Fatty acids as natural uncouplers preventing generation of $O_2^{\bullet-}$ and H_2O_2 by mitochondria in the resting state

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Received 31 July 1998

Abstract Both natural (laurate) and artificial (*m*-chlorocarbonylcyanide phenylhydrazone; CCCP) uncouplers strongly inhibit $O_2^{\bullet-}$ and H_2O_2 formation by rat heart mitochondria oxidizing succinate. Carboxyatractylate, an ATP/ADP antiporter inhibitor, abolishes the laurate inhibition, the CCCP inhibition being unaffected. Atractylate partially releases the inhibition by laurate and decelerates the releasing effect of carboxyatractylate. GDP is much less effective than carboxyatractylate in releasing the laurate inhibition of reactive oxygen species (ROS) formation. Micromolar laurate concentrations arresting the ROS formation cause strong inhibition of reverse electron transfer from succinate to NAD^+, whereas State 4 respiration and the transmembrane electric potential difference ($\Delta\Psi$) level are affected only slightly. It is suggested that (i) free fatty acids operate as natural 'mild uncouplers' preventing the transmembrane electrochemical H^+ potential difference ($\Delta\bar{\mu}_{H+}$) from being above a threshold critical for ROS formation by complex I and, to a lesser degree, by complex III of the respiratory chain, and (ii) it is the ATP/ADP-antiporter, rather than uncoupling protein 2, that is mainly involved in this antioxidant mechanism of heart muscle mitochondria.

Key words: Reactive oxygen species; Heart muscle mitochondrion; Fatty acid; ATP/ADP antiporter; Uncoupling protein

1. Introduction

The mitochondrial respiratory chain is known to form reactive oxygen species (ROS) by a mechanism strongly activated under resting (respiratory control or State 4) conditions when the electrochemical H^+ potential difference ($\Delta\bar{\mu}_{H^+}$) is high and the rate of electron transport is limited by discharge of $\Delta\bar{\mu}_{H^+}$ [1–3]. In this group, a threshold value of the electric $\Delta\bar{\mu}_{H^+}$ constituent ($\Delta\Psi$) critical for mitochondrial H_2O_2 formation was found [3]. The threshold $\Delta\Psi$ was slightly higher than the $\Delta\Psi$ level in the active (phosphorylating) state when ADP and phosphate were available and respiration was coupled to ATP synthesis. A hypothesis has been put forward that mitochondria possess a special mechanism of 'mild uncoupling' which keeps $\Delta\bar{\mu}_{H^+}$ below the threshold and, hence, prevents ROS generation by the mitochondrial respiratory chain in the resting state [4,5].

Free fatty acids were shown to be natural uncouplers mediating fast thermoregulatory uncoupling in muscles [6] and brown fat (for review, see [7]). We have postulated [8,9] that the mechanism of uncoupling by fatty acids can be described within the framework of the proton-conducting fatty acid cycle composed of (i) passive or ΔpH-driven influx of the protonated fatty acid to the mitochondrial matrix via the phospholipid bilayer of the inner mitochondrial membrane and (ii) electrophoretic efflux of the deprotonated fatty acid anion, mediated by mitochondrial anion carriers, namely uncoupling protein (UCP) [8,9], the ATP/ADP antiporter [8–12], the aspartate/glutamate antiporter [13,14] and the dicarboxylate carrier [15]. The fatty acid cycle concept was recently confirmed by several independent pieces of evidence (for reviews, see [7,16]).

In 1997 several laboratories published data indicating that various isoforms of uncoupling protein initially discovered in the brown fat mitochondria [17] are present in many animal and human tissues (reviewed in [7,16]) as well as in plants [18,19,16]. These observations strongly support the idea that fatty acids really play the role of natural uncouplers since the fatty acid-linked uncoupling seems to be the only function of UCP.

The suggestion that small concentrations of fatty acids operate as mild uncouplers preventing $\Delta\bar{\mu}_{H^+}$ from being above the ROS-generating threshold value [5] was recently confirmed by Casteilla and coworkers [20] who reported that GDP, a UCP inhibitor, caused a three-fold increase in the H_2O_2 production by mitochondria of non-parenchymal liver cells expressing UCP, but was completely ineffective on mitochondria from parenchymal liver cells deprived of UCP. Smaller but measurable GDP effects were described in brown fat, as well as in spleen and thymus mitochondria also possessing some UCP. CTP (a nucleotide which cannot affect UCP) was ineffective in stimulating the mitochondrial H_2O_2 production.

When this paper was in preparation, a publication by Vercesi and coworkers [21] appeared describing inhibition by fatty acid and stimulation by GDP of the H_2O_2 formation in potato mitochondria containing the plant UCP.

