High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria

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Abstract **Formation of H_2O_2 has been studied in rat heart mitochondria, pretreated with H_2O_2 and aminotriazole to lower their antioxidant capacity. It is shown that the rate of H_2O_2 formation by mitochondria oxidizing 6 mM succinate is inhibited by a protonophorous uncoupler, ADP and phosphate, malonate, rotenone and myxothiazol, and is stimulated by antimycin A. The effect of ADP is abolished by carboxyatractylate and oligomycin. Addition of uncoupler after rotenone induces further inhibition of H_2O_2 production. Inhibition of H_2O_2 formation by uncoupler, malonate and ADP+P_i is shown to be proportional to the $\Delta\Psi$ decrease by these compounds. A threshold $\Delta\Psi$ value is found, above which a very strong increase in H_2O_2 production takes place. This threshold slightly exceeds the state 3 $\Delta\Psi$ level. The data obtained are in line with the concept [Skulachev, V.P., Q. Rev. Biophys. 29 (1996), 169–202] that a high proton motive force in state 4 is potentially dangerous for the cell due to an increase in the probability of superoxide formation.**

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Key words: Reactive oxygen species; Protonic potential; Mitochondria; Uncoupling

1. Introduction

In 1973 Boveris and Chance [1] found that an uncoupler of oxidative phosphorylation (CCCP) or ADP+P_i inhibit H_2O_2 formation by mitochondria oxidizing succinate and glutamate. Later Liu and Huang [2,3] reported a very steep dependence of the $O_2^{-\bullet}$ and H_2O_2 production by mitochondria upon the $\Delta\Psi$ level on the mitochondrial membrane. In their experiment, $\Delta\Psi$ was decreased by adding different concentrations of malonate which decreases the state 4 respiration rate. Therefore one might suggest that formation of reactive oxygen species (ROS) is a function of respiratory chain electron transport rather than of $\Delta\Psi$.

Our group proposed [4–6] that activation of ROS production in state 4, when protonic potential is high and respiration rate is limited by lack of ADP, is due to the fact that some transients of the respiratory chain electron transport, capable of reducing O_2 to $O_2^{-\bullet}$, such as CoQH$^\bullet$, become long-lived. It was assumed that mitochondria are equipped with a special mechanism ('mild uncoupling') preventing protonic potential from being too high.

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Abbreviations: $\Delta\Psi$, transmembrane electric potential difference; BSA, bovine serum albumin; cAtr, carboxyatractylate; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; kCh, 6-ketocholestanol (5α-cholestan-3β-ol-6-one); MOPS, morpholinopropanesulfonate; SF6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile

In this paper, we re-investigate the problem of mitochondrial ROS-protonic potential relationships. It was found that any decrease in the state 4 $\Delta\Psi$ resulted in a strong inhibition of H_2O_2 formation by mitochondria independently of whether activators (uncoupler or ADP+P_i) or inhibitors (malonate) of electron transport were added to cause a lowering of the $\Delta\Psi$. A threshold $\Delta\Psi$ value required for the elevation of H_2O_2 generation was found.

2. Materials and methods

Mitochondria were isolated from rat heart muscle. Cooled muscles, purified from fat and tendons, were minced and passed through a stainless steel press with holes about 1 mm in diameter. The tissue was then homogenized for 3 min with a Teflon pestle in a glass (Pyrex) homogenizer, the tissue:isolation mixture ratio being 1:8. After the first centrifugation (10 min, $700\times g$), the supernatant was decanted and filtered through gauze, then centrifuged (10 min, $16\,000\times g$). The sediment was suspended in 1 ml isolation mixture (250 mM sucrose, 10 mM MOPS, 1 mM EDTA, pH 7.4) supplemented with BSA (3 mg/ml). Then the mixture was centrifuged once more and washed with the isolation medium without BSA (10 min, $16\,000\times g$). The final mitochondrial sediment was suspended in the isolation mixture (70–90 mg protein/ml) and stored on ice.

In all the experiments shown in the figures, the above procedure was supplemented with the treatment of mitochondria by 2 mM H_2O_2 and 7 mM aminotriazole which were added to the supernatant before the second centrifugation.

Oxygen consumption was recorded with a Clark-type oxygen electrode and a Rank Brothers polarograph.

The $\Delta\Psi$ level was estimated using safranin O. The ratio of dye (nmol) to mitochondrial protein (mg) was 20:1. It should be noted that the safranin O response has been shown to linearly depend on $\Delta\Psi$ in the region 50–170 mV [7–9].

