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ATP-synthase complex: the mechanism of control of ion fluxes induced by cumene hydroperoxide in mitochondria

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In state 4, the cumene hydroperoxide-induced decrease of mitochondrial transmembrane potential is prevented by the free radical scavenger, butylhydroxytoluene. This decrease is accompanied by accumulation of lipid hydroperoxides. Oligomycin suppresses the cumene hydroperoxide-induced uncoupling of mitochondria, but has no significant effect on lipid peroxidation. The transition of mitochondria from state 4 to state 3 by ADP addition leads to the suppression of both lipid peroxidation and mitochondrial uncoupling. This effect on lipid peroxidation is prevented by carboxyatractyloside and oligomycin.

Mitochondria; Ion transport; Hydroperoxide

1. INTRODUCTION

The ability of hydroperoxides to modify mitochondrial ion permeability is now well established [1,2]. However, the precise mechanism of its action is still controversial. Free-radical reactions [3,4], LP in particular [4], may be one of the intermediate steps in the hydroperoxide-induced increase in the ion permeability of mitochondria. This possibility is confirmed by the recently discovered correlation between the lipid peroxidation intensity in mitochondria and the magnitude of CuOOH-induced ion fluxes [5,6].

An interesting feature of hydroperoxide-activated ion permeability of mitochondria is its linkage to the operation of ATP synthase. According to the data of Marshansky et al. [5,6], addition of the substrate (ADP) or ATP synthase inhibitors (oligomycin, DCCD) causes simultaneous suppression of CuOOH-induced electrogenic ion fluxes and of accumulation of LP products. It might be suggested therefore that the ATP-synthase-controlled step in CuOOH-induced ion permeability is LP. However, the measurement of LP products in [5,6] was performed by a method which did not exclude the possibility of lipid peroxidation upon fixation of mitochondria. In this connection, in the present work we also looked into the role of LP as a step accounting for the effect of the functional state of ATP synthase on the CuOOH-induced ion permeability of mitochondria; a more adequate method was selected for the assay of LP products.

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Abbreviations: BHT, butylhydroxytoluene; CuOOH, cumene hydroperoxide; TPP^+, tetraphenylphosphonium; MDA, malonic dialdehyde; TBA, 2-thiobarbituric acid; $\Delta\psi$, mitochondrial inner-membrane potential; CATR, carboxyatractyloside; LP, lipid peroxidation

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated by differential centrifugation in a medium containing 250 mM sucrose, 250 μM EDTA and 5 mM Hepes (pH 7.4). The final washing was performed in the same medium, but without EDTA. Protein in the mitochondrial suspension was assayed by the biuret method with bovine serum albumin as a standard. TPP^+ and lipid

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hydroperoxides were assayed simultaneously in a measuring cell. $\Delta\psi$ changes were evaluated by the TPP^+ distribution between the incubation medium and the mitochondrial matrix with a TPP^+-selective electrode [7]. $\Delta\psi$ was calculated according to Rottenberg [8]. LP intensity was estimated by hydroperoxide accumulation [9]. 0.2 ml samples from the measuring cell were fixed in a mixture containing 0.2 ml of 8% Na-dodecylsulfate and 0.1 ml of 10^{-2} M ethanol solution of BHT. The samples were supplemented with 1.5 ml of 0.5% TBA and 2 ml of the mixture containing 1.6 ml of 20% acetic acid (pH 3.5), 0.3 ml of H_2O and 0.1 ml 10^{-2} M $FeCl_3$. The samples were heated in a water bath at 100°C for 15 min. Upon cooling, they were supplemented with 1 ml of ice-cold acetic acid and 2 ml of chloroform. After vigorous shaking, the samples were centrifuged for 20 min at 10000 × *g*. MDA was assayed spectrophotometrically at λ = 532 nm, using $\epsilon = 1.56 \times 10^5\ M^{-1}\cdot cm^{-1}$. Mitochondria (2 mg/ml) were incubated in a medium containing 0.1 M sucrose, 10 mM Mes, 10 mM succinate, 10 mM H_3PO_4, 2 mM $MgCl_2$, $CaCl_2$ (30 nmol/mg protein), 40 μM P^1,P^5-di(adenosine-5′) pentaphosphate, 10 mM glucose, 20 μg/ml hexokinase (81 IU/mg protein), 2 μM TPP^+ and 2 μM rotenone (t = 37°C). The incubation medium was adjusted to pH 7.4 with Tris.

