Biochemical Pharmacology, Vol. 45, No. 11, pp. 2289–2294, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

OXIDATIVE STRESS IN CULTURED HEPATOCYTES EXPOSED TO ACETAMINOPHEN

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(*Received* 4 *December* 1992; *accepted* 9 *February* 1993)

Abstract—The effect of acetaminophen (APAP) exposure on the formation of oxidized glutathione (GSSG) was investigated in cultured mouse hepatocytes to determine if oxidative damage is involved in the toxicity of this drug. Incubations of hepatocytes for 24 hr with 1 mM APAP produced a time-dependent loss of cell viability which was preceded by depletion of reduced glutathione (GSH) and an increase in GSSG formation. Pretreatment with 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) (0.1 mM) for 30 min, which irreversibly inhibited glutathione reductase (GSSG-Rd) activity, increased the extent of GSSG formation produced by APAP exposure and potentiated its cell killing. Pretreatment of hepatocytes with 20 mM deferoxamine (DFO) for 1 hr to chelate ferric iron decreased GSSG formation and cell killing produced by APAP. Pretreatment with BCNU or DFO did not affect APAP oxidation as determined by the formation of the APAP–GSH conjugate or the covalent binding of APAP metabolites to cellular protein. Hence, increasing the susceptibility of hepatocytes to an oxidative stress with BCNU increased both the formation of GSSG and cell killing produced by APAP. Conversely, decreasing their susceptibility to an oxidative stress by chelating iron with DFO decreased GSSG formation and cell injury. It follows that APAP toxicity involves oxidative processes that occur early in the poisoning process and are a major factor contributing to injury in these cells.

Acetaminophen (APAP§) overdose produces liver necrosis in humans and a number of animal species [1]. While there is general agreement that its toxicity requires that it be activated by hepatic mixed-function oxidase enzymes to *N*-acetyl-*p*-benzoquinone imine (NAPQI), the nature of the toxic lesion produced is still controversial.

At present, there appears to be two theories that propose to account for the toxic action of APAP. First, it has been demonstrated that APAP activation leads to covalent binding of NAPQI to cellular macromolecules [2]. It has been hypothesized that the interaction of NAPQI with critical sulfhydryl groups leads to inactivation of homeostatic mechanisms, such as loss of calcium ion flux across the cell membrane and this ultimately leads to loss of cell viability [3, 4]. Second, there is evidence implicating a role for oxidative stress produced by APAP. Its toxicity has been associated with lipid peroxidation [5–7], and inhibitors of the glutathione peroxidase (GSH-Px) and glutathione-reductase (GSSG-Rd) enzyme system increase APAP toxicity [8–10]. Furthermore, agents that protect from oxidative stress also prevent APAP toxicity in cultured hepatocytes [11, 12].

Oxidant injury in the hepatocyte is associated with an increase in the efflux of oxidized glutathione (GSSG) [13]. This type of injury involves the formation of reactive oxygen species, which include superoxide anions, hydroxyl radicals, hydrogen peroxide and lipid hydroperoxides [14]. The detoxification of peroxides involves their reduction by enzymes with GSH-Px activity, resulting in the concomitant formation of GSSG [15]. If the formation of GSSG exceeds the capacity of GSSG-Rd to reduce it back to reduced glutathione (GSH), then GSSG will accumulate in the cell and subsequently will be exported. Hence, the export of GSSG from the hepatocyte has been used as a sensitive indicator for the presence of an intracellular oxidative stress [13].

Therefore, if oxidative damage is a component of APAP toxicity, GSSG formation should occur as part of the early events in the poisoning process. The present study examines whether the appearance of GSSG is associated with the early stages of APAP toxicity in cultured mouse hepatocytes.

MATERIALS AND METHODS

Chemicals. RPMI 1640 culture medium was obtained from Gibco (Grand Island, NY). Collagenase type IV enzyme was purchased from the Sigma Chemical Co. (St. Louis, MO). 1,3-Bis(chloroethyl)-1-nitrosourea (BCNU, Carmustine) was obtained from Bristol Laboratories (NY). Deferoxamine-methanesulfonate (DFO) was from Ciba-Geigy (Basel, Switzerland). All other chemicals were obtained from Sigma.

