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Reversible and irreversible inhibition of hepatic mitochondrial respiration by acetaminophen and its toxic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI)

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Acetaminophen (AA) is an analgesic and antipyretic drug that has become a popular alternative to aspirin as a readily available over-the-counter pain reliever. While considered to be safe when taken in therapeutic doses, it has become evident that the drug is capable of causing severe centrilobular liver damage when taken in large quantities [1, 2]. The hepatotoxic action of AA has been suggested to be the result of activation of the drug by hepatic mixed-function oxidase (MFO) to its toxic metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) [3]. High doses of AA have been found to saturate the glucuronide and sulfate conjugating systems in the liver which allows more of the drug to be metabolized by MFO to NAPQI [4, 5]. In the presence of sufficient levels of glutathione (GSH), the NAPQI formed in the hepatocyte is thought to be detoxified through conjugation with GSH or by reduction of the NAPQI back to AA by GSH [6]. The eventual depletion of GSH from the cell, resulting from the formation and excretion of the GSH conjugate, leaves essential sulfhydryl-containing enzymes within the cell vulnerable to NAPQI. NAPQI has the potential to react with sulfhydryl groups through the formation of covalent adducts [7–10] or through sulfhydryl

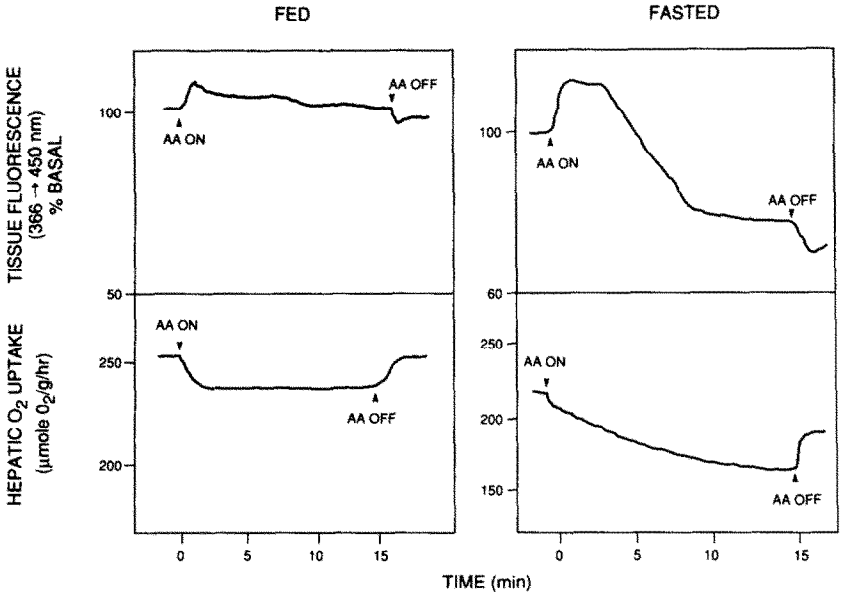

Fig. 1. Left panel: Effect of 5 mM AA perfused through a control liver. Right panel: Effect of 5 mM AA perfused through an isolated liver from a 48-hr fasted rat. Krebs–Henseleit bicarbonate buffer containing 5 mM AA, pH 7.4, was perfused through the isolated rat liver for 15 min. The resulting alterations in hepatic oxygen uptake and tissue fluorescence were determined as described in Materials and Methods. Data presented are representative of three liver perfusions for each treatment.

group oxidation [6, 11]. These processes of covalent binding and oxidative stress have both been suggested to contribute to the inactivation of critical sulfhydryl-containing enzymes within the hepatocyte which ultimately lead to the death of the cell.

An inhibition of rat kidney tubule mitochondrial respiration has been described previously as a possible mechanism contributing to the renal toxicity caused by AA overdoses [12]. Recently, Myers and coworkers [13] reported that AA inhibits mouse liver mitochondrial respiration *in vitro* and *in vivo*; however, a clear assignment of the inhibition to AA or its toxic metabolite was not made. We have also observed a similar inhibition of rat liver mitochondrial respiration by AA in the isolated perfused liver. To determine if mitochondrial alterations observed in the liver following treatment with AA were the result of the parent compound or its toxic metabolite, NAPQI, a comparison of the effects of AA and NAPQI on hepatic mitochondrial function was performed.

