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METABOLISM OF ACETAMINOPHEN BY CULTURED RAT HEPATOCYTES

DEPLETION OF PROTEIN THIOL GROUPS WITHOUT ANY LOSS OF VIABILITY

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Abstract—Over the course of 4 hr, the metabolism of acetaminophen (APAP) by cultured rat hepatocytes resulted in a depletion of protein thiols and an accumulation of oxidized glutathione (GSSG) in the medium. With 20 mM APAP, arylation and the formation of glutathione mixed disulfides accounted for a loss of 22% of the total protein thiols in the absence of any loss of viability. With 20 mM APAP and an inhibition of glutathione reductase by 1,3-(2-chloroethyl)-1-nitrosourea (BCNU), protein thiols were depleted by 40% by arylation and the formation of glutathione mixed disulfides, again without a loss of viability. With 20 mM APAP and BCNU in the presence of 20 mM deferoxamine, there was still little or no cell killing after 8 hr despite a loss now of almost 60% of the total protein thiols. These data do not support the hypothesis that a depletion of protein thiols is related to the toxicity of APAP. One millimolar APAP and BCNU killed 60% of the hepatocytes within 4 hr. In this circumstance, the loss of protein thiols was not attributable to either arylation by APAP metabolites or the formation of glutathione mixed disulfides. The antioxidant *N,N'*-diphenyl-phenylenediamine prevented the cell killing and the loss of protein thiols, a result implicating a role for lipid peroxidation in the depletion of protein-bound thiols. However, protein thiol depletion under these circumstances is not necessarily related to the lethal cell injury and most likely represents an epiphenomenon of the peroxidation of cellular lipids.

A loss of protein thiol groups is held to be a critical event in the genesis of lethal cell injury by an acute oxidative stress [1–3]. Such a depletion of protein thiols can occur by a number of different mechanisms. The major mechanism is presumed to be a direct oxidation of the thiol groups of contiguous amino acids with the formation of protein–protein disulfides [1–3]. Glutathione mixed disulfides formed in the reaction of protein thiols with oxidized glutathione (GSSG‡) [4, 5] are believed to be of quantitatively lesser importance [1]. Finally, quinones such as menadione that undergo cyclical oxidation–reduction reactions to generate reactive oxygen species can directly arylate protein thiol groups [1–3].

The killing of liver cells by the hepatotoxin acetaminophen has also been attributed to a depletion of protein thiols [6–9]. Treatment of hepatocytes with acetaminophen or the electrophilic product of its oxidative metabolism, *N*-acetyl-*p*-benzoquinone imine (NAPQI), results in a rapid depletion of protein thiol groups [6–9]. The covalent binding of NAPQI to protein accounts for only part of the total depletion of protein thiols [6, 9]; the remainder is said to result from a direct oxidation of the thiols, presumably by NAPQI itself [6]. This conclusion was based on the ability of reducing agents, such as dithiothreitol, to restore the protein thiol content of the hepatocytes [6]. However, neither protein–protein disulfides nor glutathione protein mixed disulfides were quantitated directly [6–9].

Our studies with acetaminophen that employ cultured rat hepatocytes have implicated a metabolism-dependent generation of partially reduced oxygen species in the toxicity of this compound [10–13]. Superoxide dismutase, catalase, and mannitol prevent the cell killing [11]. Pretreatment of the hepatocytes with the ferric iron chelator deferoxamine prevents the loss of viability without reducing the covalent binding [10–12]. These data document the participation in this model of activated oxygen species in the cytotoxicity of acetaminophen, and suggest that hydroxyl radicals, or an equivalent species, mediate the cell injury. One action of this species is to initiate the peroxidation of membrane lipids, a process that can be related to the loss of hepatocyte viability [13].

Using this same system, the present study evaluates the mechanisms by which acetaminophen depletes protein thiols and assesses the relationship of this depletion to the cell killing. The metabolism of acetaminophen is shown to result in a significant accumulation of GSSG. In turn, the formation of glutathione protein mixed disulfides as well as arylation by NAPQI can deplete 40–60% of the total protein thiol content of the hepatocytes under conditions where there is little or no loss of viability.