Hepatocyte preparation. Male Swiss mice (25–30 g in weight) fed a standard chow diet and tap water *ad lib.* were used to isolate hepatocytes. Hepatocytes

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§ Abbreviations: APAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinone imine; GSSG, oxidized glutathione; GSH, reduced glutathione; GSSG-Rd, GSSG reductase; GSH-Px, GSH peroxidase; BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; DFO, deferoxamine-methanesulfonate; PBS, phosphate-buffered saline; PCA, perchloric acid; and LDH, lactate dehydrogenase.

were isolated by using a collagenase-containing perfusion solution as previously described [16]. Hepatocytes were cultured in monolayer (3×10^6 cells/3 mg protein/plate) in RPMI 1640 culture medium, on collagen-coated culture plates (60 mm diameter wells, Corning) [16]. Cells were placed in an incubator (Forma Scientific, model 3164), and allowed to adhere to culture plates for 3 hr at 37° in a humidified 95% air, 5% CO_2 atmosphere. Cultures were then washed three times with 3 mL of phosphate-buffered saline (PBS) to remove nonadherent cells.

Incubations. Experiments were conducted in a buffer consisting of either RPMI 1640 culture medium or in modified Krebs–Henseleit (115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 35 mM $NaHCO_3$, 20 mM Hepes and 2 g/L glucose). BCNU was dissolved in 50 μL ethanol prior to addition to 80 mL of RPMI 1640 medium. Control cells were incubated in RPMI 1640 medium with ethanol alone. Ethanol had no effects on cell viability or on the glutathione pool (data not shown). Cells were exposed to 0.1 mM BCNU for 30 min, then washed twice with 3 mL PBS, followed by a 2-hr incubation in BCNU-free RPMI 1640 medium to allow GSH concentrations to return to the level of controls. As we have found previously [8], BCNU caused irreversible inhibition (91 ± 5%, N = 4) of GSSG-Rd activity over the 24-hr incubation period. DFO was dissolved directly into the culture medium immediately before incubation. Cells were exposed to 20 mM DFO for 1 hr, washed twice with 3 mL PBS prior to exposure to 1 mM APAP and incubation continued for up to 24 hr. APAP was dissolved directly into the incubation medium. Cell killing was monitored by measurement of lactate dehydrogenase (LDH) release [17]. Total LDH activity was not decreased by APAP, BCNU or DFO, nor did it significantly decrease during the 24-hr incubation period (data not shown). APAP metabolites were measured by HPLC analysis [18] in 50-μL aliquots of cell-free supernatant from culture dishes incubated in 1 mM APAP. Covalent binding of [^{14}C]APAP metabolites [19] and GSSG-Rd activity [8] were determined as described previously. Protein was determined using the method of Hartree [20].

GSH/GSSG assay. GSH and GSSG were measured by the method of Fariss and Reed [21] with minor modifications. Intracellular GSH/GSSG levels were determined after removal of the culture supernatant and replaced with ice-cold 10% perchloric acid (PCA) containing 2 mM EDTA. To determine extracellular levels, the culture medium was added to a separate tube containing 50 μL of 70% PCA. Following lyophilization, the residue was resuspended in 0.6 mL of 10% PCA. The PCA extracts for both intra- and extracellular samples were centrifuged at 1000 *g* for 15 min at 4°. Dinitrophenol derivatives of GSH and GSSG were then prepared from 0.5-mL aliquots of protein-free supernatant as described previously [21] and separated on a (25 cm × 4.6 mm i.d.) Spherisorb S5 Amino column (ICI Australia) by HPLC chromatography, with detection at 365 nm using gradient elution. The mobile phase was 80% methanol (A), and 0.8 M sodium acetate in 80% methanol (B). Flow was 2 mL/min isocratically for 1 min (80% A: 20% B) followed by a 5-min linear gradient to 1% A: 99% B. The retention times for authentic GSH and GSSG standards were 5.5 and 7.6 min, respectively. Area under the peaks was quantified using the data acquisition and analysis software package DAPA (DAPA Scientific Software, Western Australia).

