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Conjugative metabolism of 1,2-dibromoethane in mitochondria: disruption of oxidative phosphorylation and alkylation of mitochondrial DNA

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Abstract

1,2-Dibromoethane (DBE) is an environmental contaminant that is metabolized by glutathione *S*-transferases to a haloethane–glutathione conjugate. Since haloethane–glutathione conjugates are known to alkylate nuclear DNA and cytoplasmic proteins, these effects were investigated in isolated rat liver mitochondria exposed to DBE by measuring guanine adducts and several aspects of oxidative phosphorylation including respiratory control ratios, respiratory enzyme activity, and ATP levels. Mitochondrial large-amplitude swelling and glutathione status were assessed to evaluate mitochondrial membrane integrity and function. When exposed to DBE, mitochondria became uncoupled rapidly, yet no large-amplitude swelling or extramitochondrial glutathione was observed. Mitochondrial GSH was depleted to 2–53% of controls after a 60-min exposure to micromolar quantities of DBE; however, no extramitochondrial GSH or GSSG was detected. The depletion of mitochondrial glutathione corresponded to an increase of an intramitochondrial GSH-conjugate which, based on HPLC elution profiles and retention times, appeared to be *S*,*S*′-(1,2-ethanediyl)bis(glutathione). Activities of the NADH oxidase and succinate oxidase respiratory enzyme systems were inhibited 10–74% at micromolar levels of DBE, with succinate oxidase inactivation occurring at lower doses. ATP concentrations in DBE-exposed mitochondria in the presence of succinate were 5–90% lower than in the controls. The DNA adduct *S*-[2-(N^7-guanyl)ethyl]glutathione was detected by HPLC in mtDNA isolated from DBE-exposed mitochondria. The results suggest that respiratory enzyme inhibition, glutathione depletion, decreased ATP levels, and DNA alkylation in DBE-exposed mitochondria occur via the formation of an *S*-(2-bromoethyl)glutathione conjugate, the precursor of the episulfonium ion alkylating species of DBE.

Keywords: 1,2-Dibromoethane; Glutathione; Conjugative metabolism; Mitochondrial dysfunction; Mitochondrial DNA; Oxidative phosphorylation; Rat liver

1. Introduction

DBE has been used extensively as an agricultural fumigant, a gasoline additive, and an industrial solvent [1]. It is a mutagen and carcinogen in rodents and an acute toxicant for laboratory animals and humans [2–5]. DBE metabolism occurs either by direct conjugation with glutathione or by oxidation with mixed-function oxidases. Conjugative metabolism of DBE catalyzed by glutathione *S*-transferase yields glutathione conjugates, which results in the depletion of hepatic GSH that is associated with DBE poisoning. Such intoxication by DBE is thought to occur via an unstable *S*-(2-bromoethyl)glutathione conjugate that rearranges nonenzymatically to an electrophilic episulfonium ion, which alkylates nucleophilic sites in nucleic acids and proteins (Fig. 1) [6–9]. Specific purine adducts in nuclear DNA (Fig. 1) and model cytosolic protein adducts that result from alkylation with haloethane–glutathione conjugates have been identified [10–12]. The bioactivation events that lead to alkylation in the nucleus or cytoplasm are also probable in mitochondria given the presence of mitochondrial glutathione *S*-transferases [13], protein, and DNA.

Mitochondrial metabolism could be critical in DBE-induced hepatotoxicity, as Botti *et al.* [14] have shown that the hepatic mitochondrial pool of GSH, which normally has

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Abbreviations: DBE, 1,2-dibromoethane; GAPD, glyceraldehyde phosphate dehydrogenase; mtDNA, mitochondrial DNA; PGK, phosphoglycerate phosphokinase; and RCR, respiratory control ratio.

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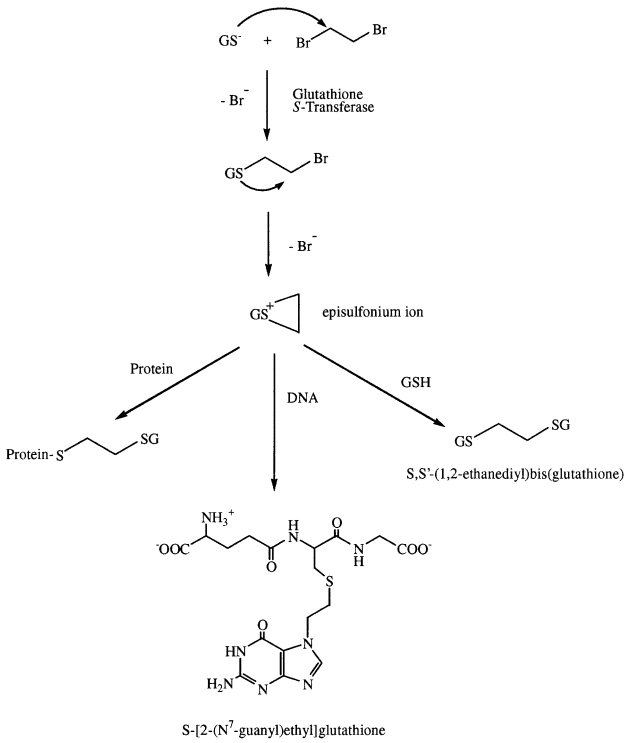

Fig. 1. Reactions, intermediates, and metabolic products from the conjugative metabolism of DBE. Reaction of DBE with the glutathione thiolate anion catalyzed by glutathione *S*-transferase leads to an electrophilic episulfonium ion that alkylates nucleophilic sites in DNA and protein. Reaction of the episulfonium ion with GSH to form *S,S′*-(1,2-ethanediyl) bis(glutathione) represents detoxication.

