Biochemical Pharmacology, Vol. 35, No. 10, pp. 1731–1735, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

AGE-RELATED TOXICITY OF PARACETAMOL IN MOUSE HEPATOCYTES

ANDREW W. HARMAN* and LORRETTA E. MCCAMISH
Department of Pharmacology, Queen Elizabeth II Medical Centre, The University of Western Australia, Nedlands, 6009 W.A., Australia

(*Received* 7 *August* 1985; *accepted* 2 *December* 1985)

Abstract—Hepatocytes from postnatal and adult mice were isolated by perfusion of the liver with a collagenase-containing bicarbonate buffer. These were allowed to attach to collagen-coated tissue culture dishes and were then examined for their susceptibility to paracetamol toxicity. After an 8 hr incubation in either 0.1 or 1.0 mM paracetamol, the extent of lactate dehydrogenase leakage and depletion of glutathione were similar in hepatocytes from young (1-, 2- and 3-week-old) mice when compared to adult mice. The covalent binding of [^{14}C]-paracetamol to protein was greater in the hepatocytes from young mice. The results indicate that while the amount of reactive metabolites free to react with cellular constituents is greater in hepatocytes from young mice, the amount of damage produced was not different than that found in those from adults.

Paracetamol causes hepatotoxicity in both man and animals when given in overdose. Paracetamol is oxidized by the hepatic microsomal mixed function oxidase enzyme system to a reactive metabolite, believed to be *N*-acetylbenzoquinoneimime [1, 2]. This metabolite is detoxified by conjugation with GSH.† However, after paracetamol overdose GSH stores are depleted and the reactive metabolite binds to other constituents of the liver cell and this is associated with the development of hepatic necrosis [3].

While much is known of the effects of paracetamol overdose in adults, information relating to the toxicity of paracetamol in children is limited. Despite its widespread use in paediatric medicine, there appears to be a low incidence of toxicity in children, although case reports of liver damage in infants are becoming more frequent [4–9]. The newborn human and many neonatal animals are deficient in their ability to metabolize a variety of drugs and chemicals [10]. Such a deficiency in the ability to oxidize paracetamol may render them less susceptible to its toxic effects. Little is known of the relative susceptibility of children to such reactive metabolites and studies in neonatal mice and rats have produced conflicting results. One study demonstrated that lethality of paracetamol was greater in young mice [11]. However, other evidence suggests that young rats and mice are less susceptible to the toxic effects of paracetamol [12–14]. Since age-related pharmacokinetic differences or age-related toxic effects on other organ systems may contribute to the lethal effect of a toxic compound, the contribution of liver damage to the overall toxicity *in vivo* can be difficult to assess. In order to examine the direct effect of paracetamol on the liver cell, the present study examines the toxicity and metabolism of paracetamol in isolated hepatocytes obtained from both young and adult mice in an attempt to determine how age-related factors may alter the response of these cells to high concentrations of paracetamol. We demonstrate that while age-related differences exist in metabolism, all age groups studied exhibit a similar toxic response to a high concentration of paracetamol.

* Author to whom correspondence should be sent.

† Abbreviations used: GSH, reduced glutathione; LDH, lactate dehydrogenase; PCA, perchloric acid; HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Chemicals. Collagenase (Worthington type CLS 11) and RPMI 1640 culture medium were obtained from Flow Labs Australasia (North Ryde, N.S.W.). [Ring-3,5-^{14}C]-paracetamol (acetaminophen; 46 mCi/mmol) was obtained from Amersham, IL, and purified by HPLC on a 30 cm × 3.9 mm (i.d.) μbondapak C_{18} column using a mobile phase of 0.5% acetic acid and a flow rate of 2 ml/min. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals. Swiss Webster mice were obtained from Animal Resources Centre (Murdoch, W.A.). Adult mice were housed in cages of not more than 5 per cage. Pregnant mice were housed separately and litters kept with their dams. The day of birth of each litter was recorded as the morning on which they were first observed. Mice were allowed food (Purina rat chow) and tap water *ad libitum* and were maintained in a constant temperature of 22° with lighting from 6.00 a.m. to 6.00 p.m.