Statistical analysis. Analysis of variance was performed to determine significant variance ratios. If a significant variance ratio was indicated, individual group differences were compared with controls by

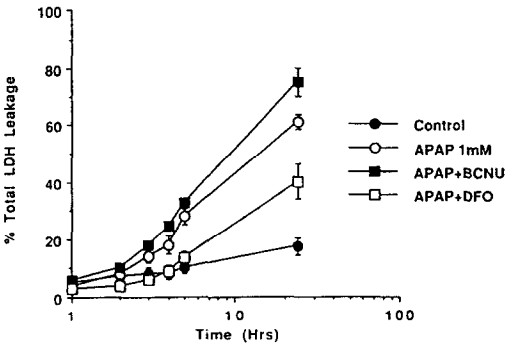

Fig. 1. Effect of 1 mM APAP on the time course of LDH leakage from non-pretreated mouse hepatocytes and from hepatocytes pretreated with BCNU (0.1 mM) or DFO (20 mM). LDH leakage from cells is expressed as activity in cell-free supernatant as a percentage of total activity. Total cellular LDH activity was 6.4 ± 0.3 nmol NADH consumed/min/10^6 cells (N = 6). BCNU pretreatment was for 30 min, followed by a 2-hr recovery in BCNU-free culture medium. DFO pretreatment was for 1 hr directly prior to APAP exposure. BCNU + APAP significantly increased, and DFO + APAP significantly decreased, LDH leakage compared to APAP alone (Newman–Keul's test, $D_{crit} = 9.0\%$). Each point is the mean ± SEM of four separate experiments.

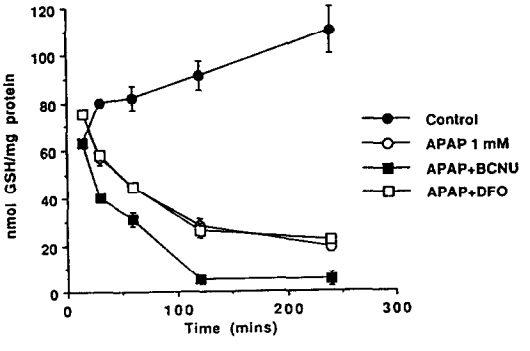

Fig. 2. Effect of APAP (1 mM) exposure on cellular GSH in non-pretreated hepatocytes, or those pretreated with either BCNU (0.1 mM) or DFO (20 mM), as in Fig. 1. Neither BCNU nor DFO alone significantly affected cellular GSH levels (data not shown). Each point is the mean ± SEM of four separate experiments.

Table 1. Covalent binding and glutathione conjugate formation of APAP metabolites in isolated hepatocytes exposed to 1 mM APAP for up to 4 hr

Treatment	GSH–APAP conjugate (nmol/mg protein)		Covalent binding (nmol/mg protein)	
	2 hr	4 hr	2 hr	4 hr
APAP	6.2 ± 0.4	13.5 ± 1.1	2.9 ± 0.3	6.4 ± 0.3
APAP + BCNU*	5.9 ± 0.2	12.8 ± 0.6	2.5 ± 0.4	5.2 ± 0.9
APAP + DFO†	6.5 ± 0.5	14.4 ± 2.6	3.2 ± 0.3	7.0 ± 0.7

Values shown are means ± SEM, N = 5.

* Hepatocytes were pretreated with 0.1 mM BCNU for 30 min, followed by a 2-hr incubation in BCNU-free medium, prior to exposure to APAP.

† Hepatocytes were exposed to APAP after a 1-hr exposure to 20 mM DFO.

Dunnett's test, or between treatment groups using Newman–Keul's test [22].