3. RESULTS AND DISCUSSION

As seen from fig.1A, curve 1, the addition of mitochondria to the incubation medium results in the decrease of TPP^+ concentration due to TPP^+ accumulation in the mitochondrial matrix. This attests to the presence of a stable (180 mV) $\Delta\psi$ on the mitochondrial membrane. CuOOH addition (curve 2) sets off a TPP^+ efflux from mitochondria, which indicates the activation of electrogenic ion fluxes and the resulting drop of $\Delta\psi$. A concurrent accumulation of lipid hydroperoxides – proof of LP activation (fig.1B, curve 2) – is observed. BHT suppresses the accumulation of lipid hydroperoxides (fig.1B, curve 3) and prevents a CuOOH-induced lowering of the mitochondrial $\Delta\psi$ (fig.1A, curve 3). This argues in favor of the supposition that the CuOOH-induced electrogenic ion fluxes in mitochondria are controlled by free-radical reactions. Thereby the suppression by the ATP synthase inhibitors (oligomycin, DCCD) and also by the ATP-synthase substrate (ADP) of hydroperoxide-induced electrogenic ion fluxes [5,6] may be caused by the effect of these agents on LP intensity. However, our experimental data show that though both oligomycin and ADP suppress the CuOOH-induced electrogenic ion fluxes, these agents exert a different effect on LP.

Oligomycin completely prevents CuOOH-induced de-energization of mitochondria (fig.1A,

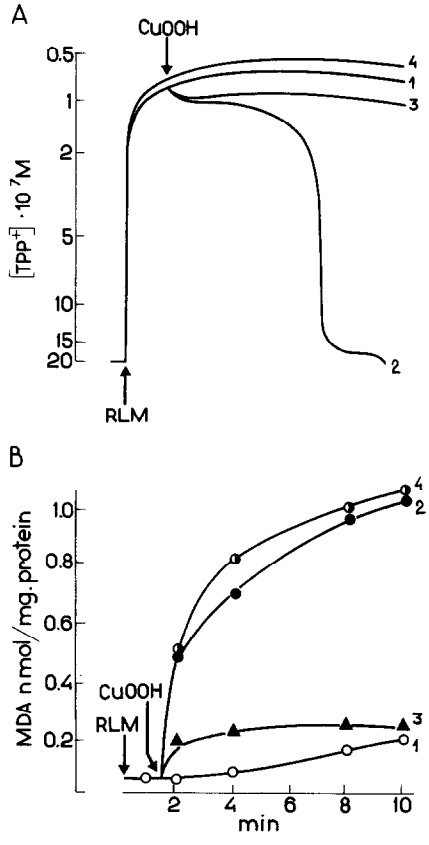

Fig.1. Effect of oligomycin and BHT on the transmembrane potential decrease (A) and the accumulation of MDA (B) induced by CuOOH. For experimental conditions, see section 2. Curve 1, control. Curves 2–4, 1.5 min after the addition of mitochondria, 250 μM CuOOH was added; curve 3, in the presence of 50 μM BHT; curve 4, in the presence of oligomycin (2 μg/mg protein).

curve 4) and has little, if any, effect on lipid hydroperoxide accumulation (fig.1B, curve 4). This means that (i) oligomycin inhibition is not due to its effect on LP and (ii) the action of LP products is not merely confined to the enhanced permeability of the mitochondrial bilayer, as it was shown on model systems [10,11]. Most probably, free-radical reactions stimulate endogenous transport systems. The mechanism of the oligomycin inhibitory action on the LP level, as observed in [5], needs special investigation. Unlike oligomycin, ADP, inhibiting electrogenic ion fluxes, simultaneously reduces the intensity of LP. Transition of mitochondria to the state of phosphorylation (from state 4 to state 3) caused by the addition of

ADP, leads to some lowering of $\Delta\psi$ (from 180 to 150 mV) due to the dissipation of a portion of the electrochemical proton potential for ATP synthesis (fig.2A, curve 2). A comparison of curve 1 (state 4) and curve 2 (state 3) shows that activation of phosphorylation prevents the induction of electrogenic ion fluxes by CuOOH and simultaneously inhibits the formation of lipid hydroperoxides in mitochondria (fig.2B, curve 2). ADP produces this effect because of its transfer across the inner mitochondrial membrane. Indeed, addition of CATR, the adenine nucleotide translocase inhibitor, reverses the ADP effect on both the CuOOH-induced electrogenic ion fluxes (fig.2A, curve 3) and the accumulation of LP products (fig.2B, curve 3). In principle, the inhibition of LP with the addition of ADP may be related either to its direct action on systems controlling the formation or utilization of LP products, or to a change of the mitochondrial functional state (transition from 4 to 3 state). One could expect in the latter case that oligomycin (which does not affect LP per se), by inhibiting the transition of mitochondria to the phosphorylating state, would prevent the inhibitory effect of ADP. This is what was observed in the experiment (fig.3A, 3B, curves 2). It can be seen that mitochondria in the phosphorylating state (state 3) are characterized by a lower intensity of lipid peroxidation than state 4 mitochondria. Consequently, we suggest that the inhibitory effect

