Toxicology Letters, 59 (1991) 203–211

TOXLET 02660

Uncoupling of oxidative phosphorylation in rat liver mitochondria by chloroethanols

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(Received 24 June 1991)
(Accepted 4 September 1991)

Key words: Chloroethanols; Uncoupling of oxidative phosphorylation; Liver mitochondria

SUMMARY

Chloroethanols are toxic chemicals used in industry and also formed as a result of the metabolism of several widely used halogenated hydrocarbons. The effect of 2-chloroethanol (CE), 2,2-dichloroethanol (DCE) and 2,2,2-trichloroethanol (TCE) on rat liver mitochondrial respiration was studied. Rat liver mitochondria were isolated in a medium consisting of 250 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Mitochondrial respiration was determined with an oxygen electrode at 30°C and the polarographic buffer consisted of 250 mM mannitol, 10 mM KCl, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 0.2 mM EDTA and 10 mM Tris–HCl (pH 7.4). With succinate as the respiratory substrate and using chloroethanols (150 mM), CE stimulated respiration by 28.2 ± 6.5% and DCE by 202.7 ± 8.2% while TCE inhibited mitochondrial respiration (>95%). The effect of change in the concentration of chloroethanols on mitochondrial respiration was also studied. CE showed maximum stimulation at 600 mM (97.6%), DCE at 150 mM (202.6%) and TCE at 30 mM (313.6%). Respiratory stimulation was independent of mitochondrial protein concentration. Chloroethanols (optimal concentrations for respiratory stimulation with succinate) inhibited mitochondrial respiration when glutamate–malate was used as the respiratory substrate. Estimation of adenosine triphosphate (ATP) showed that chloroethanols inhibited the synthesis of ATP. These results indicate that chloroethanols stimulate mitochondrial respiration by uncoupling oxidative phosphorylation and that the uncoupling potency is proportional to the extent of chlorination at the β-position of haloethanol.

INTRODUCTION

Chloroethanols are widely used toxic industrial chemicals and are common conta-

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minants in food products, medical and surgical supplies because ethylene oxide, one of the precursors of chloroethanol, is frequently used for the fumigation of food products and sterilization of surgical supplies [1–4]. Chloroethanols are also formed from the metabolism of chlorinated ethenes and ethanes [5–9]. 2-Chloroethanol (CE) is a putative intermediate in the metabolism of vinyl chloride and of 1,2-dichloroethene into acetic acid [6,10]. 2,2-Dichloroethanol (DCE) is formed by the metabolism of 1,2- and 1,1-dichloroethene [11,12]. 2,2,2-Trichloroethanol (TCE) is an intermediate of trichloroethene and tetrachlorethene metabolism [13,14]. The metabolism of several chloroethyl nitrosoureas (a family of anticancer drugs) also results in the formation of chloroethanols [15–17].

CE causes significant reduction in the activities of several drug-metabolizing enzymes and also of glucose-6-phosphatase [18]. CE is mutagenic for *Salmonella typhimurium* inducing mutations of the base-substitution type [19]. This chemical also inhibits the growth of DNA-polymerase-deficient bacteria, which indicates its ability to interact with DNA of living cells [19]. CE and DCE cause an increase in the frequency of haploid sectors and diploid non-disjunctional sectors [20] while TCE has been shown to induce somatic segregation [21].

CE, when given orally to rats, results in rapid depletion of liver glutathione with a concomitant formation of *S*-carboxymethyl glutathione [22]. Acute oral administration of CE results in a significant decrease in the mitochondrial elongation of fatty acids [23]. Chloroethanols have been shown to be metabolized to ethylene oxide and also to form esters of fatty acids [24–27]. Chloroethyl esters of several fatty acids have been isolated and identified from livers of rats treated with CE [28,29]. High levels of chloroethyl esters of fatty acids have also been found in foods treated with ethylene oxide [30]. Recently, the enzymatic formation of chloroethyl esters of different fatty acids has been demonstrated [31].

An early study suggested that CE acts as an uncoupler of oxidative phosphorylation [32]. Therefore, the purpose of the present study was to compare the effects of CE, DCE and TCE on the stimulation of mitochondrial respiration and also to derive a structure–activity relationship.