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‡ Abbreviations: GSSG, oxidized glutathione; NAPQI, *N*-acetyl-*p*-benzoquinone imine; APAP, acetaminophen; GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; BCNU, 1,3-(2-chloroethyl)-1-nitrosourea; DPPD, *N,N'*-diphenyl-phenylenediamine; and LDH, lactate dehydrogenase.

MATERIALS AND METHODS

Male Sprague–Dawley rats (175–225 g) were purchased from Charles River Laboratories, Wilmington, MA. All animals were treated with 25 mg/kg 3-methylcholanthrene (Sigma) as a 10 mg/mL solution in corn oil by intraperitoneal injection on the afternoon of the day prior to use and then fasted overnight. Isolated hepatocytes were prepared by collagenase perfusion according to Seglen [14].

Yields of $2–6 \times 10^8$ cells/liver with 85–90% viability by trypan blue exclusion were obtained routinely. The hepatocytes were plated in 25 cm^2 flasks (Corning Glass Works, Corning, NY) at a density of 1.33×10^6 cells/flask in 3 mL of Williams' E medium (GIBCO Laboratories, Chagrin Falls, OH) containing 10% heat-inactivated (56°, 15 min) fetal calf serum (Hazelton Research Products, Lenexa, KA), 10 I.U./mL penicillin, 10 μg/mL streptomycin, 0.05 mg/mL gentamicin, and 0.02 units/mL insulin (complete Williams' E medium). After incubation for 2 hr in an atmosphere of 5% CO_2–95% air, the cultures were rinsed twice with a prewarmed HEPES (Sigma) buffer (0.14 M NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$ and 2.4 mM HEPES, pH 7.4) to remove unattached dead cells. The hepatocytes were then incubated in complete Williams' E medium for 4 or 8 hr with the treatments indicated in the text. The viability of the cells was determined by the release of lactate dehydrogenase (LDH) into the culture medium [15] as described previously. Acetaminophen did not inhibit the activity of LDH at any of the concentrations used.

Acetaminophen (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and added to the cultures to yield final concentrations ranging from 1 to 20 mM (final concentration of DMSO was always 1%, v/v). 1,3-(2-Chloroethyl)-1-nitrosourea (BCNU, Bristol Laboratories) was dissolved in absolute ethanol and added to the cultures simultaneously with acetaminophen to a final concentration of 50 μM (final concentration of ethanol was 0.03%, v/v). Deferoxamine (Ciba Pharmaceutical Co., Summit, NJ) was dissolved in 0.9% saline and added to the cultures at a final concentration of 20 mM. *N,N'*-Diphenyl-phenylenediamine (DPPD, Eastman Kodak Co.) and β-naphthoflavone (Sigma) were dissolved in DMSO and added to final concentrations of 2 and 10 μM respectively.

The protein thiol content of the cultured hepatocytes was determined spectrophotometrically at 412 nm using Ellman's reagent (Sigma) according to the method of Sedlak and Lindsay [16] as modified by Albano *et al.* [6]. Glutathione protein mixed disulfides were determined according to the procedure described by Bellomo *et al.* [5] with the exception that the released glutathione was quantitated by the fluorometric method of Hissin and Hilf [17]. The protein content of the cultures was assayed by a modification of the procedure of Lowry *et al.* [18] as described by Peterson [19]. The GSSG content of the culture medium was measured by the HPLC method of Reed *et al.* [20].

The covalent binding of [^{3}H]acetaminophen to protein was measured following incubation of cultures with either 1, 10, or 20 mM acetaminophen and a total of 1 μCi/mL of [^{3}H]acetaminophen (*p*-[^{3}H(G)hydroxyacetanilide, 9.3 Ci/mmol, New England Nuclear). After 4 hr the medium was aspirated, and 5 mL of 10% trichloroacetic acid was added to the flasks. The cells were scraped from the plates, the precipitates recovered by centrifugation, and the proteins prepared according to Rao and Recknagel [21]. The protein residues were weighed in tared scintillation vials, dissolved in 1 mL of NCS tissue solubilizer (Amersham Corp.), and then counted in 15 mL of a toluene based liquid scintillation fluid.

