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Acetaminophen Toxicity Results in Site-specific Mitochondrial Damage in Isolated Mouse Hepatocytes*

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Exposure of isolated mouse hepatocytes to a toxic concentration of acetaminophen (5 mM) resulted in damage to the mitochondrial respiratory apparatus. The nature of this damage was investigated by measuring respiration stimulated by site-specific substrates in digitonin-permeabilized hepatocytes after acetaminophen exposure. Respiration stimulated by succinate at energy-coupling site 2 was most sensitive to inhibition and was decreased by 47% after 1 h. Respiration supported by NADH-linked substrates (site 1) was also decreased but to a lesser extent, while there was no decrease in the rate of ascorbate + *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD)-supported respiration (site 3). The loss of mitochondrial respiratory function was accompanied by a decrease in ATP levels and ATP/ADP ratios in the cytosolic compartment and was preceded by a loss of reduced glutathione in both the cytosol and mitochondria. All these effects occurred well before the loss of cell membrane integrity. The putative toxic metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinonimine (NAPQI), produced a similar pattern of respiratory dysfunction in isolated hepatic mitochondria. Respiration stimulated by succinate- and NADH-linked substrates was very sensitive to 50 μM NAPQI, while ascorbate + TMPD-supported respiration was unaffected. The interaction between NAPQI and the respiratory chain was further investigated using submitochondrial particles. Succinate dehydrogenase (associated with respiratory complex II) was found to be very sensitive to NAPQI, while NADH dehydrogenase (respiratory complex I) was inhibited to a lesser extent. Our results indicate that a loss of the ability to utilize succinate- and NADH-linked substrates due to attack of the respiratory chain by NAPQI causes a disruption of energy homeostasis in acetaminophen hepatotoxicity.

The hepatotoxicity of acetaminophen is known to involve the formation of a chemically reactive metabolite, *N*-acetyl-*p*-benzoquinonimine (NAPQI),[1] which is produced during cytochrome P-450-mediated oxidation of the drug. NAPQI has been reported to be highly reactive both as an electrophile and as an oxidant species (1), and it irreversibly arylates protein thiol residues (2). However, the specific nature of the critical biochemical lesions induced by NAPQI that result in cellular necrosis is the subject of continuing research.

An early histological change in the livers of mice poisoned with acetaminophen is an alteration in mitochondrial morphology (3). Recently, impairment of mitochondrial oxidative phosphorylation has been shown to be associated with the development of acetaminophen hepatotoxicity *in vivo* (4, 5). Since mitochondrial oxidative processes play a central role in the maintenance of cellular energy supply (6, 7), the deleterious effect of a xenobiotic on mitochondrial respiration may have serious consequences for the viability of the cell. We have recently shown that mitochondrial function is diminished in acetaminophen-treated isolated mouse hepatocytes prior to the development of cell membrane damage (8), and it is possible that mitochondrial damage may be a component of acetaminophen hepatotoxicity. Indeed, it has been recently demonstrated that both acetaminophen itself, and NAPQI can inhibit respiration in isolated rat and mouse liver mitochondria (9, 10).

In the present study we have examined the nature of the mitochondrial damage induced by acetaminophen. The effect of acetaminophen exposure on mitochondrial function in isolated hepatocytes was compared with the effects of NAPQI on the respiratory behavior of isolated hepatic mitochondria. The effect of NAPQI on the activity of respiratory chain enzymes in submitochondrial particles was also examined to determine whether direct interactions between the toxin and the respiratory chain might be involved in the induction of mitochondrial damage.

EXPERIMENTAL PROCEDURES

Animals—Male Swiss ARC mice (body weight 25–30 g, Animal Resources Center, Murdoch, WA), allowed free access to food and water, were used in all experiments.

Chemicals—Albumin, antimycin A, and dithiothreitol were purchased from Boehringer Mannheim (Sydney, Australia). All other chemicals were obtained from Sigma.

Mitochondrial Isolations—Mitochondria were prepared from mouse livers essentially as described previously (11). The protein content of the final mitochondrial suspension was determined using the method of Hartree (12) and was typically 15–30 mg/ml. When submitochondrial particles were prepared, mitochondrial pellets from two livers were pooled and sonicated using methodology described previously (13). The final submitochondrial particle pellet was resuspended in 0.5 ml of 0.25 M sucrose, 1 mM EDTA, 10 mM Tris buffer. The protein content of the suspension was typically 14–20 mg/ml.