a longer half-life than cytosolic GSH [15], is depleted, together with cytosolic GSH, in DBE-exposed rats. A subsequent *in vitro* study by Botti *et al.* [16] demonstrated that DBE is converted to DBE–GSH conjugates by rat liver mitochondria, presumably via the activity of mitochondrial glutathione *S*-transferases. Evidence also suggests that hepatic vitamin E depletion by DBE administration *in vivo* is related to GSH conjugation [17]. Since hepatic vitamin E is concentrated in mitochondria, these findings support the hypothesis that DBE conjugation with GSH leads to the formation of the reactive episulfonium ion in mitochondria. The role of mitochondrial damage in DBE hepatotoxicity, however, remains relatively unexplored. Masini and coworkers [18] have demonstrated that DBE causes calcium cycling and the collapse of membrane potential in isolated rat liver mitochondria. Nuclear DNA alkylation by DBE–GSH conjugates has been established [7,10,11]; however, mtDNA alkylation due to DBE exposure has not. If DBE–GSH conjugates are produced within the mitochondrial matrix, then mtDNA and protein will be susceptible to alkylation damage. When protein and DNA alkylation events are at critical sites or are numerous, such as in circumstances of acute poisoning, mitochondrial dysfunction will inevitably occur. In this paper, evidence is presented for the disruption of mitochondrial oxidative phosphorylation and alkylation of mtDNA mediated by conjugative metabolism of DBE in isolated rat liver mitochondria.

2. Materials and methods

2.1. Chemicals

DBE was purchased from the Sigma Chemical Co. DNA restriction enzymes were purchased from Boehringer Mannheim GmbH, the 1-kb DNA ladder was obtained from Gibco BRL Life Technologies, and Wizard midiprep DNA purification systems were from the Promega Corp. The Bradford protein dye (Coomassie brilliant blue G-250) was supplied by Bio-Rad. All other reagents, obtained from the Sigma Chemical Co. or the Aldrich Chemical Co., were of the highest grade possible and were used as received from the supplier.

2.2. Mitochondrial isolation

Liver mitochondria were isolated from male Sprague–Dawley rats (275–325 g) by differential centrifugation as described by Savage *et al.* [19]. Mitochondrial protein was measured by the Bradford method [20], with bovine serum albumin as a standard. All experiments with mitochondria were carried out on the day of isolation. Isolated mitochondria were incubated at 25° in a water bath with the following components at final concentrations: 15 mg mitochondrial protein/mL, 210 mM mannitol, 70 mM sucrose, 5 mM disodium succinate, and 3 mM HEPES, pH 7.0.

2.3. Exposure of isolated mitochondria to DBE

Mitochondria were exposed to DBE in 10- or 25-mL Erlenmeyer flasks covered with parafilm by adding ethanol-diluted DBE directly to the mitochondrial solution noted above (15 mg mitochondrial protein/mL, 210 mM mannitol, 70 mM sucrose, 3 mM HEPES, and 5 mM disodium succinate, pH 7.0) and were incubated without shaking for the indicated time. Control mitochondria received an equal volume of ethanol (0.13 to 1.3%, v/v). Dose–response effects were examined from 0.10 to 10.0 μmol DBE/mg mitochondrial protein, which corresponds to 1.5 to 150 mM DBE.

2.4. RCR

RCRs were measured by monitoring oxygen consumption with a Clark electrode using succinate as substrate in the presence and absence of ADP, as described by Lash and Sall [21]. The 1.8-mL reaction mixture contained isolated mitochondria (1 mg protein/mL), 27 mM succinate, 1 μM rotenone, 277 μM ADP, 250 mM sucrose, 15 mM KCl, 5 mM $MgCl_2$, 30 mM K_2HPO_4, pH 7.0, and 0.3% (w/v) bovine serum albumin (defatted).

2.5. NADH oxidase and succinate oxidase enzyme assays

NADH oxidase and succinate oxidase activities were monitored by measuring oxygen consumption with a Clark electrode, using NADH or succinate as a substrate. After exposure to DBE, mitochondria were sonicated on ice with five bursts of 5 sec to allow respiratory enzyme activity to be determined without ADP control and to allow NADH access to the matrix. The solution was centrifuged, and the pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.0. The 1.6-mL assay mixtures contained mitochondria (1 mg/mL), 50 mM potassium phosphate buffer, pH 7.0, and either NADH (1.0 mM) or succinate (5.0 mM).

2.6. Glutathione determination

Intra- and extramitochondrial GSH, GSSG, and glutathione conjugates were analyzed according to Reed *et al.* [22]. Briefly, after the addition of the internal standard γ-glutamylglutamate, supernatant and perchloric acid samples were derivatized for HPLC analysis by adding iodoacetic acid (20 mg/mL of H_2O with 0.2 M cresol purple as a pH indicator) followed by 1-fluoro-2,4-dinitrobenzene [1% (v/v) in 100% ethanol]. Samples were stored for 24 hr at ambient temperature in the dark prior to HPLC analysis. Concentrations of GSH and glutathione conjugates were calculated based on standard curves of GSH and GSSG. Since the glutathione conjugate *S*,*S*′-(1,2-ethanediyl)bis (glutathione) has two derivatizable groups, its concentration was calculated based on GSSG standard curves.

