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Induction of calcium efflux from isolated rat-liver mitochondria by 1,2-dibromoethane

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Addition of 1,2-dibromoethane to rat-liver mitochondria induces a concentration-dependent depletion of mitochondrial glutathione. This event seems to be associated with the induction of Ca^{2+} release from mitochondria pre-loaded with a low pulse of Ca^{2+}. The enhancement of the energy-dissipating process to reaccumulate the released Ca^{2+} ('Ca^{2+} cycling') results in a progressive drop of membrane potential. Addition of EGTA (ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid), when the membrane potential has reached the lowest level, restitutes it to a normal value. All these findings and the observation that Ca^{2+} release also occurs under non cycling conditions (e.g., in the presence of ruthenium red) suggest that 1,2-dibromoethane induces a Ca^{2+} efflux by activating a selective pathway which is sensitive to critical sulfhydryl groups.

Introduction

1,2-dibromoethane, a large diffused fruit and grain fumigant, has hepatotoxic, carcinogenic and mutagenic properties [1,2]. Glutathione (GSH) plays an important role in the biotransformation of 1,2-dibromoethane in the liver, either by the direct conjugation process or by the reaction with bromoacetaldehyde, an oxidation product of 1,2-dibromoethane [3]. It follows that 1,2-dibromoethane poisoning results in a rapid depletion of hepatic GSH content. More recently, it has been shown that administration of 1,2-dibromoethane to rats results in a marked decrease of liver mitochondrial GSH [4].

Enhancement of lipid peroxidation processes, as a consequence of GSH depletion, has been suggested as one of the possible mechanism of 1,2-dibromoethane hepatotoxicity in isolated hepatocytes [5]. However, the biochemical mechanism of hepatocellular injury in 1,2-dibromoethane intoxication has not yet been fully understood.

In the current study the 'in vitro' effects of 1,2-dibromoethane on GSH content, lipid peroxidation, Ca^{2+} transport and membrane potential of isolated rat liver mitochondria were investigated.

Methods

Rat liver mitochondria were prepared in 0.25 M sucrose according to a standard procedure [6].

Protein was determined by a biuret method, with bovine serum albumin as the standard.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; GSH, reduced glutathione; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; $\Delta\psi$, mitochondrial transmembrane electrical potential, negative inside.

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Mitochondrial GSH content was measured as follows: mitochondrial (6.5 mg protein/ml) were incubated at 25°C in the following medium: 120 mM sucrose/10 mM Tris-HCl (pH 7.4)/2 μM rotenone/2.5 mM succinate. Samples of 2 ml were taken at specified times and the reaction was stopped by rapid mixing with 1 ml of 5% trichloroacetic acid (w/v) and 5 mM EDTA. After centrifugation aliquots of supernatants were reacted with Ellman's reagent as described in Ref. 4.

The amount of malondialdehyde formed in mitochondria was measured by the thiobarbituric acid method [7] in the presence of 0.1% butylated hydroxytoluene and 0.5 mM $FeCl_3$ in the thiobarbituric acid reagent. Mitochondria (1 mg protein/ml) were incubated at 37°C in a medium of the same composition of that used for GSH determination. Samples of 1 ml were taken at specified time and the reaction was stopped by rapid mixing with 2 ml thiobarbituric acid mixture. The amount of malondialdehyde formed was determined, after centrifugation, spectrophotometrically at 535 nm.

Ca^{2+} movement were followed by a Ca^{2+}-selective electrode as described in detail in Ref. 8. Mitochondria (4.5 mg protein) were incubated at 25°C in a final volume of 1.5 ml in a standard medium containing 100 μM $CaCl_2$. The standard incubation medium contained: 210 mM mannitol/70 mM sucrose/10 mM Hepes (pH 7.4)/5 μM rotenone. After a preincubation period of 5 min, the initial rates of Ca^{2+} influx were measured following addition of 5 mM succinate (K^+ salt) as respiratory substrate.