All experiments were repeated at least three times. The data from one representative experiment are shown. The statistical significance of the data was determined by Student's *t*-test (unpaired).

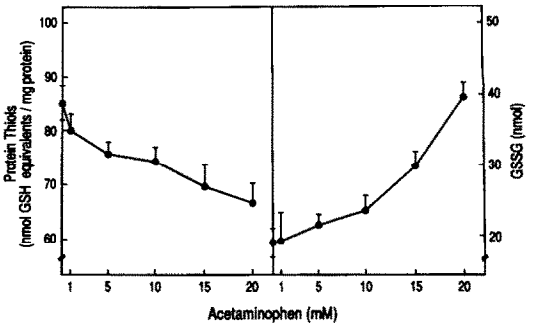

Fig. 1. Protein thiol depletion and GSSG accumulation in hepatocytes treated with acetaminophen. Hepatocytes in culture for 2 hr were washed and then treated with from 1 to 20 mM acetaminophen (APAP). The protein thiol content of the cells and the GSSG content of the medium were measured 4 hr later. Results are the means ± SD of the determinations on three separate flasks. Protein thiol levels were statistically different from control at concentrations of APAP greater than 1 mM ($P < 0.02$). GSSG was statistically different from control at concentrations of APAP greater than 5 mM ($P < 0.05$).

RESULTS

Protein thiol depletion and GSSG accumulation in hepatocytes treated with acetaminophen. Isolated hepatocytes prepared from rats induced with 3-methylcholanthrene were placed in culture for 2 hr, washed, and then treated with from 1 to 20 mM acetaminophen. Four hours later the content of protein thiols and the accumulation of GSSG in the medium were determined. There was no loss of viability with any of the concentrations of acetaminophen over the 4 hr of this experiment. However, as detailed in the left panel of Fig. 1, the content of protein thiols declined as the concentration of acetaminophen increased. With 20 mM acetaminophen, protein thiols were depleted by 18 nmol GSH equivalents/mg protein, a loss of about 22% of the total protein thiols. The addition to the culture medium of 10 μM β-naphthoflavone, an inhibitor of the mixed-function oxidase activity induced by 3-methylcholanthrene and, thus, of the metabolism of acetaminophen, did not change the content of protein thiols (84 ± 4 nmol GSH equivalents/mg protein) from that of control cultures (85 ± 3).

Table 1. GSH protein mixed disulfides and covalent binding in hepatocytes treated with acetaminophen

	GSH protein mixed disulfides (nmol GSH/mg protein)	Covalent binding (nmol/mg protein)
Control	4.8 ± 0.7	
APAP (1 mM)	5.4 ± 0.8	4 ± 1
APAP (10 mM)	6.4 ± 0.9*	11 ± 0.5
APAP (20 mM)	8.4 ± 0.8†	17 ± 1

Hepatocytes in culture for 2 hr were washed and then treated with 1, 10 or 20 mM acetaminophen (APAP). GSH protein mixed disulfides were measured 4 hr later. Other cultures were treated with similar concentrations of [^{3}H]APAP for 4 hr to determine the extent of covalent binding to protein. Results are the means ± SD of the determinations on three separate flasks.

* Significantly different from control at $P < 0.05$.

† Significantly different from control at $P < 0.01$.

Table 2. Mechanism of the loss of protein thiols in hepatocytes treated with acetaminophen

	Sum of covalent binding and mixed disulfides (nmol/mg protein)	Protein thiol loss
1 mM APAP	4.6*	5 ± 2†
10 mM APAP	12.6	11 ± 2
20 mM APAP	20.6	18 ± 4
1 mM APAP + BCNU	2.6	19 ± 3
10 mM APAP + BCNU	14.0	22 ± 3
20 mM APAP + BCNU	29.2	30 ± 3

* These data represent the calculated sum of the covalent binding and glutathione mixed disulfides in Tables 1 and 3.

