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Mitochondrial Dysfunction Is an Early Manifestation of 1,1-Dichloroethylene-Induced Hepatotoxicity in Mice

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ABSTRACT

Hepatotoxicity induced by 1,1-dichloroethylene (DCE) is mediated by cytochrome P450-dependent metabolism to reactive intermediates, including the epoxide. We have tested the hypothesis that mitochondria are a primary target of toxicity by investigating dose- and time-dependent effects of DCE on mitochondrial respiration. Hepatotoxicity, as assessed by serum alanine aminotransferase (ALT) activity, was evaluated. We have also determined the effectiveness of *N*-acetyl-L-cysteine (NAC) in protecting against respiratory perturbations and hepatotoxicity. Liver mitochondria were isolated 2 h after DCE (50, 75, 100, 125, and 150 mg/kg) treatment. Glutamate (complex I)- and succinate (complex II)-supported mitochondrial respiration was assessed by measurement of state 3 (ADP-stimulated) and state 4 (resting) rates of oxygen consumption. The corresponding respiratory control ratios (RCRs, state 3/state 4) and ADP:O ratios were then calculated. A DCE dose of 125 mg/kg significantly inhibited glutamate- and succinate-supported state 3 respiration, leading to a significant reduction in corresponding RCRs and ADP:O ratios. In time-dependent studies, state 3 respiration rates and RCRs for glutamate-supported respiration were significantly decreased as early as 20 min after DCE (125 mg/kg) treatment, whereas those for succinate-supported respiration were significantly decreased at 90 min. Additionally, ADP:O ratios for glutamate-supported respiration were significantly decreased starting at 60 min, and those for succinate-supported respiration at 90 min. Alterations in mitochondrial function preceded significant increases in ALT activity, which was first manifested at 2 h. Pretreatment with NAC (1200 mg/kg) abrogated DCE-induced GSH depletion and inhibited disturbances in mitochondrial respiration. Moreover, NAC protected against increased ALT activity, suggesting that the protective effect of NAC is due to increased GSH for conjugation reactions and/or its antioxidant property. These results showed that DCE-mediated mitochondrial dysfunction is an early event that preceded the onset of hepatotoxicity.

Liver damage involving centrilobular hepatocytes occurs as a result of exposure to 1,1-dichloroethylene (DCE), a chemical used extensively in the plastics manufacturing industry (Coleman et al., 1976; Forkert et al., 1986). It is also a degradation product of trichloroethylene and is found in conjunction with the latter as a water contaminant (Semprini, 1995). Metabolic studies have implicated covalent binding of DCE metabolites to cellular macromolecules as a mechanism responsible for the cytotoxic response. The extent of binding was dose-dependent and correlated with the severity of centrilobular necrosis (Forkert et al., 1986; Forkert and Moussa, 1991). Furthermore, immunohistochemical studies using an antibody that recognizes DCE protein adducts showed dose-dependent adduct formation that was preferentially localized in the centrilobular hepatocytes (Forkert, 1999). Subcellular fractions to which DCE was covalently bound were those from nuclei, cytosol, mitochondria, and microsomes (Okine et al., 1985; Okine and Gram, 1986). Other studies showed that DCE treatment produced morphological alterations primarily in sinusoidal and lateral plasma membranes, nuclei, and mitochondria (Reynolds et al., 1975). Early alterations involving mitochondria were manifested as swelling, disruption of cristae, and loss of mitochondrial matrix density (Reynolds et al., 1975; Reynolds and Moslen, 1977). Moreover, DCE treatment produced decreased histochemical staining of the inner mitochondrial membrane enzymes, succinate dehydrogenase and cytochrome oxidase. Staining for the outer mitochondrial membrane enzyme, monoamine oxidase, was also decreased (Chieco et al., 1982). These findings suggested that mitochondria are potential primary sites of damage in DCE-mediated hepatic injury.

The mechanism of DCE-induced hepatotoxicity involves cytochrome P450-dependent metabolism to reactive interme-

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ABBREVIATIONS: DCE, 1,1-dichloroethylene; GSH, glutathione; NAC, *N*-acetyl-L-cysteine; ALT, alanine aminotransferase; RCR, respiratory control ratio; *t*BH, *t*-butyl hydroperoxide.

diates (Forkert et al., 1986, 1987). Subsequent studies in mice revealed that CYP2E1 is a major cytochrome P450 isoform catalyzing the oxidative metabolism of DCE (Kainz et al., 1993; Lee and Forkert, 1994). The primary metabolites formed from DCE oxidation in rat and murine liver microsomal incubations were the epoxide 2,2-dichloroacetaldehyde and 2-chloroacetyl chloride (Fig. 1) (Costa and Ivanetich, 1984; Liebler et al., 1985, 1988; Dowsley et al., 1995). The DCE epoxide was the major metabolite produced, whereas 2-chloroacetyl chloride was found at minimal levels (Dowsley et al., 1995). Acetal, the hydrate of 2,2-dichloroacetaldehyde, was also detected. The secondary metabolites generated included products of conjugation with glutathione (GSH) that were identified as 2-(*S*-glutathionyl)acetyl glutathione and 2-*S*-glutathionyl acetate (Fig. 1). These conjugates were major metabolites detected and were the products of conjugation of DCE epoxide with GSH. Because GSH depletion is associated with DCE-induced toxicity, it is plausible that the epoxide is responsible for mediating centrilobular necrosis by conjugating with GSH and depleting the GSH pool. This assumption is consistent with findings showing that rodents with reduced GSH levels due to fasting or pretreatment with diethylmaleate are more vulnerable to DCE-induced hepatotoxicity (Jaeger et al., 1974; Okine et al., 1985). Similarly, pretreatment with buthionine sulfoximine decreased GSH levels and exacerbated the toxicity of hepatocytes exposed to DCE in suspension (Kainz et al., 1993). These findings strongly supported GSH conjugation of DCE metabolites as a protective mechanism against DCE-induced hepatic injury.

In the present study, our objective was to obtain evidence to demonstrate that mitochondria are an early primary target of DCE-induced hepatotoxicity. We have performed dose- and time-dependent studies in mice to determine the effects of DCE administration on mitochondrial respiratory status. Mitochondrial respiration was also investigated after pretreatment with *N*-acetyl-L-cysteine (NAC), an agent that increases the availability of intracellular GSH through provision of cysteine for GSH biosynthesis as well as functioning as an antioxidant (Corcoran et al., 1985; Cotgreave, 1997). Furthermore, all respiratory data were temporally correlated with hepatotoxicity.

Materials and Methods

Chemicals and Reagents. Chemicals and reagents were obtained from suppliers as follows: 1,1-dichloroethylene (>99% purity) (Aldrich Chemical Co., Montreal, QC, Canada); Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA); and *N*-acetyl-L-cysteine (>99% purity), succinic acid (disodium salt), L-glutamic acid (monosodium salt), rotenone (95–98% pure), D-mannitol, HEPES, ADP (sodium salt), bovine serum albumin, γ-glutamyl glutamate, GSH, and *t*-butyl hydroperoxide (Sigma-Aldrich, St. Louis, MO). All other chemicals were of reagent grade and were obtained from standard commercial suppliers.

