mice of 10 days old [4] were less susceptible to the hepatotoxic effects of paracetamol when compared to adults. In both these studies, the binding of the paracetamol reactive metabolite to liver protein was greater in the postnatal animals.

Oxidative stress may be involved in paracetamol toxicity. Antioxidants, such as 3-*O*-methylcatechin [18], vitamin E, promethazine and *N*,*N*′-diphenylphenylenediamine [19] can protect from the damage initiated by paracetamol. Further, the ferric ion chelator, desferrioxamine, which reduces susceptibility to oxidative stress, also inhibits paracetamol toxicity [20]. If reactive oxygen species were involved, then superior enzymic protective mechanisms in hepatocytes from the 2-week-old mice, even in the face of a higher concentration of reactive metabolite, may explain their reduced susceptibility.

In support of this, we found that iodoacetic acid was less toxic to the hepatocytes from the postnatal mice. Iodoacetic acid oxidises sulphydryl groups, including that of GSH-peroxidase [10, 21], and inhibits glycolysis [22]. It does not require metabolic activation to produce toxicity. Therefore, the reduced susceptibility may be due to factors in the cell that protect from the damaging effects of toxic agents.

Hepatocytes from the postnatal mice also showed

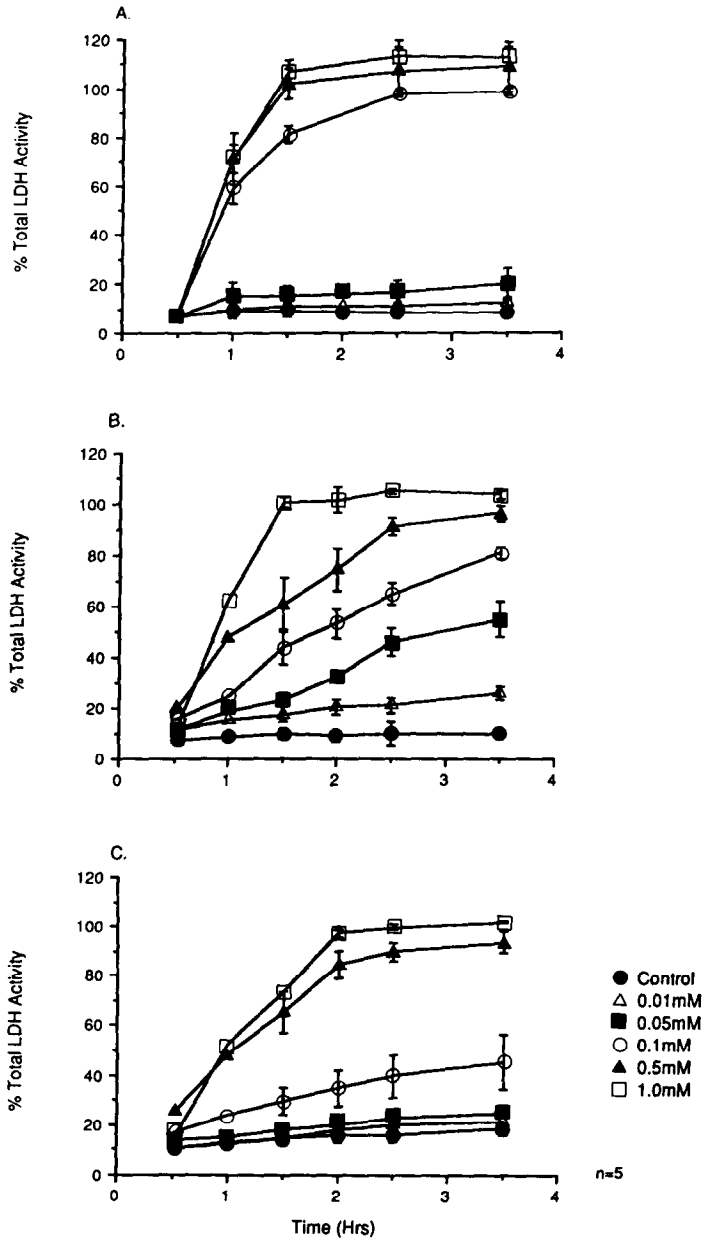

Fig. 5. The effect of t-BOOH on the time course of LDH leakage from hepatocytes isolated from adult (A), three week-old (B), and two week-old (C) mice. Hepatocytes were exposed to concentrations of 0.0–1.0 mM t-BOOH over a 3.5 hr period. Each point represents the mean ± SE of 5 experiments.