Hepatocyte Isolations—Hepatocytes were prepared by collagenase perfusion of the liver as described previously (14). Nonviable hepatocytes were excluded by differential centrifugation through Percoll (8, 15). The cells were resuspended at a concentration of 10^6 cells/ml in RPMI 1640 culture medium supplemented with 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% (w/v) bovine serum albumin. Incubations (5 ml) were carried out in stoppered 50-

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[1] The abbreviations used are: NAPQI, *N*-acetyl-*p*-benzoquinonimine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

ml Erlenmeyer flasks equilibrated with 95% O_2, 5% CO_2 in a gyratory water bath at 80 oscillations/min and 37 °C. Acetaminophen was dissolved directly in the culture media.

Measurement of Mitochondrial Respiration in Isolated Hepatocytes—Rates of site-specific mitochondrial respiration in isolated mouse hepatocytes were measured at 30 °C using a Clark-type oxygen probe apparatus described previously (8). The respiration buffer contained 250 mM sucrose, 25 mM Tris, 1 mM EGTA, and 15 μg/ml digitonin. This low concentration of digitonin permeabilizes the plasma membrane, facilitating access to such substrates as glutamate, succinate, and NADH, which do not normally cross the hepatocyte plasma membrane (16, 17). Site-specific mitochondrial respiration rates were measured in a cell pellet derived from a 3-ml aliquot of cell suspension. The cells were resuspended in 2.1 ml of respiration media and transferred to the incubation chamber of the oxygen probe apparatus. Respiration rates for each of the three energy-coupling sites were measured as the difference between the maximal rates of respiration stimulated by the addition of site-specific substrates (*i.e.* NADH + glutamate + malate, succinate, and ascorbate + TMPD) and the residual respiration remaining after the addition of the respective site-specific inhibitors (rotenone, antimycin A, and KCN). Rates were determined sequentially (sites 1–3) in the same cells and unless indicated otherwise were calculated over a 2-min period. Respiratory rates in the permeabilized cells after a 10-min incubation period were unchanged, indicating that mitochondrial respiratory function was unaffected by the digitonin treatment (data not shown). The inclusion of ADP and hexokinase in the assay media did not stimulate respiration at any of the three sites, indicating that ADP supply was not limiting (data not shown).

Cell Disruption and Mitochondrial Isolation—Isolated mouse liver cells were separated into cytosolic and mitochondrial fractions using the digitonin procedure of Meredith and Reed (18). This procedure allowed preparation of a distinct mitochondrial pellet by selective solubilization of the cell membrane in 0.5 mg/ml digitonin for 90 s followed by rapid centrifugation of the intact mitochondria through dibutyl phthalate. The pellet contained 98 ± 0.27 and 2.7 ± 0.34% cellular glutamate dehydrogenase (a mitochondrial marker) and lactate dehydrogenase (a cytosolic marker) activities, respectively (n = 8). In experiments where the subcellular compartmentation of ATP and ADP was examined, the procedure of Meredith and Reed was modified slightly. Cell fractionations were carried out at 4 °C to minimize the phosphorylation of ADP during the process (19). The inert oil employed in the fractionation procedure (dibutyl phthalate) was diluted 4:1 in acetone to overcome the increase in viscosity of the oil which occurred at 4 °C. The recovery of mitochondrial and cytosolic marker enzymes was unaffected by this modification.

Effect of NAPQI on Isolated Mitochondria—NAPQI was synthesized by silver oxide oxidation in $CHCl_3$ as described previously (20). NAPQI was dissolved in dimethyl sulfoxide and used immediately after preparation. The structure of the yellow crystalline product was confirmed by 60-MHz ^1H NMR: ($CDCl_3$) δ 2.25 (s, 3H), δ 6.50 (d, 2H, J_{BA} = 9 Hz), δ 6.90 (d, 2H, J_{BA} = 9 Hz). The purity of the preparation was determined via reverse-phase high pressure liquid chromatography using a phenyl column and a 0.1 M citrate, 20% (v/v) methanol (pH 7.2) mobile phase (20). The purity of the preparation was 95% or greater with respect to NAPQI. To examine the effect of NAPQI on respiration in isolated hepatic mitochondria, mitochondria (0.5 mg of protein/ml) were added to the incubation chamber of the oxygen probe apparatus containing respiration buffer (25 mM sucrose, 10 mM EDTA, 50 mM Tris, 25 mM KCl, 15 mM $MgCl_2$, and 5 mM KH_2PO_4). After 90 s, NAPQI was added in a 5-μl volume. After an additional 90 s, respiration was stimulated by the addition of oxidizable substrates, either glutamate + malate (both 5 mM), succinate (5 mM) + rotenone (4 μg, to prevent oxalacetate accumulation), or ascorbate (2 mM) + TMPD (200 μM). Respiration was followed for 2 min to obtain a linear oxygen utilization rate.

