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A ROLE FOR THE GLUTATHIONE PEROXIDASE/REDUCTASE ENZYME SYSTEM IN THE PROTECTION FROM PARACETAMOL TOXICITY IN ISOLATED MOUSE HEPATOCYTES

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Abstract—The role of the glutathione peroxidase/reductase (GSH-Px/GSSG-Rd) enzyme system in protection from paracetamol toxicity was investigated in isolated mouse hepatocytes in primary culture. The effect of inhibitors of these enzymes on the toxicity of paracetamol and on *t*-butylhydroperoxide (*t*-BOOH), used as a positive control, was determined. 1,3-Bis(chloroethyl)-1-nitrosourea (BCNU) was used to inhibit GSSG-Rd, and goldthioglucose (GTG) used to inhibit GSH-Px. Both these inhibitors increased cell membrane damage in response to oxidative stress initiated by *t*-BOOH. However, they also increased the susceptibility of hepatocytes to paracetamol toxicity, indicating that a component of paracetamol's toxic effect involves formation of species that are detoxified by the GSH-Px/GSSG-Rd enzymes. To further examine the role of these enzymes, age-related differences in their activity were exploited. Hepatocytes from two-week-old mice were less susceptible to both *t*-BOOH and paracetamol toxicity than were those from adult mice. This corresponds to higher activity of cytosolic GSH-Px/GSSG-Rd in this age group. However, after inhibition of GSSG-Rd with BCNU, hepatocytes from these postnatal mice were more susceptible to paracetamol toxicity. This suggests that the higher activity of GSH-Px/GSSG-Rd in hepatocytes from two-week-old mice is responsible for their reduced susceptibility to paracetamol toxicity. The data indicate that the GSH-Px/GSSG-Rd enzymes contribute to protection from paracetamol toxicity and suggest that formation of peroxides contributes to this drug's hepatotoxic effects.

The events associated with paracetamol toxicity in the liver are largely unresolved. Theories such as binding of the reactive metabolite, *N*-acetylbenzoquinone imine (NAPQI)†, to cellular macromolecules [1], lipid peroxidation [2], and oxidation of critical sulphydryl groups and alteration of calcium homeostasis [3] have been proposed, but none appear to explain comprehensively the sequence of events initiated by this drug that ultimately kills the cell.

It has been demonstrated that paracetamol oxidation in the hepatocyte initiates a sequence of events that eventually leads to cell death [4–7]. Antioxidants can inhibit these events [6] suggesting that deleterious oxidative changes are involved. The nature of these events are unknown but one possibility is that reactive oxygen species play a role. If oxidative mechanisms were involved, the activity of the glutathione peroxidase/glutathione reductase (GSH-Px/GSSG-Rd) enzyme system would be important in protecting against toxicity [8].

Dietary supplementation with selenium has been shown to reduce [9], while selenium deficiency enhances [2], sensitivity to paracetamol toxicity. Both of these regimes modulate the selenium-dependent GSH-Px activity. However, other studies have indicated that these enzymes do not play a major role, as paracetamol intoxication does not increase oxidized glutathione (GSSG) excretion from isolated hepatocytes or whole liver [10, 11].

In the present study we have exploited differences in the susceptibility of hepatocytes from postnatal and adult mice to investigate the role of the GSH-Px/GSSG-Rd enzyme system in paracetamol toxicity. We have previously shown that hepatocytes from postnatal mice are less susceptible to hepatotoxic agents, including paracetamol, compared to adults [12]. This reduced susceptibility corresponded to higher levels of GSH-Px and GSSG-Rd in the postnatal liver [12]. We postulate that higher activity of the GSH-Px/GSSG-Rd enzyme system in the liver of postnatal mice may play a role in the reduced susceptibility to chemical-induced damage.

This study has examined the influence of the GSH-Px/GSSG-Rd enzyme system on toxicity of both paracetamol and *t*-butylhydroperoxide (*t*-BOOH) in isolated hepatocytes. The latter was used as a positive control as its toxicity is due to the production of an oxidative stress [13]. In order to examine the role of these enzymes in the toxic process, we used 1,3-bis(chloroethyl)-1-nitrosourea (BCNU), a specific inhibitor of GSSG-Rd [14]. Inhibition of GSSG-Rd prevents replenishment of the reduced glutathione (GSH) pool necessary for GSH-Px activity.