The transmembrane potential $\Delta\psi$ was measured at 25°C in the standard incubation medium containing 20 μM tetrahenylphosphonium chloride and 2.5 mM succinate, in a final volume of 1.5 ml, by monitoring with a tetraphenylphosphonium-selective electrode the movements of tetraphenylphosphonium across the mitochondrial membrane as in Ref. 9.

Results

The time-course of the effect of various concentrations of 1,2-dibromoethane on the GSH content of liver mitochondria respiring with succinate is presented inFig. 1. It is seen that 1,2-dibromoethane produces a concentration-dependent reduction in the level of mitochondrial GSH. The GSH content decreases from 8.1 nmol/mg protein of the control up to 4.3 nmol/mg protein within 10 min incubation in the presence of the highest 1,2-dibromoethane concentration tested, e.g., 1.2 μmol/mg protein.

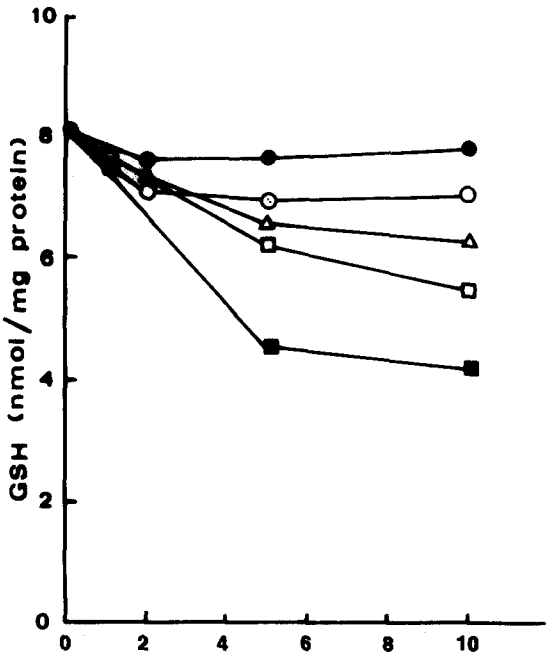

Fig. 1. Time-course of GSH depletion in rat-liver mitochondria induced by various concentrations of 1,2-dibromoethane. Mitochondria were incubated at 25°C in the presence of various concentrations of 1,2-dibromoethane (●, none; ○, 0.1 μmol/mg; △, 0.3 μmol/mg; □, 0.6 μmol/mg; ■, 1.2 μmol/mg). GSH was determined as described in Methods. The reaction was started by the addition of mitochondria.

In order to assess whether the depletion of GSH, which is well known to constitute an important antioxidant defense [10], may result in the enhancement of peroxidative reactions in the mitochondrial membrane, the malondialdehyde production was parallelly measured. It was found that no increase in malondialdehyde formation was observable also in the presence of concentrations of 1,2-dibromoethane (e.g., 1.2 μmol/mg) which deplete by 54% the mitochondrial GSH.

Since several studies have implicated sulfhydryl groups as being involved in the mechanism of Ca^{2+} release from liver mitochondria [11–19], the

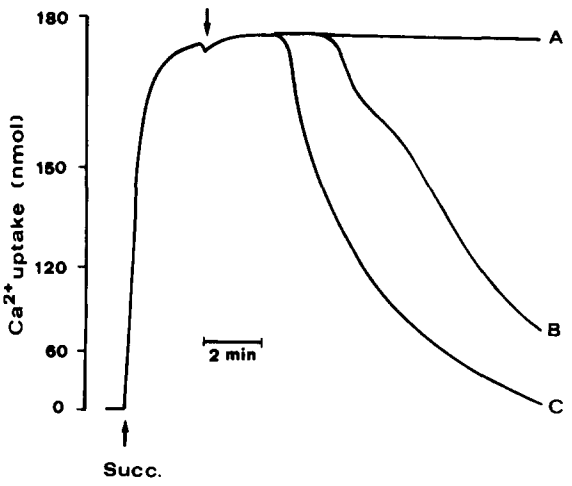

Fig. 2. 1,2-dibromoethane-induced Ca^{2+} efflux from liver mitochondria. Mitochondria were loaded with 33 nmol Ca^{2+}/mg protein in the presence of succinate (Succ.). At the arrow, 1,2-dibromoethane was added at various concentrations (A, none; B, 0.3 μmol/mg protein; C, 0.6 μmol/mg protein, respectively).

