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Mechanisms of *N*-Acetyl-*p*-benzoquinone imine Cytotoxicity

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SUMMARY

N-Acetyl-*p*-benzoquinone imine (NAPQI), a reactive metabolite of acetaminophen, rapidly reacts at physiological pH with glutathione (GSH) forming an acetaminophen-glutathione conjugate and stoichiometric amounts of acetaminophen and glutathione disulfide (GSSG). The same reaction products are formed in isolated hepatocytes incubated with NAPQI. In hepatocytes which have been treated with 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) in order to inhibit glutathione reductase, the initial rise in GSSG concentration in the presence of NAPQI is maintained, whereas GSSG is rapidly reduced back to GSH in untreated hepatocytes. Oxidation by NAPQI of GSH to GSSG and the reduction of GSSG back to GSH by the NADPH-dependent glutathione reductase appear to be responsible for the rapid oxidation of NADPH that occurs in hepatocytes incubated with NAPQI in that the effect is blocked by pretreatment of cells with BCNU. When added to hepatocytes, NAPQI not only reacts with GSH but also causes a loss in protein thiol groups. The loss in protein thiols occurs more rapidly in cells pretreated with BCNU or diethylmaleate. Whereas both of these treatments enhance cytotoxicity caused by NAPQI, BCNU pretreatment has no effect on the covalent binding of [^{14}C-*ring*]NAPQI to cellular proteins. Furthermore, dithiothreitol added to isolated hepatocytes after maximal covalent binding of [^{14}C-*ring*]NAPQI but preceding cell death protects cells from cytotoxicity and regenerates protein thiols. Thus, the toxicity of NAPQI to isolated hepatocytes may result primarily from its oxidative effects on cellular proteins.

INTRODUCTION

Acetaminophen (paracetamol) is a widely used analgesic and antipyretic drug, which is known to cause severe tissue necrosis at high doses in man and experimental animals (1–3). An electrophilic metabolite that depletes GSH and covalently binds to tissue macromolecules has been implicated in the toxic reaction (4).

One such metabolite of acetaminophen that is formed by hepatic cytochrome P-450 is the oxidation product, NAPQI[2] (5). This metabolite is highly reactive both as an electrophile and as an oxidant (5–11). Because of these properties, NAPQI covalently binds to tissue proteins (12) and also may stimulate peroxidative events (13, 14).

Although NAPQI is a potent cytotoxin (15) it is not clear how it causes cell death. Therefore, we investigated reactions of NAPQI with GSH and isolated hepatocytes in attempts to more clearly delineate the relative importance of covalent and noncovalent interactions of NAPQI in causing cell death. Results of these experiments provide further insight into mechanisms of pathogenesis of cell injury caused by acetaminophen.

MATERIALS AND METHODS

Collagenase (grade II) was obtained from Boehringer (Mannheim, FRG). Glutathione, HEPES, DTNB, dicumarol (bis-hydroxycumarin), and DEM were purchased from Sigma. BCNU was a generous gift from Bristol Laboratories (Stockholm, Sweden).

NAPQI was synthesized and purified as previously described (9) and dissolved in anhydrous dimethyl sulfoxide immediately before use. [^{14}C-*ring*]*N*-Acetyl-*p*-benzoquinone imine was produced by oxidation of [^{14}C-*ring*]acetaminophen (0.67 nmol, 0.2 mCi/mmol) with freshly prepared silver I oxide and anhydrous sodium sulfate as previously described (12).

Male Sprague-Dawley rats (200–300 g, body weight) were used in all the experiments; the rats received sodium phenobarbital (1 mg/ml) in the drinking water for 5 days prior to use. Hepatocytes were isolated by collagenase perfusion of the liver (16), and cell incubations were performed in rotating, round-bottom flasks at 37°, under an atmosphere of 95% O_2 and 5% CO_2.

To inhibit glutathione reductase (17), freshly isolated hepatocytes were incubated for 30 min with BCNU (50 μM) in a modified Krebs-

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[2] The abbreviations used are: NAPQI, *N*-acetyl-*p*-benzoquinone imine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; DTT, dithiothreitol; DEM, diethylmaleate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid; LDH, lactate dehydrogenase.