To examine the effect of NAPQI on the GSH content of isolated mitochondria, NAPQI (in 15 μl of dimethyl sulfoxide) was added to a 0.6-ml Eppendorf tube containing mitochondria (300–400 mg of protein) in 100 μl of a 250 mM sucrose, 5 mM Tris buffer, pH 7.4. The tubes were incubated in a water bath at 30 °C for 10 min. The reaction was stopped by the addition of 15 μl of 30% (w/v) perchloric acid, and the tubes were placed on ice for 20–30 min. Protein was removed during a 20-min centrifugation at 2000 × g, and the supernatant (150 μl) was assayed for GSH content (21).

Effect of NAPQI on Respiratory Chain Enzyme Activities in Submitochondrial Particles—NADH dehydrogenase activity in submitochondrial particles was determined using both menadione and ferricyanide as electron acceptors as described by Galante and Hatefi (22). The activity of succinate dehydrogenase, which is associated with respiratory chain complex II, was measured in submitochondrial particles using a colorimetric procedure with *p*-iodonitrotetrazolium violet as electron acceptor (23). Ubiquinol-cytochrome *c* reductase activity was measured using duroquinol as the reducing substrate (24). Cytochrome oxidase activity was measured using a polarographic procedure described elsewhere (25, 26). The ATPase activity in submitochondrial particles was determined using an assay described by Cain and Skilleter (13). Inorganic phosphate liberated during the ATPase reaction was measured using a colorimetric procedure (27). When the effect of NAPQI on the various respiratory chain enzymes was examined, it was added to the appropriate assay medium containing submitochondrial particles. After 90 s the enzyme assays were initiated by the addition of the corresponding substrate.

Assays—Cellular viability was assessed by measuring the extent of lactate dehydrogenase leakage from the hepatocytes into the incubation medium as a percentage of the total lactate dehydrogenase activity in cells plus medium (28). Glutamate dehydrogenase activity was measured as described previously (29). Aliquots of protein-free cell extracts were neutralized with 20% (w/v) $KHCO_3$ and were assayed for ATP and ADP content by enzymatic procedures (30, 31). The DNA content of the cell suspension was determined as a measure of cell number (32).

Statistical Analysis—Statistical analysis was performed using the statistical package GENSTAT (Rothamstead Experimental Station, Harpenden, United Kingdom). Analysis of data was by one- or two-way analysis of variance. If a significant variance ratio was observed, the data were further analyzed by Dunnett's or Tukey's tests (33, 34).

RESULTS

Acetaminophen Is Toxic to Isolated Mouse Hepatocytes—Isolated mouse hepatocytes are sensitive to high concentrations of acetaminophen. Fig. 1 details the time course of lactate dehydrogenase leakage from hepatocytes exposed to 5 mM acetaminophen. No cell killing was evident at 1.5 h. Between 2 and 4 h the lactate dehydrogenase leakage increased from 14 to 47%. There was no loss of cell viability in control cells during this time. Mitochondrial damage can be produced in these cells with concentrations as low as 0.5 mM (8). The concentration of acetaminophen used in these experiments (5 mM) was chosen on the basis of previous experiments where it produced a more consistent toxic response (8), and it is not dissimilar to the concentrations found in the livers of mice following an LD_{50} dose of acetaminophen (35).

Acetaminophen Exposure Depletes Cytosolic ATP—Since mitochondrial respiratory processes contribute the majority of the hepatocyte's ATP requirements (36), an impairment of mitochondrial respiration should be reflected in a lowering of cellular ATP levels. The effect of acetaminophen intoxication (5 mM) on ATP levels and ATP/ADP ratios in the cytosolic and mitochondrial compartments is shown in Fig. 2. Cytosolic ATP levels and ATP/ADP ratios fell by 26 and 45%, respectively, after 1.0 h (Fig. 2, *A* and *B*). Mitochondrial ATP levels

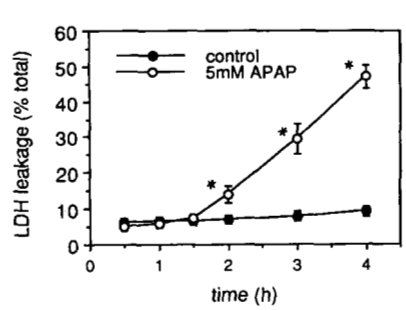

FIG. 1. **The effect of acetaminophen (*APAP*) (5 mM) on the time course of lactate dehydrogenase (*LDH*) leakage from isolated hepatocytes over a 4-h incubation period.** Values are means ± S.E. of five experiments. An *asterisk* indicates a significant difference from controls (Tukey's test, $p < 0.05$).