RESULTS

Hepatocytes from male Swiss mice are susceptible to APAP toxicity. When incubated in the presence of 1 mM APAP under culture conditions for 24 hr, over 80% of the cells were killed (Fig. 1). Hepatocytes exposed to this concentration of APAP following inhibition of GSSG-Rd activity with BCNU (0.1 mM), as described in Materials and Methods, significantly increased cell killing (Newman–Keul's test, $P < 0.05$) (Fig. 1). On the other hand, pretreatment with 20 mM DFO for 1 hr prior to APAP exposure significantly reduced cell killing (Newman–Keul's test, $P < 0.05$) (Fig. 1).

APAP toxicity in these hepatocytes was preceded by a fall in intracellular GSH levels. The intracellular GSH content fell rapidly over the first 2 hr of exposure to APAP to 28% of control values (Fig. 2). Pretreatment with BCNU increased the rate of GSH depletion (Fig. 2) (Newman–Keul's test, $P < 0.05$). However, DFO pretreatment had no effect on the fall in intracellular GSH (Fig. 2) (Newman–Keul's test, $P < 0.05$).

Table 1 shows the effects of BCNU and DFO pretreatments in the formation of the APAP–GSH conjugate and on the covalent binding of APAP metabolites to cellular protein after 2 and 4 hr of

Table 2. Effects of BCNU and DFO on intra- and extracellular levels of GSSG from isolated hepatocytes exposed to APAP (1 mM) and incubated in RPMI 1640 culture medium

Time (min)	Intracellular GSSG (nmol/mg protein)			
	Control	APAP	APAP + BCNU	APAP + DFO
0	3.2 ± 0.9	2.6 ± 1.1	1.6 ± 0.3	2.4 ± 1.0
15	3.2 ± 0.4	1.6 ± 0.2	ND*	2.1 ± 0.5
30	2.1 ± 0.4	ND	ND	2.8 ± 0.7
60	2.6 ± 1.0	ND	ND	2.0 ± 0.3
120	3.8 ± 0.5	ND	ND	1.6 ± 0.5
Time (min)	Extracellular GSSG (nmol/mg protein)			
	Control	APAP	APAP + BCNU	APAP + DFO
0	ND	ND	ND	ND
15	ND	ND	ND	ND
30	ND	ND	1.6 ± 0.5	ND
60	1.6 ± 0.8	5.0 ± 1.0	9.3 ± 2.0	ND
120	2.3 ± 0.5	6.5 ± 1.0	10.4 ± 1.3	ND

Hepatocytes were pretreated with 0.1 mM BCNU for 30 min, followed by a 2-hr incubation in BCNU-free medium, prior to exposure to APAP. Hepatocytes were pretreated with DFO (20 mM) for 1 hr. Values are means ± SEM of four separate experiments.

* Not detectable (minimum detection limit 0.5 nmol/mg protein).

exposure to 1 mM APAP. Taken together, these are an index of the amount of APAP oxidized by the microsomal mixed-function oxidase enzymes. Neither BCNU nor DFO pretreatments altered APAP–GSH conjugate formation (ANOVA, $F = 2.1$; $df = 3, 95$; $P > 0.05$) or covalent binding (ANOVA, $F = 1.6$; $df = 3, 95$; $P > 0.05$).

The levels of GSSG were determined both intracellularly and in the RPMI 1640 culture medium during a 2-hr exposure to 1 mM APAP, with and without BCNU or DFO pretreatment (Table 2). In control cells (not exposed to APAP), the GSSG level in the cells rose slightly from 3.2 ± 0.9 to 3.8 ± 0.5 nmol/mg cellular protein over the 2-hr incubation period. There was also an efflux of GSSG of 2.3 ± 0.5 nmol/mg protein into the culture supernatant (Table 2, column 1). In hepatocytes exposed to APAP, the intracellular GSSG level fell rapidly and was below the detectable limit of the assay at 30 min. After 2 hr the GSSG exported from the cells was 6.5 ± 1.0 nmol/mg protein, which was almost 3-fold greater than that from controls (Table 2, columns 1 and 2). Inhibition of GSSG-Rd activity by BCNU pretreatment increased the efflux of GSSG from cells exposed to APAP to 10.4 ± 1.4 nmol/mg protein at 2 hr (Table 2, columns 1 and 3). BCNU pretreatment itself did not increase GSSG efflux in the absence of APAP after a 2-hr incubation period (2.7 ± 0.4 nmol/mg protein) beyond that of control values (2.3 ± 0.5 nmol/mg protein). Pretreatment with DFO abolished GSSG efflux in APAP-exposed cells and reduced the level of intracellular GSSG in comparison to controls (Table 2, columns 1 and 4).