Animal Treatment. Female CD-1 mice (25–30 g) were obtained from Charles River Canada (St. Constant, QC, Canada). They were maintained on a 12-h light/dark cycle and given free access to food (Purina Rodent Chow; Ralston Purina International, Strathroy, ON, Canada) and drinking water. After acclimatization to laboratory conditions for at least 5 days, mice were randomly assigned to control or treatment groups. For dose-dependent assessment of respiratory status, mice were treated with DCE (50, 75, 100, 125, and 150 mg/kg i.p.) in corn oil and were sacrificed by cervical dislocation 2 h later. In time-course experiments, mice were treated with DCE (125 mg/kg i.p.) and sacrificed 10, 20, 30, 60, 90, and 120 min later. In studies that examined the effects of NAC (1200 mg/kg i.p. in distilled H_2O, pH 7.0) (Donnelly et al., 1993), NAC was administered 15 min before treatment with DCE (125 mg/kg), and mice were sacrificed 1 h later. For measurements of hepatic GSH content, mice were treated with DCE (125 mg/kg i.p.) and were sacrificed 10, 20, 30, 60, 120, 240, 360, and 480 min later. For measurements of serum alanine aminotransferase (ALT) activity, mice were treated with DCE (125 mg/kg) for 10, 20, 30 60, 90, 120, and 240 min. Mice were then anesthetized with sodium pentobarbital (120 mg/kg i.p.), and intracardiac blood was obtained for assessment of ALT activity. In all experiments, control mice were treated with equivalent volumes of the appropriate vehicle and were sacrificed at times corresponding to those in the experimental groups.

Isolation of Mitochondria. Mitochondria were isolated according to Johnson and Lardy (1967), with modifications. All procedures were carried out on ice or at 4°C. After cervical dislocation, livers were rapidly dissected into ice-cold isolation medium (0.25 M sucrose and 1 mM EDTA, pH 7.4). Gall bladders were removed and livers were blotted dry and weighed. Livers were then minced in fresh isolation medium and gently homogenized manually [10% (w/v) in isolation medium] using a Dounce homogenizer with a loose-fitting pestle. The resulting 10% homogenate was centrifuged at 500*g* for 10

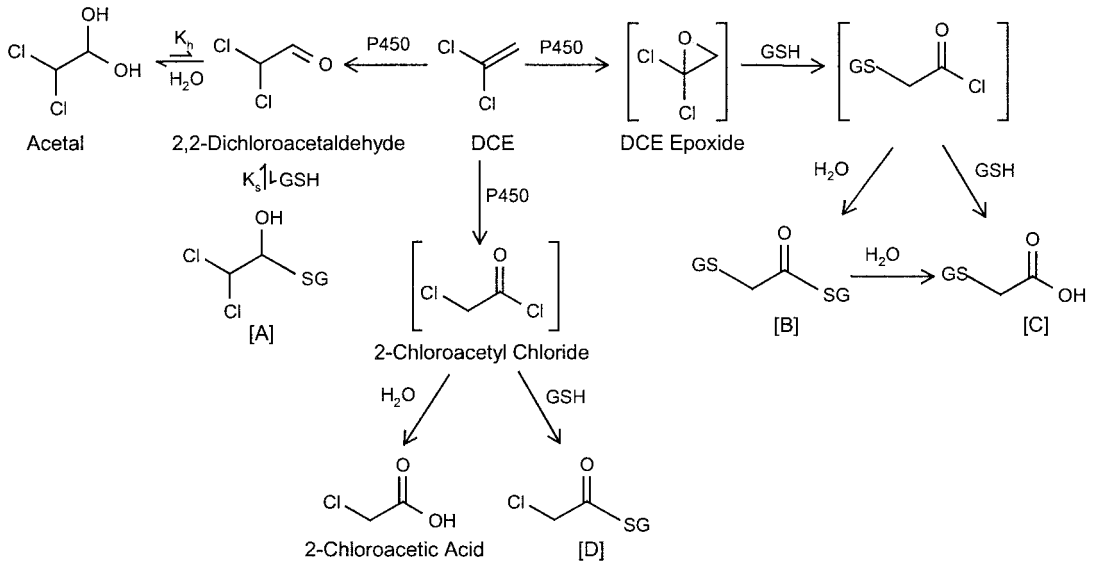

Fig. 1. Scheme of proposed pathway of DCE metabolism. The GSH conjugates are identified as follows: [A], *S*-(2,2-dichloro-1-hydroxy)-ethyl glutathione; [B], 2-(*S*-glutathionyl)acetyl glutathione; [C], 2-*S*-glutathionyl acetate; and [D], *S*-(2-chloroacetyl)glutathione.

min. The supernatant fraction was retained, whereas the pellet was washed with fresh isolation medium and recovered by centrifugation at 500*g* for 10 min. The supernatant fraction from this wash was combined with the initial supernatant fraction and again centrifuged at 500*g* for 10 min. The resulting supernatant fraction was centrifuged at 5000*g* for 15 min to obtain the mitochondrial pellet. This pellet was washed once with isolation medium and again with a respiration reaction buffer (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 0.5 mM KH_2PO_4, and 2 mM K_2HPO_4, pH 7.4), and the purified mitochondria were sedimented at 12,000*g* for 10 min. All washes were carried out in volumes amounting to 25% of the original homogenate with careful removal of lipid, which adhered to the walls of the centrifuge tubes. The final mitochondrial pellet was resuspended in respiration reaction buffer (1 ml/g tissue) to produce a suspension containing 25–40 mg of mitochondrial protein per milliliter. An aliquot was removed for determination of protein concentration by the Bradford (1976) method, using bovine serum albumin as the standard.

Polarographic Measurement of Oxygen Consumption. Oxygen consumption of isolated mitochondria was measured at 30°C using a model 5300 biological oxygen monitor and model 5331 Clarke-type polarographic oxygen probe (YSI, Inc., Yellow Springs, OH). Respiration buffer (3 ml), containing 10 mM glutamate, was added to a magnetically stirred sample chamber and allowed to equilibrate. Glutamate (complex I)-supported state 4 (resting) respiration was initiated by adding a 100-μl aliquot of the mitochondrial suspension (1.0–1.5 mg of protein) to the sample chamber. Subsequent addition of ADP (900 nmol in 50 μl of reaction medium) generated glutamate-supported state 3 (ADP-stimulated) respiration. After returning to state 4 respiration, glutamate-supported respiration was inhibited by adding rotenone (3 μM final concentration) to the sample chamber. Succinate (complex II)-supported state 3 and state 4 respiration was then assessed by consecutive addition of ADP (450 nmol in 25 μl of reaction medium) and succinate (10 μM final concentration). To assess the integrity of mitochondrial respiratory function, the respiratory control ratio (RCR, state 3/state 4) and ADP:O ratio were calculated (Estabrook, 1967).

Effects of in Vitro DCE Exposure on Mitochondrial Respiration. Isolated hepatic mitochondria were incubated with 1, 2, and 4 mM DCE at room temperature for 15, 30, and 60 min. Aliquots (100 μl) of the mitochondrial suspension were added to the sample chamber for analysis of rates of oxygen consumption. *t*-Butyl hydroperoxide (*t*BH; 1 mM), which has previously been shown to inhibit hepatic mitochondrial respiration (Lash et al., 1995), was used as a positive control in these mitochondrial toxicity assays.