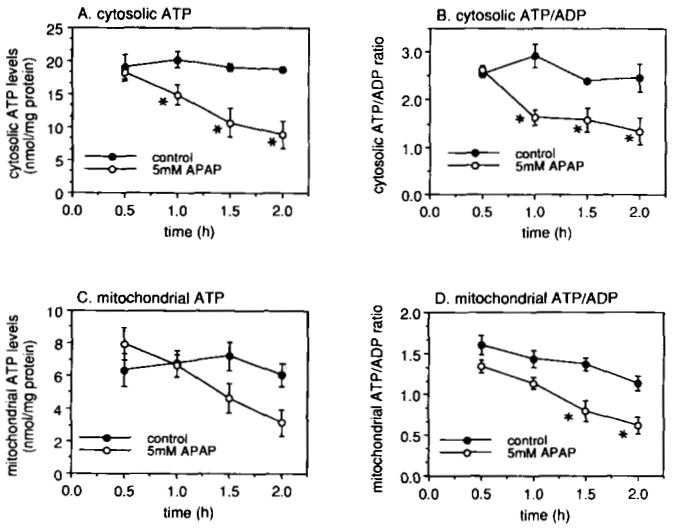

FIG. 2. **The effect of exposure time in the presence and absence of acetaminophen (*APAP*) (5 mM) on ATP levels (*A*) and ATP/ADP ratio (*B*) in the cytosolic compartment and ATP levels (*C*) and ATP/ADP ratios (*D*) in the mitochondrial compartment of isolated hepatocytes.** Cells were fractionated using the digitonin method as described under "Experimental Procedures." Values are means ± S.E. of five experiments. An *asterisk* indicates a significant difference from controls (Tukey's test, $p < 0.05$).

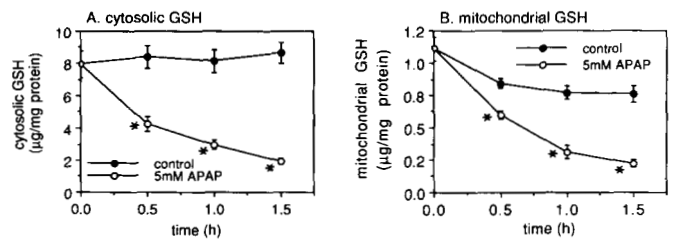

FIG. 3. **The effect of exposure time in the presence and absence of acetaminophen (*APAP*) (5 mM) on glutathione levels in the cytosolic (*A*) and mitochondrial (*B*) compartments of isolated hepatocytes.** Cells were fractionated as described under "Experimental Procedures." Values are means ± S.E. of four experiments. An *asterisk* indicates a significant difference from controls (Tukey's test, $p < 0.05$).

were not different from controls at any time point (Fig. 2*C*). Mitochondrial ATP/ADP ratios were not significantly changed after 1.0 h but were decreased in comparison with controls at later time points (Fig. 2*D*). These effects occur prior to the loss of cell membrane viability (Fig. 1).

Acetaminophen Exposure Depletes Cytosolic and Mitochondrial GSH—Depletion of intracellular GSH during acetaminophen intoxication results from the conjugation of this tripeptide with NAPQI (37). Thus, cytosolic GSH content of the mouse hepatocytes was rapidly depleted in the presence of 5 mM acetaminophen (Fig. 3*A*). After 1 h it had declined to 63% of control. The hepatocyte mitochondria are thought to contain a pool of GSH, which is metabolically distinct from the corresponding cytosolic pool (18). However, the rate of GSH depletion in the mitochondria was essentially the same as that in the cytosol. After 1 h, the GSH content of the mitochondria had declined to 60% of the control level (Fig. 3*B*). Decreases in hepatic mitochondrial GSH levels have also been shown to occur during acetaminophen intoxication in the mouse *in vivo* (38).