2.7. ATP assay

ATP levels were determined using PGK and GAPD in a coupled enzyme reaction in which NADH and ATP concentrations are stoichiometrically related (diagnostic kit from Sigma). Mitochondria were incubated with DBE (0.1, 0.5, or 1.0 μmol DBE/mg protein) for 20 min at 25°. After 20 min of conjugative metabolism, the following reagents at final concentrations were mixed to allow for ATP synthesis: DBE/mitochondrial mixture (1 mg mitochondrial protein/mL), 12.5 mM succinate, 25 mM ADP, 250 mM sucrose, 15 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 30 mM K_2HPO_4, 0.3% defatted bovine serum albumin, pH 7.0. ATP synthesis was initiated by the addition of ADP and stopped by protein precipitation with 12% (w/v) trichloroacetic acid (TCA, final concentration 6%, w/v). After centrifugation, supernatants of the TCA-treated mitochondria were analyzed immediately for ATP (μmol/mg protein) with the Sigma diagnostic kit by monitoring NADH oxidation at 340 nm (extinction coefficient 6.22 cm^{-1} mM^{-1}). A solution containing a known concentration of ATP was analyzed together with samples to verify the accuracy of the procedure. ATP loss due to the procedure did not exceed 10%.

2.8. mtDNA isolation

Isolated mitochondria were subjected to alkaline lysis, and mtDNA was alcohol-precipitated in 0.8 M LiCl according to standard protocols for isolating plasmid DNA from bacteria [23,24]. mtDNA was also purified using the Wizard midiprep purification system from the Promega Corp.

2.9. Restriction enzyme digestion of mtDNA

Restriction enzyme digests were carried out at 37° for 1.5 hr, the products were analyzed by agarose gel (0.8%, w/v) electrophoresis, and DNA was detected using ethidium bromide according to standard protocols [24].

2.10. mtDNA alkylation

DNA, isolated from DBE-exposed (30 min, 1.0 μmol DBE/mg protein) and control mitochondria, was subjected to neutral thermal hydrolysis (30 min, 95°, pH 7.0) to depurinate the DNA and release DNA adducts. Purines were separated by HPLC as described by Humphreys *et al.* [25] except that ammonium acetate replaced ammonium phosphate in the mobile phase. Briefly, purines were eluted on a 5 μm C_{18} ODS column (Custom LC with Alltech C_{18} guard column) for 5 min with 5% methanol in 10 mM ammonium acetate (pH 4.75) followed by a linear gradient to 30% methanol over 30 min at 1.0 mL/min. Purines were detected at 283 nm and identified by co-elution with standards. The *S*-[2-(N^7-guanyl)ethyl]glutathione standard was synthesized, purified by HPLC, and identified by mass spectrometry as described by Foureman and Reed [26].

2.11. Statistical analysis

Results are expressed as means ± SD. Using SPSS version 9.0 (SPSS, Inc.), means were compared by an independent samples *t*-test, a one-way ANOVA, or a multivariate analysis of variance (MANOVA). Post-hoc analyses were calculated using Tukey's *t*-tests. In all tests, P values <0.05 were considered significant.

3. Results

3.1. Mitochondrial RCR and membrane integrity after DBE exposure

For actively respiring rat liver mitochondria in which succinate oxidation is coupled to ADP phosphorylation, RCR values typically range between 5 and 8 [21]. In all experiments, RCR values of freshly isolated mitochondria respiring with succinate were in this range, indicating that mitochondria were coupled and capable of synthesizing ATP. Freshly isolated mitochondria with RCR values of less than 4 were not used. After exposure to DBE, mito-

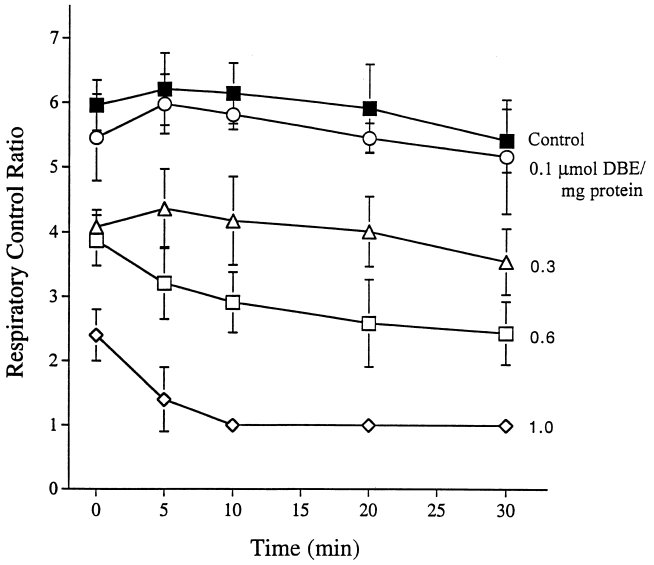

Fig. 2. RCRs in DBE-exposed mitochondria. RCRs were used to assess the extent to which succinate oxidation was coupled to ATP synthesis. Mitochondria were exposed to DBE (0.1, 0.3, 0.6, or 1.0 μmol/mg mitochondrial protein) at 25° for the indicated time. For the zero time point, mitochondria were exposed to DBE on ice (4°), the reaction components were mixed in a temperature-equilibrated water bath (25°), the reaction was started with ADP, and respiration was measured. Values are means ± SD of three to four independent determinations. Time and dose trends for RCR values were statistically significant (Repeated measures ANOVA, $P < 0.05$). The means for all DBE doses (except for 0.1 μmol DBE) were significantly different from those of time-matched controls (one-way ANOVA with post-hoc Tukey's test, $P < 0.05$).