Measurement of Hepatic GSH Content. Tissue GSH levels were measured by the method of Fariss and Reed (1987). Briefly, liver tissue samples (50–100 mg) were pulverized in liquid nitrogen and vortexed in 1 ml of 10% (v/v) perchloric acid containing 1 mM bathophenanthroline-disulfonic acid. Perchloric acid-insoluble material was removed by centrifugation (15,000*g* for 3 min), and 0.5 ml of the supernatant was added to a Wheaton vial containing 50 μl of 15 mM γ-glutamyl glutamate, which was used as the internal standard. After the addition of iodoacetic acid (50 μl, 100 mM in 0.2 mM *m*-cresol purple), the pH of the samples was adjusted to 8 to 9 with the addition of a potassium hydroxide/potassium bicarbonate solution (2 M KOH and 2.4 M $KHCO_3$). The samples were then mixed and incubated for 10 min in the dark. Subsequently, 1 ml of 1% 1-fluoro-2,4-dinitrobenzene was added to the samples that were then capped, vortexed, and stored overnight at 4°C. High-performance liquid chromatography analysis was conducted using an aminopropyl silica ion-exchange column (5 μm, 4 × 250 mm; SGE International, PTY Ltd., Ringwood, Australia). Initially, 4 parts of mobile phase A (80% methanol), and 1 part of mobile phase B (0.5 M ammonium acetate in 64% methanol) was allowed to equilibrate at a flow rate of 0.75 ml/min. After a 100-μl injection of the derivatized sample, increasing concentrations of mobile phase B (20–99%) were introduced to the column over a period of 10 min to elute the chromophore derivative. The derivatized concentrations of γ-glutamyl glutamate and GSH were determined by detection at 360 nm.

Measurement of ALT Activity. Serum ALT activity was measured by the method of Bergmeyer et al. (1978) using an ALT 20 kit (Sigma Diagnostics, St. Louis, MO). ALT activity was determined at 10, 20, 30, 60, 90, 120, and 240 min after DCE (125 mg/kg) treatment.

Statistical Analysis. Data are expressed as mean ± S.D. Data were analyzed by one-way analysis of variance followed by Tukey's test to determine significant differences between experimental groups ($p < 0.05$).

Results

Effects of in Vivo DCE Treatment on Mitochondrial Respiration. The effects of DCE on mitochondrial respiration were examined in dose- and time-dependent studies to identify early alterations in mitochondrial function in the toxic response. In dose-dependent studies, a decreasing trend was observed in state 3 respiration rates for glutamate (complex I)- and succinate (complex II)-supported respiration over the range of DCE doses administered. A DCE dose of 125 mg/kg was found to significantly inhibit glutamate- and succinate-supported state 3 respiration rates by 42 and 40%, respectively (Figs. 2A and 3A). In contrast, no significant changes in state 4 respiration rates were observed at any dose for either substrate (Figs. 2B and 3B). Glutamate- and succinate-supported respiration was found to be completely uncoupled with DCE doses of 150 mg/kg or higher (data not shown). In time-dependent studies, a marked decline in glutamate- and succinate-supported state 3 respiration rates was followed by a more level phase. State 3 respiration rates for glutamate-supported respiration were significantly decreased from 20 to 120 min after DCE (125 mg/kg) treatment, whereas those for succinate-supported respiration were significantly decreased from 90 to 120 min (Figs. 4A and 5A). No significant changes in state 4 respiration rates were observed at any time for either substrate (Figs. 4B and 5B).

RCRs, calculated by the ratio of state 3 and state 4 respiratory rates, served as an index of mitochondrial integrity. With glutamate as substrate, the presence of ADP should enhance oxygen consumption at least 3- to 4-fold above the rate of oxygen consumption in the resting state, whereas succinate oxidation should exhibit at least a 2-fold increase (Johnson and Lardy, 1967). Herein, the control RCRs with glutamate and succinate as substrates were 3.6 ± 0.3 and 3.3 ± 0.1, respectively, confirming that our mitochondria preparations were functionally intact. Dose-dependent studies revealed that a dose of 125 mg/kg significantly reduced the RCRs for glutamate and succinate oxidation by 46 and 30%, respectively (Figs. 2C and 3C). These reductions were a direct result of decreases in state 3 respiration rates, because state 4 respiration rates remained unchanged from control values. In time-dependent studies, RCRs for glutamate-supported respiration were significantly decreased from 20 to 120 min after DCE (125 mg/kg) treatment, whereas those for succinate-supported respiration were significantly decreased from 90 to 120 min (Figs. 4C and 5C).

The ADP:O ratio (i.e., mole of ADP phosphorylated per mole of atomic oxygen consumed) that is equivalent to the P:O ratio (i.e., number of molecules of inorganic phosphate incorporated into organic form per atom of oxygen consumed) was calculated and used as an index of oxidative phosphor-

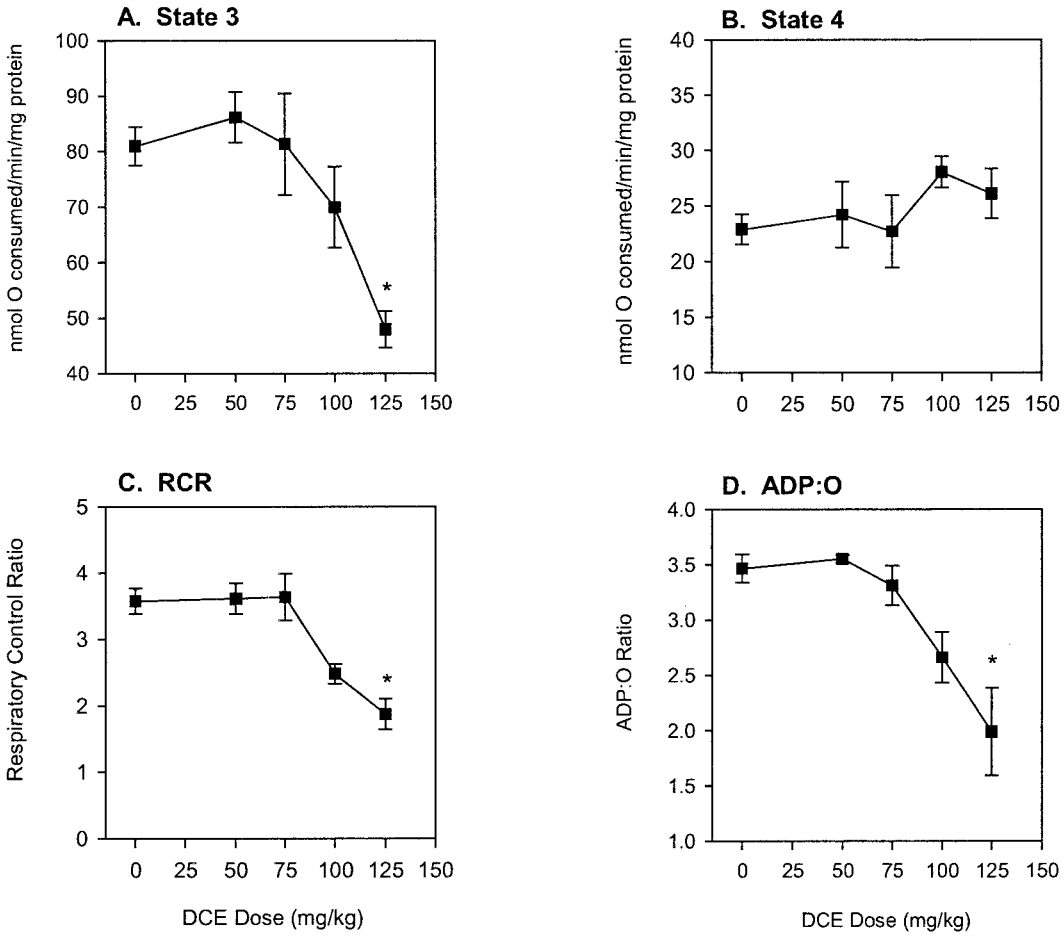

Fig. 2. Dose-dependent effects of DCE treatment (2 h) on glutamate-supported (complex I) mitochondrial respiration. Respiration was measured as state 3 (A) and state 4 (B) rates of oxygen consumption. RCR (C; state 3/state 4) and ADP:O (D) values were then calculated. Data for the control are depicted at time 0. Data are expressed as mean ± S.D. of measurements from three separate experiments. ⋆, significantly different from the control ($p < 0.001$).

ylation. The maximum ADP:O ratios for the oxidation of glutamate and succinate are generally assigned values of 3 and 2, respectively (Brand et al., 1993). In dose-response studies, a DCE dose of 125 mg/kg produced significant decreases in the ADP:O ratio for both glutamate and succinate (Figs. 2D and 3D). With glutamate as substrate, a significant decrease in the ADP:O ratio was observed from 60 to 120 min, whereas that with succinate was significantly decreased from 90 to 120 min (Figs. 4D and 5D).

