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PARACETAMOL-INDUCED HEPATIC NECROSIS IN THE MOUSE—RELATIONSHIP BETWEEN COVALENT BINDING, HEPATIC GLUTATHIONE DEPLETION AND THE PROTECTIVE EFFECT OF α-MERCAPTOPROPIONYLGLYCINE

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Abstract—The severity of hepatic necrosis in mice dosed with 3.31 m-mole/kg paracetamol was significantly greater than after a 2.65 m-mole/kg dose, and the quantity of a reactive metabolite of the drug bound to hepatocyte macromolecules was significantly greater after the higher dose. α-Mercaptopropionylglycine (α-MPG), a sulphydryl group-containing compound, together with 3.31 m-mole/kg paracetamol afforded significant protection against hepatic necrosis, as assessed histologically, but had no significant effect on the quantity of paracetamol metabolite bound within the liver. α-MPG did not increase levels of reduced glutathione in the liver, nor did it prevent the fall in hepatic glutathione content observed in animals receiving hepatotoxic doses of paracetamol without the protective agent. Furthermore, the compound had no effect on the metabolism of paracetamol, as judged by the urinary excretion of conjugates of the drug. It is concluded that binding of the reactive metabolite of paracetamol to hepatocyte macromolecules need not lead to hepatic necrosis provided that sulphydryl groups are present in the liver to prevent the deleterious effects of such binding. This appears to be the mode of action for α-MPG.

It is now well established that the hepatotoxicity of paracetamol is related to the cytochrome P-450-linked metabolism of the drug to a reactive metabolite, which binds to liver cell proteins [1, 2]. Such binding occurs when hepatic levels of reduced glutathione have become depleted [3], as a result of increased production of the cysteine and mercapturic acid conjugates of paracetamol [4], which have as their precursor a reactive intermediate which binds to glutathione in the liver.

This has provided the rationale for administering precursors of glutathione, or sulphydryl group-containing compounds, on the basis that these will either increase hepatic stores of reduced glutathione or substitute for this substance in the liver. Thus, cysteine [5], methionine [6], and cysteamine [7] have all been shown to diminish hepatocellular necrosis after high doses of paracetamol in rats and mice. Initial clinical experience with cysteamine has been encouraging [8], although the compound is not without unpleasant side-effects and, as it is not available as a pharmaceutical preparation, infusions have to be made up for individual patients. Our preliminary studies have shown that another sulphydryl-containing compound. α-mercaptopropionylglycine (α-MPG) also reduces the hepatotoxicity of paracetamol in mice. This substance is widely used on the continent for the treatment of various hepatic disorders, without reported side-effects. The present paper reports an investigation into its mode of action.

MATERIALS AND METHODS

Male albino mice (30–35 g) from an inbred colony at King's College Hospital were used throughout. Paracetamol (Sigma) dissolved in saline (150 m-mole/l) was given orally after a 2½-hr fast in doses of 2.65 or 3.31 m-mole/kg. Food was restricted for a further 2 hrs, and the effect of α-mercaptopropionylglycine (Santen Pharmaceuticals Ltd., Japan) was investigated in a further group of mice given a 3.31 m-mole/kg dose of paracetamol. α-MPG was given intraperitoneally (i.p.) at 0,2,4, and 6 hr after paracetamol in a dose of 2.63 -mole/kg diluted in saline (150 mmol/l) to give a final injection volume of 0.3 ml.

The mice were killed 48 hr after the administration of paracetamol by cervical dislocation. Livers were immediately removed, sliced, and fixed in formol saline prior to histological assessment. The extent of hepatic necrosis was graded into four categories by a histopathologist (B.P.), who was unaware of the treatment groups. Liver lesions were sometimes patchily distributed, and at least six sections from different parts of each liver were examined to establish an overall grading. Thus, animals classified as Grade 0 had normal hepatic histology on all sections examined, while those with Grade I had centrilobular necrosis affecting less than 25 per cent of the lobule as their most severe lesion. Those with confluent necrosis joining adjacent centrilobular areas were classified as Grade II, and Grade III indicated predominantly massive necrosis, with survival of only a thin rim of periportal hepatocytes.

Covalent binding studies. Covalent binding of the reactive metabolite of paracetamol to hepatocyte macromolecules was investigated in three groups of mice. One group was given paracetamol 3.31 m-mole/kg with i.p. saline (0.3 ml) at 0,2,4, and

6 hr after paracetamol alone, a second received paracetamol 3.31 m-mole/kg with i.p. α-MPG (2.63 m-mole/kg) at 0,2,4, and 6 hr after paracetamol, and a third received paracetamol in a dose of 2.65 m-mole/kg with intraperitoneal saline as above. In all these animals, the administered paracetamol was given with 2 μCi of the chromatographically pure ^{14}C ring-labelled drug (prepared by Dr. R. S. Andrews of Sterling–Winthrop Group Ltd). Animals were killed at 0.5, 1,2,4,6, and 24 hr after administration of paracetamol, and covalent binding determined as described by Jollow *et al.* [1], after protein precipitation and multiple solvent extractions.