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† Abbreviations: GSH-Px, glutathione peroxidase; GSSG-Rd, glutathione reductase BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; GTG, goldthioglucose; *t*-BOOH, *t*-butylhydroperoxide; NAPQI, *N*-acetylbenzoquinone imine; GSH, reduced glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase.

Goldthioglucose (GTG), an inhibitor of GSH-Px [15, 16] was also used to examine the influence of this enzyme on the toxicity of both paracetamol and *t*-BOOH.

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. (Freehold, NJ). BCNU was from Bristol Laboratories (NY, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Hepatocyte preparation. Hepatocytes were isolated from two-week-old and adult (8–10-week-old) Swiss mice (Animal Resources Centre, Murdoch, WA) by collagenase perfusion of the liver, as described previously [17]. 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 two-week-old mice were 90 ± 4% and 92 ± 5%, respectively (Trypan Blue exclusion). Cells were cultured in monolayer on collagen-coated culture dishes (55 mm dia.) in 3 ml culture media (4 mg wet wt cells/ml) as described previously [17]. 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 time they were washed with 2 × 3 ml phosphate buffered saline, pH 7.4, to remove non-viable cells. 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, *t*-BOOH, BCNU or GTG in RPMI 1640 medium.

Incubations. BCNU was dissolved in 50 μl ethanol prior to addition to 80 ml RPMI culture media. Control cells were incubated in RPMI containing ethanol alone. GTG, paracetamol and *t*-BOOH were all dissolved directly into RPMI culture media immediately before incubation. 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% (v/v) final concentration) as described previously [5]. Total LDH activity was not decreased by either hepatotoxin, nor did it significantly decrease during the 24 hr incubation period.

Enzyme assays. Hepatocytes 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 using a Beckman TL-100 Ultracentrifuge, at 100,000 *g* for 30 min at 4°. Aliquots of cytosolic fraction were then assayed for enzyme activity. Protein was determined using the method of Hartree [18]. GSH-Px (EC 1.11.1.9) was measured using the method of Paglia and Valentine [19]. GSSG-Rd (EC 1.6.4.2) was measured by a method adapted from Paglia and Valentine [19]. A 50 μl aliquot of sample was added to 2.95 ml reaction mixture containing 8.4 mM NADPH. 0.15 M oxidized 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°. GSH was measured by a fluorometric method [20].

Enzyme inhibition. GSSG-Rd activity was inhibited in hepatocytes from both adult and 2-week-old mice by exposing cells to BCNU at concentrations of either 0.1 mM (adults) or 0.05 mM (2-week-old), followed by a 2 hr incubation in BCNU-free culture media. Cells were then exposed to varying concentrations of either paracetamol or *t*-BOOH. GSH-Px was inhibited using GTG. Maximal inhibition was achieved after 4 hr incubation in RPMI containing 1.0 mM GTG. Hepatocytes were then exposed to either paracetamol or *t*-BOOH in culture medium containing 1.0 mM GTG.

Statistical analysis. Analysis of variance (ANOVA) was performed using the computer statistical package GENSTAT (Lawes Agricultural Trust, Rothamsted Experimental Station, U.K.). Individual group differences were compared to control using Dunnett's test [21].

RESULTS

Preincubation with BCNU irreversibly inhibited GSSG-Rd in the mouse hepatocytes (Table 1). Hepatocytes were exposed to BCNU for 30 min then washed and incubated in fresh media for up to 24 hr. GSSG-Rd was inhibited by 89 ± 3% with 0.1 mM BCNU in hepatocytes from adults. Hepatocytes from two-week-old mice were more sensitive to BCNU and GSSG-Rd activity was inhibited by 90 ± 2% with 0.05 mM BCNU. This inhibitory effect was irreversible over a subsequent 24 hr incubation in BCNU-free medium in hepatocytes from both age groups (data not shown). BCNU was not toxic at these concentrations. The toxic threshold concentrations of BCNU were 0.1 mM and 0.5 mM for two-week-old and adults, respectively (data not shown). In order to examine the effect of GSSG-Rd inhibition on the toxicity of *t*-BOOH and paracetamol, hepatocytes were pretreated with either 0.05 mM (two-week-old) or 0.1 mM BCNU (adult) for 30 min. These concentrations of BCNU produced a similar degree of GSSG-Rd inhibition in the two age groups without producing cell membrane damage. Since exposure to BCNU decreased GSH levels, the hepatocytes were washed in phosphate buffered saline and then incubated for 2 hr in fresh media in order to allow GSH to return to control levels (data not shown). The susceptibility of control (non-pretreated) and BCNU-pretreated hepatocytes to *t*-BOOH and paracetamol were then compared.