Acetaminophen Inhibits Mitochondrial Respiration in Hepatocytes—Acetaminophen can impair cellular respiration in isolated mouse hepatocytes before the loss of cell membrane viability (8). This effect of acetaminophen depends on the oxidation of acetaminophen by the microsomal oxidase enzymes and is not related to the direct inhibitory effect that acetaminophen itself has on respiration (8, 9). Fig. 4 details the effect of acetaminophen exposure on mitochondrial respiration stimulated by site-specific substrates. At various times after exposure to acetaminophen, mitochondrial respiration was stimulated by either site 1 (NADH + glutamate + malate), site 2 (succinate), or site 3 (ascorbate + TMPD) substrates in digitonin-permeabilized hepatocytes. Within 1 h of exposure to 5 mM acetaminophen, respiration stimulated at energy-coupling site 1 was inhibited by 30% (Fig. 4*A*), and succinate-supported respiration was reduced by 47%, compared with corresponding controls (Fig. 4*B*). The respiratory depression produced at sites 1 and 2 occurred before a loss of cell viability was evident (see Fig. 1). However, respiration supported by energy-coupling site 3-specific substrates (ascorbate + TMPD) was unaffected by acetaminophen exposure

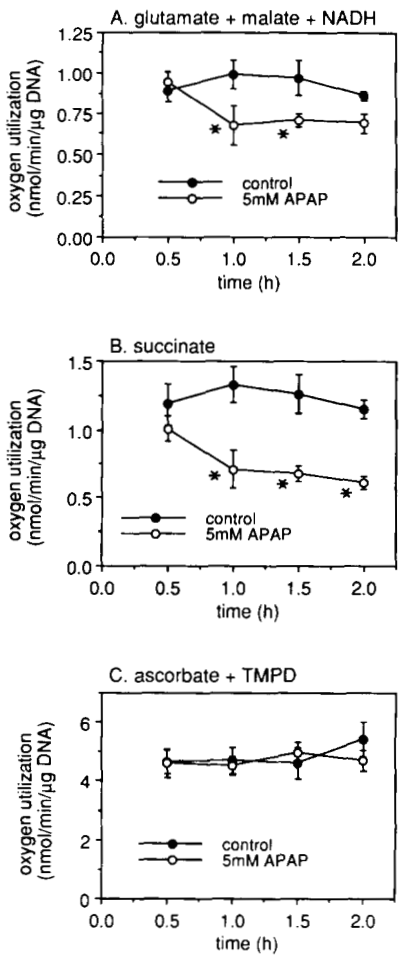

FIG. 4. **The effect of exposure time in the presence and absence of acetaminophen (*APAP*) (5 mM) on cellular respiration rates in digitonin-permeabilized hepatocytes with site-specific substrates, namely NADH + glutamate + malate for energy-coupling site 1 (*A*), succinate for site 2 (*B*), or ascorbate + TMPD for site 3 (*C*).** Respiration at each energy-coupling site was stimulated by the addition of the reducing substrates. After a linear oxygen utilization rate was attained, respiration was inhibited by the addition of the appropriate site-specific inhibitor, namely rotenone (*A*), antimycin A (*B*), or KCN (*C*). The difference between the respiratory rate observed in the presence of substrate and that remaining after the addition of the inhibitor was taken to be the respiratory rate for a given site. Values are means ± S.E. of four experiments. An *asterisk* indicates a significant difference from controls (Tukey's test, $p < 0.05$).

(Fig. 4*C*). There was no effect on site 3-supported respiration even at 2 h, a time when there was significant cell killing by acetaminophen. These *in vitro* findings resemble those reported by others using the intact mouse model. Meyers and associates (4) showed that an inhibition of respiration supported at complexes I and II precedes frank hepatic necrosis in mitochondria prepared from the livers of mice treated with a toxic dose of acetaminophen.

Effect of NAPQI on Mitochondrial Function—The inhibitory effects of acetaminophen exposure on mitochondrial respiration could be reproduced by exposing isolated mouse liver mitochondria to NAPQI for 90 s. Since the half-life of NAPQI in the presence of tissue fractions is a matter of seconds (39), it would be expected that no unreacted NAPQI would remain at this time. Fig. 5 documents the effect of NAPQI on mitochondrial respiration stimulated at each of the three energy-coupling sites. Site 1-stimulated respiration was inhibited by 83% with 50 μM NAPQI (Fig. 5*A*). Respiration stimulated at site 2 by succinate dehydrogenase was also sensitive to NAPQI exposure and was inhibited by 44 and 85% at NAPQI concentrations of 25 and 50 μM respectively (Fig. 5*B*). However, NAPQI had no effect on site 3-supported respiration. At a concentration that virtually abolished site 1- and site 2-supported respiration (50 μM), there was no change