The RPMI 1640 culture medium contains a low concentration of GSH; therefore the GSSG appearing in the culture supernatant could result from the oxidation of either intra- or extracellular GSH. To determine the source of the GSSG appearing in the supernatant, the experiments described in Table 2 were repeated in hepatocytes using Krebs–Henseleit buffer (KHB) as the incubation medium (Table 3). As KHB does not contain GSH or precursors for *de novo* GSH synthesis, the GSSG appearing in the supernatant can only be derived from existing intracellular glutathione pools. In hepatocytes not exposed to APAP, no GSSG could be detected in the supernatant during a 2-hr incubation period. Intracellular GSSG levels in these cells remained relatively constant at about 2.0 nmol/mg protein (Table 3, column 1). In hepatocytes exposed to 1 mM APAP, the GSSG level in the culture supernatant increased to 4.7 ± 0.6 nmol/mg protein after 2 hr (Table 3, column 2). Pretreatment with BCNU coupled with APAP exposure almost doubled the amount of GSSG appearing in the supernatant at 2 hr compared with that from cells exposed to APAP alone (Table 3, columns 2 and 3). On the other hand, extracellular GSSG could not be detected following APAP exposure in DFO-pretreated cells (Table 3, column 4).

DISCUSSION

The present study demonstrated that there is substantial GSSG formation during APAP toxicity, which occurs well before the loss of cell membrane integrity. When GSSG-Rd activity was inhibited by pretreatment with BCNU, the cell killing induced by APAP increased as did the amount of GSSG formed. By contrast, when APAP toxicity was ameliorated with DFO, GSSG formation was decreased.

Results from our laboratory have indicated a role for oxidative damage in APAP toxicity in cultured mouse hepatocytes. Antioxidants, such as vitamin E, *N,N'*-diphenylphenylenediamine and promethazine, protect from APAP damage [11]. Conversely, inhibition of GSH-Px or GSSG-Rd activity enhances susceptibility to APAP toxicity [8]. We have also found that hepatocytes from 2-week-old mice are less susceptible to APAP toxicity compared with adults and that this can be attributed to higher activity of the GSH-Px/GSSG-Rd enzyme system in hepatocytes from these postnatal animals [8]. If this protective enzyme system is important in determining susceptibility to APAP toxicity, it follows that oxidative damage must play a role in the toxic process.

The present study further implicates oxidative injury as a contributing factor to APAP toxicity. Pretreatment with BCNU inhibits GSSG-Rd and increases susceptibility to agents that induce damage by oxidative stress [13, 23]. BCNU pretreatment has been shown to increase susceptibility to APAP in both mouse [8] and rat [9] hepatocytes, and *in vivo* in rats [10]. Consistent with these findings, the present study demonstrates that BCNU pretreatment potentiated APAP toxicity in cultured mouse hepatocytes. Pretreatment of hepatocytes with DFO resulted in the chelation of an intracellular iron pool, and protects against damage induced by agents that induce oxidative stress via iron-catalyzed reactions [24]. DFO pretreatment has been shown to protect from APAP toxicity in cultured rat hepatocytes [25]. In the present study, DFO decreased cell killing produced by APAP exposure. The protective effect of DFO and the potentiating effect of BCNU were not due to alterations of the rate of APAP oxidation in these cultured mouse hepatocytes. The appearance of the GSH conjugate and binding of reactive metabolite to cellular protein were unchanged by these pretreatments (Table 1).