Below the effect of lauric acid on generation of $O_2^{\bullet-}$ and H_2O_2 by rat heart muscle mitochondria will be described. It is found that low (micromolar) concentrations of laurate arrest superoxide and H_2O_2 production in these mitochondria oxidizing succinate under resting conditions. This effect correlates with inhibition of the reverse electron transfer from succinate to NAD^+. The laurate inhibition of H_2O_2 production is

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Abbreviations: $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical H^+ potential difference; $\Delta\Psi$, transmembrane electric potential difference; Atr, atractylate; cAtr, carboxyatractylate; BSA, bovine serum albumin; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; ROS, reactive oxygen species; UCP, uncoupling protein

PII: S0014-5793(98)01073-4

abolished by carboxyatractylate (cAtr), a specific inhibitor of the ATP/ADP antiporter.

2. Materials and methods

Mitochondria were isolated from rat heart muscle as described previously [3]. The isolation procedure included treatment of mitochondria with 2 mM H_2O_2 and 10 mM aminotriazole to inhibit matrix catalase and glutathione peroxidase and to exhaust the matrix pool of reduced glutathione.

Oxygen consumption was measured using a Clark-type oxygen electrode and a Rank Brothers polarograph.

$\Delta\Psi$ level estimations were carried out using safranin O [3].

H_2O_2 generation was measured by the scopoletin/horseradish peroxidase method [1].

Superoxide generation was assayed by ESR spectroscopy with Tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid) as a spin trap. The samples were placed into a gas-permeable thin wall teflon capillary (Norell, USA) and kept under conditions of continuous aeration at 25°C. ESR measurements were carried out in the X-Band E-109E spectrometer (Varian, USA) with 100 kHz modulation frequency, 0.5 G modulation amplitude, 5 mV microwave power, 5 G/min scan rate (time constant, 0.032 s).

The rate of the reverse electron transfer was estimated by measuring the increase in the level of fluorescence of endogenous mitochondrial nicotinamide nucleotides after addition of succinate.

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

Lauric acid, antimycin A, and rotenone were dissolved in twice distilled ethanol; fatty acid-free BSA and carboxyatractylate were dissolved in bidistilled water; scopoletin was dissolved in dimethylsulfoxide.

3. Results

In Fig. 1 it is shown that addition of 5 μM laurate strongly inhibits H_2O_2 production by mitochondria in the resting state (no ADP and phosphate are present). This inhibition is immediately and completely abolished by BSA or cAtr. Atr also activates H_2O_2 production but partially. Addition of cAtr after Atr caused complete deactivation of H_2O_2 formation, but in this case a lag phase takes place.

The data of Fig. 1 are consistent with the assumptions that (i) a small amount of laurate decreases $\Delta\overline{\mu}_{H^+}$ below the threshold level required to stimulate H_2O_2 production in the respiratory chain and (ii) this effect is mediated by the ATP/ADP antiporter. In this context, it should be noted that cAtr completely and Atr partially reverse the fatty acid-induced uncoupling, and Atr added before cAtr decelerates development of the cAtr effect [10].

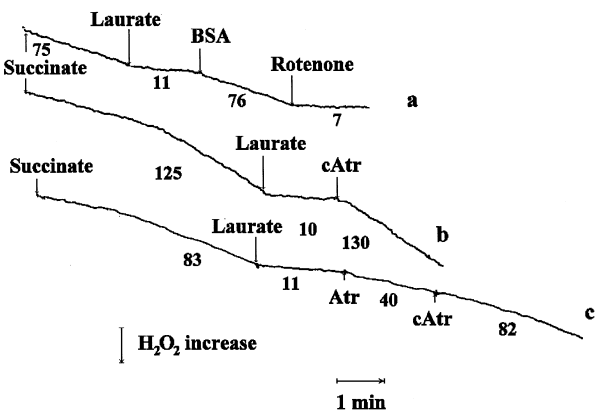

Fig. 1. Effect of laurate on H_2O_2 generation by rat heart mitochondria. The incubation medium contained 0.25 M sucrose, 10 mM MOPS (pH 7.3), 0.5 mM EGTA, 10 mM KCl, horseradish peroxidase (25 U), 1.2×10^{-6} M scopoletin, 5 mM succinate and rat heart mitochondria (1 mg protein/ml). Additions: 5×10^{-6} M laurate; BSA, 0.5 mg/ml; 2×10^{-7} M rotenone; 2×10^{-6} M cAtr; 6×10^{-6} M Atr. Figures near curves are rates of H_2O_2 production (pmol of H_2O_2/mg protein/min).