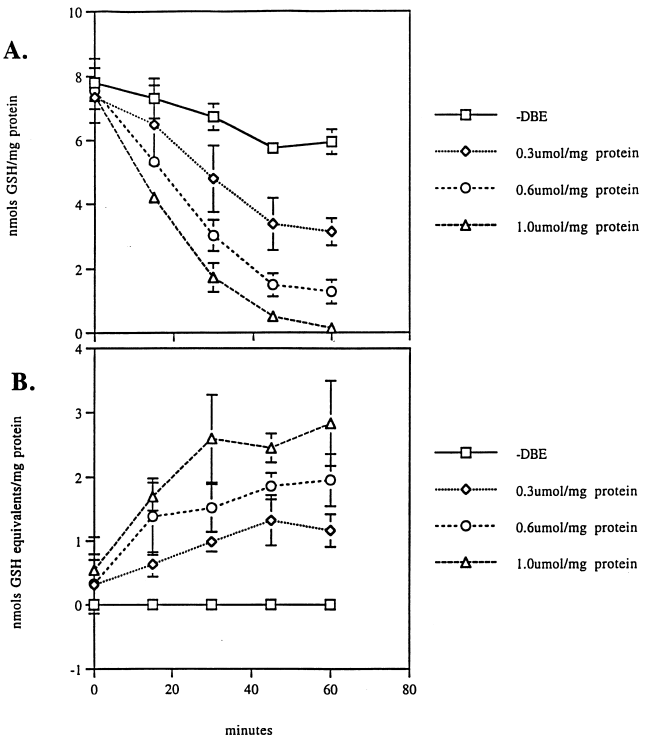

Fig. 3. Depletion of intramitochondrial GSH and formation of a GSH-conjugate in DBE-exposed mitochondria. Mitochondria were incubated with DBE at 25° for the indicated time, and intra- and extramitochondrial glutathione levels were determined. (A) Intramitochondrial GSH. (B) Intramitochondrial GSH-conjugate, the putative *S, S′*-(1,2-ethanediyl)bis-(glutathione), expressed as GSH equivalents. Values (nmol GSH/mg protein) are means ± SD of five independent determinations. Time and dose trends for GSH depletion and GSH-conjugate increases were statistically significant (MANOVA with Wilks' Lambda criteria, $P < 0.001$). At time zero, the DBE and control means were not statistically different. At all non-zero time points, DBE means were significantly different from time-matched controls except for the 0.3 μmol DBE dose at 15 min (one-way ANOVA with post-hoc Tukey's test, $P < 0.05$).

chondria rapidly became uncoupled in a dose- and time-dependent manner (Fig. 2). The lowest dose, 0.1 μmol DBE/mg protein, had little effect on mitochondria over the 30-min time course, as RCR values were not significantly different from controls. The largest dose, 1.0 μmol DBE/mg protein, immediately decreased RCR values to 44% of time-matched controls, and by 10 min the mitochondria were completely uncoupled. RCR values are a ratio of state 3 to state 4 respiration (respiration in the presence or absence of ADP, respectively). Decreased RCR values due to DBE exposure resulted from an increase in state 4 respiration (data not shown). Although mitochondria became uncoupled after exposure to DBE, membranes remained intact, as spectroscopic studies (540 nm) showed no large-amplitude swelling at the highest DBE dose (1.0 μmol DBE/mg protein) over a 30-min period (data not shown).

3.2. Depletion of mitochondrial glutathione

Depletion of intracellular GSH without a concomitant increase of GSSG is indicative of conjugative metabolism. Exposure of freshly isolated mitochondria to DBE caused a time- and dose-dependent loss of GSH (Fig. 3A). At the highest dose (1.0 μmol DBE/mg protein), GSH was depleted completely within 60 min (2% of time-matched controls). Although lower DBE doses did not exhaust mitochondrial GSH, statistically significant depletion was observed. After 60 min of exposure to 0.3 μmol DBE/mg protein, mitochondrial GSH values were 53% of controls and for 0.6 μmol DBE/mg protein, 22% of controls. No concomitant increase in either intra- or extramitochondrial GSSG was observed. However, HPLC chromatograms of derivatized glutathione metabolites exhibited a stable GSH-conjugate peak with shorter retention times than GSSG. Due to its distinct yet similar retention time to that of the GSSG standard, the conjugate was assumed to be *S,S′*-(1,2-ethanediyl)bis(glutathione). Calculations to quantify the GSH-conjugate were based on nanomoles of GSH equivalents and are shown in Fig. 3B. No GSH-conjugate appeared in control mitochondria. Although no GSSG was detected, an increase in the GSH-conjugate was observed (Fig. 3B). GSH-conjugate formation did not account for total GSH loss from the mitochondria, however. At 1.0 μmol DBE/mg protein, the amount of GSH-conjugate formed accounted for only 50–64% of lost GSH, at 0.6 μmol DBE/mg protein 55–73% of GSH was accounted for, and at 0.3 μmol