† These data represent the depletion of protein thiols shown in Figs. 1 and 2. Values are means ± SD, N = 3.

The right panel of Fig. 1 details the accumulation of GSSG in the culture medium. No significant accumulation of GSSG was observed in those cultures treated with either 1 or 5 mM acetaminophen. However, with concentrations greater than 5 mM, the GSSG content of the medium was increased significantly over that seen with untreated cells. With 20 mM acetaminophen, the amount of GSSG present in the medium after 4 hr was 40 nmol/flask, a level 2-fold greater than that of untreated cultures (19 nmol/flask). This accumulation of GSSG also depended upon the metabolism of acetaminophen. The presence of 10 μM β-naphthoflavone reduced the accumulation of GSSG to that of controls (20 nmol/flask).

The content of glutathione protein mixed disulfides in untreated cells (Table 1) was similar to that reported previously with suspensions of freshly isolated hepatocytes [1]. Whereas there was no significant increase with 1 mM acetaminophen, 10 and 20 mM acetaminophen increased the cellular content of glutathione protein mixed disulfides. Thus, concentrations of acetaminophen (10 and 20 mM) that produced a significant increase in the formation of glutathione protein mixed disulfides also resulted in a significant accumulation of GSSG (Fig. 1).

Table 1 also details the extent of the covalent binding to total hepatocyte proteins of the metabolites of radiolabeled acetaminophen. Increasing concentrations of acetaminophen were accompanied by increased covalent binding. In addition, the sum of the covalent binding and the increase in glutathione mixed disulfides can account for the entire loss of protein thiols with each concentration of acetaminophen (Table 2). With 1 mM acetaminophen, covalent binding (arylation) was the major mechanism depleting protein thiols. By contrast, formation of glutathione mixed disulfides and arylation together accounted for the depletion of protein thiols with 10 and 20 mM acetaminophen.

Protein thiol depletion and GSSG accumulation in hepatocytes treated with acetaminophen and BCNU. As shown in Fig. 2 (left panel), acetaminophen had a biphasic effect on cell viability in the presence of BCNU, an inhibitor of glutathione reductase. Concentrations of 1 and 5 mM acetaminophen killed a substantial proportion of the cells over the 4-hr course of the experiment. By contrast, higher concentrations of acetaminophen were significantly less

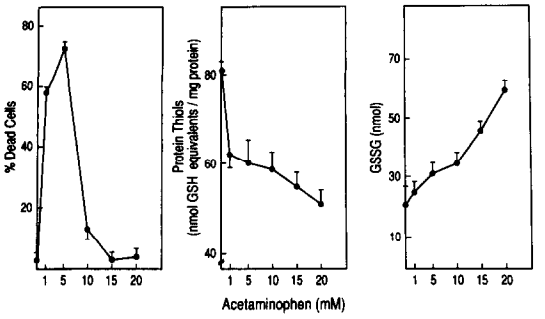

Fig. 2. Cell killing, protein thiol depletion, and GSSG accumulation in hepatocytes treated with acetaminophen and BCNU. Hepatocytes in culture for 2 hr were washed and placed in fresh medium containing 50 μM BCNU, and then treated with from 1 to 20 nM acetaminophen (APAP). The release of LDH, the protein thiol content of the cells, and the GSSG content of the medium were measured 4 hr later. Results are the means ± SD of the determinations on three separate flasks. Protein thiol levels were statistically different from control at all concentrations of APAP ($P < 0.01$). GSSG was statistically different from control at concentrations of APAP greater than 1 mM ($P < 0.05$).