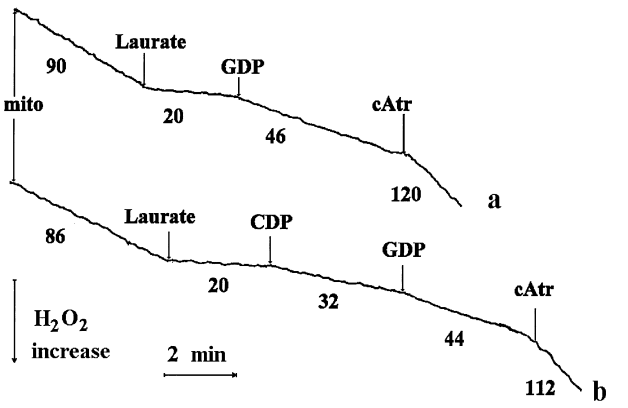

Fig. 2. Effect of cAtr and GDP on H_2O_2 generation by heart muscle mitochondria from rats exposed for 2 days to 6°C. For conditions, see Fig. 1. GDP and CDP concentrations were 0.5 mM.

Exposure of rats for two days to 6°C, which according to Boss et al. [23] causes a 4.3-fold increase in the UCP2 mRNA level in heart muscle, allowed us to find some GDP effect. It was variable but always much smaller than that of cAtr. CDP was less efficient than GDP. An example of the experiment on mitochondria from the cold-exposed rats is shown in Fig. 2. It is noteworthy that the combined action of GDP and cAtr results in a faster H_2O_2 production rate than before the laurate addition. This is apparently due to the fact that mitochondria from the cold-exposed animals have an increased level of endogenous fatty acids [6] that may lower the basal rate of the ROS formation in the cAtr- and GDP-sensitive fashion.

In Fig. 3 effects of various laurate concentrations on rates of H_2O_2 production, reverse electron transfer, $\Delta\Psi$ and State 4 respiration are compared. It is seen that inhibition of H_2O_2 formation occurs at laurate concentrations similar to those causing inhibition of the reverse electron transfer. This effect is accompanied by slight stimulation of respiration and small $\Delta\Psi$ decrease, which is in line with data obtained in this group in experiments with an artificial uncoupler [3].

In Fig. 4 effects of natural (laurate) and artificial (*m*-chlorocarbonylcyanide phenylhydrazone; CCCP) uncouplers on the superoxide level in mitochondria are compared. One can see that both uncouplers strongly lower this level and cAtr abolishes the effect of laurate, but not of CCCP. These relationships confirm our previous observations that the ATP/ADP antiporter is not involved in uncoupling by CCCP [10]. Moreover, the above data indicate that the uncoupler-

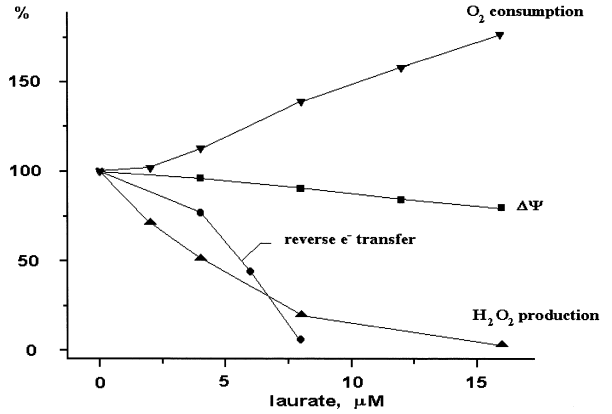

Fig. 3. Effect of laurate on H_2O_2 generation, reverse electron transfer, membrane potential and O_2 consumption by mitochondria. For conditions, see Fig. 1.

induced inhibition of H_2O_2 formation is localized at the superoxide-generating step, i.e. before superoxide dismutase.

4. Discussion

The data show that uncoupling caused by low concentrations of free fatty acids prevents formation of reactive oxygen species in heart muscle mitochondria. This effect is mediated mainly by the ATP/ADP antiporter as judged from the effect of cAtr. As to UCP2, which is also competent in facilitating the fatty acid-induced uncoupling, it usually plays a minor role since the antiporter inhibitor cAtr appears to be much more efficient than the UCP inhibitor GDP. However, the UCP role seems to increase under conditions where uncoupling potency increases, e.g. under cold adaptation (Fig. 2) accompanied by strong increase in the UCP2 level in heart muscle [23]. As was quite recently found in this group [24], a 48-h exposure of rats to 6°C resulted in appearance of the coupling effect (the $\Delta\Psi$ increase) of GDP on rat heart mitochondria, CDP being ineffective. In the control animals, both GDP and CDP were without influence. As for cAtr, it increased $\Delta\Psi$ in both exposed and control groups, the effect being larger in the former case.