Hepatocyte isolation. Hepatocytes were isolated from 1-, 2- and 3-week-old mice and from adult male (8–10 weeks) as follows: The mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital (250 mg/kg) containing heparin (1000 U/kg). A longitudinal incision was made into the abdomen and chest cavity. The vena cava was occluded with an artery clamp in a position rostral to the hepatic vein and the abdominal vena cava then cannulated with a 26 gauge needle. The portal vein

was then severed and the liver perfused in a retrograde fashion with a modified Hanks buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_3, 26 mM $NaHCO_3$, 20 mM HEPES, pH 7.3, 37°) containing 0.1 mM EGTA for 3 min. Perfusion was then continued for a further 8 min with modified Hanks buffer containing 1 mM $CaCl_2$ and either 0.4 mg/ml collagenase for 1-, 2- and 3-week-old mice or, 0.2 mg/ml collagenase for adult mice. Flow rates were controlled by a perfusion pump (Masterflex, Model 7013, Cole Palmer Instrument Co., Chicago, U.S.A.) and were 0.75, 0.90, 1.0 and 4.0 ml/min for 1-, 2-, 3-week-old and adult mice respectively. After perfusion the liver was excised and gently teased apart using a blunt spatula. The resulting cell suspension was filtered through two layers of nylon mesh (250 μm, 100 μm) to remove undigested material. The cell suspension was centrifuged at 40 *g* for 2 min and the cell pellet resuspended in Hank's buffer. The centrifugation and washing procedure was repeated three times. The cells were then resuspended into RPMI 1640 culture medium containing 10^{-4} M 5-aminolevulinic acid, 10^{-4} hydrocortisone-21-hemisuccinate, 10^{-6} M insulin, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 mM HEPES, pH 7.4. Three-ml aliquots of cell suspension (30 mg wet weight cells/ml) were added onto a thin coating of collagen on 60 mm diameter culture dishes prepared as described previously [15]. These were then placed in an incubator (Forma Scientific, Model 3164) maintained at 37° in a humidified 95% air : 5% CO_2 atmosphere.

Hepatocyte incubation. The hepatocytes were allowed to equilibrate for 4 hr, after which they were washed with 4 × 3 ml phosphate buffered saline to remove cells that had not attached to collagen. The remaining hepatocytes that had adhered to the dish were then incubated for up to a further 8 hr in either control media (no paracetamol) or media containing either 0.1 mM or 1.0 mM paracetamol.

Assays. At 1, 2, 4 and 8 hr after adding paracetamol, the leakage of the cytosolic enzyme LDH (EC 1.1.1.27) was determined by measuring its activity in an aliquot of cell free supernatant as a percentage of total activity after treating the cells with Triton X-100 (0.01% final concentration) using a method described previously [15]. The GSH content of the cells was determined in separate dishes. In this case, hepatocytes were washed with 3 ml phosphate buffered saline and then resuspended in 3.0 ml of ice-cold 0.3 M PCA. They were then transferred to a centrifuge tube, centrifuged at 3000 *g* for 10 min at 4° and an aliquot of the protein-free supernatant was assayed for GSH [16]. The DNA content of the resulting pellet was determined as described previously [17]. Paracetamol metabolites were determined in 0.1 ml aliquots of cell-free supernatant by a HPLC method [18] after incubating hepatocytes for 4 hr in 1.0 mM paracetamol containing 1.0 μCi of [^{14}C]-paracetamol. Covalent binding of radiolabelled paracetamol metabolites to protein was determined as previously described [15].

Statistical analysis. Analysis of variance was performed using the computer statistical package GENSTAT (Rothamsted Experimental Station, U.K.). If a significant variance ratio was indicated, differences between individual groups were investigated using a multiple range test (either Newman-Keuls or Dunnett's test) [19].