H_2O_2 generation was measured by the scopoletin/horseradish peroxidase method [10]. Scopoletin fluorescence response was calibrated with glucose/glucose oxidase [11] and by adding known amounts of H_2O_2. In Figs. 3 and 4, hydrogen peroxide production rates are expressed as percent of the rate which was obtained in state 4 mitochondria. This rate was 0.14 ± 0.04 nmol H_2O_2/min/mg protein.

Oligomycin, antimycin A, SF6847, and rotenone were dissolved in twice distilled ethanol. All reagents were first tested to avoid interference with the scopoletin/peroxidase assay.

EDTA, EGTA, rotenone, and scopoletin were from Serva; ADP, oligomycin, MOPS, fatty acid-free BSA, catalase, antimycin A, and aminotriazole were from Sigma; 30% H_2O_2 was from Merck.

3. Results

In the literature there is no consensus about the ability of mitochondria to produce measurable amounts of ROS in state 4. Some authors describe such a production [1,10–12], others fail to do this [13] or succeeded under special conditions (e.g. only in the case when high succinate concentration was used [14]).

In our hands, the rate of H_2O_2 production in state 4 was negligible but increased during storage of isolated mitochon-

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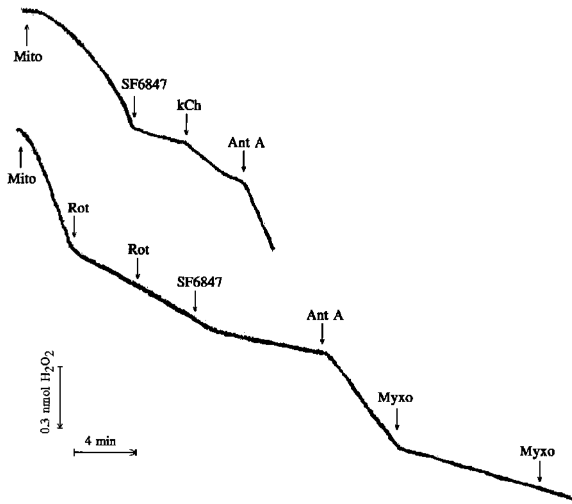

Fig. 1. Effects of uncoupler SF6847, recoupler 6-ketocholestanol (kCh), and respiratory chain inhibitors on H_2O_2 formation by rat heart mitochondria. Incubation mixture: 250 mM sucrose, 1 mM EGTA, 10 mM KCl, 10 mM MOPS (pH 7.3), 6 mM succinate, 1.2 μM scopoletin, horseradish peroxidase (10 U). Additions: rat heart mitochondria (1 mg protein/ml), 1 nM SF6847, 25 μM kCh, 1 μM antimycin (Ant), A, 4 μM rotenone (Rot), 2 μM myxothiazole (Myxo).

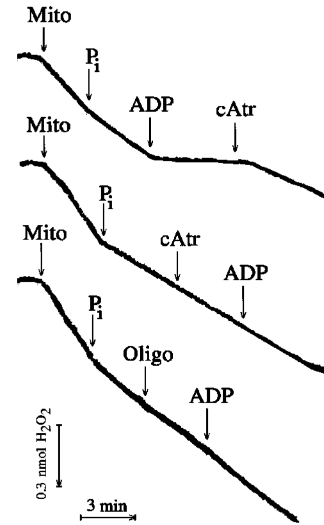

Fig. 2. Effects of P_i, ADP, carboxyatractylate and oligomycin on the mitochondrial production of H_2O_2. The incubation mixture was as in Fig. 1. Additions: rat heart mitochondria (1 mg protein/ml), 5 mM P_i, 100 μM ADP, 2 μM carboxyatractylate (cAtr), 2 μM oligomycin (Oligo).

dria. It was suggested that such an increase is due to exhaustion of endogenous mitochondrial antioxidants (e.g. reduced glutathione). To stimulate this process, we modified the procedure of isolation of mitochondria to include a short H_2O_2 treatment at the stage of washing of mitochondria with the BSA solution in the cold. The washing mixture was supplemented with 2 mM H_2O_2 and 7 mM aminotriazole, the catalase inhibitor. This modification gave mitochondria which always produce H_2O_2 at a measurable rate when oxidizing 6 mM succinate in state 4. The H_2O_2 treated mitochondria show a 3.5–4-fold stimulation of state 4 respiration by uncouplers.