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Henseleit buffer, pH 7.4, supplemented with 25 mM HEPES and an amino acid mixture as described in a previous paper (18). Under these conditions BCNU inhibited the glutathione reductase activity by about 90%, without affecting the cell viability. After 30 min of treatment with BCNU, the hepatocytes were washed and resuspended in the same incubation medium as above, but without BCNU, and further incubated for 2 hr in order to restore GSH levels. During a standard experiment both BCNU-treated and untreated hepatocytes were incubated concurrently.

To prevent reactions of NAPQI with amino acids, the incubation medium was changed to Krebs-Henseleit buffer without amino acids immediately prior to addition of substrate. Small volumes of a 100 mM solution of NAPQI in dimethyl sulfoxide were then added to 10 ml of hepatocyte suspension (10^6 cells/ml) to obtain final concentrations of 100 and 250 μM NAPQI. Assays were performed as follows: GSH and GSSG were measured by high-performance liquid chromatography, as described by Reed *et al.* (19). In order to minimize reactions of NAPQI with GSH during the extraction procedures, 50 mM *N*-ethylmaleimide was used instead of iodoacetic acid to derivatize GSH. Acetaminophen and its glutathione conjugate were determined as described by Moldéus (20); here again the reactions of the benzoquinone imine with GSH were blocked by derivatizing GSH with 10 mM *N*-ethylmaleimide. NADPH and NADH were determined by the method of Klingenberg (21). Hepatocyte integrity was monitored by the release of LDH (EC 1.1.1.27) as previously described (14) or by the exclusion of trypan blue.

For the determination of covalent binding [^{14}C]NAPQI (final concentration 250 μM) was incubated with 10 ml of isolated hepatocyte suspension. Every 5 min, 2-ml aliquots were withdrawn and the protein precipitated with 2 ml of 10% trichloroacetic acid. Protein pellets were processed as described by Streeter *et al.* (12) to eliminate unbound NAPQI and were redissolved in 1 ml of 1 N NaOH and neutralized with HCl to negate chemiluminescence. Aliquots were taken for protein determination and liquid scintillation counting. The radioactivity was measured in a LKB 1217 Rackbeta liquid scintillation counter, using Instagel as scintillation mixture.

Protein sulfhydryl groups were determined using Ellman's reagent according to the method described by Sedlak and Lindsay (22) modified as follows. 0.5 ml of cell suspension were centrifuged at low speed and the supernatant discarded. The cell pellet was treated with 1 ml of 5% trichloroacetic acid, 5 mM EDTA and the protein precipitate washed twice with the same trichloroacetic acid-EDTA solution. When DTT was used this procedure was repeated four times. Protein was redissolved in 3 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 5 mM EDTA and 0.5% sodium dodecyl sulfate. Aliquots of this solution were reacted with 0.1 mM (final concentration) DTNB in 2 ml of Tris-EDTA buffer, pH 8.6. Extinctions were measured at 412 nm and subtracted from blank values obtained by treating sample aliquots with 5 mM *N*-ethylmaleimide before reaction with DTNB. The values are expressed as nanomoles of SH equivalents/mg of protein using GSH as a standard. Proteins were assayed with a modified Lowry procedure, according to Peterson (23).

Reactions of NAPQI with GSH were performed using various concentrations of GSH and NAPQI dissolved in a final volume of 1 ml of 0.05 M Tris-HCl buffer, pH 7.4. The incubations were carried out for 15 min at room temperature, and aliquots of the reaction mixture were assayed for GSH/GSSG and acetaminophen/acetaminophen-glutathione as described above.