Materials and methods

Materials. Female Sprague–Dawley rats, weighing between 150 and 225 g, were purchased from Taconic Farms (Germantown, NY). Acetaminophen, NADH, pyruvate, malate, succinate, and β-hydroxybutyrate were purchased from the Sigma Chemical Co. (St Louis, MO). All other chemicals were of the highest grade commercially available.

Experimental methodology. Rat livers were perfused using the method of Scholz *et al.* [14]. The perfusate used was Krebs–Henseleit bicarbonate buffer, pH 7.4, at 37° saturated with 95% O_2/5% CO_2. The outflow oxygen tension was measured continuously with a Clark-type oxygen electrode (Bachofer Laboratory Equipment Co., Reutlingen, West Germany) which had been calibrated with gas mixtures containing 0, 20, 60, and 95% oxygen. The rate of hepatic oxygen uptake was calculated from the inflow–outflow oxygen concentration difference, the flow rate, and the liver wet weight and is expressed as micromoles of O_2 consumed per gram of liver per hour.

The pyridine nucleotide redox state in the isolated perfused liver was monitored noninvasively using the surface fluorescence techniques of Chance and coworkers [15].

Mitochondria were isolated from rat liver using the method of Pedersen *et al.* [16]. Measurement of the acceptor control ratios (ACRs) of rat liver mitochondria was also performed using the method of Pedersen *et al.* [16]. Mitochondrial respiration was monitored using a Clark-type oxygen electrode and chamber from Diamond Electrotech Inc. (Ann Arbor, MI).

NAPQI was synthesized by the method of Dahlin and Nelson [17]. The synthesized NAPQI/ether solution was used in experimentation following evaporation of the ether vehicle from the test vessels by N_2. Isolated mitochondria were added to the test vessel containing NAPQI and were allowed to incubate for 3 min prior to placement into the test chamber for respiratory measurements.

Results and discussion

Following infusion of AA into the isolated perfused rat liver, two distinct effects on hepatic respiration were observed which depended on the glutathione status of the liver. It was found that the infusion of 5 mM AA through control livers resulted in an immediate inhibition of respiration which remained stable for the duration of the 15-min infusion period (Fig. 1). A corresponding increase in tissue pyridine nucleotide fluorescence was also observed, indicative of a reduction of pyridine nucleotides. When the infusion of the 5 mM AA through the livers was stopped, both parameters returned rapidly to control values.

When 5 mM AA was infused through livers from rats fasted for 48 hr or pretreated with diethylmaleate to deplete glutathione stores, the rapid inhibition of respiration was still observed. However, it was followed by a slowly developing inhibition of respiration which increased over the 15-min AA infusion period (Fig. 1). Cessation of the AA infusion at the end of 15 min revealed that the inhibition of respiration observed over this time period was partially irreversible. In a manner similar to control livers, the tissue pyridine nucleotide fluorescence initially increased following AA infusion. However, during the development of the slow inhibition of hepatic respiration, the fluorescence signal began to decrease, indicating that an oxidation of pyridine nucleotides was occurring within the hepatocytes. These observations in the perfused rat liver suggest that AA is capable of inhibiting mitochondrial respiration both before and after its activation to NAPQI provided the hepatocytes are made susceptible to NAPQI through the depletion of cellular glutathione. To test this conclusion directly, the effects of acetaminophen and NAPQI on isolated rat liver mitochondria were compared.

