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SHORT COMMUNICATIONS

Differences in glutathione synthesis and glutathione-*S*-transferase activities in hepatocytes from postnatal and adult mice

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While it has been shown that many drugs and chemicals can be activated in the liver to electrophilic metabolites capable of producing hepatic damage, little is known of the susceptibility of young animals to this kind of toxicity. Compounds such as paracetamol and bromobenzene, which are activated to reactive arylating metabolites, are thought to be less toxic to the postnatal rat [1, 2] and mouse [3]. We have recently shown that while the binding of the reactive metabolite of paracetamol to protein in hepatocytes from postnatal mice was twofold greater than in adults, there was no concomitant increase in toxicity [4]. Hence, it appears that the tissues of young animals are less susceptible than those of adults to the toxic effects of these electrophilic metabolites. The present study investigated the role that glutathione (GSH) may play in protecting young mice from hepatotoxic metabolites. GSH has the ability to limit chemically induced injury to cells. It can act as a reductant with GSH peroxidase to detoxify H_2O_2 and organic hydroperoxides [5]. Alternatively, it can detoxify reactive metabolites via the GSH-*S*-transferase enzyme system. These are a family of inducible enzymes with differing substrate specificities that can act on a variety of substrates [6]. They can detoxify electrophilic drug metabolites by catalysing the formation of glutathione conjugates. This enzyme system relies on adequate amounts of GSH being available to conjugate with these reactive species. It has been found that depletion of GSH in the presence of such reactive metabolites is rapidly followed by their binding to other sites in the cell, and eventually cell death [7, 8]. Hence, the ability of the cell to maintain adequate GSH stores is of great importance. In the face of a toxic insult by an agent that depletes GSH, the level of GSH will be determined by both its rate of utilization and its rate of synthesis. We have measured two aspects of the role GSH might play in protecting young animals. Firstly, the activity of the GSH-*S*-transferases were measured in livers from postnatal and adult mice, and secondly, the ability of hepatocytes from these animals to resynthesize GSH following depletion with diethylmaleate (DEM) was determined as an index of the rate of GSH synthesis. Enhanced activity of either of these two systems in young animals may play a role in protecting hepatocytes from electrophilic metabolites.

Materials and methods

Swiss mice were obtained from Animal Resources Centre (Murdoch, W.A.). Collagenase (Worthington Type CLSII) and RPMI 1640 culture medium were obtained from Flow Labs Australasia (North Ryde, N.S.W.). GSH, 1-chloro-2,4-dinitrobenzene (CDNB), *p*-nitrobenzylchloride (*p*-NBC) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (ENPP) and DEM were obtained from Sigma Chemical Co. (St. Louis, MO).

Hepatocytes were isolated from 1-, 2-, 3-week-old and adult male mice (8–10 weeks) as described previously [4]. After isolation they were suspended into RPMI 1640 medium containing 0.1 mg/ml streptomycin, 100 U/ml penicillin and 20 mM HEPES, pH 7.4, to a concentration of 30 mg wet weight/ml. Aliquots (1.5 ml) were added onto a thin layer of collagen on 25 mm diameter Linbro culture dishes (Flow Labs, Australasia) prepared as described previously [9]. These were incubated at 37° in a humidified 95% air:50% CO_2 atmosphere in an incubator (Forma Scientific, Model 3164). After 4 hr the dead cells were removed by washing with 4 × 2 ml phosphate buffered saline (PBS), pH 7.4, and fresh culture medium added.