MATERIALS AND METHODS

Chemicals

2-Chloroethanol, 2,2-dichloroethanol, 2,2,2-trichloroethanol (purity ~99%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). ADP, ATP, antimycin A, oligomycin and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). The solvents used were of the high-performance liquid chromatography (HPLC) grade. All other reagents were of the highest quality commercially available.

Preparation of mitochondria

Rat liver mitochondria were prepared from 2-month-old male Sprague-Dawley rats (Harlan, Indianapolis, IN) using a slight modification of the method described by

Asimakis and Aprille [33]. Briefly, 5 g of liver tissue were placed in ice-cold 250 mM sucrose, 1 mM EDTA and 10 mM Tris–HCl buffer (pH 7.4). The tissue was minced, suspended in an amount of fresh sucrose–EDTA–Tris equal to 5 ml/g wet wt. and homogenized using 5–6 up/down strokes of a motor-driven, tight-fitting Teflon pestle. The homogenate was centrifuged at 600 × *g* for 5 min and then the 600 × *g* supernatant was centrifuged at 8000 × *g* for 10 min. The 8000 × *g* pellet was washed once with sucrose–EDTA–Tris buffer and suspended in sucrose–Tris to about 10-12 mg protein/ml. Protein was estimated by the Lowry method using crystalline bovine serum albumin, fraction V as a standard [34].

Polarographic measurement of respiration

The rates of oxygen utilization were measured polarographically using a Clark oxygen electrode in a 1.8 ml water-jacketed chamber maintained at 30°C [33]. The respiratory medium consisted of 250 mM mannitol, 10 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.2 mM EDTA and 10 mM Tris–HCl (pH 7.4); 50 μl of respiratory substrate (300 mM succinate or 150 mM glutamate + 150 mM L-malate) was added to the respiratory medium followed by mitochondrial suspension. Test chemicals CE, DCE and TCE were added to determine the effect on respiratory rates; 5 μl of rotenone (1 μg/ml) was also added when succinate was used as the respiratory substrate.

Measurement of adenosine triphosphate (ATP)

ATP formation was estimated by high-performance liquid chromatography (HPLC). HPLC was performed on a Beckman Model 160 liquid chromatograph connected to an Ultrasphere ODS column. 30 mM KH_2PO_4, 15 mM tetrabutyl hydrogen ammonium sulfate and 19% acetonitrile (all at pH 6.75) at a flow rate of 1 ml/min was used as the mobile phase [35]. The eluant was monitored at 254 nm. The sample was prepared by adding ice-cold perchloric acid (1 N) to an aliquot of the mitochondrial reaction mixture. After thorough mixing, the sample was centrifuged at 8000 × *g* for 10 min. The pH of the 8000 × *g* supernatant was adjusted between 7.1 and 7.3 with ice-cold KOH and after letting it stand in ice for 15 min, again centrifuged at 8000 × *g* for 10 min. The supernatant was filtered through a 0.2-μm filter and subjected to HPLC. Quantification was carried out by comparing the elution profiles of standards versus samples.

RESULTS

Figure 1 shows the effect of chloroethanols on mitochondrial respiration using succinate as the respiratory substrate. When CE, DCE and TCE were tested at 150 mM concentration, CE stimulated mitochondrial respiration by 28.2 ± 6.5% and DCE by 202.7 ± 8.2% whereas TCE at this concentration inhibited oxygen consumption (>95%). Ethanol at this concentration did not stimulate mitochondrial respiration.

The effect of change in the concentrations of chloroethanols on mitochondrial

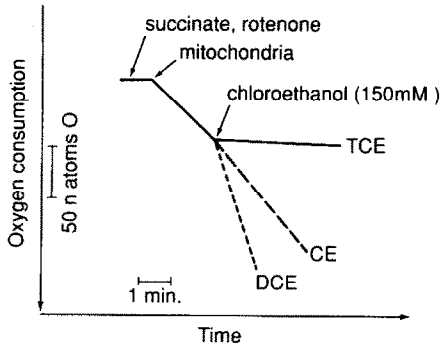

Fig. 1. Effect of 2-chloroethanol (CE), 2,2-dichloroethanol (DCE) or 2,2,2-trichloroethanol (TCE) on mitochondrial respiration. Chloroethanols were used at 150 mM concentration and succinate at 300 mM. Conditions and buffer used for polarographic measurements are given in the text.