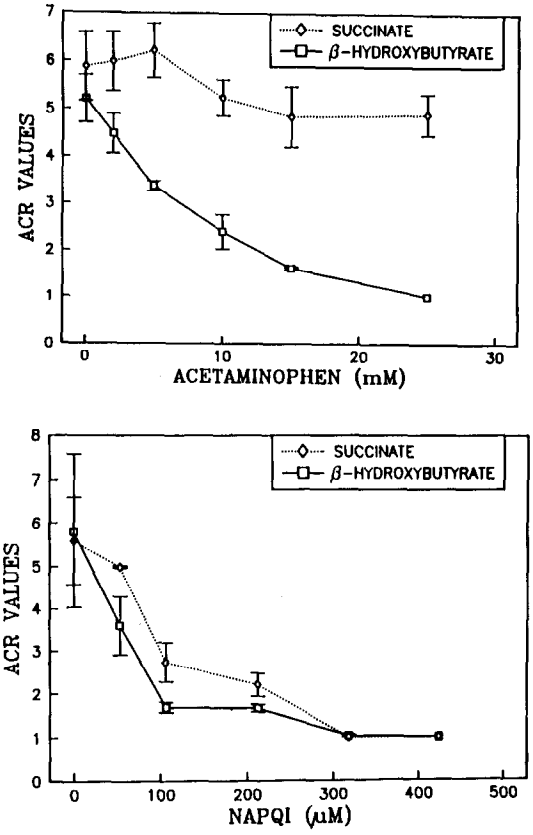

Fig. 2. Effect of 0–25 mM AA (upper panel) and 0–424 μM NAPQI (lower panel) on the acceptor control ratios (ACR) of mitochondria utilizing β-hydroxybutyrate or succinate as respiratory substrate. Treatment of mitochondria with AA consisted of adding 50 mg of mitochondria to the respiratory chamber (0.6 ml total volume) which contained a mannitol, sucrose, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, required substrates and the desired acetaminophen concentration. In the case of NAPQI, the mitochondria + buffer suspension was added to a vessel containing NAPQI, and the suspension was then allowed to incubate for 3 min prior to transfer to the respiratory chamber. In both cases, respiratory measurements were initiated immediately following the introduction of the mitochondria into the chamber. (25 mM AA = 45.4 μg AA/mg mitochondrial protein; 424 μM NAPQI = 0.758 μg NAPQI/mg mitochondrial protein.) ACR values were determined as described in Materials and Methods and are the means ± SE of three experiments.

AA caused an inhibition of respiration in rat liver mitochondria utilizing NAD-linked respiratory substrates such as β-hydroxybutyrate (Fig. 2) or pyruvate and malate (data not shown). The basis of the inhibition was found to be a concentration-dependent inhibition of state 3 respiration with significant inhibition observed at AA concentrations of 2 mM and higher (data not shown). Succinate-supported respiration was not affected by AA (Fig. 2) nor was NAD-linked state 4 respiration (data not shown). AA (0–25 mM) added to submitochondrial particle suspensions also caused a concentration-dependent inhibition of NADH oxidase activity, indicating that the inhibition was at the level of the mitochondrial electron transport chain (data not shown). This inhibition was found to be glutathione (5 mM) and dithiothreitol (3 mM) insensitive (data not shown).

NAPQI (0–424 μM) was found to cause a concentration-dependent inhibition of NAD-linked mitochondrial respiration in a manner similar to AA (Fig. 2). However, NAPQI also caused a concentration-dependent inhibition of succinate-linked respiration. Additionally, both state 3 and state 4 respiration supported by both substrates were affected by NAPQI (data not shown), whereas only state 3 respiration supported by NAD-linked substrate was inhibited by AA. Although NAPQI inhibited NADH-oxidase activity in submitochondrial particles, this inhibition was found to be largely prevented by the inclusion of either glutathione (5 mM) or dithiothreitol (3 mM) in the submitochondrial suspension (data not shown).

To investigate further the nature of the inhibition of hepatic mitochondrial respiration, the reversibilities of the AA-mediated and the NAPQI-mediated inhibition of mitochondrial respiration were compared. The inclusion of either 25 mM AA or 424 μM NAPQI in mitochondrial suspensions was found to be sufficient to inhibit 100% of the ADP-stimulated state 3 mitochondrial respiration. Following pelleting of the mitochondrial suspension via centrifugation, one wash of the AA-treated mitochondrial pellet with AA-free buffer was sufficient to totally restore respiration in the mitochondria following resuspension (Fig. 3). In contrast, under identical conditions, a single wash of NAPQI-free buffer had no effect on the NAPQI-treated mitochondria with state 3 respiration remaining totally inhibited (Fig. 3). It is clear that AA was inhibiting hepatic mitochondrial respiration in a highly reversible manner, whereas the NAPQI-mediated inhibition was found to be irreversible.