The effect of BCNU-pretreatment in combination with *t*-BOOH exposure on cell viability of hepatocytes from two-week-old and adult mice incubated for up to 3.5 hr is shown in Fig. 1. Three major conclusions can be drawn from these data. (1) Non-pretreated hepatocytes from adult mice (Fig. 1A) were more susceptible to *t*-BOOH than those from two-week-olds (Fig. 1B). Comparison of data in Fig.

Table 1. The effect of BCNU and GTG exposure on GSSG-Rd and GSH-Px activities in isolated hepatocytes*

BCNU conc. (mM)	GSSG-Rd activity (nmol/min/mg protein) Adult	Two-week-old	GTG conc. (mM)	GSH-Px activity (nmol/min/mg protein) Adult	Two-week-old
0.0	32 ± 4	55 ± 4	0.0	331 ± 10	905 ± 60
0.01	17 ± 3	7 ± 4	0.1	264 ± 35	487 ± 27
0.05	7 ± 2	5 ± 1	0.5	172 ± 59	413 ± 17
0.1	4 ± 1	6 ± 1	1.0	106 ± 40	400 ± 34

* Hepatocytes were exposed to either BCNU for 30 min, washed and then incubated in fresh medium for 2 hr, or incubated in GTG for 4 hr, prior to determination of enzyme activities. All values are mean ± SE of four separate determinations expressed as nmol NADPH consumed/min/mg cytosolic protein.