Oxidative injury is associated with an increase in the formation of GSSG due to the detoxification of peroxides produced from the formation of reactive oxygen species [14]. Hence, the formation and/or efflux of GSSG from whole liver or from hepatocytes has been used as a sensitive indicator for the presence of oxidative stress [13]. In the present study, APAP exposure was associated with an increase in GSSG formation and efflux from the hepatocytes. This amount of GSSG formed during APAP exposure represents a significant proportion of the glutathione equivalents of the cell. Hepatocyte GSH content fell markedly over the first 2 hr after APAP exposure. To gain a more comparative understanding of the state of the glutathione redox balance in the cells, ratios of GSSG versus total remaining free GSH + GSSG (intra- plus extracellular pools) were determined, to establish the proportion of glutathione being oxidized during exposure to APAP. These calculations do not include glutathione consumed by

Table 3. Effects of BCNU and DFO on intra- and extracellular GSSG levels in isolated hepatocytes exposed to acetaminophen (1 mM) and incubated in Krebs–Henseleit buffer

Time (min)	Intracellular GSSG (nmol/mg protein) Control	APAP	APAP + BCNU	APAP + DFO
0	2.0 ± 0.3	2.1 ± 0.6	ND*	1.1 ± 0.3
15	1.6 ± 0.3	1.3 ± 0.6	ND	1.8 ± 0.2
30	1.4 ± 0.5	ND	ND	2.0 ± 0.6
60	1.8 ± 0.8	ND	ND	1.5 ± 0.5
120	2.1 ± 0.6	ND	ND	2.3 ± 0.8
Time (min)	**Extracellular GSSG (nmol/mg protein) Control**	**APAP**	**APAP + BCNU**	**APAP + DFO**
0	ND	ND	ND	ND
15	ND	ND	ND	ND
30	ND	ND	ND	ND
60	ND	1.6 ± 0.3	4.2 ± 0.8	ND
120	ND	4.7 ± 0.6	7.3 ± 1.5	ND

Treatments were as described in Table 2, except that exposure to APAP was carried out in Krebs–Henseleit buffer. Values are means ± SEM of four separate experiments.
* Not detectable (minimum detection limit 0.5 nmol/mg protein).

conjugate formation. Therefore, at 2 hr, the fraction of GSH equivalents appearing as GSSG [total GSSG (6.1 × 2) divided by GSH + GSSG (93 + 12.1), expressed by weight] was 0.11 in control cells, and 0.35 [total GSSG (6.5 × 2) divided by GSH + GSSG (25 + 13)], in cells exposed to APAP (data from Fig. 2 and Table 2). This indicates that a marked change in the redox state had occurred in the glutathione pool of hepatocytes during the first 2 hr of APAP exposure, as a larger proportion of the glutathione pool had been converted to the oxidized form. These changes were seen well before the loss of cell viability, as cell killing did not become evident until after 3 hr (Fig. 1). Therefore, these oxidative events cannot be attributed to changes in hepatocytes that occur as a consequence of cell death.

The extent of GSSG formation coupled with the depletion of GSH by covalent binding with NAPQI did not completely account for the loss of GSH in the cells. The unaccounted loss of GSH may be explained by the formation of glutathione mixed disulfides resulting from oxidation of protein thiol groups by NAPQI. In rat hepatocytes exposed to APAP, formation of glutathione mixed disulfides accounted for a loss of 22% of total protein thiols [26]. These authors also reported some formation of GSSG in hepatocytes exposed to APAP, which supports the findings in the present study [26].

Inhibition of GSSG-Rd with BCNU has been shown to potentiate the toxicity of a number of compounds that induce damage via oxidative mechanisms [13, 26, 27]. BCNU produced marked inhibition of GSSG-Rd activity in mouse hepatocytes without an effect on APAP oxidation. Both the cell killing and GSSG formation induced by APAP were potentiated markedly by BCNU. In these cells the fraction of GSH equivalents appearing as GSSG after 2 hr was 0.59 (data from Fig. 2 and Table 2), indicating that BCNU further potentiates APAP-induced GSSG formation.