Effects of in Vitro DCE Exposure on Hepatic Mitochondria. Isolated hepatic mitochondria were incubated with 1, 2, and 4 mM DCE for 15, 30, and 60 min to determine whether the parent compound, exclusive of its P450-derived metabolites, produced mitochondrial perturbations. No effects on glutamate- or succinate-supported state 3 and state 4 respiration were observed, regardless of DCE concentration or incubation time; as a result RCRs and ADP:O ratios remained unaffected (data not shown). In contrast, 1 mM *t*BH inhibited glutamate-supported state 3 respiration rates by 32, 45, and 65% at 15, 30, and 60 min, respectively. Succinate-supported state 3 respiration rates were inhibited by 27, 48, and 64% at 15, 30, and 60 min, respectively. Also, at these time-points, glutamate-supported state 4 respiration rates were increased by 14, 20, and 22%, and succinate-supported state 4 respiration rates were increased by 9, 15, and 24%, respectively. As a consequence of *t*BH-induced alterations in state 3 and state 4 respiration rates, the control RCR (3.7 ± 0.4) for glutamate oxidation was decreased to 2.2 ± 0.3, 1.7 ± 0.4, and 1.2 ± 0.2 at 15, 30, and 60 min, respectively. The control RCR (3.3 ± 0.2) for succinate oxidation was decreased to 2.5 ± 0.4, 1.7 ± 0.3, and 1.2 ± 0.3 at 15, 30, and 60 min, respectively.

Effects of DCE and/or NAC on GSH Content. To identify a potential relationship between DCE-induced mitochondrial disturbances and hepatic GSH levels, GSH levels were measured at 10 to 480 min after treatment with 125 mg/kg DCE or the vehicle (Fig. 6). GSH levels were significantly reduced at 30 and 60 min by 28 and 37%, respectively. Thereafter, GSH levels gradually increased and at 4 h returned to control levels. Additionally, GSH levels were determined at 1 h in mice treated with 1200 mg/kg NAC or 125 mg/kg DCE and NAC. GSH levels were similar in control mice and mice treated with NAC or DCE and NAC (Fig. 7).

Effect of NAC on Mitochondrial Dysfunction. To further investigate the early effects of DCE treatment on hepatic mitochondrial respiration, 1200 mg/kg NAC was administered 15 min before 125 mg/kg DCE and the respiratory status was evaluated. In preliminary experiments, it was determined that NAC alone had no effect on any of the respiratory parameters assessed. DCE-induced inhibition of glutamate- and succinate-supported state 3 respiration rates was diminished by pretreatment with NAC (Fig. 8A). State 3 respiration rates for mice treated with DCE and NAC were less than the controls and were significantly higher than in mice treated with DCE alone. Glutamate- and succinate-supported state 4 respiration rates were similar for all treatment groups (Fig. 8B). Additionally, NAC treatment also

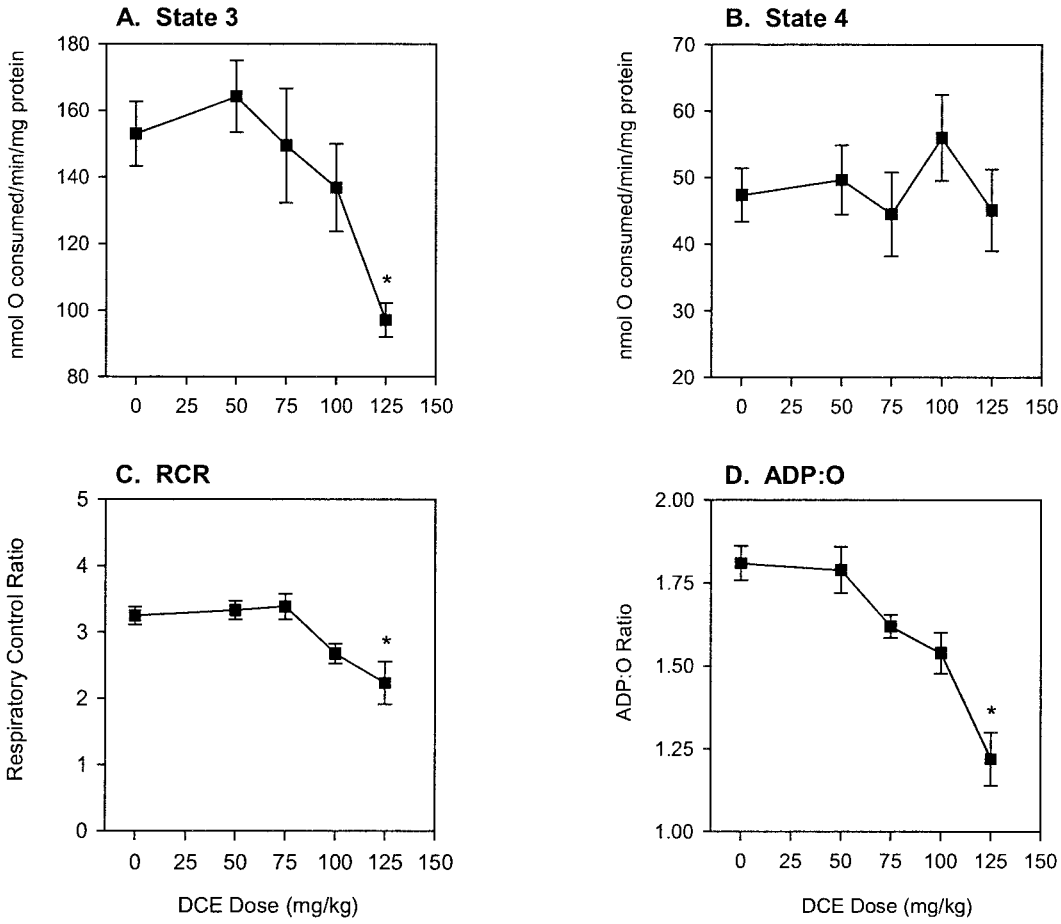

Fig. 3. Dose-dependent effects of DCE treatment (2 h) on succinate-supported (complex II) mitochondrial respiration. Respiration was measured as state 3 (A) and state 4 (B) rates of oxygen consumption. RCR (C; state 3/state 4) and ADP:O (D) values were then calculated. Data for the control are depicted at time 0. Data are expressed as mean ± S.D. of measurements from three separate experiments. ⋆, significantly different from the control ($p < 0.001$).

decreased inhibition of RCRs and ADP:O ratios for glutamate and succinate oxidation (Fig. 8, C and D).