RESULTS

After isolation, the viability of the hepatocytes was between 50 and 70% for all age groups, as judged by Trypan Blue exclusion (0.1% w/v). Initial cell yields were 0.82, 0.94, 0.95 and 0.84 g wet weight of cells per g liver for 1-, 2-, 3-week-old and adult mice livers respectively. After 4 hr preincubation on collagen-coated tissue culture dishes, greater than 90% of the cells that had adhered to the collagen excluded Trypan Blue. The relationship between dry weight of cells (mg) : protein content (mg) : DNA content (μg) per number of cells for hepatocytes from adult mice was 8.8 mg : 3.2 mg : 33 μg per 10^6 cells. The corresponding relationships for hepatocytes from 1-, 2- and 3-week-old mice were 3.9 mg : 0.9 mg : 61 μg, 4.0 mg : 1.3 mg : 25 μg and 4.1 mg : 1.3 mg : 25 μg per 10^6 cells, respectively. The wet weight : dry weight ratio was 5:1.

At 1, 2, 4 and 8 hr of incubation with paracetamol the leakage of the cytosolic enzyme LDH was determined as an index of plasma membrane integrity. These data are shown in Fig. 1. Three classification analysis of variance was used to determine effects of paracetamol concentration, incubation time and age on the extent of LDH leakage. As the data suggests there were significant effects of paracetamol concentration ($F = 122$, $P < 0.001$) and incubation time ($F = 144$, $P < 0.001$) on the extent of LDH leakage. Damage increased with incubation time and the extent of LDH leakage was greater at 1.0 mM paracetamol compared to 0.1 mM. There was also an effect of age ($F = 12.6$, $P < 0.001$) and further analysis using a multiple range test indicated that the

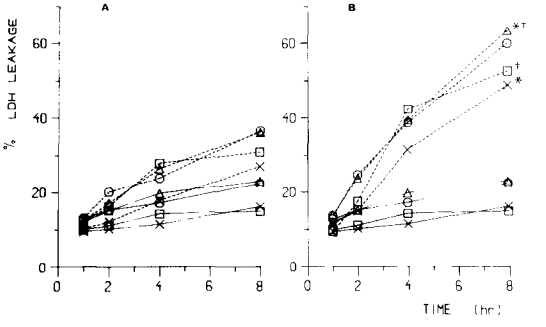

Fig. 1. The effect of paracetamol on the time course of LDH leakage from hepatocytes isolated from 1-week (×), 2-week (△), 3-week (○) old and adult (□) mice. The LDH leakage from the cells is expressed as activity in the cell-free supernatant as a percentage of the total activity. (A) Comparison of the effect of 0.1 mM paracetamol (broken line) and control (no paracetamol; continuous line). (B) Comparison of the effect of 1.0 mM paracetamol (broken line) and control (no paracetamol; continous line). Individual data points represent the means of 6 separate experiments. The standard error of the difference between any two means was 4.4. Significant differences between means are denoted with either (*) or (†), (Newman-Keuls test, $P < 0.05$).

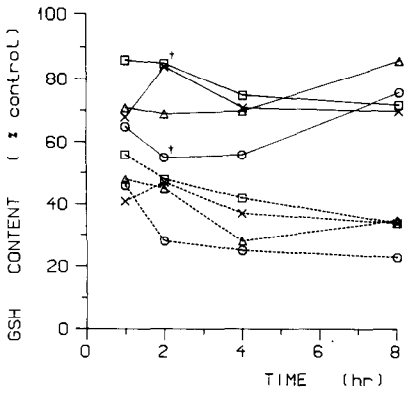

Fig. 2. The effects of either 0.1 mM (continous line) or 1.0 mM (broken line) paracetamol on the time course of GSH levels in hepatocytes isolated from 1-week (×), 2-week (△), 3-week (○) old and adult (□) mice. The GSH levels are expressed as a percent of corresponding controls not exposed to paracetamol. Individual data points represent the means of 4 separate experiments. The standard error of the difference between any two means is 9.3. Significant differences between means are denoted by (†) (Newman-Keuls test, $P < 0.05$).

only differences in LDH leakage were (a) between hepatocytes from 1- and 2-week-old mice, and (b) 2-week-old and adult mice both at the 8 hr incubation time after exposure to a 1.0 mM paracetamol concentration. Damage was not observed at concentrations at or below 0.05 mM (data not shown).