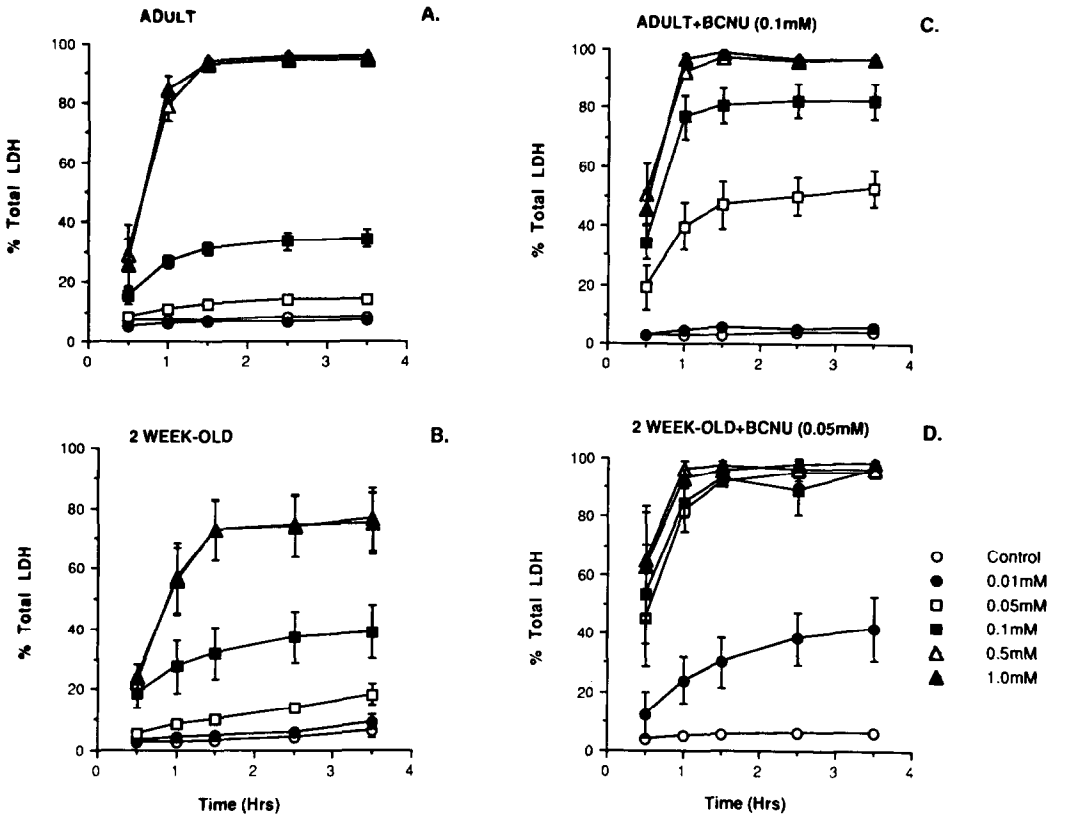

Fig. 1. The effect of *t*-BOOH on the time course of LDH leakage from non-pretreated hepatocytes isolated from adult (A) and two-week-old mice (B), and on hepatocytes pretreated with BCNU, from adult (C) and two-week-old mice (D). The LDH leakage from cells is expressed as activity in the cell-free supernatant as a percentage of total activity. Hepatocytes were exposed to *t*-BOOH (0.01–1.0 mM) for up to 3.5 hr. BCNU-pretreatment was for 30 min, followed by a 2 hr recovery period to allow GSH levels to return to control values prior to exposure to *t*-BOOH. Individual data points represent the mean ± SE of four separate experiments.

1A with Fig. 1B by ANOVA indicated a significant effect of age ($F = 410$; $df = 1,415$; $P < 0.01$). (2) BCNU-pretreatment potentiated the toxicity of *t*-BOOH in hepatocytes from both adult and two-week-old mice. Comparison of data in Figs 1A with 1C ($F = 165$; $df = 1,177$; $P < 0.01$), and Figs 1B with 1D ($F = 458$; $df = 1,177$; $P < 0.01$) using ANOVA indicated a significant effect of BCNU-pretreatment on *t*-BOOH-induced toxicity. (3) Following BCNU-pretreatment, the susceptibility of hepatocytes from two-week-old mice was now greater than in those from adult. Comparison of data in Figs 1C to 1D using ANOVA indicated a significant effect of age ($F = 43$; $df = 1,415$; $P < 0.01$). Thus, prior to BCNU-pretreatment the toxic response to *t*-BOOH in hepatocytes from two-week-old mice was less than