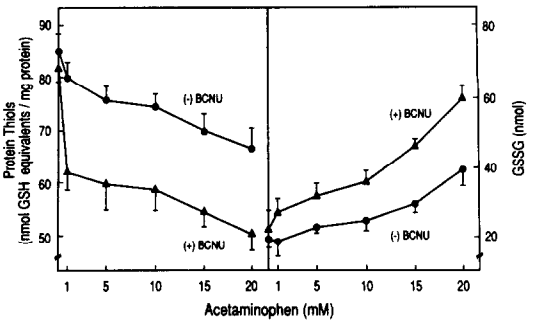

Fig. 3. Effect of acetaminophen on protein thiol depletion and GSSG accumulation in the presence or absence of BCNU. Hepatocytes in culture for 2 hr were washed and then treated with from 1 to 20 mM acetaminophen (APAP) in the presence or absence of 50 μM BCNU. The protein thiol content of the cells and the GSSG content of the medium were measured 4 hr later. Results are the means ± SD of the determinations on three separate flasks. The protein thiol content in the presence of BCNU was statistically different from that in its absence at all concentrations of APAP ($P < 0.01$). The accumulation of GSSG in the presence of BCNU was statistically different from that in its absence at concentrations of APAP greater than 1 mM ($P < 0.05$).

toxic. Indeed, there was no cell killing with 15 and 20 mM acetaminophen. This result is similar to that reported previously [13] and is attributable to the antioxidant action of higher concentrations of acetaminophen [22, 23].

The effect of acetaminophen in the presence of BCNU on the protein thiol content of hepatocyte cultures is illustrated in the center panel of Fig. 2. Acetaminophen at 1 mM abruptly depleted protein thiols by 19 nmol GSH equivalents/mg of protein thiol. With concentrations of acetaminophen greater than 5 mM, protein thiol levels declined further despite the decreasing cell killing. Importantly, with 20 mM acetaminophen the protein thiol content was reduced by nearly 40% without any loss of viability.

The accumulation of GSSG in the cultures exposed to acetaminophen and BCNU is shown in the right panel of Fig. 2. Again treatment with acetaminophen resulted in a concentration-dependent increase in GSSG with significantly greater accumulations at all concentrations of acetaminophen above 1 mM. The accumulation of GSSG in the presence of acetaminophen plus BCNU, as well as the depletion of protein thiols, was dependent upon the oxidative metabolism of acetaminophen. Addition of 10 μM β-naphthoflavone to the culture medium prevented the increase in GSSG and the loss of protein thiols with each concentration of acetaminophen.

Figure 3 compares the effect of acetaminophen in the presence or absence of BCNU on both the loss of protein thiols and the accumulation of GSSG. As illustrated in the left panel, BCNU was without effect on the protein thiol content of native hepatocytes. However, BCNU potentiated the loss of protein thiols at all concentrations of acetaminophen studied. Similarly, BCNU had no effect on the accumulation of GSSG in control cultures, but potentiated the accumulation of GSSG in response to acetaminophen at concentrations greater than 1 mM.

Table 3. GSH protein mixed disulfides and covalent binding in hepatocytes treated with acetaminophen and BCNU

	GSH protein mixed disulfides (nmol GSH/mg protein)	Covalent binding (nmol/mg protein)
Control	5.2 ± 1	
APAP (1 mM)	5.3 ± 1	2.5 ± 1
APAP (10 mM)	8.9 ± 1*	10 ± 1
APAP (20 mM)	19.4 ± 4†	15 ± 1

Hepatocytes in culture for 2 hr were washed and then fresh medium containing 50 μM BCNU was added. The cells were then treated with 1, 10 or 20 mM acetaminophen (APAP); GSH protein mixed disulfides were measured 4 hr later. Other cultures were treated with similar concentrations of [^{3}H]APAP for 4 hr to determine the extent of covalent binding to protein. Results are the means ± SD of the determinations on three separate flasks.

* Significantly different from control at $P < 0.02$.

† Significantly different from control at $P < 0.01$.

Table 4. Effect of deferoxamine on the loss of protein thiols in hepatocytes treated with 20 mM acetaminophen and BCNU

	% Dead cells	Protein thiols (nmol GSH/mg protein)
Control	4 ± 2	85 ± 2
APAP	70 ± 2	35 ± 4
APAP + deferoxamine	15 ± 4	33 ± 5

Hepatocytes in culture for 2 hr were washed, fresh medium containing 50 μM BCNU was added and then the cultures were treated with 20 mM acetaminophen (APAP) in the presence or absence of 20 mM deferoxamine. The release of LDH and the content of protein thiols were measured 8 hr later. Results are the means ± SD of the determinations on three separate flasks.