In similar experiments the effect of paracetamol on the content of intracellular GSH was assessed. These data are shown in Fig. 2. Data were expressed as percent of control (not exposed to paracetamol). The GSH contents in controls at the zero time point were 0.26 ± 0.01, 0.33 ± 0.04, 0.36 ± 0.02 and 0.63 ± 0.02 $\mu g/\mu g$ DNA (mean ± S.E.) for 1-, 2-, 3-week-old and adult mice, respectively. Three classification analysis of variance indicated significant effects of paracetamol concentration ($F = 207.0$, $P < 0.001$), incubation time ($F = 3.5$, $P < 0.05$) and

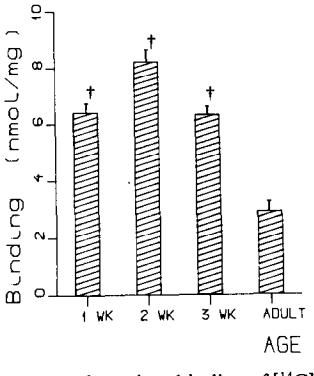

Fig. 3. The amount of covalent binding of [^{14}C]-paracetamol to hepatocytes isolated from 1-, 2-, 3-week-old and adult mice. Hepatocytes were incubated for 4 hr in 1.0 mM paracetamol containing 1.0 μCi of [^{14}C]-paracetamol. Results are mean ± S.E. of 4 separate experiments and are expressed in nmol paracetamol bound per mg protein. A (†) indicates significant difference from adults (Dunnett's test, $P < 0.05$).

Table 1. Paracetamol metabolites formed by isolated hepatocytes*

Age	Metabolites		
	Sulphate	Glucuronide	Glutathione
1 week	10 ± 1	17 ± 1†	45 ± 5
2 weeks	35 ± 5†	70 ± 17†	167 ± 55
3 weeks	23 ± 2	59 ± 19†	126 ± 24
Adult	13 ± 3	157 ± 34	57 ± 9

* Isolated hepatocytes from 1-, 2-, 3-week-old and adult mice were incubated for 4 hr in a medium initially containing 1.0 mM paracetamol. Values are mean ± S.E. of 4 separate experiments expressed in nmol/mg protein.

† Significantly different from adult, Dunnett's test, $P < 0.05$.

age ($F = 7.6$, $P < 0.001$). The fall in GSH was greater at the higher paracetamol concentration. Although there was an overall effect of incubation time, the GSH levels did not change appreciably after the initial fall at 1 hr. Further analysis of the differences between age groups using a multiple range test indicated only a minor effect, the only difference being between hepatocytes from three-week-old mice and adults after two hours of incubation in 0.1 mM paracetamol.

The extent of covalent binding of paracetamol metabolites to cellular protein was determined after incubating cells in [^{14}C]-paracetamol (Fig. 3). At 1.0 mM paracetamol binding of [^{14}C]-paracetamol metabolites to protein in adult mouse hepatocytes was 2.9 ± 0.4 nmol/mg. However, the binding in hepatocytes from 1-, 2-, 3-week-old mice was more than twofold greater than in adults.

Hepatocytes from all four age groups studied produced the three major hepatic metabolites of paracetamol (glucuronide, sulphate and glutathione conjugates). In addition, the cysteine conjugate was also detected and values for this have been included with those of the glutathione conjugate (since the cysteine conjugate is formed by the further metabolism of the glutathione conjugate). Table 1 shows the values for the metabolites obtained after incubation in 1.0 mM paracetamol for 4 hr. The amount of sulphate conjugate produced by hepatocytes from 2-week-old mice was greater than in adults. The ability of cells to form the glucuronide conjugate increased with age. The amount of this metabolite formed by the hepatocytes from young mice was significantly less than that formed by adults. On the other hand, glutathione conjugate formation in the young was at least as high as that found in adults.

