INHIBITION OF CATION EFFLUX BY ANTIOXIDANTS DURING OSCILLATORY ION TRANSPORT IN MITOCHONDRIA

Wolfgang AUGUSTIN, Frank GELLERICH, Ingrid WISWEDEL, Yuri EVTODIENKO+ and Valeri ZINCHENKO+

Institute of Physiological Chemistry, Medical School, 301 Magdeburg, Leipziger Str. 44, GDR and *+Institute of Biological Physics, USSR Academy of Sciences, Pushchino, Moscow Region, USSR*

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1. Introduction

It is well known that biological membranes are susceptible to lipid peroxidation [1]. Effects of peroxidative damage on energy-linked functions and membrane enzymes of mitochondria were reported [2]. In the presence of pro-oxidants isolated mitochondria swell and lyse after incubation in salt media and concomitantly lipid peroxides were accumulated [3]. Inhibitory actions of antioxidants on these processes were noted [4]. Probably one of the earliest events following lipid peroxidation is an alteration in ionic permeability of the mitochondrial membrane.

It was shown recently that during oscillations of ion fluxes in mitochondrial suspensions, periodic increases and decreases of membrane permeability and passive ion fluxes occur [5]. The obvious advantages of such a reversible system of mitochondrial ion transport prompted us to study the influence of different antioxidants on the process of mitochondrial oscillatory ion fluxes. The main effect of all antioxidants studied was the inhibition of ion efflux during the oscillatory cycle without significant influences on either active transport or oxidative phosphorylation

2. Materials and methods

2.1. *Chemicals*

α-Tocopherol (Merck), butylated hydroxytoluene (Calbiochem), *tert.* butyl-4-hydroxyanisol (Serdary Res.) and α-naphthol (Sigma) were applied as solutions in freshly distilled ethanol. Thiobarbituric acid was purified according to [6]. Sucrose was purified by ion exchange chromatography on columns of Dowex 50 (Serva). All other reagents were of analytical grade.

2.2. *Preparation of mitochondria*

Rat liver mitochondria (RLM) were isolated in a solution containing 300 mM sucrose, 0.5 mM EDTA and 5 mM Tris–HCl (pH 7.5). RLM were washed with the same medium containing no EDTA, suspended in sucrose–Tris–HCl and kept at 2–4°C.

2.3. *Measurement of ion fluxes and oxygen consumption*

The activity of K^+ and Sr^{2+} was measured by ion-selective electrodes [7]. Oxygen concentration in medium was monitored with a closed Clark-type electrode. All ion-flux measurements were done in an open cell with continuous mixing at room temperature (23°C). Incubation medium: 20 mM sucrose, 1 mM KCl, 5 mM succinate, 12.5 mM Tris–HCl (pH 7.4) and 5 mg mitochondrial protein/ml. Mitochondrial respiration was measured in a closed system at room temperature in an incubation medium containing 135 mM mannitol, 10 mM KH_2PO_4, 0.5 mM EDTA–Na_2, 60 mM KCl, 1 mM TRA-buffer, 20 mM Hepes, 5.6 mM $MgCl_2$ (pH 7.4) and ~3.5 mg mitochondrial protein/ml with 5 mM succinate as substrate either in the presence or absence of ADP.

2.4. *Iron-ascorbate-induced lipid peroxidation*

For measurement of malondialdehyde formation, an incubation medium containing 0.1 M KCl, 10^{-2} M Tris-buffer, 2×10^{-4} M $SrCl_2$, 5×10^{-4} M ascorbic

acid, 2×10^{-5} M $FeCl_2$ (pH 7.5) and 1 mg mitochondrial protein/ml was used. After 20 min incubation with constant shaking 1.0 ml samples of mitochondrial suspension were mixed with 0.3 ml 30% trichloroacetic acid, centrifuged and 0.5 ml supernatant was added to 1 ml thiobarbituric acid solution. Colour was developed by incubation for 20 min in a boiling water bath and products were measured at 532 nm. Protein determination was performed with a modified biuret-method [8].