effect of 1,2-dibromoethane on the capability of mitochondria to retain accumulated Ca^{2+} was parallelly tested. Fig. 2 shows that 1,2-dibromoethane induces a Ca^{2+} release from energized liver mitochondria which had previously accumulated a low pulse of Ca^{2+}, i.e., 33 nmol/mg protein. It also appears that the time of onset and the rate of Ca^{2+} release depends on the amount of 1,2-dibromoethane added. 1,2-dibromoethane at a concentration of 0.1 μmol/mg protein does not cause any appreciable efflux of Ca^{2+} at least during the period of the incubation tested (not shown).

In order to evaluate whether the 1,2-dibromoethane-induced Ca^{2+} release may result from irreversible damage to the inner mitochondrial membrane, the membrane potential $\Delta\psi$ was measured under the same experimental conditions. Fig. 3 shows that, upon addition of the same low pulse of Ca^{2+} to energized mitochondria, there is a sudden drop of $\Delta\psi$ which corresponds to the

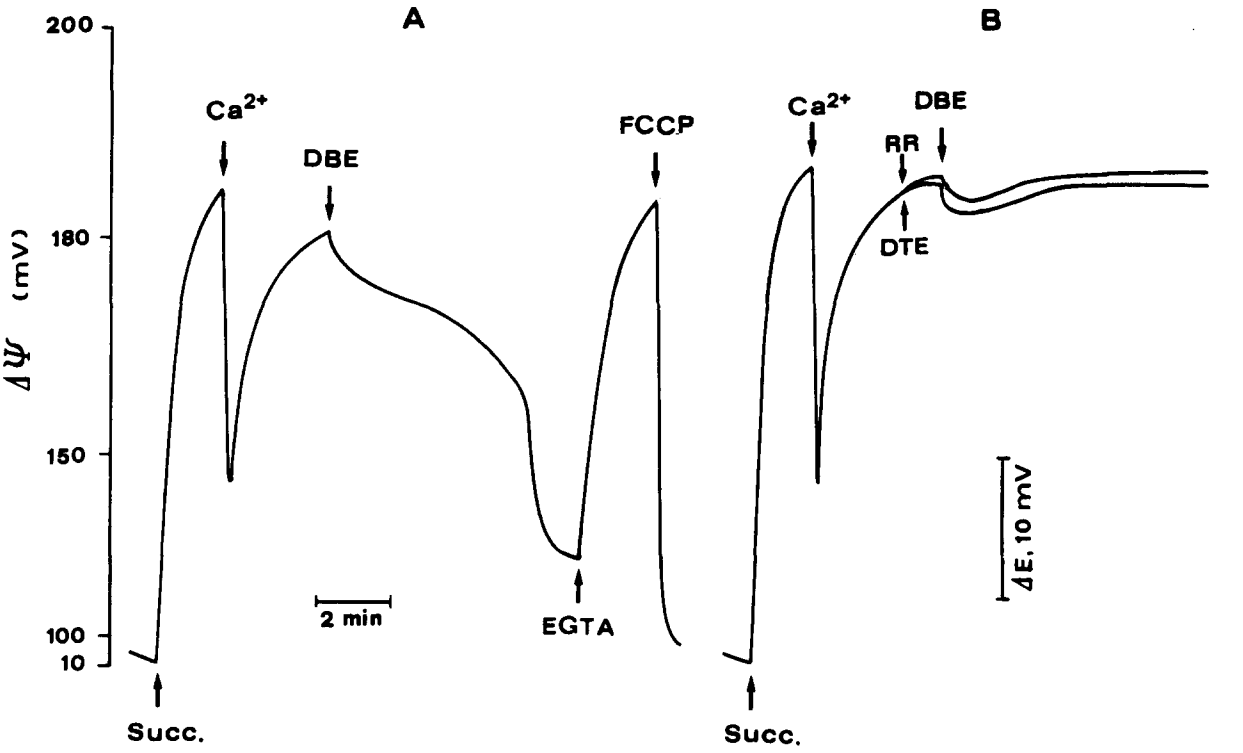

Fig. 3. 1,2-dibromoethane (DBE)- induced changes of membrane potential. Mitochondria were energized by the addition of 2.5 mM succinate (Succ.). The arrows in Fig. 3A indicate the following additions: 33 nmol Ca^{2+}/mg protein; 0.6 μmol 1,2-dibromoethane/mg protein; 0.5 mM EGTA; 0.5 μM FCCP. The arrows in Fig. 3B indicate the addition of either 2 μM ruthenium red (RR) or 2 mM dithioerythritol (DTE). ΔE, electrode potential.

energy required for Ca^{2+} accumulation. When Ca^{2+} uptake is complete, trace reverses to a new steady-state almost identical to the pre-Ca^{2+} level. At this point 1,2-dibromoethane addition induces a progressive decrease of $\Delta\psi$ (Fig. 3A). When $\Delta\psi$ has reached the lowest value, addition of the Ca^{2+} chelator EGTA results in a full restoration of the membrane potential. This indicates that the inner mitochondrial membrane is not irreversibly depolarized and that the 