DISCUSSION

The results indicate that hepatocytes isolated from young mice and from adult mice exhibit similar susceptibility to paracetamol toxicity. The toxicity was assessed by measuring the extent of leakage of LDH as an index of plasma membrane integrity. Paracetamol toxicity *in vivo* is characterized by an initial fall in intracellular GSH followed by the appearance of liver damage [3]. In each age group 0.1 mM and 1.0 mM concentrations of paracetamol produced a

fall in GSH levels which preceded the loss of plasma membrane integrity. Damage was greater at the higher concentration. Except in two instances, the extent of LDH leakage produced by paracetamol was similar in each age group at both paracetamol concentrations. Consistent with this, the extent of GSH depletion caused by paracetamol was essentially the same for each age group. Hence the effect of age on the toxic response of the hepatocytes to paracetamol is minor and the small number of differences seen do not follow a pattern that would suggest that any one age group responds differently to paracetamol toxicity.

The metabolism of paracetamol in hepatocytes was studied by determining the metabolites formed by either direct conjugation of paracetamol with sulphate and glucuronic acid, as well as the amount of paracetamol oxidized by the cytochrome P450 enzyme system to the reactive metabolite. The latter was estimated by measuring both the amount of glutathione conjugate formed and the binding of [^{14}C]-paracetamol metabolites to protein. It has been shown that the sulphation pathway for many compounds is fully developed in neonatal mice [20]. Consistent with this, hepatocytes from the young mice produced at least as much of the paracetamol–sulphate conjugate as did adults. The ability to form glucuronide conjugates is low in many animal species, including man and mouse [10]. In these experiments glucuronidation of paracetamol was low in hepatocytes from the 1-week-old mice and the rate increased with age. A similar pattern has been noted in humans where glucuronidation of paracetamol does not reach adult levels until after 9 years of age [21]. The rate of development of the cytochrome P450 enzyme system varies with different species. In the mouse, it is known to be low at birth, but develops rapidly during the first week [10]. Hepatocytes from 1-week-old mice were capable of oxidizing paracetamol and the hepatocytes from all three age groups of young mice produced at least as much of the paracetamol–glutathione conjugate as did adults. Furthermore, the amount of [^{14}C]-paracetamol metabolites bound to protein was more than twofold greater in these young hepatocytes compared to those from adults. So it appears that hepatocytes from young mice produce at least as much of the reactive metabolites as do adults. The value of 3 nmol paracetamol metabolites bound/mg protein in hepatocytes from adult mice found in the present study after 4 hr of incubation is similar to values reported by others in mouse hepatocytes [22] and *in vivo* [18].

It has been proposed that the extent of covalent binding of reactive metabolite is proportional to the amount of damage that results in the liver [23, 24]. This has been the basis of the theory that the damaging event in paracetamol toxicity is the binding of the reactive metabolite to critical macromolecules in the cell. Since the amount of binding is greater in hepatocytes from young mice in the present study, one would expect these cells to exhibit greater toxicity compared to adults. However, there is a growing body of evidence that suggests that the mechanism of paracetamol toxicity is not simply due to the interaction of the reactive metabolite with macromolecules. Experiments have dissociated this binding event from the toxicity that develops in the hepatocyte [15, 25, 26]. It may be that the extent of binding of reactive metabolite to protein is only an index of how much reactive metabolite is formed, rather than being an index of damage.

Results from other workers have shown that the developing liver may be resistant to the damaging effect of some hepatotoxins. Despite the fact that the neonatal rat appears to be capable of oxidizing paracetamol to its reactive metabolite to a greater extent than the adult rat, it was found that it exhibited lower susceptibility to the hepatotoxic effects of this drug, as well as to two other hepatotoxins, bromobenzene and tannic acid [14]. In the present study, the binding of the paracetamol reactive metabolite to protein in hepatocytes from young mice was greater and the amount of glutathione conjugate formed was at least as great as in adults, yet the toxic response to paracetamol in all age groups was similar. This suggests that the amount of reactive metabolite free to bind to cellular macromolecules does not necessarily correlate with the extent of damage and that the sensitivity of the tissue to attack by electrophilic metabolites may be a function of other factors. Differences in susceptibility in the young animal may be related to the altered rates of many of the cellular processes that occur during development. For example, during the developmental stage the metabolic rate of the liver is higher [27]. A greater rate of protein turnover may enable the cells to repair damage more effectively. Another explanation may be that protective mechanisms in the cell play an important role in protecting young liver cells. Evidence suggests that the rate of GSH resynthesis is greater in hepatocytes from the younger animals such as the rat [28] and mouse (unpublished observation). An understanding of how these factors influence the toxic response in the young animal is required to enable one to make predictions on differences in susceptibility between the young and adult animal.