3. Results

3.1. *Oscillatory ion fluxes in mitochondria*

Typical oscillations of K^+ and Sr^{2+} fluxes are depicted in fig.1A. Two stages of the oscillatory process can be easily distinguished. Stage 1 represents the stage of active ion transport, the cation influx. After cation accumulation stage 2 of the oscillatory cycle starts, representing the efflux of K^+ and Sr^{2+} from the mitochondria down to the concentration gradient. Figure 1B demonstrates the effect of a relatively low concentration of the natural antioxidant α-tocopherol on the ion fluxes. The rates of the cation influx ($I^{K^+}_{in}$) apparently are not changed in the presence of the antioxidant, whereas the rates of cation efflux ($I^{K^+}_{out}$) are inhibited. The oscillations proceed with an increased period and a small decrease in the amplitude. Figure 1C shows the influence of a relatively high concentration of α-tocopherol. In this case full

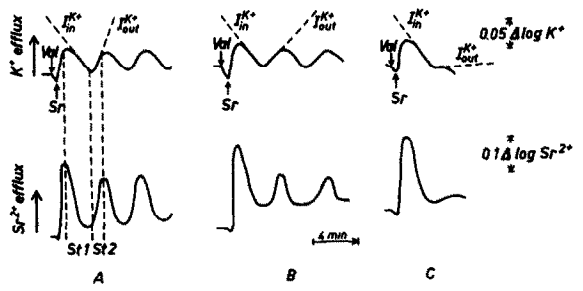

Fig.1. Effect of α-tocopherol on K^+- and Sr^{2+}-fluxes in oscillating mitochondrial suspensions. Conditions as described in section 2. Val, addition of valinomycin (6 ng/mg protein); Sr, addition of Sr^{2+} (320 μmol/l); St 1 and St 2, stage 1 and stage 2 of the oscillatory cycle. (A) Without α-tocopherol; (B) 130 μmol/l α-tocopherol; (C) 150 μmol/l α-tocopherol.

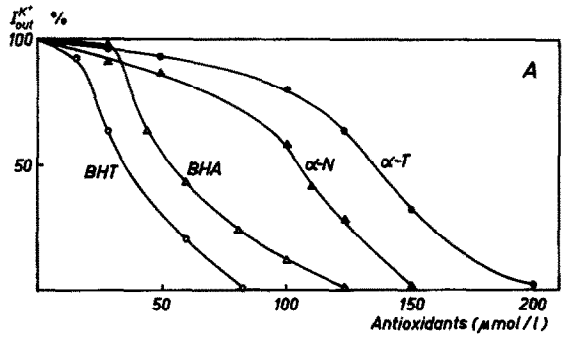

Fig.2. Inhibition of mitochondrial K^+-efflux by different antioxidants during oscillatory ion fluxes. Conditions and calculation as described in fig.1. Rate of K^+-efflux without antioxidants $I^{K^+}_{out}$ = 100% equals 80 nmol/mg protein × min.

inhibition of cation efflux occurs. Consequently, the oscillations of ion fluxes are abolished completely.

3.2. *Influence of different antioxidants on oscillatory ion fluxes*

The influence of one natural antioxidant, α-tocopherol (α-T), and three synthetic antioxidants, butylated hydroxytoluene (BHT), *tert.* butyl-4-hydroxyanisol (BHA) and α-naphthol (α-N) was evaluated on oscillating ion fluxes.

Figure 2 illustrates the dependence of the relative rate of K^+-efflux on the concentration of antioxidants. The efficiency of the antioxidants tested was in the sequence BHT > BHA > α-N > α-T. The antioxidant concentrations yielding half-maximal inhibition of K^+-efflux in nmol/mg protein were 9.4 for BHT, 10.7 for BHA, 21 for α-naphthol and 37 for α-tocopherol. The sensitivity of Sr^{2+}-efflux to antioxidants was essentially the same as for the K^+-efflux. Active cation transport and oxidative phosphorylation, as indicated by the respiratory rate in the presence and absence of ADP, were not markedly influenced within this concentration range.