Measurement of ALT Activity. The hepatotoxic effect of DCE was observed over time by measurement of serum ALT activity. ALT activity was found to be significantly elevated at 2 h after DCE administration and was further increased after 4 h (Fig. 9). The effectiveness of NAC in protecting against DCE-induced hepatotoxicity was also investigated by measuring serum ALT activity. Pretreatment with NAC did not produce an increase in serum ALT activity (8.7 ± 2.7 U/l), compared with the control (4.2 ± 0.8 U/l).

Discussion

The liver has been identified as a major target of DCE-induced cytotoxicity. Yet, despite a well characterized hepatic lesion, the early events critical to the onset of toxicity remained unclear. Morphological, histochemical, and covalent binding studies have implicated mitochondria in the early pathogenesis of DCE-mediated hepatotoxicity (Reynolds et al., 1975; Reynolds and Moslen, 1977; Chieco et al., 1982; Okine et al., 1985; Okine and Gram, 1986); however, biochemical data are lacking in this respect. In this study, we examined the early effects of DCE on respiratory parameters indicative of mitochondrial integrity and function. Isolated mouse liver mitochondria were used to examine the effects of DCE treatment on mitochondrial respiration. Dose-response experiments were initially performed to establish a dose that produced early, moderate inhibition of respiratory function at 2 h, a time point at which alterations in mitochondrial morphology were manifested (Reynolds et al., 1975; Kanz and Reynolds, 1986). A DCE dose of 125 mg/kg significantly reduced glutamate- and succinate-supported state 3 respiration rates as well as RCRs, and ADP:O ratios (Figs. 2 and 3). As a result, this DCE dose was used in subsequent studies. To evaluate the early effects of DCE on mitochondrial function, respiratory parameters were assessed in time-course experiments. State 3 respiration rates and RCRs for glutamate-supported respiration were significantly decreased as early as 20 min after DCE treatment, whereas those for succinate-supported respiration were significantly decreased at 90 min (Figs. 4 and 5). These findings are consistent with results of previous studies (Jaeger, 1977) showing a significant reduction in pyruvate- and malate-supported state 3 respiration in murine liver 1 h after inhalation exposure of DCE. To further assess the effects of DCE on mitochondrial function, the ADP:O ratio was determined and used as an index of oxidative phosphorylation. The ADP:O ratio reflected how efficiently mitochondria used the molecular substrate O_2 for flavoprotein-mediated ATP synthesis (Toyomizu et al., 1992). As early as 60 min after DCE treatment, the ADP:O ratio for glutamate oxidation was significantly decreased (Fig. 4). Succinate oxidation was not affected as rapidly as was glutamate oxidation, and the ADP:O ratio was significantly reduced by 90 min (Fig. 5). Hence, significant decreases in oxidative phosphorylation, as assessed by ADP:O ratios, were noted at 60 to 90 min after DCE treatment. The functional alterations in the mitochondria seemed to precede hepatic necrosis, as assessed by measurement of

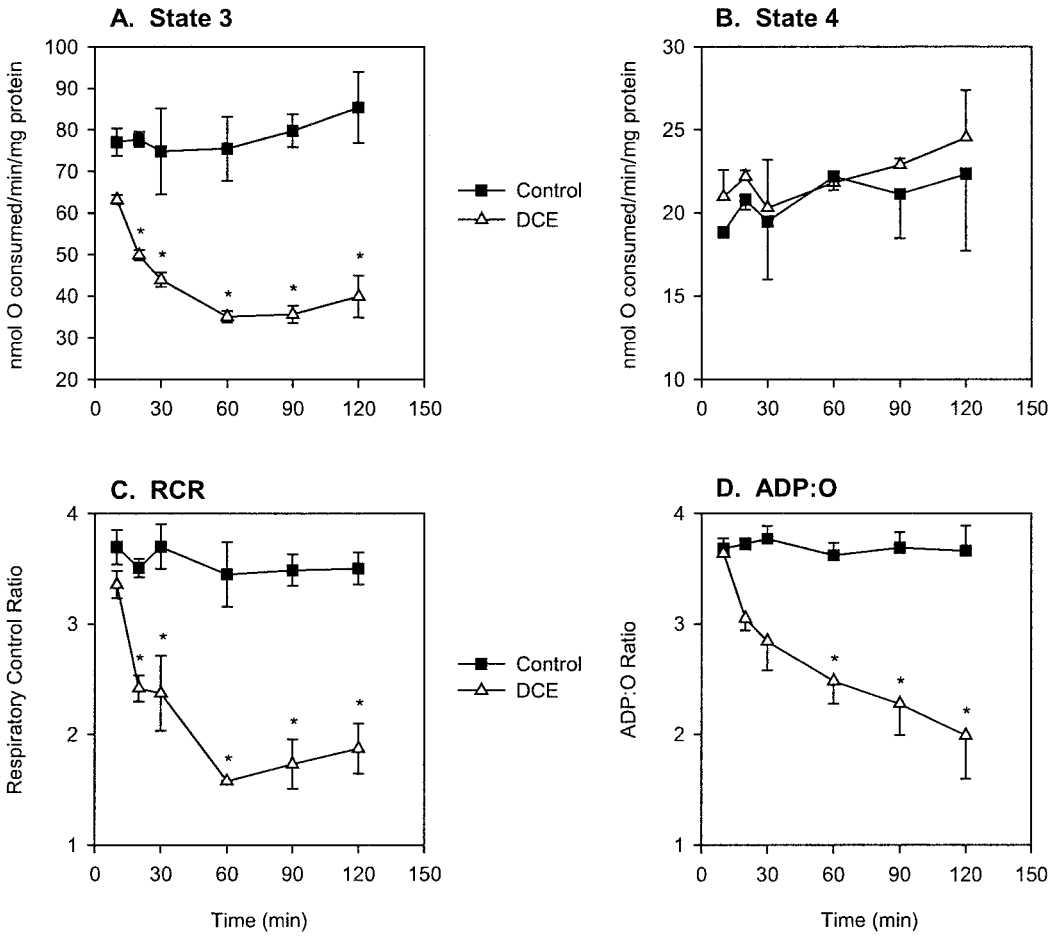

Fig. 4. Time-dependent effects of 125 mg/kg DCE treatment on glutamate-supported (complex I) mitochondrial respiration. Respiration was measured as state 3 (A) and state 4 (B) rates of oxygen consumption. RCR (C; state 3/state 4) and ADP:O (D) values were then calculated. Control mice were treated with corn oil. Data are expressed as mean ± S.D. of measurements from three separate experiments. ⋆, significantly different from the controls ($p < 0.001$).

serum ALT activity. Serum ALT activity was significantly elevated at 2 h, whereas mitochondrial perturbations were evident as early as 20 min after DCE exposure (Figs. 4 and 9). Additionally, centrilobular necrosis was observed 24 h after treatment of mice with 125 mg/kg DCE (Forkert et al., 1986). Moreover, it has been reported that decreases in histochemical staining of mitochondrial membrane enzymes preceded increases in serum transaminase activities, a biomarker of hepatic injury (Chieco et al., 1982). Thus, these data suggested that mitochondrial alterations are associated with the initiation and/or early development of DCE-induced hepatotoxicity.