In conclusion, it appears that acetaminophen and NAPQI are both capable of independently inhibiting mitochondrial respiration. This conclusion is based on the fundamentally different characteristics of the inhibition mediated by the two chemicals. The AA-mediated inhibition was found to be (1) specific for NAD-linked mitochondrial substrates, (2) readily reversible, (3) unaffected by hepatocyte glutathione levels, and (4) immediate and accompanied by a relatively stable increase in the level of reduced pyridine nucleotides in the isolated perfused liver. In contrast, NAPQI was found to inhibit mitochondrial respiration in a manner which was characterized as being (1) nonspecific with respect to respiratory substrates, and (2) totally irreversible. Further, in glutathione-depleted perfused livers, a secondary irreversible inhibition of respiration which was accompanied by an oxidation of pyridine nucleotides developed which may be attributable to the oxidative properties of NAPQI within the hepatocyte. The

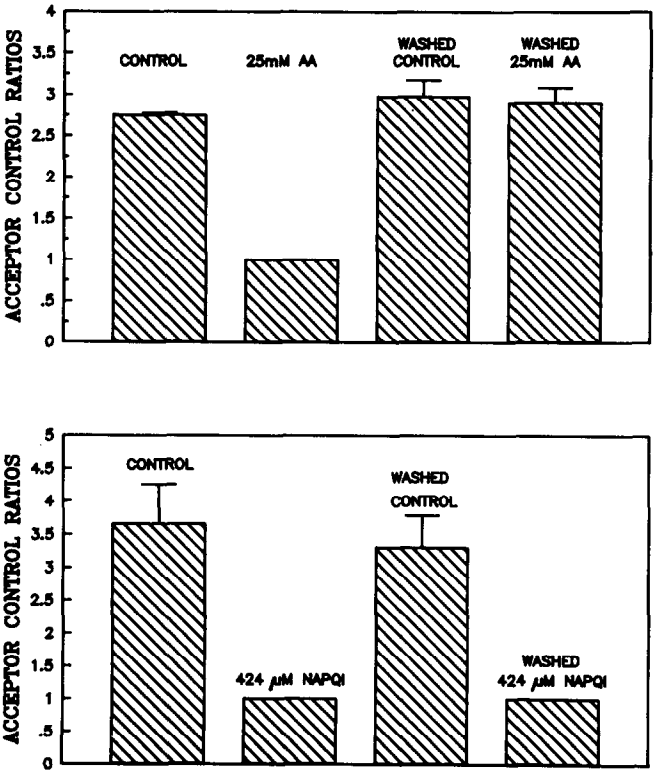

Fig. 3. Reversibility of 25 mM AA-mediated inhibition (upper panel) or 424 μM NAPQI-mediated inhibition (lower panel) of mitochondrial respiration utilizing pyruvate and malate as respiratory substrates. Washed ACR values were determined following a single wash of the isolated mitochondria with control buffer. Results are the mean ACR ± SE of three experiments.

inhibitions of mitochondrial respiration by AA and NAPQI may both contribute to the toxicity observed following AA overdose.

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Metabolic alterations resulting from the inhibition of mitochondrial respiration by acetaminophen *in vivo*

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Acetaminophen (AA) is considered to be a relatively safe drug when taken in therapeutic doses which generally range from 650 to 1000 mg taken every 4 hr. Toxic doses of AA vary depending on a variety of conditions such as the blood levels of AA attained after overdose, the rate of disposition of the drug, the activity of the mixed-function oxidase system and the level of glutathione stores in the liver at the time of the AA overdose. In general, a single dose of over 15 g will lead to hepatotoxicity in most individuals [1].

Within the first 24 hr following AA overdose, symptoms such as pallor, anorexia, right hypochondrial tenderness, nausea and vomiting may occur [1, 2]. Often, however, early clinical signs and symptoms are absent making AA overdoses extremely difficult to diagnose in the absence of patient cooperation. In cases of severe overdose (plasma AA concentrations > 800 mg/l. or approximately 5 mM), coma and metabolic acidosis have been reported in the hours immediately following the overdose in the absence