The toxicity of agents that induce an oxidative stress depend upon cellular sources of iron [24]. Chelation of ferric iron with DFO in cultured rat hepatocytes prevents the toxicity of both hydrogen peroxide [23] and *tert*-butyl hydroperoxide [13]. In the present study, pretreatment with DFO abolished GSSG production induced by APAP, and substantially reduced the cell killing. This demonstrates that the formation of GSSG as a result of APAP exposure is dependent upon the presence of iron. These findings are supported by a previous study comparing the toxicity of APAP with its putative toxic metabolite NAPQI. The chelation of intracellular iron by deferoxamine causes a marked reduction in the extent of cytotoxicity in hepatocytes [28].

Experiments were repeated in KRH buffer to determine changes in the glutathione pool in the absence of any *de novo* synthesis. APAP induced an increase in GSSG formation and efflux, clearly showing that APAP causes a marked intracellular oxidative stress that leads to significant oxidation of the existing glutathione pool, and is not merely a result of increased glutathione synthesis. GSSG formation in these incubation conditions was not due to overt cell killing as APAP did not cause any cell death in hepatocytes until after 3 hr of exposure (data not shown).

In summary, the killing of cultured mouse hepatocytes by APAP was accompanied by an increase in the formation of GSSG. The amount of GSSG formed represented a substantial fraction of the total cellular glutathione pool. Inhibition of GSSG-Rd increased GSSG formation and

cytotoxicity induced by APAP, whereas chelation of iron with DFO decreased GSSG formation and APAP toxicity. Whether the oxidation of glutathione is a result of APAP-induced reactive oxygen intermediate generation or of oxidations induced by the quinone imine metabolite is yet to be determined. However, the formation of GSSG occurred well before overt cell killing, and it follows that the oxidative damage produced by APAP in these hepatocytes is an early event in the poisoning process and contributes to its severity.

Acknowledgements—This work was supported by a grant from the National Health and Medical Research Council of Australia and the TVW Telethon Foundation.