The time-dependent alterations of mitochondrial function produced by DCE have provided some insight into the sequence of events leading to the toxic outcome. Initial decreases of state 3 respiration rates without concomitant decreases of state 4 respiration rates suggested specific inhibition of enzymes in the respiratory chain proper and/or inhibition of utilization of nucleotides (i.e., the adenine nucleotide translocase). Further investigation, however, is required to discern the precise site(s) of inhibition. At times subsequent to 120 min after DCE treatment, oxidation in the respiratory chain was found to be uncoupled from ADP phosphorylation. As a result, respiration became uncontrolled and the concentration of ADP or P_i was no longer rate limiting (data not shown). Thus, complete uncoupling of oxidative phosphorylation occurred subsequent to inhibition of specific components of the respiratory chain.

Because DCE is readily metabolized in target tissues, it was of interest to determine whether the parent compound or its metabolites were associated with the observed mitochondrial dysfunction. To this end, isolated liver mitochondria were incubated with 1, 2, and 4 mM DCE for 15, 30, and 60 min, and mitochondrial respiration was assessed. Inhibition of respiratory parameters was not identified under these conditions. To validate the assay for mitochondrial toxicity in vitro, isolated mitochondria were incubated with *t*BH, a known mitochondrial toxicant, and this system was used as a positive control (Lash et al., 1995; Cawthon et al., 1999). *t*BH significantly inhibited state 3 respiration rates and increased state 4 respiration rates, resulting in significantly reduced RCRs. Hence, the in vivo metabolism of DCE seemed to be a necessary event in the development of mitochondrial toxicity.

In the present study, DCE-induced functional alterations in hepatic mitochondria coincided with changes in cellular GSH levels. Both the impairment of mitochondrial respiration and decreased GSH levels occurred rapidly and were maximal at approximately 1 h after DCE exposure (Fig. 6). However, whereas GSH levels were fully restored by 4 h, mitochondrial uncoupling was still evident at this time (data not shown). Thus, a relationship between mitochondrial dysfunction and hepatic GSH levels seemed to exist in the early time period after DCE treatment. To investigate the relationship between mitochondrial alterations, GSH and hepatotoxicity, respiration, GSH levels, and ALT activity were assessed after NAC pretreatment. Our results revealed that GSH levels were similar in control mice and mice treated with DCE and NAC (Fig. 7). Thus, NAC in providing cysteine

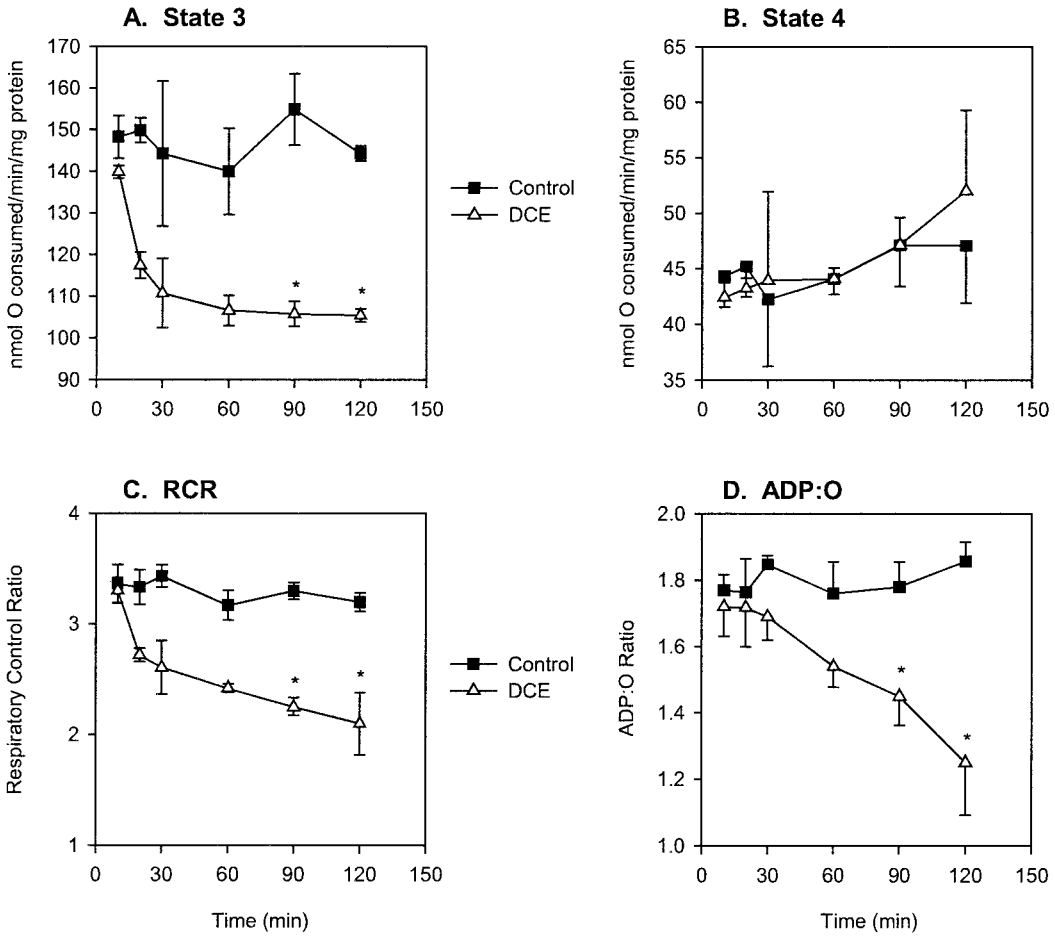

Fig. 5. Time-dependent effects of 125 mg/kg DCE treatment on succinate-supported (complex II) mitochondrial respiration. Respiration was measured as state 3 (A) and state 4 (B) rates of oxygen consumption. RCR (C; state 3/state 4) and ADP:O (D) values were then calculated. Control mice were treated with corn oil. Data are expressed as mean ± S.D. of measurements from three separate experiments. ⋆, significantly different from the controls ($p < 0.001$).

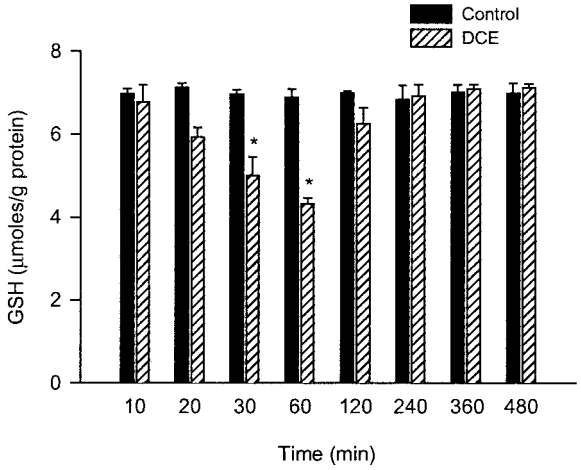

Fig. 6. Time course of alterations in tissue GSH in livers of control and 125 mg/kg DCE-treated mice. Control mice are treated with corn oil. Data are expressed as mean ± S.D. of measurements from three separate experiments. ⋆, significantly different from the controls ($p < 0.001$).