3.3. *Effect of antioxidants on iron-ascorbate supported lipid peroxidation in mitochondria*

In order to check the antioxidative effect of the substances their influence on the formation of the peroxidation product malondialdehyde (MDA) was measured (fig.3). The same order in the efficiency of antioxidants as in the suppression of cation efflux,

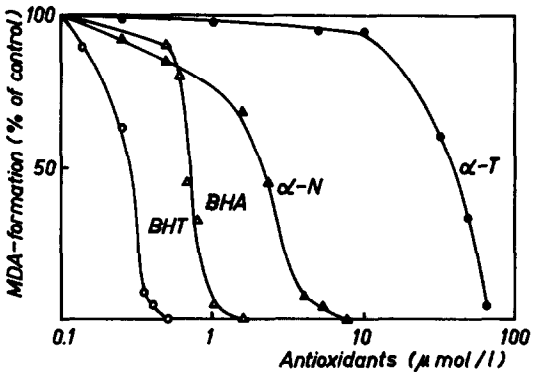

Fig.3. Inhibition of malondialdehyde (MDA) formation by different antioxidants during iron-ascorbate-induced lipid peroxidation in mitochondria. Conditions as described in section 2. The concentrations of antioxidants are depicted in a logarithmic scale. Malondialdehyde formation in the absence of antioxidants (100%) was 12 nmol/mg protein during the incubation time.

namely BHT > BHA > α-N > α-T was established during the iron-ascorbate-supported peroxidation of mitochondrial lipids. Half maximal inhibition of MDA formation, expressed in nmol antioxidant/mg protein, was attained at 0.5 for BHT, 1.0 for BHA, 2.4 for α-N and 43 for α-T. Because ion fluxes and MDA formation were measured under different conditions, the latter process was found, except α-T, more sensitive against antioxidants.

4. Discussion

It has been shown in this paper that four different antioxidants prevent the accumulation of malondialdehyde and the cation efflux under oscillating conditions within a similar efficiency range in the order of BHT > BHA > α-N > α-T in rat liver mitochondria. Influences of antioxidants on properties of phospholipid bilayers [9] and on activities of microsomal enzymes [10], apparently not connected with their antioxidatic potential, were observed at higher concentrations of antioxidants than those applied in the experiments reported above. It seems therefore justified to conclude that the effects of antioxidants are specifically attributed to their inhibitory action on lipid peroxidation. As antioxidants specifically prevent the cation efflux and exert no influence on active ion transport and oxidative phosphorylation as well, it follows that one essential step in the postulated mechanism of oscillating ion transport [11] is represented by lipid peroxidation, which may initiate the transition of the membrane from the 'intact' (stage 1) to the 'permeable' state (stage 2), allowing the cations to leak out.

The following possibilities may be considered for the inherent mechanisms:

(i) Products of lipid peroxidation are themselves active as ionophores and/or by the introduction of polar groups into hydrophobic regions during peroxidation, gates or channels are opened within the membrane. Compounds with ionophoretic properties resembling peroxidation products have been already isolated from mitochondria [12] and the appearence of a higher ionic permeability in lipid bilayers after peroxidation has been observed [13].

(ii) Lipid peroxidation causes changes in membrane proteins, leading to changes in ionic permeability. Reactions of free radicals of peroxidizing lipids, lipohydroperoxides and reactive carbonyls with proteins are well documented [1,14].

(iii) Perturbations of the membrane structure by lipid peroxidation make the phospholipids more susceptible to the attack by enzymes, as already reported for phospholipase A_2 in liver mitochondria [15]. In this way an accumulation of lysocompounds may occur, which have been shown recently to induce a high K^+-conductivity in bilayer membranes [16]. Results of current investigations on phospholipid turnover during the oscillatory ion transport [17], pointing to cyclic acylation–deacylation reactions of certain mitochondrial phospholipids parallel to the cyclic permeability changes, are also compatible with the third possibility.

As oxygen radicals inducing lipid peroxidation obviously can be generated at the level of the mitochondrial electron transport chain also in vivo [18], it may be anticipated that changes in mitochondrial ion transporting systems can be mediated by peroxidations in vivo as well and thus may constitute one factor in the regulation of mitochondrial ion transport in living cells.

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