RESULTS

It has previously been reported (8, 10) that GSH can react with NAPQI both to form a glutathione conjugate and acetaminophen through reduction of the quinone imine. The stoichiometry of these reactions was determined and the results presented in Fig. 1. In the presence of either excess GSH or equimolar amounts of GSH, NAPQI partitions in a ratio of about 3:2 between conjugate addition product and reduction product. GSSG is

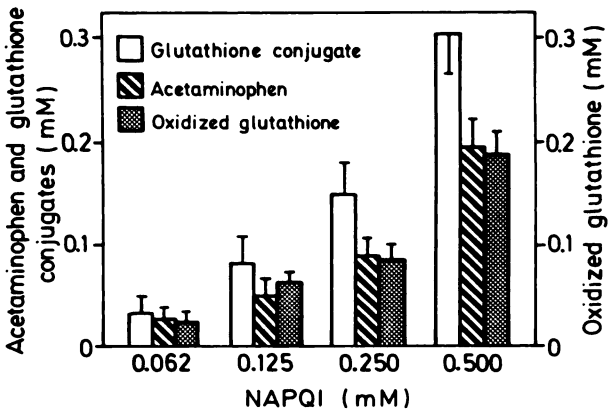

FIG. 1. *Reaction of NAPQI with GSH*

The effect of increasing concentrations of NAPQI on the formation of acetaminophen and its glutathione conjugate and on the oxidation of GSH to GSSG were studied by adding NAPQI to 0.05 mM Tris-HCl buffer, pH 7.4, containing GSH (0.5 mM). Products were analyzed as described under "Materials and Methods." The data are means of three different experiments ± S.D.

formed in amounts stoichiometric with acetaminophen. In the presence of excess NAPQI, comproportionation reaction of acetaminophen and NAPQI leads to radical recombination products (9)[3] but the ratio of acetaminophen glutathione conjugate to GSSG remains at 3:2 (data not shown).

When added to isolated hepatocytes NAPQI reacts very rapidly to decrease intracellular GSH concentrations to about 50% of control values at 100 μM NAPQI and 30% at 250 μM NAPQI. The data obtained in the chemical system indicated that the glutathione depletion may result not only from conjugation reactions but also from redox reactions. Indeed, intracellular GSSG is increased following the addition of NAPQI to the hepatocytes (Fig. 2B), but most is rapidly reduced back to GSH. Only if the hepatocytes are pretreated with BCNU, which results in a more than 90% inhibition of glutathione reductase do intracellular GSSG concentrations remain elevated (Fig. 2B).

The rapid decrease in cellular GSH is paralleled by the formation of the acetaminophen glutathione conjugate (Fig. 3). The amount of conjugate increases up to 5 min of incubation, a time course which is somewhat delayed when compared to the almost immediate depletion of GSH and increase in GSSG. In the BCNU-treated cells, considerably less of the glutathione conjugate is formed apparently because less GSH is available in cells treated with BCNU as a result of inhibition of glutathione reductase.

Recently the oxidation of NADPH by NAPQI has been observed in the presence of purified cytochrome P_{450} reductase (11). Addition of NAPQI to isolated hepatocytes caused a transitory decrease in NADPH content that could be prevented by BCNU pretreatment (Fig. 4). No variation in intracellular NADH was observed (data not shown). Thus, the changes in NADPH redox state in isolated hepatocytes are apparently a consequence of

[3] E. Albano, M. Rundgren, P. J. Harvison, S. D. Nelson, and P. Moldéus, unpublished observations.