The protein precipitate (200 mg) was dissolved in 2 ml Packard Soluene and 10 ml scintillation fluid containing 80% Toluene, 20% Triton, 2.5-diphenoxazole (PPO; 5 g/l) and 0.5 g/l bis-MSB in a Packard Tricarb Scintillation Counter. Radioactivity was corrected for background and quench (internal standardisation with [^{14}C]toluene) and reproducibility was ±10%. The quantity of paracetamol metabolite covalently bound was calculated by reference to the specific activity of the administered drug.

Hepatic glutathione concentrations. Two groups of mice received paracetamol (3.31 m-mole/kg), with either i.p. α-MPG at 0,2,4, and 6 hr, or i.p. saline. Mice were killed at 1,2,4,5,6, and 24 hr after paracetamol for estimation of hepatic reduced glutathione, by the method of Tietze [9]. The presence of α-MPG was shown not to interfere with the glutathione assay.

Glutathione was also determined in two further groups of animals, which did not receive paracetamol, but were given four 2-hourly i.p. injections of α-MPG (2.63 m-mole/kg), or saline.

Urinary paracetamol conjugates. Two groups of mice were given ^{14}C labelled paracetamol (3.31 m-mole/kg, 2 μCi per mouse) with and without α-MPG in the same doses as above, and were housed in metabolic cages for 24 hr so that urine could be collected. Total urinary paracetamol conjugates were determined by counting 0.1 ml aliquots of each specimen after the addition of 0.9 ml distilled water, and 9 ml of the scintillation medium above. Individual conjugates were isolated using two-dimensional thin-layer chromatography (Andrews *et al.* [10] and following location under short-wave u.v. light, each conjugate was scraped off the plate into a counting vial containing the scintillation fluid. Background and quench corrections were made as above, and the relative proportions of each metabolite calculated. Student's *t* test or the chi-square test were used for statistical analysis.

RESULTS

α-Mercaptopropionylglycine afforded significant protection against the hepatotoxicity of paracetamol when given in doses of 3.31 m-mole/kg (Table 1). The extent of hepatic necrosis following a lower dose of paracetamol (2.65 m-mole/kg) was less severe than after the 3.31 m-mole/kg dose, but slightly greater than that associated with the high dose given together with the protective agent.

The quantity of ^{14}C labelled paracetamol bound to hepatocyte macromolecules increased progressively with time in all groups of animals studied to reach peak values at 2–4 hr after the administration of the drug. In keeping with the less severe hepatic necrosis produced by 2.65 m-mole/kg paracetamol, covalent binding was significantly less after this dosage than that observed after 3.31 m-mole/kg dose (Fig. 1).

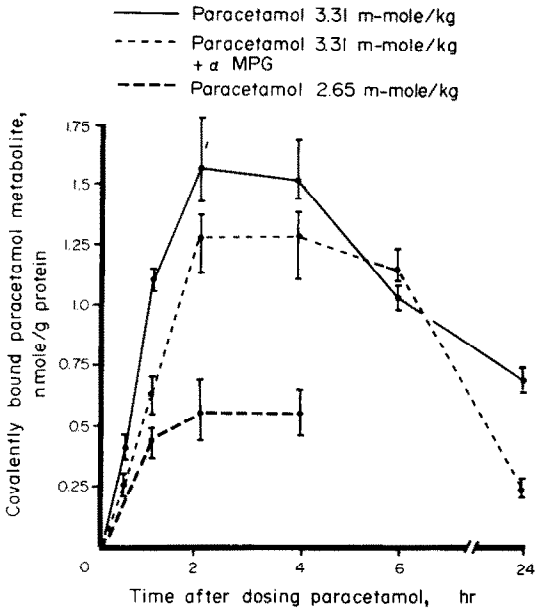

Fig. 1. The effect of α-MPG (2.63 m-mole/kg at 0,2,4,6, hr) on covalent binding following oral paracetamol administration. Each point represents mean ± S.E.M. for four animals.

Table 1. Hepatic histology in mice given paracetamol with and without α-mercaptopropionylglycine

	Paracetamol (m-mole/kg)		
	2.65		3.31 + α-MPG 2.63 3.31m-mole/kg at 0,2,4,6 hr
Hepatic necrosis	Number and (%) of animals		
Grade 0–1	15 (62.5%)	5 (21%)*	20 (100%)†‡
Grade II–III	9 (37.5%)	19 (79%)	0 (0%)
	N = 24	N = 24	N = 24

* $p < 0.01$ compared with 2.65 m-mole/kg.
† $p < 0.005$ compared with 3.31 m-mole/kg without α-MPG.
‡ $p < 0.05$ compared with 2.65 m-mole/kg.