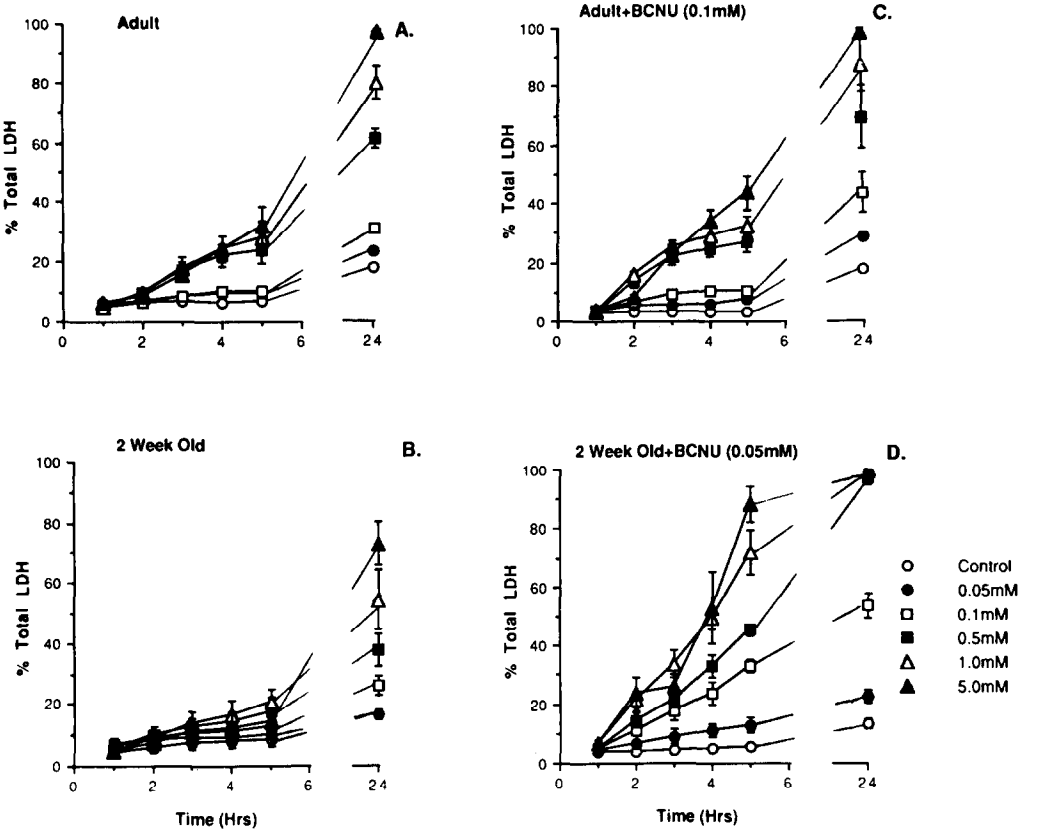

Fig. 2. The effect of paracetamol exposure on the time course of LDH leakage from non-pretreated hepatocytes isolated from adult (A) and two-week-old mice (B) and on hepatocytes pretreated with BCNU, from adults (C) and two-week-olds (D). Hepatocytes were exposed to paracetamol (0.01–5.0 mM) for up to 24 hr. BCNU-pretreatment was for 30 min, followed by a 2 hr recovery period to allow GSH levels to return to control values prior to exposure to paracetamol. Data points represent the mean ± SE of four separate experiments.

that in adults, but after BCNU-pretreatment the susceptibility in the two-week-olds was now greater than in adults.

The combination of paracetamol exposure and BCNU-pretreatment produced similar alterations as seen with *t*-BOOH described above. (1) Non-pretreated hepatocytes from adult mice (Fig. 2A) were more susceptible to paracetamol toxicity than those from two-week-old mice (Fig. 2B) ($F = 14$; df = 1,454; $P < 0.01$). (2) BCNU-pretreatment potentiated the toxicity of paracetamol in hepatocytes from both adult (Fig. 2A vs Fig. 2C; $F = 6$; df = 1,213; $P < 0.01$) and two-week-old mice (Fig. 2B vs Fig. 2D; $F = 235$; df = 1,213; $P < 0.01$). (2) Following BCNU-pretreatment, the susceptibility of hepatocytes from two-week-old mice (Fig. 2D) was greater than that in adults (Fig. 2C) ($F = 110$; df = 1,454; $P < 0.01$). Again, this indicates that BCNU-pretreatment resulted in a greater increase in susceptibility of hepatocytes from two-week-old mice to paracetamol toxicity than in adults.

Inhibition of GSH-Px was also found to enhance the effects of paracetamol and *t*-BOOH toxicity. GTG (1.0 mM) inhibited cytosolic GSH-Px by approximately 60% (Table 1). This concentration of GTG was not toxic to these cells (data not shown). Despite producing only 60% inhibition of the GSH-Px activity, the susceptibility of hepatocytes from two-week-old and adult mice to paracetamol in the presence of GTG was increased (Fig. 3). A similar effect of GTG on susceptibility to *t*-BOOH toxicity in hepatocytes from two-week-old and adult mice was also seen (data not shown).

It is well known that paracetamol exposure depletes GSH levels in hepatocytes. In order to determine the effect of BCNU-pretreatment or the presence of GTG on GSH levels in the cell during paracetamol intoxication, hepatocytes from adult mice were exposed to paracetamol (5 mM) for up to 24 hr with or without BCNU-pretreatment (Fig. 4A) or the presence of GTG (Fig. 4B). BCNU-pretreatment alone did not result in lower GSH levels after the 2 hr recovery period, but did increase the rate of loss of GSH in the presence of paracetamol. GTG did not alter the effect of paracetamol on GSH depletion. Similar results were obtained when these experiments were performed in hepatocytes from two-week-old mice (Fig. 5).

DISCUSSION

In a previous study we demonstrated that hepa-

Table 2. Influence of α-naphthylacetic acid, phenolphthalein and sulfobromophthalein on uptake of [^{14}C]mersalyl and of mersalyl on uptake of [^{14}C]α-naphthylacetic acid in the isolated perfused liver

Addition to perfusion medium	[^{14}C]Mersalyl uptake nmoles/min/g liver
None	3.68 ± 0.23*
20 μM α-naphthylacetic acid	1.88 ± 0.41*
20 μM phenolphthalein	3.65 ± 0.43
20 μM sulfobromophthalein	3.45 ± 0.62
	[^{14}C]α-Napthylacetic acid uptake nmoles/min/g liver
None	2.18 ± 0.68*
20 μM mersalyl	1.02 ± 0.40*

Uptake values were obtained after 3 min perfusion with either 2 μM [^{14}C]mersalyl or 2 μM [^{14}C]α-naphthylacetic acid in presence of indicated compounds.