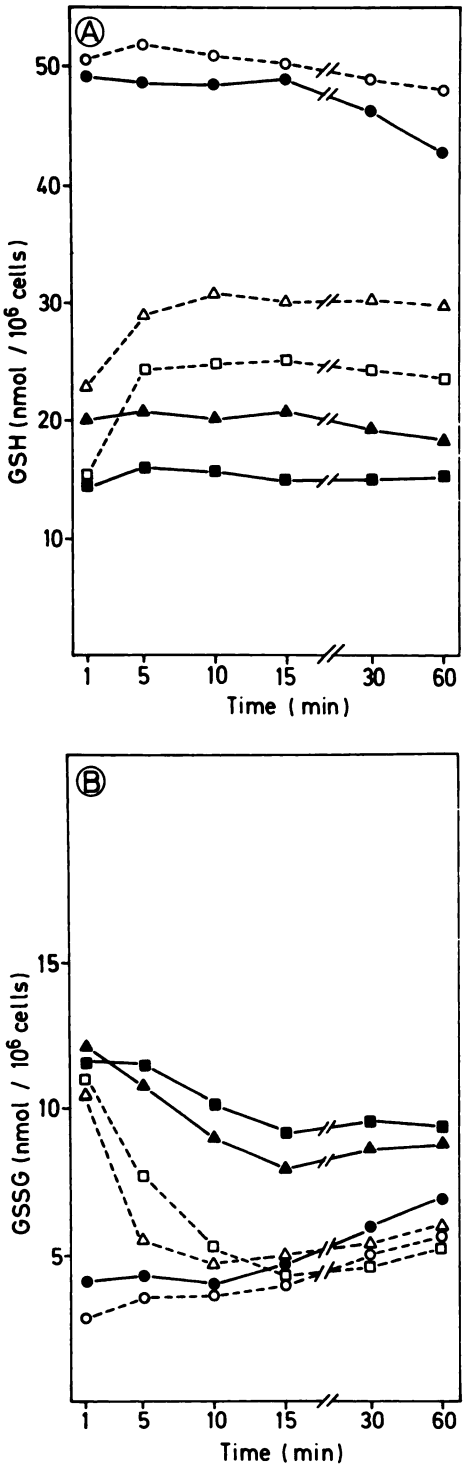

FIG. 2. *Effect of NAPQI on glutathione redox state in isolated hepatocytes*

Intracellular concentrations of GSH (A) and GSSG (B) were determined in liver cells preincubated with (*filled symbols*) or without (*open symbols*) BCNU. NAPQI concentrations were: none (○, ●), 100 μM (△, ▲), 250 μM (□, ■). One experiment typical of five.

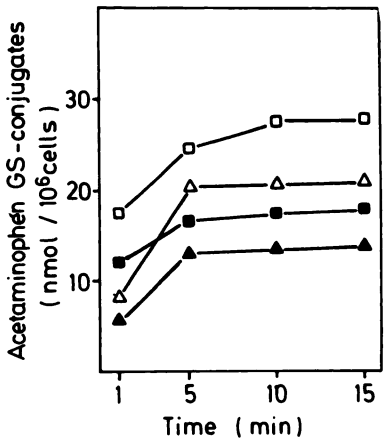

FIG. 3. *Formation of acetaminophen glutathione conjugate in isolated hepatocytes incubated with NAPQI*

NAPQI concentrations were 100 μM (△, ▲) and 250 μM (□, ■). *Open symbols* represent untreated cells and *filled symbols* cells pretreated with BCNU. One experiment typical of five.

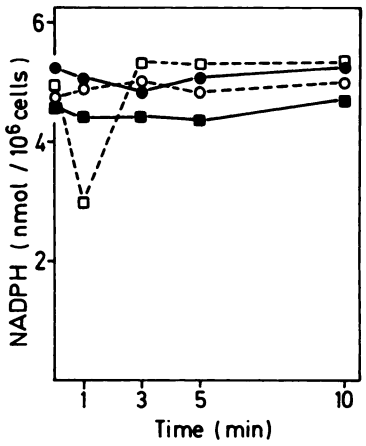

FIG. 4. *Changes in intracellular NADPH content in isolated hepatocytes incubated withNAPQI*

Control cells (○, ●) and hepatocytes receiving 250 μM NAPQI (□, ■). *Open symbols* represent untreated cells and *filled symbols* cells pretreated with BCNU. One experiment typical of three.

increased utilization of NADPH by glutathione reductase for reduction of GSSG, rather than the result of direct NADPH oxidation by NAPQI. Consistent with these observations is the lack of effect of dicumarol, an inhibitor of DT-diaphorase (NAD(P)H:quinone-oxidoreductase, EC 1.6.4.3) (24) on the amount of acetaminophen or glutathione conjugate that is formed from NAPQI (data not shown).

The arylation of liver cell macromolecules is one mechanism by which an acetaminophen reactive metabolite may cause hepatic injury (1–4). Therefore, we compared covalent binding of radiolabeled NAPQI to hepatocyte protein in relation to the occurrence of hepatocyte damage. As shown in Fig. 5, the binding of radiolabel to cell proteins is rapid, maximal binding occurring 5 min after the addition of [^{14}C-*ring*]NAPQI. Binding was equivalent in BCNU-treated cells. Cells preincubated for 30 min with diethylmaleate (0.2 mM) to lower GSH concentrations showed enhanced binding levels.