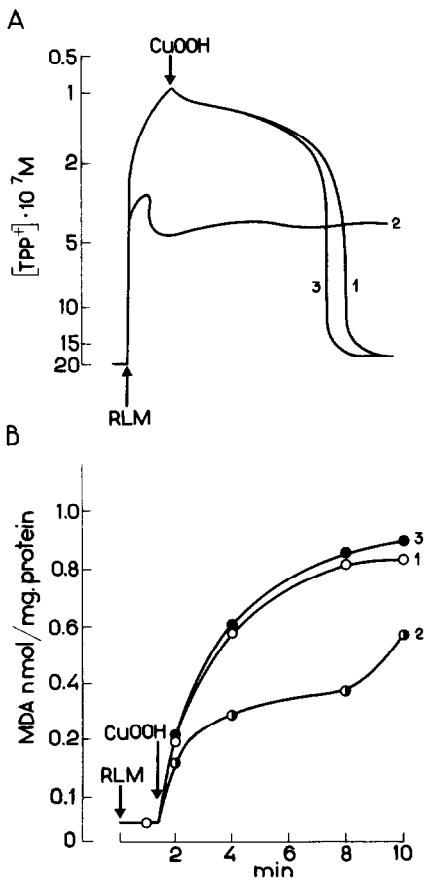

Fig.2. Effect of carboxyatractyloside on suppression by ADP of the transmembrane potential decrease (A) and the accumulation of MDA (B) induced by CuOOH. For experimental conditions, see section 2. After the addition of mitochondria, 250 μM CuOOH was added. Curve 1, control. Curve 2, in the presence of 200 μM ADP. Curve 3, in the presence of 200 μM ADP and 5 μM CATR.

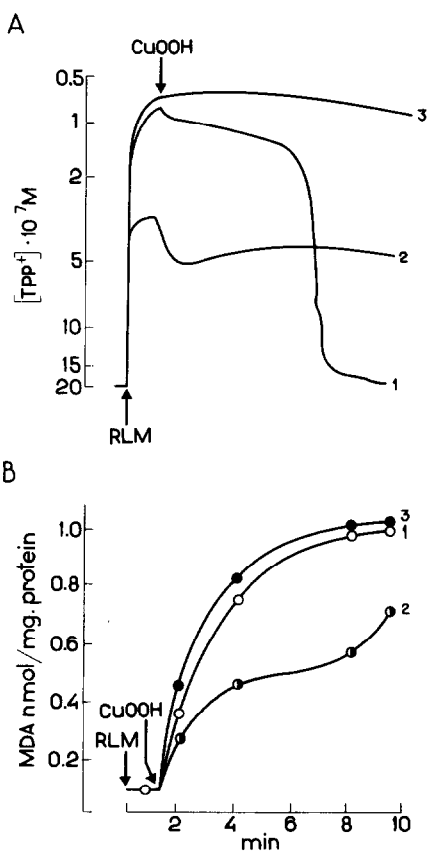

Fig.3. Effect of oligomycin on suppression by ADP of the transmembrane potential decrease (A) and accumulation of MDA (B) induced by CuOOH. For experimental conditions, see section 2. After the addition of mitochondria, 250 μM CuOOH was added. Curve 1, control. Curve 2, in the presence of 200 μM ADP. Curve 3, in the presence of 200 μM ADP and oligomycin (2 μg/mg protein).

of ADP on the CuOOH-induced ion fluxes is caused by a change of LP.

The available data suggest that the ATP synthase complex may control hydroperoxide-induced ion fluxes at least via two different mechanisms. One of them involves a change in lipid peroxidation intensity upon state 4 to state 3 transition. The other does not involve free-radical reactions and may be evaluated by the effect of oligomycin. Previously, it has been shown that oligomycin also inhibits the Ca^{2+}/P_i-induced cation fluxes and its action was reversed by the uncoupler CCCP [12]. These data indicate that in both cases the effect of oligomycin involves the modulation of $\Delta\bar{\mu}H^+$ as an intermediate step and thus, direct interaction of the ATP synthase complex with the ion transport system may be excluded.

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