REFERENCES

1. Prescott LF, Paracetamol: A model for toxic injury mediated by the formation of active metabolites. In: *Liver Cells and Drugs* (Ed. Guillouzo A), Vol. 164, pp. 153–160. Colloque INSERM/John Libbey Eurotext Ltd., London, 1988.
2. Potter WZ, Davis DC, Mitchell JR, Jollow DJ, Gillette JR and Brodie BB, Acetaminophen-induced hepatic necrosis. III. Cytochrome P-450-mediated covalent binding *in vitro*. *J Pharmacol Exp Ther* **187**: 203–210, 1973.
3. Moore M, Thor H, Moore G, Nelson S, Moldeus P and Orrenius S, The toxicity of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca^{2+}. *J Biol Chem* **260**: 13035–13040, 1985.
4. Tsokos-Kuhn JO, Hughes H, Smith CV and Mitchell JR, Alkylation of the liver plasma membrane and inhibition of the Ca^{2+} ATPase by acetaminophen. *Biochem Pharmacol* **37**: 2125–2131, 1988.
5. Wendel A, Feuerstein S and Heinz-Konz K, Acute paracetamol intoxication of starved mice leads to lipid peroxidation *in vivo*. *Biochem Pharmacol* **28**: 2051–2055, 1979.
6. Wendel A and Feuerstein S, Drug-induced lipid peroxidation in mice–I. Modulation by monooxygenase activity, glutathione and selenium status. *Biochem Pharmacol* **30**: 2513–2520, 1981.
7. Wendel A, Jaeschke H and Gloger M, Drug-induced lipid peroxidation in mice—II. Protection against paracetamol-induced liver necrosis by intravenous liposomally entrapped glutathione. *Biochem Pharmacol* **31**: 3601–3605, 1982.
8. Adamson GM and Harman AW, A role for the glutathione peroxidase/reductase enzyme system in the protection from paracetamol toxicity in isolated mouse hepatocytes. *Biochem Pharmacol* **38**: 3323–3330, 1989.
9. Farber JL, Leonard TB, Kyle ME, Nakae D, Serroni A and Rogers SA, Peroxidation-dependent and peroxidation-independent mechanisms by which acetaminophen kills cultured rat hepatocytes. *Arch Biochem Biophys* **267**: 640–650, 1988.
10. Nakae D, Oakes JW and Farber JL, Potentiation in the intact rat of the hepatotoxicity of acetaminophen by 1,3-bis(2-chloroethyl)-1-nitrosourea. *Arch Biochem Biophys* **267**: 651–659, 1988.
11. Harman AW, The effectiveness of antioxidants in reducing paracetamol-induced damage subsequent to paracetamol activation. *Res Commun Chem Pathol Pharmacol* **49**: 215–228, 1985.
12. Lauriault VVM and O'Brien PJ, Molecular mechanism for prevention of *N*-acetyl-*p*-benzoquinone imine cytotoxicity by the permeable thiol drugs diethyldithiocarbamate and dithiothreitol. *Mol Pharmacol* **40**: 125–134, 1991.
13. Sies H, *Oxidative Stress* (Ed. Sies H), pp. 73–90. Academic Press, London, 1985.
14. Cadenas E, Biochemistry of oxygen toxicity. *Annu Rev Biochem* **58**: 79–110, 1989.
15. Jones DP, Eklow L, Thor H and Orrenius S, Metabolism of hydrogen peroxide in isolated hepatocytes: Relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated H_2O_2. *Arch Biochem Biophys* **210**: 505–516, 1981.
16. Harman AW, McCamish LE and Henry CA, Isolation of hepatocytes from postnatal mice. *J Pharmacol Methods* **17**: 157–163, 1987.
17. Harman AW and Fischer LJ, Hamster hepatocytes in culture as a model for acetaminophen toxicity: Studies with inhibitors of drug metabolism. *Toxicol Appl Pharmacol* **71**: 330–341, 1983.
18. Harman AW and McCamish LE, Age-related toxicity of paracetamol in mouse hepatocytes. *Biochem Pharmacol* **35**: 1731–1735, 1986.
19. Fischer LJ, Green MD and Harman AW, Levels of acetaminophen and its metabolites in mouse tissues after a toxic dose. *J Pharmacol Exp Ther* **219**: 281–286, 1981.
20. Hartree EF, Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal Biochem* **48**: 422–427, 1972.
21. Fariss MW and Reed DJ, Measurement of glutathione and glutathione disulfide efflux from isolated hepatocytes. In: *Isolation, Characterization, and Use of Hepatocytes* (Eds. Hariss RA and Cornell NW), pp. 349–355. Elsevier, New York, 1983.
22. Grimm H, Analysis of variance. In: *Biostatistics for Pharmacology* (Ed. Delaunois AL), pp. 675–716. Pergamon Press, Oxford, 1973.
23. Rubin R and Farber JL, Mechanisms of the killing of cultured hepatocytes by hydrogen peroxide. *Arch Biochem Biophys* **228**: 450–459, 1984.
24. Starke PE and Farber JL, Endogenous defenses against cytotoxicity of hydrogen peroxide in cultured rat hepatocytes. *J Biol Chem* **260**: 10099–10104, 1985.
25. Gerson RJ, Casini A, Gilfor D, Serroni A and Farber JL, Oxygen-mediated cell injury in the killing of cultured hepatocytes with acetaminophen. *Biochem Biophys Res Commun* **126**: 1129–1137, 1985.
26. Miccadei S, Nakae D, Kyle ME, Gilfor D and Farber JL, Oxidative cell injury in the killing of cultured hepatocytes by allyl alcohol. *Arch Biochem Biophys* **265**: 302–310, 1988.
27. Kyle ME, Sakaida I, Serroni A and Farber JL, Metabolism of acetaminophen by cultured rat hepatocytes: Depletion of protein thiol groups without any loss of viability. *Biochem Pharmacol* **40**: 1211–1218, 1990.
28. Harman AW, Kyle ME, Serroni A and Farber JL, The killing of cultured hepatocytes by *N*-acetyl-*p*-benzoquinone imine (NAPQI) as a model of the cytotoxicity of acetaminophen. *Biochem Pharmacol* **41**: 1111–1117, 1991.