1,2-dibromoethane-induced decrease of the membrane potential is not due to damage to the mitochondria, but rather to a continuous and energy-draining Ca^{2+} cycling. This conclusion is further supported by the experiment depicted in Fig. 3B. It appears that ruthenium red, a specific inhibitor of the electrophoretic Ca^{2+} uptake, completely prevents $\Delta\psi$ drop when added before 1,2-dibromoethane. The same figure also shows that similar effects on $\Delta\psi$ are obtained upon addition of dithioerythritol, a chemical reductant of thiol groups, which is able to prevent the 1,2-dibromoethane effect on $\Delta\psi$ when added before 1,2-dibromoethane. By contrast, the addition of an antioxidant such as buthylated hydroxytoluene fails to prevent the 1,2-dibromoethane effect on $\Delta\psi$ (not shown).

Relevant to the above considerations are the results presented in Fig. 4. It can be seen that

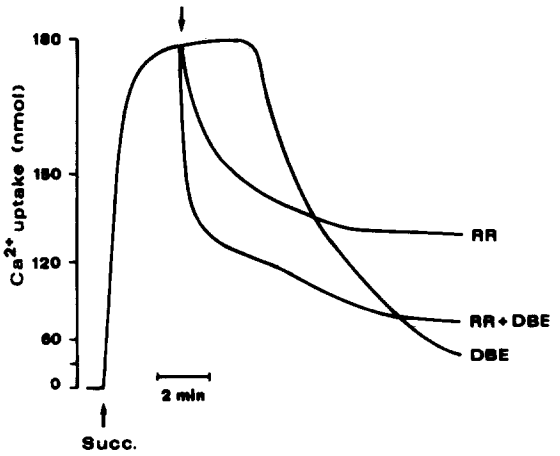

Fig. 4. Effect of ruthenium red on 1,2-dibromoethane (DBE)-induced Ca^{2+} release from liver mitochondria. Mitochondria were loaded with 33 nmol Ca^{2+}/mg protein in the presence of succinate (Succ.). At the arrow, either 2 μM ruthenium red (RR), 0.6 μmol 1,2-dibromoethane/mg protein, or rutheium red together with 1,2-dibromoethane (RR + DBE) were added.

when ruthenium red is added to Ca^{2+}-loaded mitochondria (33 nmol Ca^{2+}/mg protein) an immediate and slow release of Ca^{2+} occurs. When 1,2-dibromoethane is added instead of ruthenium red, a significant net release of Ca^{2+} is observable only after some lag time. When 1,2-dibromoethane is added together with ruthenium red, Ca^{2+} release is both immediate and fast. These results support the view that the 1,2-dibromoethane-induced Ca^{2+} release is followed by a fast Ca^{2+} reuptake ('Ca^{2+} cycling') until at some critical point the release process becomes faster than the re-uptake, and consequently a net Ca^{2+} release is observed.