The enhanced accumulation of GSSG in the presence of BCNU was, in turn, accompanied by an increased formation of glutathione protein mixed disulfides. Table 3 details the formation of glutathione protein mixed disulfides and the extent of covalent binding with 1, 10 and 20 mM acetaminophen in the presence of BCNU. A comparison with Table 1 shows that BCNU increased the formation of glutathione protein mixed disulfides with 10 and 20 mM acetaminophen. By contrast, BCNU did not increase the extent of covalent binding (Tables 1 and 3). This is consistent with the previous demonstration of a slight inhibitory effect of BCNU on the oxidative metabolism of acetaminophen [13].

It is important to note that the sum of the covalent binding and the glutathione mixed disulfides accounted almost completely for the depeletion of 40% of the total protein thiols with 20 mM acetaminophen in the presence of BCNU (Table 2). Again, there was no cell killing under these conditions. With 10 mM acetaminophen and BCNU, the sum of the covalent binding and the formation of glutathione mixed disulfides accounted for only 65% of the total protein thiol depletion (Table 2). At this concentration 18% of the cells died over the 4-hr course of the experiment. With 1 mM acetaminophen plus BCNU 58% of the cells were killed. However, only 14% of the depletion of protein thiols was accounted for by arylation and glutathione mixed disulfide formation (Table 2).

The loss of 40% of the total protein thiols at 4 hr in heptatocytes treated with 20 mM acetaminophen plus BCNU is not necessarily related to the loss of viability upon a more prolonged incubation of the cells. As shown in Table 4, incubation of cultured heptatocytes with 20 mM acetaminophen plus BCNU killed 70% of the cells after 8 hr. This loss of viability was accompaned by a depletion of nearly 60% of the total protein thiol content (Table 4). Addition of the ferric iron chelator deferoxamine prevented the death of 55% of the hepatocytes. Hmwever, chelation of iron had no effect on the extent of protein thiol depletion. Thus, 60% of the total protein thiols was lost by treatment of hepatocytes with 20 mM acetaminophen in the presence of BCNU and deferoxamine without a substantial loss of viability for up to 8 hr.

Effect of DPPD on the depletion of protein thiols and accumulation of GSSG. Table 5 documents the effect of the antioxidant DPPD on both the depletion of protein thiols and the accumulation of GSSG after treatment of the hepatocytes with either a lethal (1 mM) or non-lethal (20 mM) concentration of acetaminophen in the presence of BCNU. The addition of 2 μM DPPD to the culture medium prevented the loss of viability with 1 mM acetaminophen. DPPD also prevented the depletion of protein thiols. By contrast, DPPD had no effect on the extent of protein thiol depletion produced by 20 mM acetaminophen. Furthermore, DPPD had no effect on the accumulation of GSSG (Table 5), the extent of covalent binding (Table 6), or on the formation of glutathione protein mixed disulfides (Table 6) in hepatocytes treated with either 1 or 20 mM

Table 5. Effect of DPPD on the cytotoxicity, protein thiol depletion and GSSG accumulation produced by acetaminophen and BCNU

	% Dead cells	Protein thiols (nmol GSH/mg protein)	GSSG (nmol/flask)
Control	4 ± 1	81 ± 2	23 ± 5
APAP (1 mM)	58 ± 2	62 ± 5*	28 ± 4
APAP (1 mM) + DPPD	4 ± 2	80 ± 3	27 ± 3
APAP (20 mM)	6 ± 2	51 ± 3†	58 ± 4†
APAP (20 mM) + DPPD		52 ± 1†	59 ± 4†

Hepatocytes in culture for 2 hr were washed, fresh medium containing 50 μM BCNU was added, and then the cultures were treated with 20 mM acetaminophen (APAP) in the presence or absence of 2 μM DPPD. The release of LDH and the protein thiol content and the GSSG level in the medium were measured 4 hr later. Results are the means ± SD of the determinations on three separate flasks.

* Significantly different from control at $P < 0.01$.