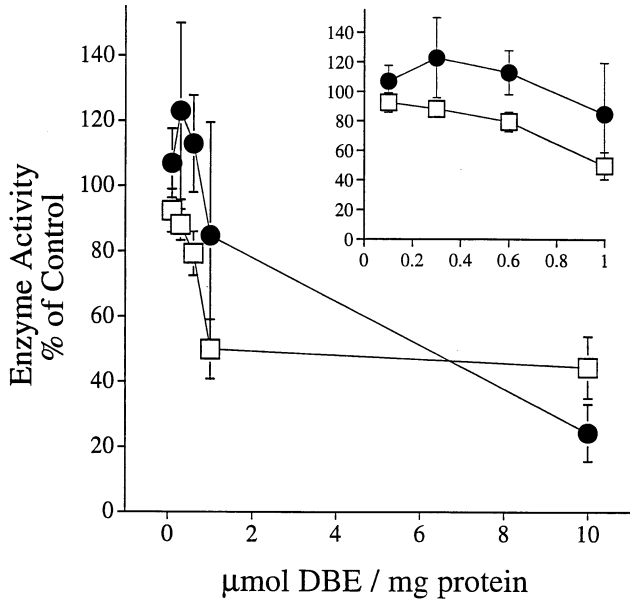

Fig. 4. NADH oxidase and succinate oxidase enzyme activities in DBE-exposed mitochondria. Isolated mitochondria were exposed to DBE at 25° for 15 min before assaying for enzyme activity. Closed circles, NADH oxidase; open squares, succinate oxidase. The insert shows an expanded x-axis view of the data between 0.1 and 1.0 μmol DBE/mg protein. Values are means ± SD of four independent determinations. Control sample specific activities were (N = 20): NADH oxidase, 0.15 ± 0.02 μg atoms $O \cdot min^{-1} \cdot mg\ protein^{-1}$; and succinate oxidase, 0.29 ± 0.07 μg atoms $O \cdot min^{-1} \cdot mg\ protein^{-1}$. At each dose except for 1.0 μmol DBE/mg protein, NADH oxidase and succinate oxidase activity values were significantly different (independent samples *t*-test; $P < 0.05$).

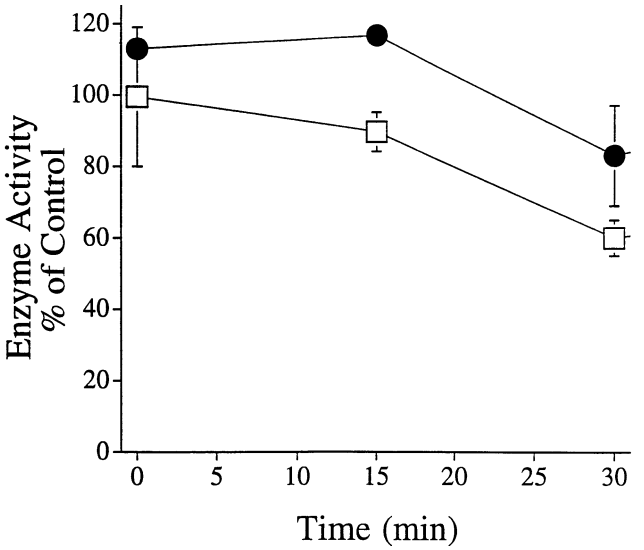

Fig. 5. Time course of NADH oxidase and succinate oxidase enzyme inhibition in DBE-exposed mitochondria. Isolated mitochondria were exposed to 0.6 μmol DBE/mg protein at 25° for the indicated time before assaying for enzyme activity. Closed circles, NADH oxidase; open squares, succinate oxidase. Values are means ± the range of two independent determinations. Control sample specific activities were (N = 6): NADH oxidase, 0.19 ± 0.02 μg atoms $O \cdot min^{-1} \cdot mg\ protein^{-1}$; and succinate oxidase, 0.35 ± 0.03 μg atoms $O \cdot min^{-1} \cdot mg\ protein^{-1}$.

DBE/mg protein 72–97% of GSH was recovered in the conjugate.

No extramitochondrial GSH, GSSG, or GSH-conjugates were observed, suggesting that DBE-exposed mitochondria did not undergo a permeability transition. These data are consistent with the observed absence of large-amplitude swelling in DBE-exposed mitochondria noted above.

3.3. Respiratory enzyme activity

Since haloethane–GSH conjugates are known to alkylate cytosolic proteins [12,27], the effect of mitochondrial DBE metabolism on respiratory enzyme activity was investigated. After a 15-min exposure to DBE, mitochondria were sonicated to eliminate ADP control of electron transport and to stop conjugative metabolism [16] before enzyme assay reagents were added. Mitochondrial metabolism of DBE caused a dose-dependent inhibition of the two respiratory enzyme systems, NADH oxidase and succinate oxidase (Fig. 4). At the lower DBE doses (Fig. 4, insert), succinate oxidase was more susceptible to inhibition, with 52% of control activity remaining after the 15-min incubation with 1.0 μmol DBE/mg protein. At 10.0 μmol DBE/mg protein, NADH oxidase experienced the greatest inhibition, with 24% of control enzyme activity remaining.

If protein inactivation was due to alkylation by a GSH-haloethane intermediate, then allowing more time for metabolite formation should accentuate protein dysfunction. Consistent with this idea, longer incubation times (30 min) of mitochondria with a constant dose of DBE (0.6 μmol DBE/mg protein) did enhance enzyme inhibition (Fig. 5).