Correlation of the inhibition of (i) reverse electron transfer and (ii) H_2O_2 formation (Fig. 3) is noteworthy. This fact is in line with our previous observation that rotenone causes a 80–90% inhibition of the H_2O_2 generation in State 4 mitochondria oxidizing succinate (see Fig. 1, curve a, and [3]). Previously a strong inhibitory effect of rotenone on ROS formation by succinate-oxidizing mitochondria was revealed by Hansford et al. [25]. In both Hansford's and our experiments, the NAD^+-linked substrates failed to substitute for succinate in supporting the ROS formation and what is more, they inhibit the succinate-induced generation of H_2O_2 (S.S. Korshunov, in preparation). In this connection, the original observation by Hinkle et al. [26] should be mentioned. They showed that addition of succinate and ATP to the cyanide-treated beef heart submitochondrial particles resulted in H_2O_2 formation which was sensitive to rotenone, rutamycin and uncouplers, being unaffected by antimycin A. It was concluded that production of H_2O_2 is somehow coupled to reverse electron transfer from succinate to complex I.

All the above data would be explained within the framework of Vinogradov's suggestion [27] that the forward and reverse electron transfers in complex I of the respiratory chain proceed via different pathways. If this were the case, one would assume that the reverse pathway is involved, whereas the forward pathway is not involved, in H_2O_2 formation by complex I. However, this cannot account for the facts reported in the mentioned paper by Hinkle et al. that (i) NADH added to the cyanide-treated particles without ATP supports the same rate of H_2O_2 formation as the ATP-dependent reverse electron transfer from succinate, and (ii) this rate is not inhibited but rather slightly stimulated by 5×10^{-6} M rotenone. The mechanism of ROS generation by the com-

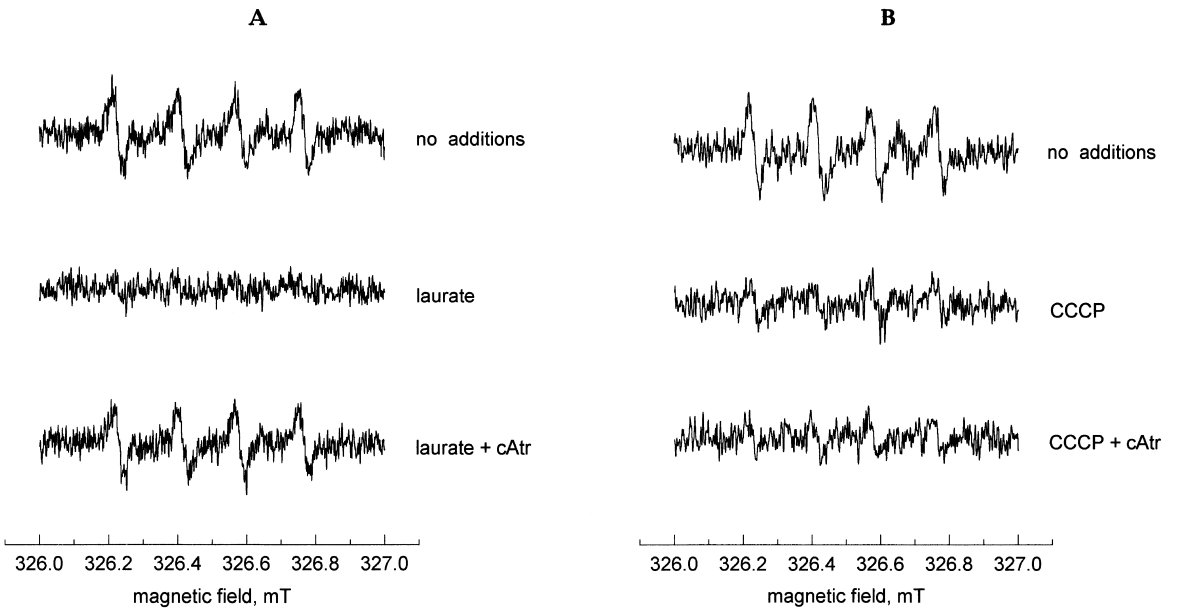

Fig. 4. Superoxide level in rat heart mitochondria. The incubation medium contained 0.25 M sucrose, 10 mM HEPES (pH 7.4), 0.2 mM EGTA, 4 mM KH_2PO_4, 2 mM $MgCl_2$, 10 mM Tiron, and mitochondria (2 mg protein/ml). Subsequent additions: 5 mM succinate; 4×10^{-6} M laurate (A) or 4×10^{-7} M CCCP (B); 2×10^{-6} M cAtr.

plex I segment of the respiratory chain is now under investigation in our group.

Acknowledgements: This work was supported by RFBR Grants N 95-04-12799, N 96-04-00022, and N 96-15-98070.

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