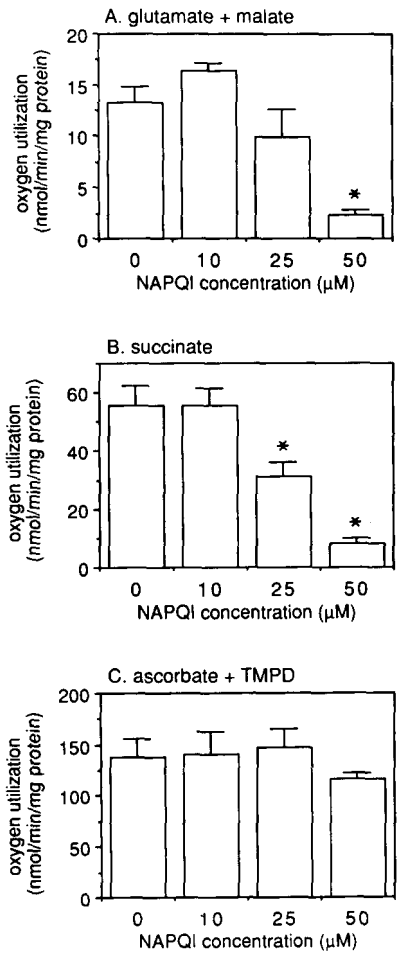

FIG. 5. **The effect of NAPQI concentration on respiratory rates in isolated hepatic mitochondria respiring on glutamate + malate (*A*), succinate (*B*), or ascorbate + TMPD (*C*).** Mitochondria (1.0 mg of protein) were incubated in the chamber of the oxygen probe in the presence of NAPQI for 90 s before the addition of the appropriate reducing substrates. Values are means ± S.E. of four to six preparations. An *asterisk* indicates a significant difference from controls (Dunnett's test, $p < 0.05$).

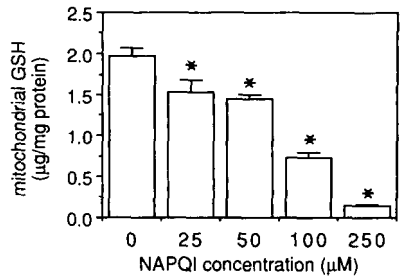

FIG. 6. **The effect of NAPQI concentration on GSH levels in isolated hepatic mitochondria after a 10-min incubation at 30 °C as described under "Experimental Procedures."** Values are means ± S.E. of four preparations. An *asterisk* indicates a significant difference from controls (Dunnett's test, $p < 0.05$).

in the rate of site 3-supported respiration (Fig. 5*C*).

The effect of NAPQI exposure on the GSH pool of isolated mitochondria was also examined. Fig. 6 shows that 10 min after exposure to 25 μM NAPQI there was a significant decrease in the GSH content of the isolated mitochondria. The extent of this depletion increased with increasing amounts of NAPQI.

NAPQI Interferes with the Activity of Certain Respiratory Chain Enzymes in Submitochondrial Particles—The site at which NAPQI disrupts the electron transport chain was investigated using submitochondrial particles. These consist of partially purified inner membrane vesicles and lack matrix and outer membrane enzymes (13).

NADH dehydrogenase activity was decreased only moderately by micromolar concentrations of NAPQI in these vesicles (Table I). In the presence of 50 μM NAPQI, NADH dehydrogenase activity was decreased by 19% with ferricyanide as the electron acceptor. A similar result was observed using menadione as the electron acceptor (data not shown). In contrast, succinate dehydrogenase activity was very sensitive to the toxin and was inhibited by 45% by 10 μM NAPQI (Table I). In the presence of 50 μM NAPQI the activity of this enzyme was virtually abolished. However, the activity of two other respiratory chain enzymes, namely ubiquinol-cytochrome *c* reductase and cytochrome oxidase, was unaffected in submitochondrial particles after exposure to NAPQI (Table I), consistent with a lack of effect of NAPQI on respiratory complexes III and IV. Similarly, exposure of submitochondrial particles to NAPQI over a 10–100 μM range did not affect ATPase activity (Table I). This indicates that the ATP-synthesizing component of the respiratory chain does not appear to be a likely target for direct damage by acetaminophen's toxic metabolite.