Acknowledgements—This work was supported by grants from the TVW Telethon Foundation of W.A. and the National Health and Medical Research Foundation.

REFERENCES

1. J. Canalese, A. E. S. Gimson, M. Davis and R. Williams, *Br. Med. J.* **282**, 199 (1981).
2. D. J. Miner and P. T. Kissinger, *Biochem. Pharmac.* **28**, 3285 (1979).
3. D. J. Jollow, S. S. Thorgeirsson, W. Z. Potter, M. Hashimoto and J. R. Mitchell, *Pharmacology* **12**, 251 (1974).
4. G. A. D. McPherson, I. S. Benjamin and L. H. Blumgart, *Lancet* **2**, 1153 (1982).
5. N. L. Golden, K. C. King and R. J. Sokol, *Clin. Pediatrics* **21**, 752 (1982).
6. S. Lederman, W. J. Fysh, M. Tredger and H. R. Gamsu, *Arch. Dis. Child.* **58**, 631 (1983).
7. G. B. Hickson, J. W. Greene, F. K. Ghishan and L. T. Craft, *Am. J. Dis. Child.* **137**, 917 (1983).
8. P. F. Agran, K. E. Zenk and S. G. Romansky, *Am. J. Dis. Child* **137**, 1107 (1983).
9. S. M. Swetnam and A. L. Florman, *Clin. Pediatrics* **23**, 104 (1984).

10. W. Kingler, *Pharmac. Ther.* **6**, 337 (1982).
11. E. Alhava, K. Hassinen and E. Nieminen, *Acta pharmac. toxic.* **42**, 317 (1978).
12. J. G. Hart and J. A. Timbrell, *Biochem. Pharmac.* **28**, 3015 (1979).
13. R. E. Mancini, B. R. Sonawane and S. J. Yaffe, *Res. Comm. Chem. Path. Pharmac.* **27**, 603 (1980).
14. M. D. Green, T. K. Shires and L. J. Fischer, *Toxic. appl. Pharmac.* **74**, 116 (1984).
15. A. W. Harman and L. J. Fischer, *Toxic. appl. Pharmac.* **71**, 330 (1983).
16. P. J. Hissin and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
17. S. L. Cookson and D. O. Adams, *J. Immunol. Methods* **23**, 169 (1978).
18. L. J. Fischer, M. D. Green and A. W. Harman, *J. Pharmac. exp. Ther.* **219**, 281 (1981).
19. H. Grimm, in *Biostatistics in Pharmacology*, Vol. 2, p. 675. Pergamon Press, Oxford (1973).
20. A. K. Percy and S. J. Yaffe, *Pediatrics* **33**, 965 (1964).
21. R. P. Miller, R. J. Roberts and L. J. Fischer, *Clin. Pharmac. Ther.* **19**, 284 (1976).
22. T. E. Massey and W. J. Racz, *Toxic. appl. Pharmac.* **60**, 220 (1981).
23. J. R. Mitchell and D. J. Jollow, *Gastroenterology* **68**, 392 (1975).
24. J. R. Mitchell, C. V. Smith, B. H. Lauterberg, H. Hughes, G. B. Corcoran and E. C. Horning, in *Drug Metabolism and Drug Toxicity* (Eds. J. R. Mitchell and M. G. Horning), p. 301. Raven Press, New York (1984).
25. J. L. Devalia, R. C. Ogilvie and A. E. M. McLean, *Biochem. Pharmac.* **31**, 3745 (1982).
26. R. J. Gerson, A. Casini, D. Gilfor, A. Serroni and J. L. Farber, *Biochem. biophys. Res. Comm.* **126**, 1129 (1985).
27. R. D. Conde and D. A. Scornik, *Biochem. J.* **166**, 115 (1977).
28. B. H. Lauterberg, Y. Vaishnav, W. G. Stillwell and J. R. Mitchell, *J. Pharmac. exp. Ther.* **213**, 54 (1980).