TABLE I

EFFECT OF CHANGE IN THE CONCENTRATION OF CHLOROETHANOLS ON MITOCHONDRIAL RESPIRATION

Concentration (mM)	Ratio (b/a)	% Stimulation
2-Chloroethanol		
150	1.282	28.2
300	1.658	65.8
450	1.926	92.6
600	1.976	97.6
1500	0.199	−79.1
2,2-Dichloroethanol		
37.5	1.430	43.0
75	2.831	183.1
150	3.026	202.6
225	1.427	42.7
300	0.348	−65.2
600	0.168	−83.2
2,2,2-Trichloroethanol		
15	2.356	135.6
30	4.134	313.6
60	2.419	141.9
90	0.414	−58.6
120	0.133	−86.7

b = rate of respiration after the addition of the chemical; a = rate of respiration before the addition of the chemical

TABLE II

EFFECT OF CHANGE IN PROTEIN (MITOCHONDRIAL) CONCENTRATION ON STIMULATION OF RESPIRATION

Stimulant	% Stimulation				
	Protein (mg)				
	1	2	3	4	5
CE	77	74	53	33	46
DCE	209	139	164	129	131
TCE	241	270	261	226	214

The concentrations of chloroethanols which showed maximum stimulation were used. CE = 2-chloroethanol; DCE = 2,2-dichloroethanol; TCE = 2,2,2-trichloroethanol.

respiration was studied (Table I). The concentrations used were: CE, 150–1500 mM; DCE, 37.5–600 mM and TCE 15–120 mM. CE showed maximum stimulation at 600 mM (97.6%), DCE at 150 mM (202.6%) and TCE at 30 mM (313.6%). As is apparent from Table I, TCE shows an inhibitory effect on mitochondrial respiration at 90 mM or higher concentration, whereas at 30 mM it shows maximum stimulation among the chloroethanols studied. Mitochondria seem to tolerate higher concentrations of DCE or CE. Table II shows the effect of change in the concentration of mitochondrial protein on the stimulation of respiration using the concentrations which showed maximum stimulation. Respiratory stimulation appeared to be independent of mitochondrial protein concentration. When antimycin was added before the addition of chloroethanols (using the concentrations of chloroethanols which had shown maximum stimulation) there was no stimulation of respiration or increase in oxygen consumption.

Chloroethanols (optimal concentrations for respiratory stimulation with succinate)

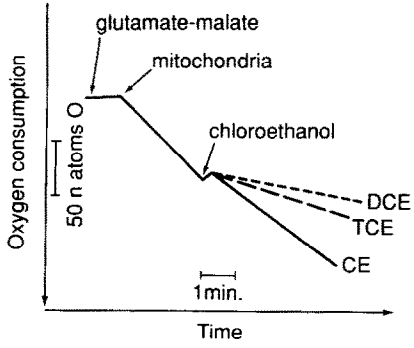

Fig. 2. Effect of 2-chloroethanol (CE), 2,2-dichloroethanol (DCE) and 2,2,2-trichloroethanol (TCE) on the respiratory stimulation using glutamate–malate as the respiratory substrate. The concentrations of chloroethanols which showed maximum respiratory stimulation with succinate as the respiratory substrate were used.