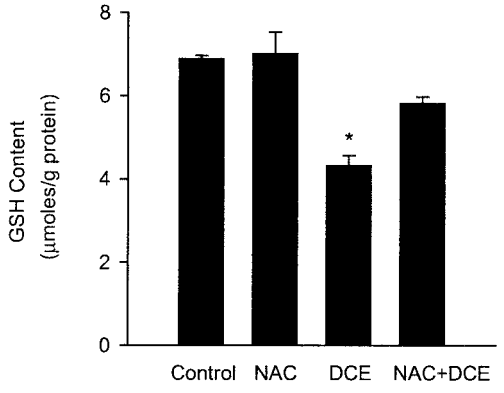

Fig. 7. Liver glutathione levels in control, NAC-, DCE-, and NAC + DCE-treated mice. Mice were treated with 1200 mg/kg i.p. NAC or the vehicle at 15 min before treatment with 125 mg/kg i.p. DCE or corn oil, and sacrificed 1 h later. Data are expressed as mean ± S.D. of measurements from three separate experiments. ⋆, significantly different from the control ($p < 0.001$).

for GSH synthesis, increased GSH levels and abrogated GSH diminution by DCE. Pretreatment with NAC inhibited both the impairment of mitochondrial respiration observed at 1 h after DCE treatment (Fig. 8) and the increase in serum ALT activity seen at 2 h. This association between early mitochondrial alterations and decreased GSH levels suggested that DCE-mediated GSH depletion may play a role in the onset of mitochondrial dysfunction and subsequent hepatotoxicity.

Although our data indicated that mitochondrial dysfunction is an early event in the development of DCE-induced hepatotoxicity, the precise underlying mechanism remains unclear. It is conceivable that DCE-derived metabolites arylate and/or oxidize essential protein sulfhydryls in the mitochondrial respiratory chain, thereby disrupting cellular energy homeostasis. Indeed, it has been shown previously that DCE metabolites, including the epoxide, conjugated readily in vivo and in vitro with GSH, the most abundant intracellular nonprotein sulfhydryl (Dowsley et al., 1995; Forkert,

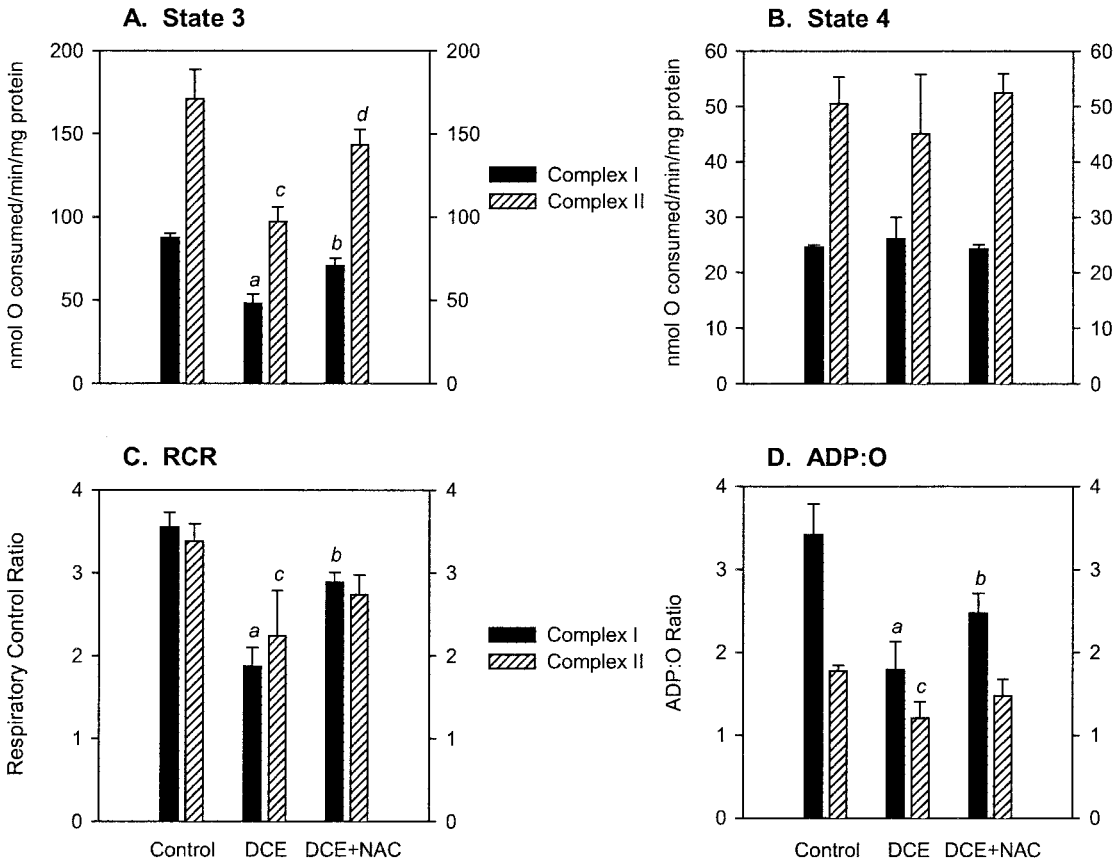

Fig. 8. Effect of 125 mg/kg DCE treatment on glutamate (complex I)- and succinate (complex II)-supported respiration after pretreatment with 1200 mg/kg NAC. Mice were treated with NAC or the vehicle 15 min before DCE treatment and sacrificed 1 h later. The following respiratory parameters were then measured: state 3 respiration (A), state 4 respiration (B), respiratory control ratio (C), and ADP:O ratio (D). Data are expressed as mean ± S.D. of measurements from three separate experiments. a and c, significantly different from the corresponding controls ($p < 0.001$). b and d, significantly different from DCE alone ($p < 0.001$).

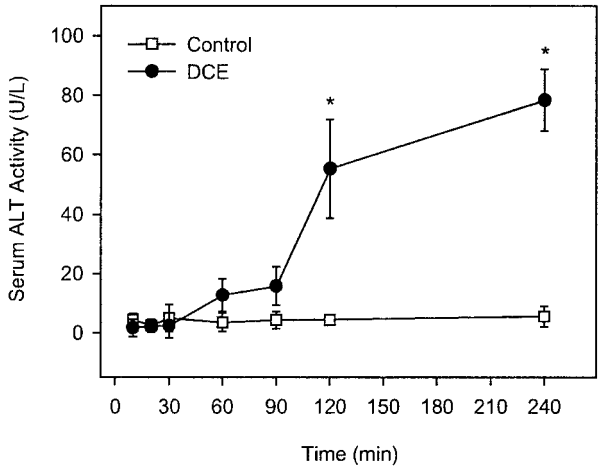

Fig. 9. Time course of serum ALT activity after 125 mg/kg DCE treatment. Control mice were treated with corn oil. Data are expressed as mean ± S.D. of measurements from three separate experiments. ⋆, significantly different from the control ($p < 0.001$).

1999). Alternately, oxidation of protein sulfhydryls may occur but seems a less likely occurrence because alterations to mitochondrial function outlast GSH recovery. Because GSH is the most abundant intracellular antioxidant, after its recovery, mitochondrial perturbations resulting from oxidation might be expected to recuperate. However, this response may not occur if the damage to mitochondria is sufficiently severe so that recovery is obviated. Both NADH dehydrogenase and succinate dehydrogenase contain several cysteine-rich iron-sulfur clusters that function in electron transport (Hatefi, 1985; Murray et al., 2000), and the activity of both enzyme complexes in this study has been shown to be inhibited. Decreased ATP production, subsequent to inhibition of these enzymes, is likely to have deleterious effects on numerous cellular functions, including Ca^{2+} homeostasis and maintenance of plasma membrane integrity (Dills and Klaassen, 1986). Such alterations may lead to cellular necrosis. However, there is currently no evidence to support a DCE-mediated mechanism of ATP depletion, and further investigation is warranted.