Discussion

The present findings show that addition of 1,2-dibromoethane to liver mitochondria results in a concentration-dependent depletion of mitochondrial GSH. This indicates that 1,2-dibromoethane is able to penetrate into the mitochondrial matrix space, and therefore it can be classified as a penetrant thiol reagent [20]. These data confirm the previously reported 'in vivo' depletion of mitochondrial GSH induced by 1,2-dibromoethane [4] and support the view that 1,2-dibromoethane itself may be the chemical thiol reactant.

The reaction of 1,2-dibromoethane with mitochondrial sulfhydryl groups brings about the induction of Ca^{2+} efflux from mitochondria and a parallel drop in the membrane potential. The importance of reduced thiol groups in modulating the capability of mitochondria to retain Ca^{2+} is further supported by the results obtained in the presence of dithioerythritol. Indeed this chemical reductant of thiol groups completely prevents the 1,2-dibromoethane effect on the membrane potential of Ca^{2+}-loaded mitochondria (Fig. 3B). The data on malondialdehyde production do not support a possible involvement of lipoperoxidative reactions, following GSH depletion by 1,2-dibromoethane, in inducing the Ca^{2+} release. This conclusion is further supported by the finding that a free radical scavenger, such as buthylated hydroxytoluene, is not able to prevent the 1,2-dibromoethane-induced drop of $\Delta\psi$.

The membrane potential measurements (Fig. 3A and 3B) clearly indicate that the 1,2-dibromo-

ethane-induced Ca^{2+} release is not due to a non-specific increase in the inner membrane permeability [21], but rather to the activation of a selective pathway of Ca^{2+} efflux sensitive to critical sulfhydryl groups. Indeed the collapse of membrane potential here observed, following 1,2-dibromoethane addition, is not the cause of Ca^{2+} release, but rather it is actually the consequence of this process. This is in agreement with previous reports on the effect of either thiol reagents, hydroperoxides, menadione, divicine and ferric iron complex on mitochondrial Ca^{2+} transport [11,15, 18,19,22–27]. This conclusion is strongly supported also by the results showing that Ca^{2+} efflux occurs in the presence of ruthenium red (Fig. 4), which specifically inhibits the Ca^{2+} efflux when it occurs via a reversal of the electrophoretic uptake route ('uniport'). Therefore the 1,2-dibromoethane-induced Ca^{2+} efflux here observed under non-cycling conditions seems to indicate that this occurs via a distinct electroneutral 'antiport' according to Ref. 28.

All these results support the view that sulfhydryl groups play a primary role in the regulation of mitochondrial Ca^{2+} transport [13,16], although a possible involvement of oxidation and hydrolysis of pyridine nucleotides [29], a condition which can follow GSH depletion by 1,2-dibromoethane, may not be excluded in the mechanism underlying the 1,2-dibromoethane-induced Ca^{2+} efflux. As to this point, an aspect which merits consideration is the correlation between the extent of GSH depletion and the rate of calcium release. This relationship seems to hold only when the extent of GSH depletion is larger than 10%. Indeed, when the GSH decrease is of the order of 10%, no Ca^{2+} release is observable, in agreement with previous findings obtained by the use of *N*-ethylmaleimide [19].

As to the mechanism of in vivo 1,2-dibromoethane hepatotoxicity, this perturbation in mitochondrial Ca^{2+} transport here reported may be a factor in the onset of cell damage in this pathology. Indeed the activation of a Ca^{2+} release route brings about the induction of a forced Ca^{2+} cycling which inevitably leads to serious mitochondrial damages.

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