3.4. ATP levels

Since DBE uncoupled mitochondria and inhibited respiratory enzyme function, a reasonable consequence would be lowered ATP levels. To test this hypothesis, ATP levels were measured in control and DBE-exposed (0.1, 0.5, or 1.0 μmol DBE/mg protein) mitochondria using the PGK/GAPD coupled enzyme diagnostic kit from Sigma. Mitochondria were exposed to DBE at 25° for 20 min to allow for conjugative metabolism. Then reagents required for ATP synthesis were added, and the reaction mixture was incubated at 25° for 1–10 min. During ATP synthesis, conjugative metabolism also continued so that 30 min of conjugative metabolism had occurred at the 10-min endpoint shown in Table 1. At the highest dose tested, 1.0 μmol DBE/mg protein, ATP levels were 50–90% less than those in the controls (Table 1). In addition, at that dose ATP levels decreased with increased incubation times, with the lowest level observed at 10 min. By comparing RCR (Fig. 2), GSH depletion (Fig. 3), and ATP level (Table 1) data, it is evident that 30 min of conjugative metabolism with 0.3 to 0.6 μmol DBE/mg protein depleted 29–55% of mitochondrial GSH without completely uncoupling mitochondria or depleting ATP. However, 30 min of conjugative metabolism with 1.0

Table 1
ATP levels in respiring mitochondria exposed to DBE

DBE (μmol/mg protein)	ATP (% of control)		
	1 min	5 min	10 min
0.1	101 ± 16.6*	78.5 ± 29.7*	94.8 ± 25.2*
0.5	82.5 ± 18.6	55.8 ± 28.1	72.1 ± 20.3**
1.0	48.9 ± 10.1*	21.2 ± 10.0*	9.44 ± 9.1*,**

Mitochondria were exposed to DBE at the doses indicated for 20 min and were subsequently incubated at 25° with reagents required for mitochondrial ATP synthesis. All enzymatic reactions were stopped by protein precipitation at 1, 5, and 10 min, and ATP levels were determined. Control ATP concentrations were (μmol/mg protein): 1 min, 1.2 ± 0.62; 5 min, 1.7 ± 0.18; 10 min, 1.4 ± 0.23 (means ± SD of three independent determinations). Percent of control values are means ± SD of three independent determinations.

*,** Significantly different time-matched means (one-way ANOVA with a post-hoc Tukey's test, $P < 0.05$). Time was a significant factor (repeated measures ANOVA, $P < 0.05$).

μmol DBE/mg protein was sufficient to deplete 75% of mitochondrial GSH, uncouple mitochondria, and drop ATP levels to less than 10% of control.

3.5. Isolation of mtDNA and determination of mtDNA guanine adducts

DNA adducts of DBE have been identified in nuclear DNA [10,11]. The only significant DNA adduct identified was *S*-[2-(N^7-guanyl)ethyl]glutathione with an estimated alklyation both *in vivo* and *in vitro* of 1 nmol/mg of DNA [10]. Since mitochondria are capable of haloethane conjugative metabolism, DNA-alkylating intermediates could form and accumulate as mtDNA adducts. To test for mtDNA adducts, DNA was isolated from mitochondria and subjected to neutral thermal hydrolysis (95°, 30 min, pH 7.0) to depurinate the DNA. The mtDNA adduct *S*-[2-(N^7-guanyl)ethyl]glutathione was identified in depurinated DNA samples by HPLC analysis. A representative HPLC chromatogram (Fig. 6B) of depurinated mtDNA from DBE-exposed mitochondria showed three compounds identified at 283 nm as guanine, *S*-[2-(N^7-guanyl)ethyl]glutathione, and guanosine by co-elution with standards. No DNA adduct peak was observed in chromatograms from control mitochondria (Fig. 6A). To verify that the mtDNA isolated from rat liver mitochondria was not contaminated with nuclear DNA, mtDNA restriction fragments were analyzed by electrophoresis. In Fig. 7, lane 1 shows undigested rat liver mtDNA of approximately 15,000 base pairs (15 kb), which is consistent in size with other rat mitochondrial DNAs [28]. Mitochondrial DNA restriction fragment lengths from *Bam*HI, *Eco*RI, *Hin*dIII and *Xba*I digests shown in Fig. 7 were consistent with fragment sizes of Sprague–Dawley rat liver mtDNAs digested with other restriction enzymes [28] and ranged between 2 and 10 kb.

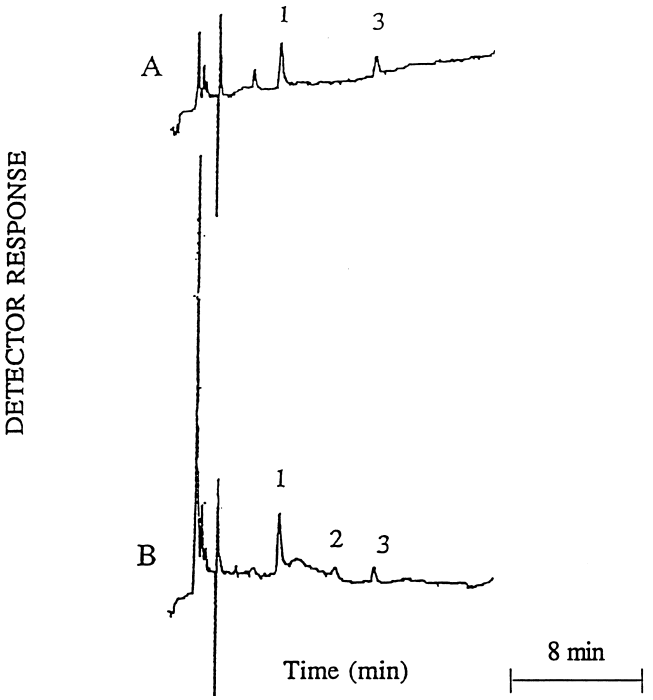

Fig. 6. HPLC chromatograms of depurinated mtDNA isolated from livers of Sprague–Dawley rats. (A) mtDNA from control mitochondria. (B) mtDNA from DBE-treated mitochondria (8.5 μmol DBE/mg protein). DNA was isolated as described in "Materials and methods" and subjected to neutral thermal hydrolysis (95°, 30 min) to depurinate the DNA. The HPLC peaks are numbered and identified by co-elution with standards: 1, guanine; 2, alkylated guanine derivative, *S*-[2-(N^7-guanyl)ethyl]glutathione; and 3, guanosine. The *S*-[2-(N^7- guanyl)ethyl]glutathione standard was synthesized and identified by mass spectrometry as described by Foureman and Reed [26].