GSH resynthesis was determined essentially as described by Hogberg and Kristoferson [10]. Hepatocytes were exposed to freshly distilled DEM (0.1 μl/ml) in 10 μl of dimethylsulphoxide and replaced in an incubator. Controls received dimethylsulphoxide only. After 10 min the cells were washed with 2 × 2 ml PBS and incubation continued. At various times (0, 10, 20, 30, 40, 60 min) cells were removed from the incubator and quickly washed with 2 ml PBS followed by addition of 1 ml ice-cold 0.3 M perchloric acid. The cells were scraped from the dish with a rubber policeman and transferred to a glass centrifuge tube using a pasteur pipette. The dish was rinsed with another 1 ml of perchloric acid and this was added to the first. After centrifugation at 3000 *g* for 15 min at 4°, the protein-free supernatant was assayed for GSH using the method of Hissin and Hilf [11]. To determine GSH-*S*-transferase activities, the livers of mice from the same age groups were quickly perfused *in situ* with ice-cold 0.15 KCl, excised and homogenized in 4 vol. of 0.25 M sucrose using an Ultraturrex blender. This was centrifuged at 10,000 *g* for 30 min and the supernatant centrifuged at 105,000 *g* for 60 min. The cytosolic fraction was assayed for GSH-*S*-transferase activities which were determined kinetically at 37° as described by Clifton *et al.* [12] for *p*-NBC and ENPP and by Habig *et al.* [6] for CDNB. Protein content [13] and DNA [14] were assayed as described previously.

Statistical analysis was performed by analysis of variance. If a significant variance ratio was indicated, differences between treatment groups were investigated using a multiple range test (Newman–Keuls test) [15].

Results and discussion

The results indicate that the rate of GSH resynthesis in hepatocytes from postnatal mice is greater than in those from adult mice after DEM exposure. The addition of DEM depleted GSH in hepatocytes from all age groups during a 10 min incubation (Fig. 1). The extent of depletion differed, from 53 ± 5% of control (mean ± SEM) in hepatocytes from 1-week-old mice to 11 ± 4% of control in 3-week-old mice. The levels of GSH in control hepatocytes (not treated with DEM) were 0.26 ± 0.02, 0.33 ± 0.04, 0.35 ± 0.04 and 0.56 ± 0.03 μg/μg DNA (mean ± SEM) for 1-, 2-, 3-week-old and adults, respectively, and these values did not change during the 60 min incubation. In hepatocytes from adult mice pretreated with DEM, GSH levels rose slowly from an initial level of 19% to 55% of control at 60 min. It took between 2 and 3 hr for the GSH level in these cells to return to control values (data not shown). On the other hand, in hepatocytes from the young mice, the GSH level rebounded to above control levels after only 10 min. In hepatocytes from 1-week-old mice the value reached a maximum of 209% of control after 10 min. In 2- and 3-week-old mice the GSH level continued to rise over the 60-min period up to 252% and 202% of control,

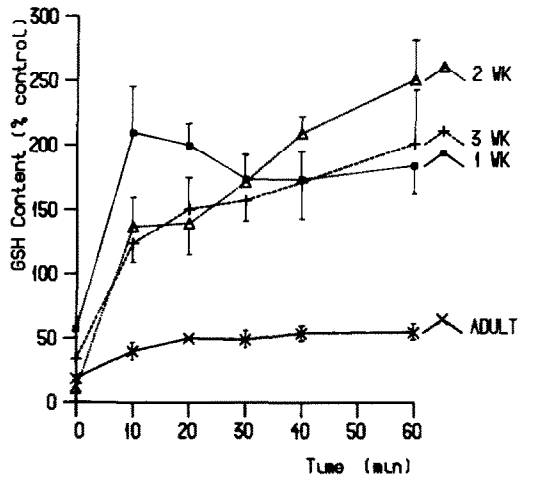

Fig. 1. GSH resynthesis in hepatocytes from 1-, 2-, 3-week-old and adult mice. Hepatocytes were exposed to DEM (0.1 μl/ml) for 10 min, washed to remove DEM, and incubation continued for up to 60 min in fresh culture medium. Values are mean ± SEM of five separate experiments. Values in DEM pretreated cells are expressed as a percentage of controls (not exposed to DEM).

respectively. When the results are standardized on the basis of cell mass (i.e. by protein content), the results indicate that the rate of GSH resynthesis in the first 10 min was highest in hepatocytes from 1-week-old mice. These values were 25.9 ± 5.3, 7.6 ± 2.3, 7.4 ± 1.8 and 2.4 ± 0.4 μg GSH/mg protein/10 min (mean ± SEM) for 1-, 2-, 3-week-old and adult mice, respectively. Analysis with a multiple range test indicated that the rate in 1-week-old mice was greater than in 2- and 3-week-old, which in turn were greater than in adults (Newman–Keuls test, $P < 0.05$). The fact that GSH synthesis is greatest in hepatocytes from 1-week-old mice may explain why the GSH depletion induced by DEM was not as great in this group (Fig. 1), as it would be more difficult to deplete GSH in the face of continued rapid resynthesis.