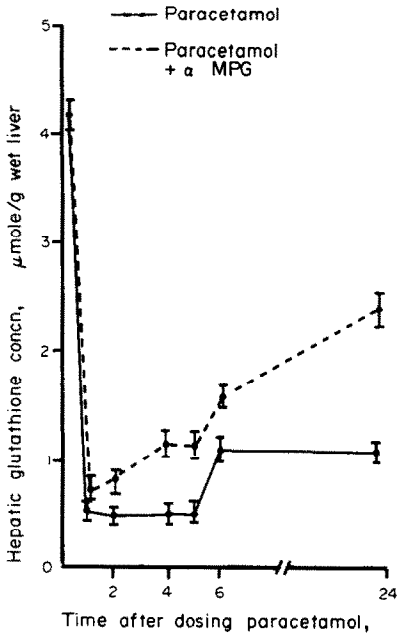

Fig. 2. The effect of α-MPG (2.63 m-mole/kg at 0,2,4,6 hr) on hepatic glutathione levels following oral paracetamol administration (3.31 m-mole/kg). Each point represents mean ± S.E.M. for four animals.

However, despite the significant histological protection afforded by α-MPG, covalent binding in animals receiving this agent did not decrease in proportion, and values at 2 and 4 hr, although slightly lower in the α-MPG treated group, were not significantly different from those receiving paracetamol (3.31 m-mole/kg) alone (1.30 ± 0.02 m-mole/kg protein and 1.50 ± 0.20, respectively, at 4 hr). Similarly, the quantity of reactive metabolite bound after paracetamol 3.31 m-mole/kg and α-MPG was at all times significantly greater than that observed after a 2.65 m-mole/kg dose of paracetamol given without α-MPG. When covalent binding was measured at 24 hr, however, significantly lower levels were seen in mice given paracetamol (3.31 m-mole/kg) together with α-MPG than in those receiving this dose of paracetamol alone (0.30 ± 0.06 m-mole/g protein and 0.70 ± 0.06 respectively, $p < 0.001$).

Serial measurements of hepatic-reduced glutathione showed that levels had fallen to less than 15 per cent of control values an hour after giving the larger dose of paracetamol, and a similar precipitous drop was observed in those who also received α-MPG (0.50 ± 1 S.E.M. 0.05 μmole/g wet liver and 0.54 ± 0.04 respectively, $p > 0.1$) (Fig. 2). However, while these low levels were maintained for 5 hr in the group receiving paracetamol alone, in the animals treated with α-MPG hepatic glutathione content had increased significantly after 4 hr to 1.30 ± 0.15 μmole/g wet liver ($p < 0.005$) compared with the group not receiving α-MPG. Twenty-four hr after the administration of paracetamol, concentrations had returned to 48 per cent of initial values in mice given α-MPG (2.40 ± 0.14 μmole/g wet liver), whereas in those controls given paracetamol alone, levels were only 25 per cent of normal (1.05 ± 0.09 μmole/g wet liver, $p < 0.001$).

Hepatic glutathione concentrations in animals not receiving paracetamol were not significantly altered by the administration of α-MPG (controls 4.20 ± 1 S.E.M. 0.16 μmole/g wet liver, α-MPG-treated 4.90 ± 0.50, $p > 0.1$).

The urinary excretion of total paracetamol conjugates during the first 24 hr after administration of the drug was similar in the control animals and in those given α-MPG, at 85–90 per cent of the administered dose. Administration of α-MPG did not give rise to any new urinary conjugates of paracetamol, and the pattern of urinary metabolites was similar to that seen in animals receiving paracetamol alone (Fig. 3). Although there was a slight increase in the proportion appearing as cysteine and mercapturic acid conjugates in the α-MPG group compared with controls 16 and 12 per cent of the total drug excreted respectively), and there was a concomitant decrease in the proportion of free paracetamol (13 and 17 per cent respectively), in neither instance did these differences reach statistical significance.

DISCUSSION

The pattern of events observed after hepatotoxic doses of paracetamol in the present study is similar to that previously described [1, 3] with rapid deple-

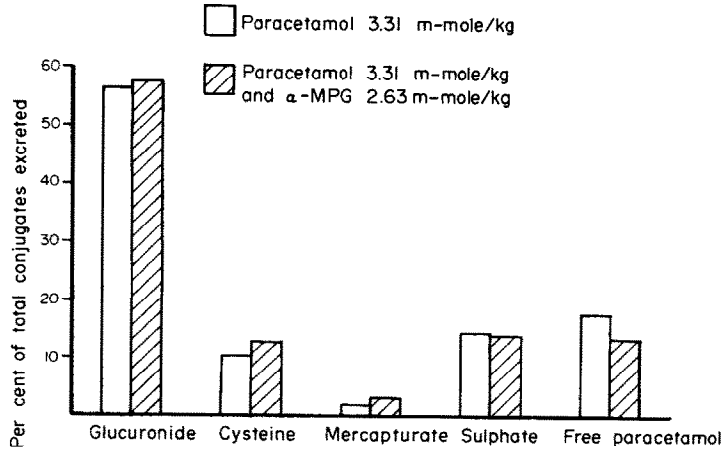

Fig. 3. The percentage excretion of paracetamol conjugates in controls and animals treated with α-MPG.