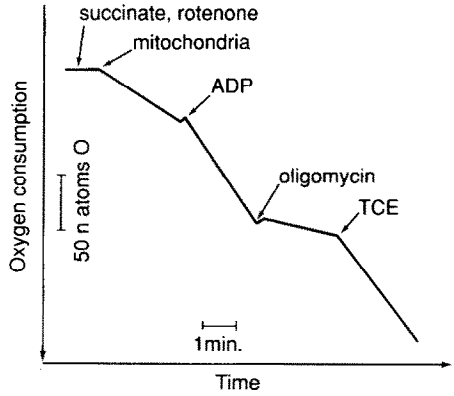

Fig. 3. Effect of trichloroethanol (TCE) on oxygen utilization after the addition of oligomycin to stop state 3 respiration. Addition of TCE (30 mM) stimulated oxygen consumption but did not stimulate more ATP formation.

inhibited mitochondrial respiration when glutamate–malate was used as the respiratory substrate (Fig. 2). The percent inhibitions were 10.0 ± 1.6, 81.3 ± 2.0 and 73.0 ± 1.0 for CE, DCE and TCE, respectively. Addition of chloroethanols inhibited the formation of ATP. For ATP determination, chloroethanol was added first, followed by the addition of ADP and the percent of ATP formed was 39.35, 34.48 and 23.78 with CE, DCE and TCE respectively, relative to control (100%) at concentrations which showed maximum respiratory stimulation with succinate as the respiratory substrate. In a separate experiment, first ADP was added, then oligomycin was added to block state 3 respiration, followed by the addition of chloroethanol. As shown in Figure 3, the addition of TCE after the addition of oligomycin stimulated oxygen consumption, but this stimulation did not result in the increase of ATP synthesis. These results indicate that chloroethanols stimulate mitochondrial respiration by uncoupling oxidative phosphorylation and that the uncoupling potency is proportional to the extent of chlorination.

DISCUSSION

Because of the presence of chloroethanols as contaminants in food products, medical and surgical supplies, studies related to the toxicity of these haloethanols are of great interest and importance. The ability of halogen to cause effective perturbation of the configuration of the hydrophobic groups in a protein has been suggested [36]. In recent years, the effect of chemicals on the respiratory activity of mitochondria has been determined in order to assess their cytotoxicity [37,38]. Ebina and Nagai have shown that CE stimulated state 4 respiration and released oligomycin inhibition of state 3 respiration [32]. This study was however done at a single concentration of CE (0.79 vol%) and using only one respiratory substrate. In our present study, we have

assessed the effect of increase in the number of chlorine atoms at the β-position of chloroethanol on the respiratory stimulation of rat liver mitochondria at different concentrations and using succinate as well as glutamate–malate as the respiratory substrate. The respiratory stimulation was found to be dose-dependent. Only 30 mM concentration of TCE was required for maximum respiratory stimulation, in contrast to 150 mM for DCE and 600 mM for CE. The maximum respiratory stimulation was 313% for TCE, whereas for DCE it was 202% and for CE 97% (Table I) when succinate was used as the respiratory substrate. However, chloroethanols (optimal concentrations for respiratory stimulation with succinate) inhibited mitochondrial respiration when glutamate–malate was used as the respiratory substrate. These results indicate that chloroethanols stimulate oxygen consumption in the case of NAD-linked substrates whereas they inhibit oxygen consumption for FAD-linked substrates. Change in the protein concentration did not show any appreciable effect on the stimulation of respiration (Table II). Stimulation of respiration and inhibition of ATP formation by these chloroethanols provides a direct indication that these chloroethanols stimulate mitochondrial respiration by uncoupling oxidative phosphorylation and that the uncoupling potency is proportional to the extent of chlorination. It has been suggested that uncouplinig by haloethanols may occur because of perturbation of the structure of the inner membrane of mitochondria, which results in leakage of protons from the outside to the inside [32]. Since most of the ATP necessary for cellular functions is produced by mitochondrial oxidative phosphorylation, impairment of the oxidative phosphorylation potential of mitochondria may result in deleterious effects on cellular activities. However, the effect of chloroethanols on the uncoupling of oxidative phosphorylation using isolated mitochondria needs to be compared with in vivo studies in rats in order to evaluate the efficacy of the present observations.

ACKNOWLEDGEMENTS

This research was supported by grant ES 04815 awarded by the National Institute of Environmental Health Sciences and by grant OH 02149 awarded by the National Institute for Occupational Safety and Health of the Center for Disease Control. The skillful technical assistance of Karen Inners-McBride is highly appreciated.

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