Fig. 6 shows the results of these treatments on LDH release from the hepatocytes. In contrast to the effects on covalent binding, the BCNU- and DEM-pretreated cells were more susceptible to the toxic effects of NAPQI than the control cells. Thus, total covalent binding to

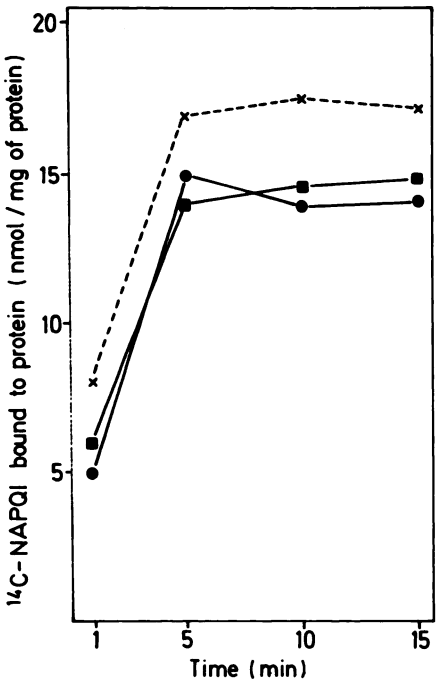

FIG. 5. *Covalent binding of [^{14}C]NAPQI in isolated hepatocytes*
Radioactive NAPQI (250 μM) was given to untreated liver cells (●) or to hepatocytes pretreated with BCNU (■) or DEM (×). One experiment typical of three.

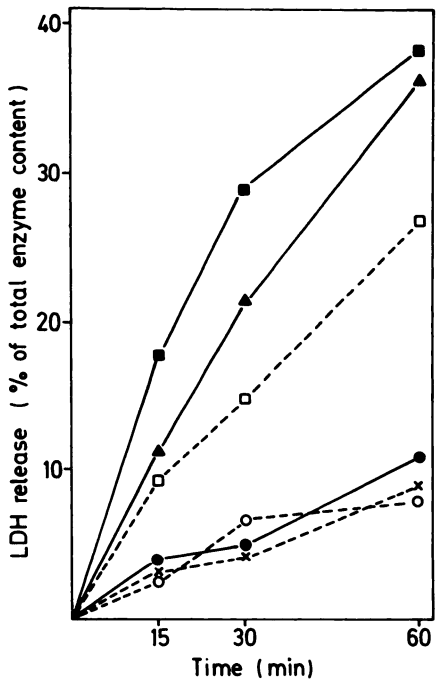

FIG. 6. *NAPQI-induced cytotoxic effects in isolated hepatocytes*
Symbols represent controls from untreated (○), BCNU (●), or DEM (×) pretreated cells, and the effects of 250 μM NAPQI in untreated (□), BCNU (■), or DEM (▲) pretreated hepatocytes. LDH release is expressed as per cent of the total enzyme content of the hepatocytes, assayed after cell destruction with 0.5% Triton X-100. One experiment typical of five.

cell proteins of NAPQI does not seem to correlate well with its cytotoxicity.

As shown in Fig. 7, cytotoxicity appears to correlate better with decreases in total protein thiol groups. NAPQI (250 μM) decreases protein thiols by approximately 25%, a decrease which is enhanced by pretreatment of cells with either BCNU or DEM. Based on the covalent binding data, arylation of proteins by NAPQI would probably not account for the total decrease in protein thiols, at least in the BCNU-treated cells.