Hence, the main effect of NAPQI on the respiratory chain appears to be an inhibition of respiratory complexes I (NADH-ubiquinone oxidoreductase) and II (succinate-ubiquinone oxidoreductase). However, the effect of the toxin is more pronounced at complex II than at complex I.

DISCUSSION

Several recent studies have implicated a role for mitochondrial damage in the toxic process initiated by acetaminophen in liver cells. High doses of acetaminophen in the mouse *in vivo* have been shown to induce hepatic mitochondrial dysfunction (4). We have recently reported that acetaminophen damages mitochondria in isolated mouse hepatocytes and that this occurs prior to the appearance of cell membrane damage (8). In addition, exposure of both rat and mouse liver mitochondria to NAPQI has been shown to result in the irreversible inhibition of mitochondrial respiration (9, 10).

The present study demonstrates that this acetaminophen-

TABLE I

The effect of NAPQI on the activity of five oxidative phosphorylation enzymes in submitochondrial particles

Enzyme activity[a]	NAPQI			
	0	10	25	50
			μM	
NADH dehydrogenase	530 ± 15[b]	490 ± 13[c]	490 ± 9.8[c]	450 ± 8.0[c]
Succinate dehydrogenase	8.3 ± 0.6	4.5 ± 0.5[c]	2.0 ± 0.08[c]	0.39 ± 0.02[c]
Ubiquinol-cytochrome *c* oxidoreductase	0.14 ± 0.01	0.14 ± 0.002	0.17 ± 0.02	0.17 ± 0.01
Cytochrome oxidase[d]	410 ± 73	ND[e]	ND	378 ± 34
ATPase	3.6 ± 0.03	3.7 ± 0.07	3.7 ± 0.07	3.5 ± 0.09

[a] Submitochondrial particles were exposed to NAPQI for 90 s (or 5 min for the ATPase assay) before the addition of the appropriate assay reagents. Enzyme activities are expressed as μmol/min/mg of protein (or as nmol of O_2/min/mg of protein for cytochrome oxidase).

[b] Values are mean ± S.E. of three to four preparations.

[c] Significantly different from controls (*i.e.* no NAPQI).

[d] The cytochrome oxidase activities shown were measured in the presence of 1.0 μM cytochrome *c*. The activity was also determined using 0.1 and 10 μM cytochrome *c*, and a similar result was obtained (data not shown).

[e] ND, not determined.

induced mitochondrial damage is specific for particular sites on the respiratory chain. Respiration supported at energy-coupling sites 1 and 2 of the respiratory chain was decreased in cells incubated with 5 mM acetaminophen, and this occurred prior to the loss of cell membrane integrity. There was no inhibition of respiration supported at energy-coupling site 3. A decrease in the functional capacity of the mitochondrial respiratory chain should be associated with a decrease in ATP production. Indeed, we found that this loss of function was accompanied by a decrease in ATP levels and ATP/ADP ratios in the cytosolic compartment. These changes in adenine nucleotides were similar to those reported recently in the livers of mice exposed to acetaminophen (40). In the present study, changes in the mitochondrial adenine nucleotide pool did not correlate well with corresponding changes in the cytosol. This may suggest that mitochondrial ATP levels and ATP/ADP ratios are not sensitive indicators of oxidative phosphorylation in the organelle. Others have reported a lack of correlation between mitochondrial ATP/ADP ratios and respiration rates in the perfused rat liver after exposure to respiratory toxins (41).