GSH-*S*-transferase activities were low in livers from the young mice. There was no age-related difference in K_m values for any of the substrates. However, the V_{max} values were significantly less in the livers of the younger mice compared to adults (Table 1). This finding is not surprising, since similar age-related differences have been observed in GSH-*S*-transferase activities in the rat [16].

The lack of susceptibility of foetal and neonatal animals to the toxic effects of electrophilic metabolites has been attributed to the immaturity of the drug metabolizing enzymes that activate these compounds in the liver [17]. Postnatal rats and mice also appear to be less sensitive to these toxic metabolites [1–3]. However, the activity of the drug metabolizing enzymes in postnatal rats and mice appear to be fully developed. For example, young rats and mice produce at least as much of the paracetamol reactive metabolite as do adults [2, 4]. Hence, this lack of tissue sensitivity cannot be explained by a lack in the ability of these young animals to generate these reactive metabolites.

In the present study, hepatocytes from young mice have a greater capacity to resynthesize GSH after depletion with DEM. It is known that DEM rapidly alkylates GSH in the hepatocyte [18]. Since the GSH level in the cell is controlled by a non-allosteric feedback mechanism [19], when GSH levels suddenly fall, the hepatocyte will attempt to resynthesize more GSH. Despite the immaturity of the GSH-transferase enzymes, the ability to rapidly resynthesize GSH may explain why postnatal mice are less susceptible to toxic electrophilic metabolites compared to adults. The rapid replenishment of GSH stores could aid in the protection of vital nucleophilic sites from electrophilic attack by these reactive metabolites. It has been shown that certain antioxidants and thiol-containing agents can protect against paracetamol toxicity by ameliorating toxic events that occur after the generation of the reactive metabolite and its binding in the cell [20–22]. Hence, another possibility is that the increased thiol content in the hepatocyte of these young mice may protect from these secondary toxic processes initiated by reactive species that eventually result in cell death.

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Table 1. Comparison of V_{max} values estimated for GSH-*S*-transferase activities in livers from 1-, 2-, 3-week-old and adult mice

Substrate	1 week	2 weeks	3 weeks	Adult
CDNB	52.9 ± 8.5*	27.4 ± 0.9	47.1 ± 8.0	184.9 ± 46.5†
p-NBC	2.5 ± 0.8	3.5 ± 0.5	4.0 ± 0.5	9.1 ± 1.3†
ENPP	3.0 ± 0.1	4.2 ± 0.8	5.9 ± 1.2	9.7 ± 2.3†

* Values are mean ± SEM of five separate experiments in units of nmol/mg protein/min. Estimates for V_{max} were obtained by curve fitting the data to the Michaelis–Menton equation using NONLIN.

† Significantly greater than either 1-, 2-, or 3-week-old, Newman–Keuls test, $P < 0.05$.

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Inhibition of rat lung *S*-adenosylmethionine decarboxylase by *N,N'*-dimethyl-4,4'-dipyridyl dichloride (paraquat)

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Polyamines are ubiquitously distributed endogenous compounds that have been associated with the regulation of numerous cellular functions [1–4]. Although simple in structure, these compounds bind to many classes of macromolecules. The binding capabilities of the polyamines appear to be related to the critical intramolecular distance between the nitrogen moieties which are positively charged at physiological pH. Modulation of polyamine synthesis by specific enzyme inhibitors and precursor analogs has shown that cellular polyamine levels are important determinants for RNA synthesis [5], cellular proliferation [2], differentiation [2] and membrane functions [6].