† Significantly different from control at $P < 0.001$.

Table 6. Effect of DPPD on GSH protein mixed disulfides and covalent binding in hepatocytes treated with acetaminophen and BCNU

	GSH protein mixed disulfides (nmol GSH/mg protein)	Covalent binding (nmol/mg protein)
Control	5.2 ± 1	
APAP (1 mM)	5.3 ± 1	2.5 ± 1
APAP (1 mM) + DPPD	6.2 ± 1*	3.8 ± 1
APAP (20 mM)	19.4 ± 4	15.0 ± 1
APAP (20 mM) + DPPD	18.4 ± 2†	15.0 ± 3

Hepatocytes in culture for 2 hr were washed, and fresh medium containing 50 μM BCNU was added. The cells were then treated with 1 or 20 mM acetaminophen (APAP) in the presence or absence of 2 μM DPPD. GSH protein mixed disulfides were measured 4 hr later. Other cultures were treated with similar concentrations of [^{3}H]APAP for 4 hr to determine the extent of covalent binding to protein. Results are the means ± SD of the determinations on three separate flasks.

* Not significantly different from 1 mM APAP alone.

† Not significantly different from 20 mM APAP alone.

acetaminophen. Thus, in a situation associated with cell killing (1 mM acetaminophen plus BCNU), protein thiol depletion occurred by a mechanism sensitive to the antioxidant DPPD. On the other hand, in the absence of cell killing (20 mM plus BCNU), the depletion of protein thiols was insensitive to the presence of DPPD.

DISCUSSION

We have quantitated the changes in protein thiols that occur upon the metabolism of acetaminophen by cultured rat hepatocytes. In the absence of an inhibition of glutathione reductase with BCNU, the metabolism of acetaminophen was not accompanied by cell killing over the course of a 4-hr incubation. By contrast, in the presence of BCNU, there was cell killing within 4 hr with relatively low concentrations of acetaminophen (1–5 mM), but not with higher concentrations (15–20 mM). Thus, we could relate a loss of protein thiols to the metabolism of acetaminophen under conditions where there was or was not associated cytotoxicity. The data presented indicate that the mechanism by which protein thiols are depleted under conditions in which cell killing occurs is different from that mediating the loss of protein thiols in the absence of toxicity.

The metabolism of acetaminophen generates two products that react with protein thiol groups. Acetaminophen is oxidized by the loss of 2 electrons to the quinone imine, NAPQI. In turn, NAPQI covalently binds to protein thiol groups. The metabolism of acetaminophen also generates GSSG, a product that reacts with protein thiols to form glutathione mixed disulfides. In the absence of BCNU, the metabolism of 20 mM acetaminophen resulted in the depletion of 22% of the total protein thiols without any loss of viability. The arylation of protein thiols by NAPQI and the formation of glutathione mixed disulfides accounted entirely for this loss of protein thiols (Table 2).

Inhibition of glutathione reductase by BCNU prevents the reduction of GSSG to GSH. As a result GSH is depeleted more rapidly [12, 13], a consequence that is reflected in a greater accumulation of GSSG. In the presence of BCNU, the fate of the hepatocytes depended upon the concentration of acetaminophen. Concentrations in the range of 1–5 mM were accompanied by substantial cell killing within 4 hr. By contrast, concentrations in the range of 10–20 mM were not toxic to the cells over this same time course. Interestingly, the decreased toxicity of acetaminophen with the higher concentrations occurred despite a greater formation of NAPQI, a fact indicated by the increased covalent binding with 20 mM as opposed to 1 mM acetaminophen (Table 3). The increased formation of NAPQI with 20 mM acetaminophen was paralleled by an increased formation of GSSG and, in turn, glutathione mixed disulfides.