Fig. 1 shows the effects of uncouplers and respiration inhibitors on the H_2O_2 production by the rat heart mitochondria pretreated with H_2O_2 and aminotriazole. It is seen (Fig. 1, upper curve) that a very low concentration of uncoupler (1×10^{-9} M SF6847) strongly inhibits state 4 H_2O_2 production. This effect seems to be due to the SF6847 uncoupling activity rather than to other possible effects of this compound since 6-ketocholestanol, a recoupler specific for SF6847 and carbonylcyanide phenylhydrazone derivatives [15,16], caused a transient activation of the H_2O_2 generation inhibited by SF6847. Subsequent antimycin A addition strongly stimulated the H_2O_2 production, this fact confirming the original observation of Boveris and Chance [1].

Hansford and coworkers [14] reported that ROS production by state 4 mitochondria in the presence of a high succinate concentration is arrested by rotenone. They assumed that NADH-CoQ reductase is responsible for all the ROS formation by the respiratory chain. We found (Fig. 1, lower curve) that rotenone is really inhibitory for production of H_2O_2 by state 4 succinate oxidation but a measurable rate (about 20%)

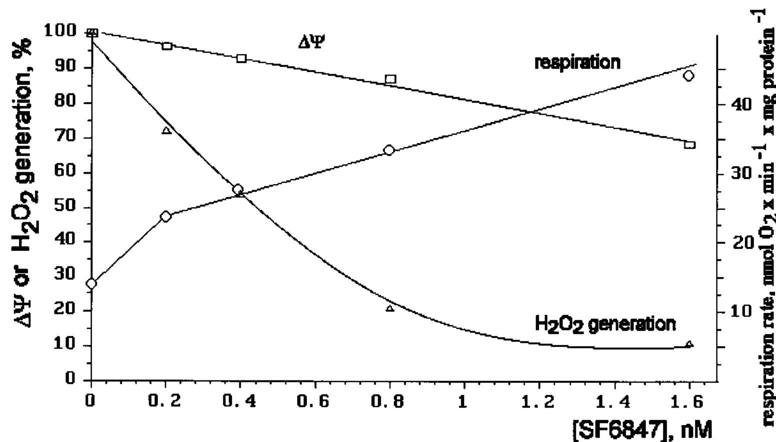

Fig. 3. Effects of SF6847 on respiration, ΔΨ, and H_2O_2 production by rat heart mitochondria. Incubation mixture: 250 mM sucrose, 1 mM EGTA, 10 mM KCl, 10 mM MOPS (pH 7.3), 6 mM succinate, rat heart mitochondria (0.8 mg protein/ml), 16 μM safranin O, 1.6 μM scopoletin, horseradish peroxidase (10 U). For H_2O_2 production measurements, safranin O was omitted, and mitochondrial protein was 1 mg/ml. H_2O_2 production and ΔΨ levels in samples without SF6846 were taken as 100%.

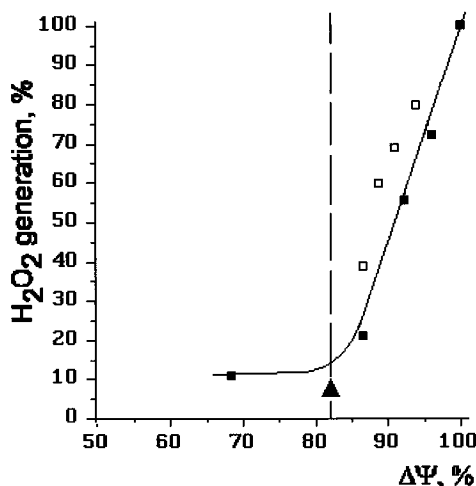

Fig. 4. H_2O_2 formation as a function of ΔΨ. Incubation mixture as in Fig. 3. The ΔΨ level was varied by adding different concentrations of SF6847 (black squares and solid line), malonate (white squares) or 100 μM ADP and 5 mM P_i (triangle). Dashed line, the state 3 ΔΨ level.

of H_2O_2 formation is still observed after addition of a saturating rotenone concentration. After rotenone, additions of SF6847 and antimycin A were inhibitory and stimulatory, respectively. In line with some previous observations (for review, see [17]), myxothiazol added after antimycin A strongly inhibited the H_2O_2 production.