4. Discussion

The current study demonstrates that DBE depletes GSH in rat liver mitochondria with concomitant formation of GSH–DBE conjugates, which alkylate mtDNA and disrupt oxidative phosphorylation. Decreased RCR values, ATP levels, and respiratory enzyme activities were indicative of dysfunctional oxidative phosphorylation. DBE rapidly and irreversibly uncoupled succinate oxidation from ADP phosphorylation without disrupting mitochondrial membrane integrity. The lack of large-amplitude swelling and extramitochondrial GSH or GSH-conjugates argues against a permeability transition of the inner mitochondrial membrane. The initial loss of respiratory control in DBE-exposed mitochondria occurred quickly (within 3–5 min); it may not be entirely explained by conjugative metabolism but could be partially due to solvent effects. Direct solvent effects of halogenated methane molecules on isolated hepatocytes are known to inhibit cellular respiration [29]. The inhibition of respiration in hepatocytes reported by Berger and Sozeri [29] reversed within 10 min when the incubation chamber was opened, allowing evaporation. In contrast,

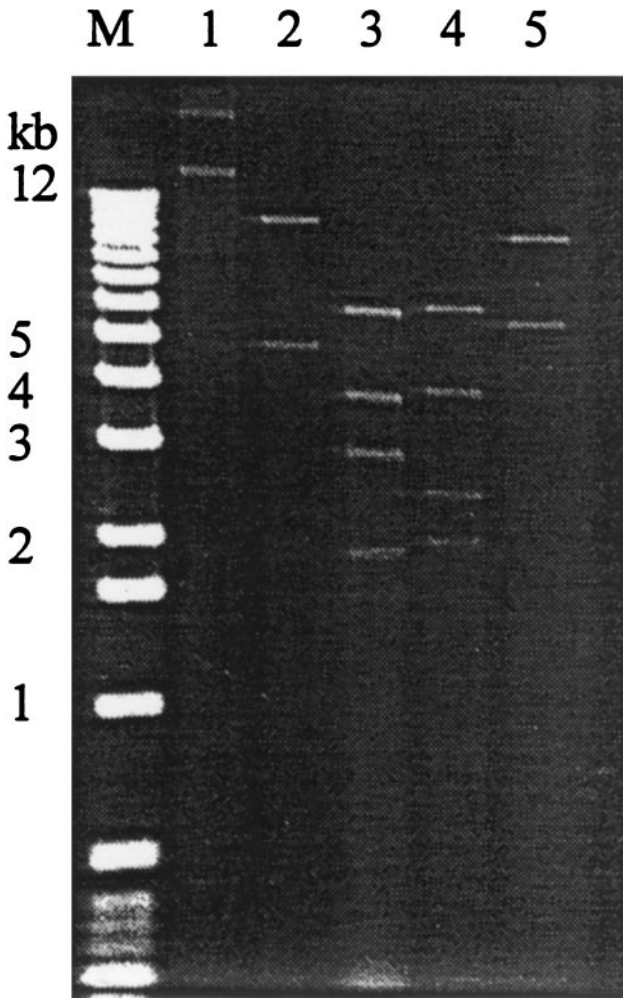

Fig. 7. Restriction enzyme digestion patterns of mtDNA isolated from Sprague–Dawley rat liver mitochondria. Electrophoresis was carried out on 0.8% agarose gels, and DNA was stained with ethidium bromide. Lanes: M (markers), 1-kb DNA ladder (Gibco BRL); 1, undigested mtDNA; 2–5 digested mtDNA (2, *Bam*HI; 3, *Eco*RI; 4, *Hin*dIII; 5, *Xba*I).

RCR values of DBE-exposed mitochondria did not return to control values during the 30-min incubation period. Instead, RCR values continued to decline and progressed toward a value of 1 at which the mitochondria were completely uncoupled. Solvent effects may partially explain the initial drop in RCR values but not the decreased RCR values over time. Masini *et al.* [18] have demonstrated that DBE causes calcium cycling and membrane depolarization in isolated rat liver mitochondria incubated with 100 μM exogenous Ca^{2+}. Cycling of endogenous calcium also lowers mitochondrial membrane potential [16]. A decrease in the mitochondrial membrane potential caused by DBE-induced cycling of endogenous calcium could partially account for the decreased RCR values observed. In mitochondria using membrane potential energy for calcium uptake, a decline in ADP phosphorylation rates would be expected and would explain lower RCR values.