Means of 4 expts ± SD, *$P < 0.05$.

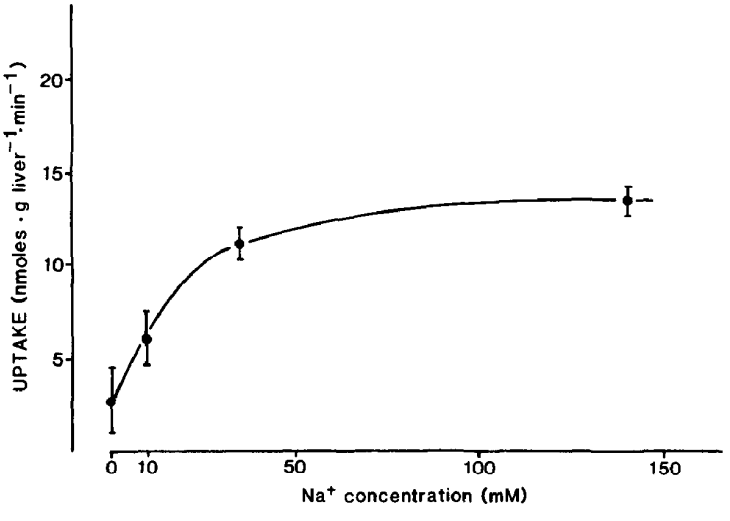

Fig. 2. Stimulation of mersalyl uptake in the isolated perfused liver at different Na^+ concentrations. Uptake values were obtained after 3-min perfusion with 10 μM [^{14}C]mersalyl. Na^+ was replaced in equimolar amounts by choline.

0°) was therefore determined in each experiment. This value was subtracted from uptake values at 25° when transmembrane transport function was assayed.

Transport of mersalyl into basolateral membrane vesicles exhibits saturation kinetics within the concentration range studied (2.5–250 μM). As already seen in the perfused liver, uptake into vesicles at low concentrations (below 10 μM) is lower than expected for first-order kinetics, revealing also a Hill coefficient of 1.5 (Fig. 1B). From the corresponding Eadie–Hofstee plot $(S)_{0.5}$ = 28 μM and V_{max} of 1.6 nmoles/min/mg protein were calculated, values in good agreement with those obtained for uptake in the perfused organ.

Extravesicular sodium concentration stimulates uptake of mersalyl into basolateral membrane vesicles. This was analysed at a mersalyl concentration of 200 μM in the presence of 120 mM NaSCN or 120 mM KSCN either outside the membrane alone or 60 mM on both sides of the membrane. Compared to KSCN, NaSCN on both sides stimulates temperature-sensitive initial uptake by 30%, whereas an increase by 40% is seen when NaSCN was only present in the extravesicular medium (these two values being statistically different at a level of $0.2 > P > 0.1$; Student's *t*-test). These data suggest, that extravesicular sodium increases substrate affinity and, in addition, that the transmembrane sodium gradient provides a driving force for substrate transport.

Uptake of mersalyl is subjected to transstimulation, i.e. preloading of membrane vesicles with non-radioactive mersalyl increases uptake of the radiolabelled compound. Under this condition uptake is also stimulated by the presence of sodium (Fig. 3).