The contention that acetaminophen toxicity results in inhibition of electron transport by site-specific damage to the mitochondria was supported by experiments that examined the effect of NAPQI on respiration in isolated mitochondria. While addition of NAPQI in a bolus dose may not completely model its sustained generation by cytochrome P-450 in the cell, the characteristics of mitochondrial inhibition produced are qualitatively similar. Previous exposure of isolated mitochondria to NAPQI resulted in inhibition of respiration at energy-coupling sites 1 and 2 but had no effect on respiration stimulated at site 3. This confirms recent reports of an inhibitory effect of NAPQI on respiration supported at complexes I and II in hepatic mitochondria from rats (9) and mice (10). We observed a similar pattern of site-specific respiratory inhibition in intact hepatocytes after exposure to acetaminophen. Hence, the lesions produced by acetaminophen in hepatocytes were qualitatively the same as those produced by NAPQI in isolated mitochondria. Furthermore, exposure of submitochondrial particles to NAPQI inhibited NADH dehydrogenase and succinate dehydrogenase activities, associated with respiratory complexes I and II, respectively, while having no effect on the activities of either ubiquinol-cytochrome *c* oxidoreductase (complex III) or cytochrome oxidase (complex IV). Again, this is consistent with the effects observed in hepatocytes. Energy-coupling site 1 is associated with respiratory complex I (NADH dehydrogenase) (42). Hence, the decrease in NADH dehydrogenase activity in submitochondrial particles exposed to NAPQI is consistent with the reduced respiratory rates with NADH-linked substrates observed in acetaminophen-poisoned hepatocytes and in mitochondria exposed to NAPQI. Site 2 is associated with respiratory complex III, but in these experiments it was assayed using succinate as the electron donor. This substrate donates reducing equivalents to complex III via the non-proton-translocating complex II (43). The finding that succinate-supported respiration was decreased both in hepatocytes exposed to acetaminophen and in mitochondria exposed to NAPQI could thus be due to damage at complex II or III. However, the fact that NAPQI did not affect ubiquinol-cytochrome *c* oxidoreductase activity in submitochondrial particles indicates that NAPQI's effect was at complex II.

The concentrations of NAPQI that inhibited succinate dehydrogenase were comparable with those that interfered with respiration in intact mitochondria. Succinate dehydrogenase is known to contain a number of cysteine-rich sulfur clusters (44) and can be inhibited by a number of agents that modify sulfhydryl groups, such as aklylating agents, oxidizing agents, arsenicals, and metal ions (45). It seems feasible that NAPQI may directly interact with sulfhydryl groups on succinate dehydrogenase, causing the loss of activity. This proposal may be consistent with the finding that significant covalent binding of the reactive metabolite to mitochondrial proteins occurs during paracetamol hepatotoxicity in mice *in vivo* (46, 47). It may well be that such interactions between NAPQI and the mitochondria are of relevance to the toxic process. It was shown recently that the increased level of covalent binding to mitochondrial proteins was one of the main differences between the subcellular binding pattern produced by acetaminophen and that produced by its nontoxic regioisomer 3′-hydroxyacetanilide (38).

However, the finding that respiratory complex II (succinate dehydrogenase) was more sensitive to NAPQI than complex I (NADH dehydrogenase) in our *in vitro* system suggests that direct interactions between the toxic metabolite and respiratory chain may not solely account for the mitochondrial damage that occurs during acetaminophen hepatotoxicity *in vivo*. For example, it has been shown by others that the respiratory capacity of hepatic mitochondria from acetaminophen-poisoned mice is more or less equally impaired with both succinate and NADH-linked substrates (4). Thus it could be that events associated with the toxic process may contrib-

ute to the damage to the respiratory chain. A number of toxic changes have been reported to occur during acetaminophen hepatotoxicity, including calcium dyshomeostasis (48), oxidative stress and lipid peroxidation (49), and also protein thiol oxidation (50). It may be that one or more of these toxic mechanisms contributes to the damage to respiratory complex I during acetaminophen hepatotoxicity in addition to direct attack by the toxic metabolite.

In order for NAPQI to damage the mitochondria it must be sufficiently long-lived to have access to the inner membrane. Since the mitochondria are believed to contain a discrete GSH pool in the hepatocyte (18), depletion of this GSH pool may result from its interaction with NAPQI. The results shown in Fig. 3 indicate that NAPQI generated by endoplasmic reticulum mixed function oxidases was capable of depleting mitochondrial GSH. The finding that a loss of mitochondrial glutathione preceded the loss of respiratory function is consistent with the protective role of glutathione in this organelle. It is possible that the mitochondrial pool of GSH may protect critical nucleophilic sites on respiratory chain proteins against toxic electrophilic metabolites such as NAPQI. On the other hand, mitochondrial injury has been associated with mitochondrial GSH depletion *per se* (51), and this may also be important in the etiology of acetaminophen toxicity.

In summary, acetaminophen toxicity resulted in an impairment of mitochondrial respiration in isolated mouse hepatocytes, which involved an inhibition of respiration supported at energy-coupling sites 1 and 2. The impairment of succinate-supported respiration appeared to be due to an inhibition of succinate dehydrogenase, since this enzyme was very sensitive to NAPQI, acetaminophen's putative toxic metabolite. Since mitochondrial dysfunction occurred prior to irreversible cell injury, it is likely that interactions between NAPQI and these respiratory components contribute to the damaging effects of acetaminophen.

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