less susceptibility to oxidative stress induced by t-BOOH. Organic hydroperoxides are reduced to their corresponding alcohols by GSH-peroxidase. High concentrations of t-BOOH result in depletion of the GSH pool [11] and an inhibition of GSH-peroxidase [11]. The subsequent decrease in GSH-peroxidase activity and increase in free peroxides results in an oxidative stress in the cell. Hepatic GSH-peroxidase and GSSG-reductase activity in 2-week-old mice are approximately three fold greater than in adult liver. GSH-peroxidase activity in hepatocytes was also higher in the 2-week-old mice, demonstrating that a difference in peroxidase activity is maintained after hepatocyte isolation. This would suggest that hepatocytes from 2-week-old mice have a greater capacity for protection against the accumulation of toxic levels of lipid hydroperoxides or H_2O_2. SOD activity was also higher in 2-week-old mice than in adults, in both whole liver and in hepatocytes. Higher levels of SOD activity in the postnatal mice would also enhance protection against superoxide radical formation.

Furosemide is also activated by hepatic mixed function oxidase enzymes to a reactive metabolite [9]. It is thought to differ from paracetamol in that

Table 1. Activities of catalase, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-Rd) in whole liver and in isolated hepatocytes from 2-week-old (2WO), 3-week-old (3WO) and adult mice

		Whole liver	Hepatocytes
Catalase	2WO	88 ± 3*,¶	45 ± 1‖,¶
	3WO	62 ± 2¶	68 ± 4¶
	ADULT	384 ± 9	453 ± 39
SOD	2WO	14 ± 0.5*,¶	10 ± 1‖,¶
	3WO	3 ± 0.3	8 ± 1¶
	ADULT	11 ± 0.5	3 ± 1
GSH-Px	2WO	440 ± 14†,¶	550 ± 45§,¶
	3WO	260 ± 17¶	460 ± 40
	ADULT	151 ± 32	370 ± 35
GSSG-Rd	2WO	111 ± 15†,¶	55 ± 4§,¶
	3WO	36 ± 2¶	41 ± 6
	ADULT	27 ± 3	32 ± 5

All values are mean ± SE of 4 separate determinations. For determination of enzyme activity in isolated hepatocytes, cells were incubated for 4 hr in RPMI 1640 culture medium following isolation, prior to enzyme analysis.

* I.U./min/mg liver protein; † nmol NADPH consumed/min/mg liver protein; ‖ I.U./min/mg cell protein; § nmol NADPH consumed/min/mg cell protein; ¶ significantly different from adult, Dunnett's test, $P < 0.05$.

its reactive metabolite is not detoxified by GSH [9]. As was the case with paracetamol toxicity, hepatocytes from the 2-week-old mice were less susceptible to furosemide-induced damage. Therefore, it seems that protective mechanisms other than those dependent upon GSH conjugation may play a role in reducing susceptibility of hepatocytes from 2-week-old mice to hepatotoxic agents. This is consistent with our previous findings, that GSH-transferase activity is lower in livers from postnatal mice than in adult livers [23].

Catalase is unlikely to be an important enzyme in the protection against the toxicity of these four agents. Catalase activity is lower in the livers of 2-week-old mice compared to adults. Also, it has been shown that GSH-peroxidase is more important than catalase in detoxifying cytoplasmic H_2O_2 [24].

If these protective enzyme systems are a major factor responsible for the reduced susceptibility of hepatocytes from the 2-week-old mice, then it would be expected that as these enzyme activities approach those found in adult liver, the susceptibility to the toxins should be similar to that of the adult. We found that hepatocytes from 3-week-old mice were intermediate in their susceptibility to t-BOOH between the responses of 2-week-old and adult mice. This is consistent with the levels of GSH-peroxidase and GSSH-reductase found in the livers of these mice. At 3 weeks old the activity of these enzymes was intermediate between those of 2-week-old and adult mice.

In conclusion, it appears that liver cells from 2-week-old postnatal mice are less susceptible to the toxic effects of four hepatotoxins. This is consistent with *in vivo* findings that both young mice [4] and rats [2, 3] are less susceptible to the hepatotoxic effects of certain hepatotoxins. This reduced susceptibility may be the result of greater activity of hepatoprotective enzymes, namely GSH-peroxidase, GSSG-reductase and superoxide dismutase at this age.

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