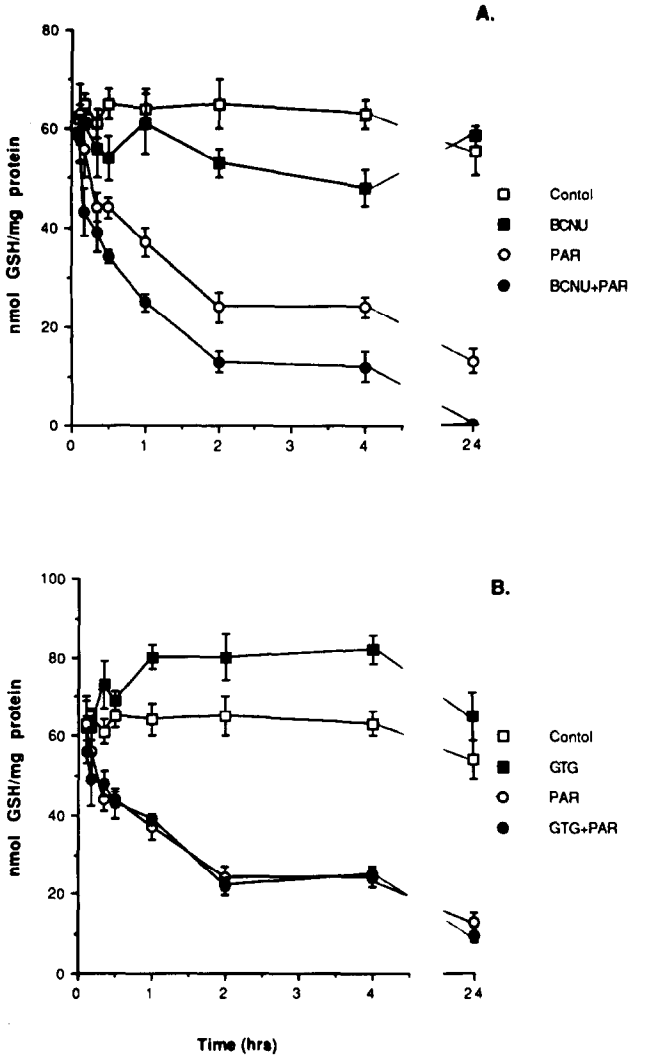

Fig. 4. (A) The effect of BCNU-pretreatment on paracetamol-induced GSH depletion in hepatocytes isolated from adult mice. Non-pretreated hepatocytes (PAR) or BCNU-pretreated hepatocytes (PAR + BCNU) were exposed to paracetamol (5 mM) for up to 24 hr. BCNU-pretreatment was for 30 min, followed by a 2 hr recovery period. The GSH content in cells not exposed to paracetamol or BCNU (control) and not exposed to paracetamol but pretreated with BCNU (BCNU) is also shown. Each data point represents mean ± SE of four separate experiments. (B) The effect of GTG treatment (1 mM) on paracetamol-induced GSH depletion in hepatocytes isolated from adult mice. Non-pretreated hepatocytes (PAR) or GTG-treated hepatocytes (PAR + GTG) were exposed to paracetamol (5 mM) for up to 24 hr. The GSH content in cells not exposed to paracetamol or GTG (control) and not exposed to paracetamol but GTG-treated (GTG) is also shown. Each data point represents mean ± SE of four separate experiments.

The increase in paracetamol toxicity in hepatocytes from adult mice after BCNU-pretreatment was not pronounced. However, in two-week-old mice, BCNU-pretreatment resulted in a marked increase in susceptibility to paracetamol and to *t*-BOOH. In fact, the increase in susceptibility was so large that after BCNU-pretreatment, it changed from being less than that in adults to being considerably more susceptible. This difference in the magnitude of the change in susceptibility between the two age groups was not due to a difference in the degree of inhibition of GSSG-Rd, as this enzyme activity was inhibited to the same extent in both. It can be explained if GSSG-Rd is important in protection from paracetamol toxicity. It is known that hepatocytes from two-week-old mice have a faster rate of production of NAPQI than do adults [12]. If GSSG-Rd is important in protection against the damaging effects of NAPQI, then inhibition of this enzyme to the same extent in hepatocytes from both age groups might be expected to have a greater effect on the extent of the toxic response in the postnatal mice, which produce more NAPQI, than in the less metabolically active hepatocytes from adults.