tion of reduced glutathione in the liver, associated with the binding of a metabolite of the drug to hepatocyte macromolecules. Similarly, the higher, more hepatotoxic doses of the drug, when given without the protective agent, gave rise to a greater degree of binding than the lower dose, which produced less liver damage. It is of considerable interest, therefore, that while α-MPG protected significantly against hepatic necrosis, it had little effect on covalent binding. If this had been reduced in proportion to the degree of protection conferred, binding comparable to, or even less than, that seen after the lower dose of paracetamol would have been expected. These findings contrast markedly with those of Mitchell *et al.* [7] for cysteamine, as the protective effect of this compound was parallelled by a decrease in the quantity of the reactive paracetamol metabolite bound within the liver.

The nature of the paracetamol metabolite which binds to liver cell macromolecules is not known, but it is thought to be an unstable oxide of the drug [1], and its formation appears to be mediated by microsomal P-450-dependent mixed function oxidases [2]. Both α-MPG and cysteamine are sulphydryl group-containing compounds and similar substances have been shown to be capable of combining with, and inhibiting the action of, haem-containing enzymes [11] such as cytochrome P-450, and the effect of this would be to diminish the formation of the reactive metabolite of paracetamol. Alternatively, such compounds could act, by virtue of their sulphydryl groups, as reducing agents to convert the reactive metabolite back to the parent drug. However, while both these mechanisms could explain the action of cysteamine, they are unlikely to be important for α-MPG. Not only would a decrease in the binding of the reactive metabolite within the liver have been expected, but also a decrease in the proportion of the drug excreted as the cysteine and mercapturic acid conjugates, which are formed via this intermediate, and neither of these changes were observed. Furthermore, the failure to demonstrate any new urinary conjugates after administration of α-MPG excludes the possibility that the compound was conjugating directly with the reactive metabolite of paracetamol.

Previous studies have shown that administration of cysteamine to mice results in an increased hepatic content of reduced glutathione [12], either by acting as a precursor of this compound, or by stimulating its release from stores, such as mixed disulphide complexes with liver proteins. In marked contrast, no increase in hepatic glutathione content was observed in the present studies after the administration of α-MPG indicating a further difference in action between this compound and cysteamine. Furthermore, α-MPG failed to prevent the fall in hepatic glutathione concentration observed in animals receiving paracetamol without this protective agent. In contrast, studies using cysteamine have indicated that this compound apparently leads to a preservation of glutathione levels within the liver [5]. However, in these latter studies no information was given on the potential interference of cysteamine in the assay used for glutathione and, because of this, artefactually high estimates for hepatic glutathione cannot be excluded.

It appears from the present study, therefore, that α-MPG acts by preventing deleterious effects on hepatocellular function caused by binding of the reactive paracetamol metabolite, rather than by modifying its production or elimination. Such a mode of action would allow hepatocytes to continue glutathione and protein synthesis at more normal rates than those damaged by the reactive paracetamol metabolite. This would explain why the rates of decrease in covalent binding and restitution of glutathione levels were more rapid and complete in the livers of animals receiving α-MPG.

It has previously been assumed that the factor determining the degree of hepatocellular injury after paracetamol overdose is the total quantity of reactive metabolite of the drug which becomes bound to macromolecules within liver cells. However, the present findings indicate that this concept must be an oversimplification. One explanation for our results is that only a very small proportion of the reactive metabolite bound to certain macromolecules, is responsible for initiating cell necrosis, and α-MPG may act specifically by preventing binding at these sites. Alternatively, the compound may act by stabilising cell constituents against the effects of binding, in a manner analogous to that proposed for reduced glutathione after administration of carcinogens [13] and carbon tetrachloride [14].

Indeed, it is possible that hepatic reduced glutathione itself might have the capacity to afford such protection after paracetamol overdose. This would explain an earlier experimental observation that a small quantity of bound paracetamol metabolite could be detected in the livers of rats given doses of the drug which did not lead to histological evidence of hepatic necrosis [15]. However, following hepatotoxic doses of paracetamol, glutathione is predominantly utilised for conjugation with the reactive metabolite of paracetamol, and the depleted levels of this compound may then be inadequate to afford significant protection. One possible mode of action for α-MPG would be to substitute for hepatic reduced glutathione in this latter function.

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