N-substituted 4,4'-dipyridyl analogs represent a class of compounds similar in structure to putrescine having two charged nitrogens separated by an intramolecular distance of approximately 6.9 A [7]. Previous studies have shown that the *N,N'*-dimethyl analog (paraquat), a commonly used herbicide, is actively accumulated into both human and animal lung tissue by a process that is inhibited by the endogenous polyamines [8, 9]. It has been suggested that the lungs contain a specific transport mechanism for polyamines that recognises paraquat. Moreover, several cellular processes thought to be under polyamine regulation [5, 10] are affected by paraquat. These include DNA synthesis [11, 12] and DNA repair [13]. It is conceivable that *N*-substituted 4,4'-dipyridyl analogs may represent a class of polyamine inhibitors.

The biosynthesis of polyamines requires the decarboxylation of *S*-adenosylmethionine to *S*-adenosyl-5'-deoxy-(5')-3-methyl-thiopropylamine. The latter substrate is the donor of a propylamine group to putrescine during the synthesis of spermidine, and to spermidine during the synthesis of spermine. The decarboxylation reaction is catalysed by *S*-adenosylmethionine decarboxylase (SAMDC*: EC 4.1.1.50), a cytosolic enzyme that is dependent upon putrescine for activation [14]. Putrescine decreases the Michaelis constant K_m with little effect on the maximum velocity of SAMDC. These data suggest that putrescine increases the affinity of substrate for the active site on the enzyme.

The principal aim of the present study was to investigate whether paraquat, and its parent compound dipyridyl, bound to the specific polyamine binding site(s) on SAMDC and whether such an interaction modulated the activity of the enzyme. Experiments were undertaken using rat lung tissue since the lungs are the major site of paraquat accumulation and toxicity [8]. The data showed that the herbicide was an effective inhibitor of the enzyme. However, the nature of inhibition suggested that paraquat did not simply displace putrescine from its enzymatic binding site.

Materials and methods

Preparation of tissue. Lungs from male Sprague–Dawley rats (200–250 g) were flushed with 10 ml 0.9% saline and homogenised at 4° in 4 vol. 100 mM phosphate buffer (pH 7.2) containing 0.1 mM ethylenediamine tetraacetic acid and 1 mM dithiothriotol. The tissue was centrifuged at 3000 *g* for 10 min followed by 110,000 *g* for 60 min at 4°. The resulting supernatant was recovered for SAMDC determinations. Protein content was estimated by the Bradford method [15].

Determination of SAMDC activity. Lung supernatant (0.5 ml) was incubated with 1 mM pyridoxal-5-phosphate, 1 mM putrescine, 0.2 μCi ^{14}C-SAM, paraquat (50–1000 μM) and phosphate buffer containing 1 mM dithiothriotol and 0.1 mM EDTA to a final volume of 1 ml. The incubations were carried out in a 20 ml glass scintillation vial at 37° for 60 min with constant shaking. An aliquot (50 μl) of ethanolamine was placed on a small square of filter paper lodged in the cap of the vial. After 60 min, 0.5 ml 6 M HCl was added to each vial which were then shaken for a further 30 min. The vial caps containing the filter paper were transferred to scintillation vials containing 15 ml scintillant (Aquasol) and ^{14}C content determined by scintillation counting. Appropriate blanks containing either boiled tissue or no tissue were concurrently run with the samples.

Initial studies established that the reaction was linear up to 90 min over a protein concentration range of 2–8 mg/ml, SAM concentration range of 1–400 μM and putrescine concentration range of 50–3000 μM.

In some experiments, superoxide dismutase (1 mg/ml), catalase (10 μg/ml) or 1,3-dimethylurea (1 mM) was added to the incubation. Final volume remained at 1 ml.

Kinetic parameters were determined by least squares regression analysis assuming the rate of reaction obeyed

* Abbreviations used: SAMDC, *S*-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; MGBG, 1,1'-[(methylethanediylidene)dinitrilo]diguanidine.