An indication that oxidation of protein thiols is responsible for some of the loss in protein thiols comes from experiments with DTT (Fig. 8). Addition of DTT (10 mM) to the hepatocytes either 2 or 4 min after the

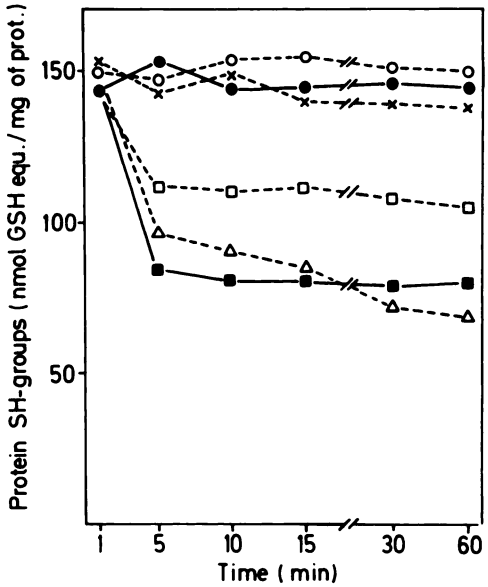

FIG. 7. *NAPQI-induced changes in protein thiols*
Symbols represent controls from untreated (○), BCNU (●), or DEM (×) pretreated cells and the effect of 250 μM NAPQI in untreated (□), BCNU (■), or DEM (▲) treated hepatocytes. One experiment typical of five.

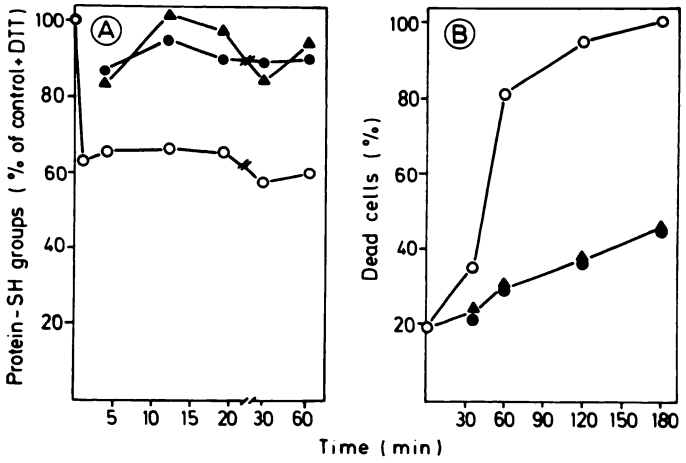

FIG. 8. *Effect of DTT on NAPQI-induced decrease in protein thiols (A) and cytotoxicity (B)*
Hepatocytes were treated with NAPQI (400 μM) and then with DTT (10 mM). Symbols represent DTT added after 2 min (▲); DTT added after 4 min (●); NAPQI alone (○). One experiment typical of three.

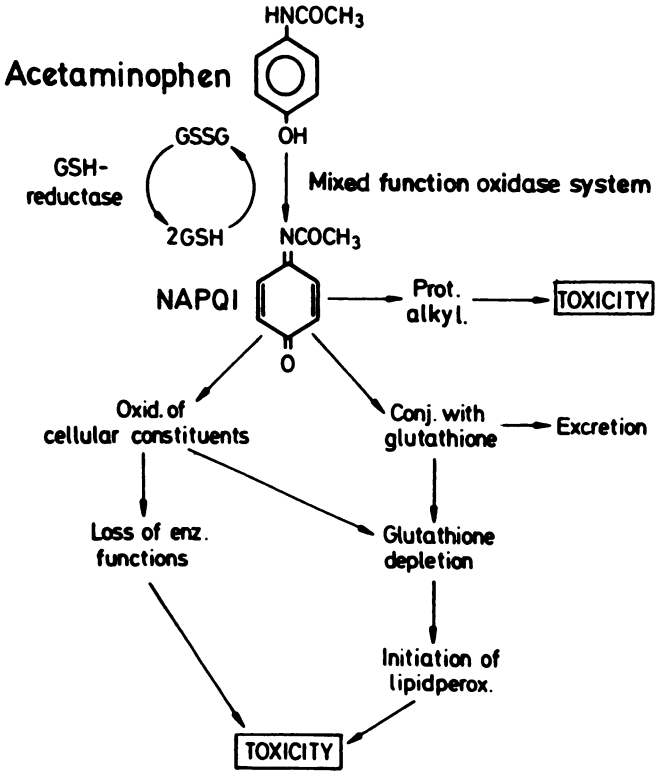

FIG. 9. *Possible mechanisms of acetaminophen-induced hepatic injury*

addition of NAPQI (400 μM) resulted in a dramatic decrease in cell death, as assessed by trypan blue uptake. DTT also prevented the NAPQI-induced loss of total protein thiols.