The state 4-state 3 transition also inhibited H_2O_2 generation by heart mitochondria. Fig. 2 shows that addition of P_i and ADP almost completely abolished H_2O_2 production. Inhibitors of ATP synthase (oligomycin) or of ADP/ATP translocase (carboxyatractylate) prevented the ADP effect.

The effects of various concentrations of SF6847 on mitochondrial respiration, the ΔΨ level, and the rate of H_2O_2 generation are compared in Fig. 3. It is seen that H_2O_2 formation is the most sensitive parameter. For instance, 8×10^{-10} M SF6847 caused a small decrease in membrane potential (about 13%), a 2.2-fold increase in respiration rate and about 80% inhibition of H_2O_2 production.

A very steep dependence of H_2O_2 formation upon ΔΨ is illustrated by Fig. 4. The ΔΨ level was varied by adding ADP and P_i or various concentrations of SF6847 or malonate.

4. Discussion

The above data show that rat heart mitochondria exhausted in their antioxidants by a short H_2O_2 treatment at low temperature can be used as a model to study ROS generation in state 4. This generation was found to strongly depend upon the ΔΨ level on the mitochondrial membrane. As is seen in Fig. 4, there is a threshold ΔΨ value above which even a small increase in ΔΨ gives rise to a large stimulation of the H_2O_2 production by mitochondria. This threshold is above the state 3 ΔΨ level. Importantly, such relationships could be shown by varying the ΔΨ values by agents causing both stimulation and inhibition of the respiratory chain electron transport. Stimulation was caused by uncouplers (CCCP [12] or SF6847, this paper) or ADP+P_i ([18] and this paper). As for inhibition, it was induced by malonate ([2,3] and this paper). Thus, under the conditions used, ROS formation is a function of ΔΨ rather than of the electron transport rate. The described effect can be explained assuming that $CoQH^{\bullet}$ and some other electron transport intermediates competent in the one electron O_2 reduction become long lived when ΔΨ is sufficiently high. In the Q-cycle this is a consequence of inhibition by ΔΨ of the heme $b_l \rightarrow$heme b_h electron transport step. This results in complete reduction of b_l which now cannot oxidize $CoQH^{\bullet}$ formed in the Q-cycle [5]. Apparently, similar relationships are also inherent in NADH-CoQ reductase producing a large portion of ROS generated by state 4 mitochondria [14].

It is not surprising that some special conditions such as H_2O_2 and aminotriazole pretreatment and high succinate concentration are required to obtain measurable H_2O_2 generation by mitochondria in state 4; otherwise the cell would be poisoned by ROS in any case when ADP was exhausted. Such exhaustion occurs quite often, e.g. in muscle under the activity-to-rest transition. This is why we postulated [4–6] the existence in mitochondria of a mechanism (called 'mild uncoupling') which makes the maintenance of protonic potential impossible at a level essentially higher than the threshold value critical for fast ROS formation (Fig. 4). The mechanism in question was assumed to be under thyroid control being related to non-ohmic resistance of the mitochondrial membrane [5,6].

Non-ohmicity of the inner mitochondrial membrane (strong increase in H^+ conductance at high protonic potential levels) was suggested to be the first line of antioxygen defence of the aerobic eukaryotic cell. It fails to save the cell, e.g. when the respiratory chain is inhibited by antimycin A or (in the case of NAD-linked substrates) by rotenone. Such inhibition is caused by a rather wide range of hydrophobic xenobiotics.

On the other hand, the antimycin A-induced H_2O_2 generation is strongly inhibited by myxothiazol ([17] and this paper) which prevents the $CoQH_2$ oxidation to $CoQH^{\bullet}$ in the Q-cycle. The same effect is caused by cyanide [19] and extraction of cytochrome *c* ([17] and Simonyan, Skulachev and Starkov, in preparation). Moreover, intermembrane cytochrome *c* can (i) organize a bypass of the superoxide-producing steps of the respiratory chain via NADH-cytochrome b_5 reductase and cytochrome b_5 in some tissues, and (ii) oxidize $O_2^{-\bullet}$ to O_2, preventing H_2O_2 formation.

It was recently reported that release of mitochondrial cytochrome *c* is a physiological phenomenon involved in apoptosis [20–24]. On the other hand, we have suggested that ROS-induced apoptosis, a mechanism of purification of a tissue from ROS-producing cells, represents the last line of antioxygen defence of organisms [4–6]. Thus the following chain of events may be postulated to be involved in antioxygen defence: (i) mild uncoupling, (ii) release of cytochrome *c*, (iii) cytochrome *c*-mediated apoptosis.

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