In conclusion, our results demonstrated that DCE-induced mitochondrial dysfunction occurs early in the toxic response and is inhibited under experimental conditions that prevent depletion of intracellular GSH. Thus, these data suggested that GSH plays an important role in conjugation and detoxification of DCE metabolites that might otherwise elicit mitochondrial toxicity.

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References

Bergmeyer HU, Scheibe P, and Wahlefeld AW (1978) Optimization of methods for aspartate aminotransferase and alanine aminotransferase. *Clin Chem* **24:**58.

Brand MD, Harper M, and Taylor HC (1993) Control of the effective P/O ratio of oxidative phosphorylation in liver mitochondria and hepatocytes. *Biochem J* **291:** 739–748.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* **72:**248–245.

Cawthon D, McNew R, Beers KW, and Bottje WG (1999) Evidence of mitochondrial dysfunction in broilers with pulmonary hypertension syndrome (ascites): effect of *t*-butyl hydroperoxide on hepatic mitochondrial function, glutathione and related thiols. *Poultry Sci* **78:**114–124.

Chieco P, Moslen MT, and Reynolds ES (1982) Histochemical evidence that plasma and mitochondrial membranes are primary foci of hepatocellular injury caused by 1,1-dichloroethylene. *Lab Invest* **46:**413–421.

Coleman WE, Lingg RD, Melton RG, and Kopfler FC (1976) The occurrence of volatile organics in five drinking water supplies using gas chromatography/mass spectrophotometry, in *Identification and Analysis of Organic Pollutants in Water* (Keith LH ed) pp 305–327, Ann Arbor Science Publishers, Ann Arbor, MI.

Corcoran GB, Todd EL, Racz WJ, Hughes H, Smith CV, and Mitchell JR (1985) Effects of *N*-acetylcysteine on the disposition and metabolism of acetaminophen in mice. *J Pharmacol Exp Ther* **232:**857–863.

Costa AK and Ivanetich KM (1984) Chlorinated ethylenes: their metabolism and effect on DNA repair in rat hepatocytes. *Carcinogenesis* **5:**1629–1636.

Cotgreave IA (1997) *N*-Acetylcysteine: pharmacological considerations and experimental and clinical applications. *Adv Pharmacol* **38:**205–227.

Dills RI and Klaassen CD (1986) The effect of inhibitors of mitochondrial energy production on hepatic glutathione, UDP-glucuronic acid and adenosine 3′-phosphate-5′-phosphosulfate concentrations. *Drug Metab Dispos* **14:**190–196.

Donnelly PJ, Walker RM, and Racz WJ (1993) Inhibition of mitochondrial respiration in vivo is an early event in acetaminophen-induced hepatotoxicity. *Arch Toxicol* **68:**110–118.

Dowsley TF, Forkert PG, Benesch LA, and Bolton JL (1995) Reaction of glutathione with the electrophilic metabolites of 1,1-dichloroethylene. *Chem Biol Interact* **95:**227–244.

Estabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios, in *Methods in Enzymology* (Estabrook RW and Pullman ME eds) pp 41–47, Academic Press, New York.

Fariss MW and Reed DJ (1987) High-performance liquid chromatography of thiols and disulfides: dinitrophenol derivatives. *Methods Enzymol* **143:**101–109.

Forkert (1999) In vivo formation and localization of 1,1-dichloroethylene epoxide in murine liver: identification of its glutathione conjugate 2-*S*-glutathionyl acetate. *J Pharmacol Exp Ther* **290:**1299–1306.

Forkert PG and Moussa M (1991) 1,1-Dichloroethylene elicits dose-dependent alterations in covalent binding and glutathione in murine liver. *Drug Metab Dispos* **19:**580–586.

Forkert PG, Hofley M, and Racz WJ (1987) Metabolic activation of 1,1-dichloroethylene by mouse lung and liver microsomes. *Can J Physiol Pharmacol* **65:**1496–1499.

Forkert PG, Stringer V, and Troughton KM (1986) Pulmonary toxicity of 1,1-dichloroethylene: correlation of early changes with covalent binding. *Can J Physiol Pharmacol* **64:**112–121.

Hatefi Y (1985) The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem* **54:**1015.

Jaeger RJ (1977) Effect of 1,1-dichloroethylene exposure on hepatic mitochondria. *Res Commun Chem Pathol Pharmacol* **18:**83–94.

Jaeger RJ, Conolly RB, and Murphy SD (1974) Effect of 18 hr fast and glutathione depletion on 1,1-dichloroethylene-induced hepatotoxicity and lethality in rats. *Exp Mol Pathol* **20:**187–198.

Johnson D and Lardy H (1967) Isolation of liver and kidney mitochondria. *Methods Enzymol* **10:**94–96.

Kainz A, Cross H, Freeman S, Gescher A, and Chipman JK (1993) Effects of 1,1-dichloroethylene and of some of its metabolites on the functional viability of mouse hepatocytes. *Fundam Appl Toxicol* **21:**140–148.

Kanz MF and Reynolds ES (1986) Early effects of 1,1-dichloroethylene on canalicular and plasma membranes: ultrastructure and stereology. *Exp Mol Pathol* **44:**93–110.

Lash LH, Xu Y, Elfarra AA, Duescher RJ, and Parker JC (1995) Glutathione-dependent metabolism of trichloroethylene in isolated liver and kidney cells of rats and its role in mitochondrial and cellular toxicity. *Drug Metab Dispos* **23:**846–853.

Lee RP and Forkert PG (1994) In vitro biotransformation of 1,1-dichloroethylene by hepatic cytochrome P-450 2E1 in mice. *J Pharmacol Exp Ther* **270:**371–376.

Liebler DC, Latwesen DG, and Reeder TC (1988) *S*-(2-Chloroacetyl)glutathione, a reactive glutathione thiol ester and a putative metabolite of 1,1-dichloroethylene. *Biochemistry* **27:**3652–3657.

Liebler DC, Meredith MJ, and Guengerich FP (1985) Formation of glutathione conjugates by reactive metabolites of vinylidene chloride in microsomes and isolated hepatocytes. *Cancer Res* **45:**186–193.

Murray RK, Granner DK, Mayes PA, and Rodwell VW (2000) The respiratory chain and oxidative phosphorylation, in *Harper's Biochemistry* (Barnes DA, Ransom J and Roche J eds) pp 137–148, Appleton and Lange, Stamford, CT.

Okine LK and Gram TE (1986) Tissue distribution and covalent binding of [^{14}C]1,1-dichloroethylene in mice: in vivo and in vitro studies. *Adv Exp Med Biol* **197:**903–910.

Okine LK, Goochee JM, and Gram TE (1985) Studies on the distribution and covalent binding of 1,1-dichloroethylene in the mouse. Effect of various pretreatments on covalent binding in vivo. *Biochem Pharmacol* **34:**4051–4057.

Reynolds ES and Moslen MT (1977) Damage to hepatic cellular membranes by chlorinated olefins with emphasis on synergism and antagonism. *Environ Health Perspect* **21:**137–147.

Reynolds ES, Moslen MT, Szabo S, Jaeger RJ, and Murphy SD (1975) Hepatotoxicity of vinyl chloride and 1,1-dichloroethylene. Role of mixed function oxidase system. *Am J Pathol* **81:**219–236.

Semprini L (1995) In Situ Bioremediation of Chlorinated Solvents. *Environ Health Perspect* **103:**101–105.

Toyomizu M, Kirihara D, Tanaka M, Hayashi K, and Tomita Y (1992) Dietary protein level alters oxidative phosphorylation in heart and liver mitochondria of chicks. *Br J Nutr* **68:**89–99.

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