DISCUSSION

NAPQI, a reactive metabolite of acetaminophen reacts with GSH in at least two ways. It forms a glutathione conjugate and it oxidizes GSH to its disulfide GSSG with the concomitant production of acetaminophen.

The same reactions occur when NAPQI is added to isolated hepatocytes. An extremely rapid decrease in intracellular GSH is accompanied by formation of a glutathione conjugate and GSSG. Interestingly, the initial ratio (determined after incubation for 1 min) of acetaminophen glutathione conjugate to GSSG is about the same as that determined in the chemical reaction of NAPQI and GSH. However, the amount of the conjugate almost doubles again in the next 4 min of incubation, possibly indicating an entrapped pool of NAPQI (GSH transferase bound?) which is more slowly released and then conjugated with GSH. Moreover, it is interesting to note that the decrease in GSH is not dose dependent and even though more than 30% of GSH still remains after addition of 100 mM NAPQI, increasing the NAPQI concentration 2.5-fold only causes a small further decrease of intracellular GSH. Thus it appears that part of the intracellular GSH is not readily available for reaction with NAPQI, possibly due to compartmentation.

In cells with a functioning glutathione reductase, the rapid increase in GSSG is transient and the GSSG is rapidly reduced back to GSH. However, inhibition of glutathione reductase by BCNU maintained the increased level of GSSG caused by NAPQI. Thus, glutathione reductase is apparently a very efficient enzyme which may explain why only glutathione conjugate formation is observed during acetaminophen metabolism in isolated hepatocytes (25). Under these conditions of continuous production from acetaminophen, only low levels of NAPQI would be present at any time and consequently steady state concentrations of GSSG would increase very little though GSH oxidation probably occurs. Alternatively, when formed intracellularly the NAPQI may preferentially undergo enzyme-mediated conjugation rather than reduction by GSH.

NAPQI also can be reduced to acetaminophen by NADPH both chemically (5) and enzymatically (11). However, the transient oxidation of NADPH caused by NAPQI in isolated hepatocytes does not appear to result from reduction of NAPQI by NADPH. Rather, the cellular shift in redox state of NADPH probably results from NADPH-dependent glutathione reductase-catalyzed reduction of GSSG, inasmuch as the shift is prevented by BCNU pretreatment of the cells. Thus in the intact hepatocyte, GSH seems to be the primary reducing agent for NAPQI. In agreement with these findings, NAPQI is apparently not metabolized by DT-diaphorase, which catalyzes the 2-electron reduction of a variety of quinones. No effect of dicumarol, an inhibitor of DT-diaphorase was observed on metabolism or toxicity caused by NAPQI in our preparations of isolated hepatocytes.

Mechanisms by which acetaminophen cause its cytotoxic effects are still unknown. Several possibilities are diagrammed in Fig. 9. Covalent binding of radiolabeled acetaminophen to cellular proteins has been used as an index of cytotoxicity although such an index is not directly correlated with cell death (26). Our studies with NAPQI have shown that this reactive metabolite of acetaminophen also covalently binds to proteins in isolated hepatocyte preparations under conditions where it is cytotoxic. However, we also have found that the binding and cytotoxic effects of NAPQI are apparently dissociable in that BCNU-pretreated cells are significantly more susceptible to the cytotoxic effects of NAPQI than untreated cells, whereas covalent binding is unaffected by BCNU pretreatment. We cannot rule out the possibility that covalent interaction with specific macromolecules is an important determinant of cytotoxicity.

Because protein thiol groups are important targets quantitatively for reaction with NAPQI (12), we investigated the effects of NAPQI on total cellular protein thiols. Protein thiols were decreased by NAPQI, and this decrease was enhanced by pretreatment of the hepatocytes with either BCNU or DEM, which correlates with the effects of both of these pretreatments on cytotoxicity. Results of further investigations with DTT, which protected hepatocytes against the cytotoxic effects of NAPQI with no effect on covalent binding, indicate that oxidation of protein thiols may be an important component of the mechanism by which this metabolite of acetaminophen causes cytotoxicity.

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