Thus, in the presence of BCNU the metabolism of 20 mM acetaminophen resulted in the depletion of 40% of the total protein thiol content. Covalent binding to NAPQI as well as the formation of glutathione mixed disulfides again accounted entirely for this loss. However, these processes occurred without any loss of viability within 4 hr. Prolonged exposure of these cells to 20 mM acetaminophen and BCNU was toxic. After 8 hr, 70% of the cells were dead and protein thiol levels declined by nearly 60%. But even this extent of the loss of protein thiols could be dissociated form the loss of viability. Whereas this cell injury over an 8 hr time course depended on a cellular source of ferric iron, the depletion of protein thiols was iron independent. Thus, hepatocytes could be depleted of 60% of their total protein thiols without a substantial loss of viability for up to 8 hr. Such a result does not support the hypothesis that a depletion of protein thiols is a mechanism of lethal cell injury by acetaminophen.

Previous studies of both acetaminophen and

NAPQI that used suspensions of freshly isolated hepatocytes [6–8] attributed the observed cell killing to a loss of protein thiols. When added to hepatocytes, 400–500 μM NAPQI causes a 40–50% loss of protein thiols [6–8], a depletion similar to the present result with 20 mM acetaminophen and BCNU. However, with 400–500 μM NAPQI essentially all the hepatocytes are killed within 3 hr [6–8]. Our data indicate that 20 mM acetaminophen can deplete 40% of the total protein thiols over a similar time course, and 60% of the total over twice the time course, without any loss of viability. Thus, it would seem that either the results with NAPQI are not necessarily relevant to the toxicity of acetaminophen, or the loss of protein thiols with NAPQI is unrelated to its toxicity. The present data suggest that the latter is likely the case.

In the presence of BCNU, 1 mM acetaminophen killed a substantial proportion of the cells within 4 hr. This cell killing was accompanied by a loss of about 25% of the total protein thiol content. Under these conditions, the loss of protein thiols could not be attributred to either the covalent binding of NAPQI or the formation of glutathione protein mixed disulfides (Table 2). Both the loss of viability and the loss of protein thiols produced by 1 mM acetaminophen in the presence of BCNU was prevented by the antioxidant DPPD. By contrast, DPPD had no effect on the protein thiol loss that occurred in the absence of cell killing with 20 mM acetaminophen and BCNU. Thus, there is a mechanism independent of arylation and the formation of glutathione mixed disulfides that depletes protein thiols during the course of cell killing by 1 mM acetaminophen.

We have shown previously that in the presence of BCNU the killing of cultured hepatocytes by 1 mM acetaminophen is related to the peroxidation of membrane lipids [13]. Such a relationship explains the ability of DPPD to prevent the killing of hepatocytes seen here and in the previous report [13]. The peroxidation of the lipids of cultured hepatocytes produces soluble products that react and thereby deplete protein thiols [24]. These products, perhaps reactive aldehydes, deplete protein thiols without an accompanying loss of viability [24]. Thus, the loss of protein thiols that occurs with 1 mM acetaminophen and BCNU is probably a consequence of the peroxidation of cellular lipids. However, there is no evidence that this loss of protein thiols is necessarily related to the cell killing. More likely, the depletion of protein thiols is simply an epiphenomenon of the lethal peroxidation of membrane lipids.

Finally, some mention must be made of the source of the GSSG shown here to form as a result of the metabolism of acetaminophen. In the test tube, NAPQI can oxidise GSH to GSSG with the formation of acetaminophen [6]. Alternatively, the GSSG could result from the reduction of hydrogen peroxide formed as a consequence of the oxidation of acetaminophen to NAPQI [25]. This latter mechanism would be consistent with the ability of catalase to prevent the toxicity of acetaminophen [11].

The ability of NAPQI to oxidize GSH has been used to argue that the loss of protein thiols is also a result of their oxidation to protein–protein disulfides by NAPQI [6]. The observation that dithiothreitol restored much of the protein thiols was used to support such an interpretation [6]. However, thiol reducing agents, such as dithiothreitol, are equally effective in reducing glutathione protein mixed disulfides. Thus, it remains likely that the previous loss of protein thiols attributed to protein–protein disulfides may actually reflect, at least in part, the formation of glutathione mixed disulfides. In this regard it is noteworthy that the formation of glutathione mixed disulfides reported here with 20 mM acetaminophen accounts entirely for that proportion of the loss of protein thiols not due to covalent binding.

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