The DBE-induced GSH depletion observed in the current study is consistent with previously reported data on the conjugative metabolism of DBE in mitochondria [16] and suggests the activity of mitochondrial glutathione *S*-transferases, which have been described [13]. Botti and co-workers [16] demonstrated that approximately 50% of intramitochondrial GSH is depleted after a 10-min incubation with 1.2 μmol DBE/mg mitochondrial protein. In the current work, the time–course study was extended, showing that intramitochondrial GSH was exhausted after 60 min of conjugative metabolism at the highest dose of DBE tested (1.0 μmol/mg protein). Metabolism of DBE depleted intramitochondrial GSH without the formation of GSSG. Instead of GSSG, a stable intramitochondrial conjugate was formed, also noted by Botti and co-workers [16], which based on HPLC retention times appears to be the bis(glutathione)-conjugate, *S,S′*-(1,2-ethanediyl)bis(glutathione). *S,S′*-(1,2-Ethanediyl)bis(glutathione) has been identified in rat liver samples after oral administration of DBE [30] and in rat hepatocytes treated with DBE [31]. Although no concomitant increase in GSSG was observed with GSH depletion, an increase was observed in the bis(glutathione)-conjugate. The increase in the GSH-conjugate did not account for all of the intramitochondrial GSH lost, however. Since DBE metabolites bind macromolecules in rats and rat hepatocytes [6,31], it is reasonable to predict that the remaining GSH in the form of the episulfonium ion (Fig. 1) alkylated mitochondrial protein and DNA.

Protein alkylation can disrupt structure and eventually lead to inactivation of protein function. Therefore, mitochondrial respiratory chain enzyme activities were measured to indirectly assess the level of protein alkylation and monitor its effect on mitochondrial function. The two respiratory enzyme systems studied, NADH oxidase and succinate oxidase, each contain three integral membrane protein complexes of the mitochondrial electron transport chain that are exposed to the matrix. NADH oxidase is comprised of complexes I, III, and IV [NADH:ubiquinone oxidoreductase (EC 1.6.5.3), ubiquinol:ferricytochrome-*c* oxidoreductase (EC 1.10.2.2), and ferrocytochrome-*c*:oxygen oxidoreductase (EC 1.9.3.1), respectively]. Succinate oxidase is comprised of complexes II, III, and IV [succinate:ubiquinone oxidoreductase (EC 1.3.5.1), ubiquinol:ferricytochrome-*c* oxidoreductase, and ferrocytochrome-*c*:oxygen oxidoreductase, respectively]. Episulfonium ions generated in the mitochondrial matrix would have access to sites on these protein complexes that are exposed to the matrix. Both NADH oxidase and succinate oxidase were inhibited with dose- and time-dependency in DBE-exposed mitochondria. Succinate oxidase was more sensitive to inactivation than NADH oxidase. The oxidases are separate branches of the respiratory chain that share electron transport reactions from ubiquinol through cytochrome-*c* oxidase (ferrocytochrome-*c*:oxygen oxidoreductase) and differ only at their respective dehydrogenases, NADH dehydrogenase (NADH:ubiquinone oxidoreductase) and succinate dehydrogenase (succinate:ubiquinone oxidoreductase). Differences in the inhibition patterns of the two respiratory enzyme systems must

reside in the dehydrogenases that are unique to each enzyme system. Studies of haloethane–glutathione conjugates and model proteins have shown that protein thiols are particularly susceptible to alkylation [12,27]. Since succinate dehydrogenase contains a thiol group critical for enzyme activity [32], alkylation at this site would explain the inactivation observed at the lower DBE doses.

Evidence for the formation of the mtDNA alkylation product *S*-[2-(N^7-guanyl)ethyl]glutathione *in situ* in isolated rat liver mitochondria exposed to DBE is presented here for the first time. *S*-[2-(N^7-guanyl)ethyl]glutathione is the nuclear DNA adduct known to occur in greatest yield in DBE studies [10]. The presence of this mtDNA alkylation product is not surprising, since mitochondria possess the enzymes, cofactors, and substrates required for DBE bioactivation and subsequent DNA alkylation.

Our findings suggest that glutathione depletion, inhibition of respiratory enzymes, lowered ATP levels, and DNA alkylation in mitochondria exposed to DBE occurred via an *S*-(2-bromoethyl)glutathione conjugate, the precursor of the episulfonium ion alkylating species of DBE (Fig. 1). It is noteworthy that functions of mitochondrial oxidative phosphorylation were resistant to DBE-induced toxicity at lower doses. The high GSH concentrations in the mitochondrial pool [15] could favor detoxication of the episulfonium ion at low doses of chemical insult. By reacting with GSH instead of cellular macromolecules, the episulfonium ion forms the stable GSH-conjugate and the putative detoxication product, *S*,*S*′-(1,2-ethanediyl)bis(glutathione) (Fig. 1). Only after 30 min of conjugative metabolism at a high DBE dose (1.0 μmol DBE/mg protein; 15 mM DBE) were mitochondrial glutathione and ATP levels exhausted in the current study. Inactivation of NADH oxidase to less than 50% of control values required an excessive concentration of DBE (10.0 μmol DBE/mg protein or 150 mM). Clearly, chemically induced damage depleted a significant portion of the mitochondrial pool of GSH before complete dysfunction of oxidative phosphorylation was observed. In mitochondria, bioactivation of DBE occurred simultaneously with detoxication, and both processes required glutathione. Our findings also suggest that when acute poisoning involves mitochondrial conjugative metabolism, glutathione can be depleted rapidly, with reactive intermediates escaping detoxication to alkylate DNA and protein. Proteins with accessible thiol groups are particularly sensitive to alkylation and inactivation. If DBE is considered a model penetrant thiol reagent [18], then in cases of acute poisoning with such reagents onset of mitochondrial dysfunction should be expected to closely parallel mitochondrial glutathione depletion.

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