It is known that synthetic NAPQI will directly

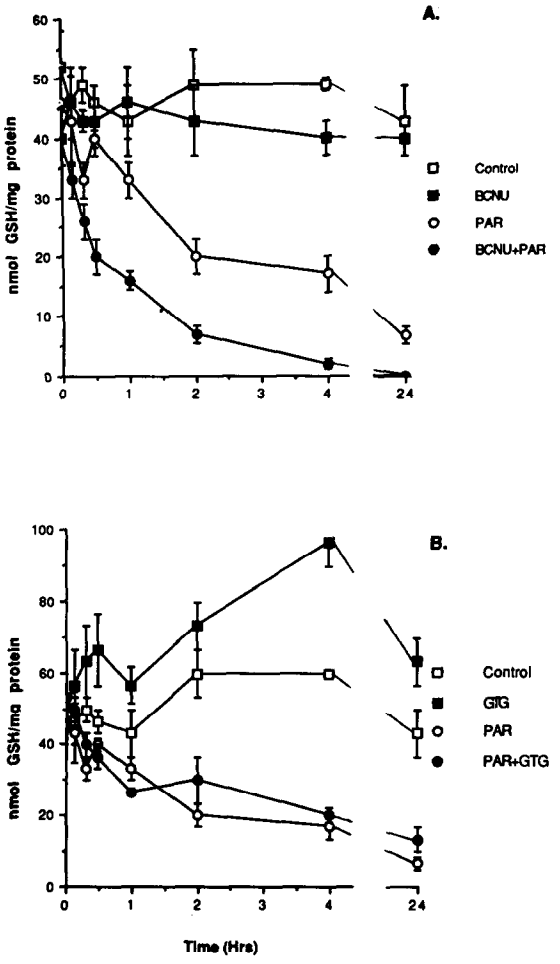

Fig. 5. The effect of BCNU-pretreatment (A) and the presence of GTG (B) on paracetamol-induced GSH depletion in isolated hepatocytes from two-week-old mice. Experimental conditions are the same as in Fig. 4.

oxidize GSH to GSSG [24]. As GSSG is reduced to GSH by GSSG-Rd, inhibition of this enzyme by BCNU may increase susceptibility to paracetamol simply by depleting the cell of GSH and rendering it more susceptible to electrophilic attack. Indeed, it was found that paracetamol exposure increased the rate of decline of GSH after BCNU-pretreatment in cells from both adult and two-week-old mice (Figs 4 and 5). However, part of this increased rate of depletion after BCNU-pretreatment would be due to the faster onset of cell damage, particularly in the hepatocytes from two-week-old mice. In order to ascertain whether the effects of BCNU were due to inhibition of the GSH-Px/GSSG-Rd enzyme system as a whole, and not simply GSSG-Rd itself, the experiments were repeated using the GSH-Px inhibitor, GTG. Whilst the inhibition of GSH-Px activity by GTG was not as great as that seen with BCNU on GSSG-Rd activity, the overall effects on paracetamol toxicity (Fig. 3) and *t*-BOOH were similar. GTG increased susceptibility to paracetamol and *t*-BOOH in hepatocytes from both two-week-old mice and adults. Hence inhibition of GSH-Px activity produced qualitatively similar effects as inhibition of GSSG-Rd. Furthermore, the loss of GSH after paracetamol exposure in the presence of GTG was the same as with paracetamol-alone (Figs 4 and 5). Since GTG also enhanced susceptibility to paracetamol but did not lower GSH levels below those produced by paracetamol alone, it is unlikely that the difference in the rate of GSH depletion in BCNU-pretreated cells is the only factor contributing to increased susceptibility.

Alkylation of key cellular proteins by NAPQI [25], the loss of calcium homeostasis following protein thiol oxidation [3] and oxidative stress [2, 26, 27] have been proposed as mechanisms of paracetamol toxicity. The evidence for a role of oxidative stress is controversial. It has been discounted by some, as there is no evidence of increased efflux of GSSG into the bile in whole rats [11]. On the other hand, lipid peroxidation has been found to precede liver damage in the isolated mouse liver [28]. In hepatocytes from the rat, BCNU has been found to either have no effect [10] or to potentiate [26, 27] paracetamol toxicity. As discussed above, potentiation of toxicity by BCNU does not necessarily implicate a role for peroxides in the toxic process, as irreversible oxidation of GSH in the face of electrophilic insult could account for increased toxicity. The present study used inhibitors of both enzymes in the glutathione redox cycle and found that both inhibitors increased susceptibility to paracetamol toxicity. Furthermore, the large increase in susceptibility of hepatocytes from two-week-old mice to both paracetamol and *t*-BOOH after inhibition by BCNU is consistent with a major role for the GSH-Px/GSSG-Rd enzymes in protection from toxicity. Hepatocytes from two-week-old mice may be less susceptible to hepatotoxins by virtue of having higher activity of GSH-Px/GSSG-Rd [12]. While these results do not rule out involvement of either protein alkylation or altered calcium homeostasis in the development of cell injury, they are consistent with findings that indicate that reversible oxidative processes are involved in the secondary events that occur after NAPQI has initiated the toxic process [4–7].

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