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Fast cold-induced activation of the external pathway of NADH oxidation in liver mitochondria of hyperthyroid rats

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The exposure of a cold-adapted rat for 15 min at 4°C results in the appearance of measurable oxidation of added NADH via the external amytal- and antimycin-A- resistant pathway in liver mitochondria. This effect increases if the animal has been treated with thyroid gland preparation for 4–5 days. In vitro, the addition of Mg^{2+} or increase in the tonicity of incubation mixture suppresses external NADH oxidation. Addition of 70 mg · ml^{-1} serum albumin or 70 mg · ml^{-1} polyvinylpyrrolidone in the presence of Mg^{2+} and addition of 20 μM palmitic or 20 μM oleic acids induces to some extent external NADH oxidation in the mitochondria of control (nontreated) and of cold-adapted, thyroidgland-preparation-treated rats. It is concluded that hyperthyroid rats can be used as a model of cold-induced initiation of the external pathway of NADH oxidation. A relation between the increase of exogenous NADH oxidation and phospolipase A_2 activity in liver mitochondria is discussed.

Introduction

The rate of exogenous NADH oxidation in intact rat liver mitochondria is very low, but the addition of cytochrome *c* stimulates NADH oxidation via the external pathway, resistant to amytal and antimycin A [1,2]. Beyer explains a decrease in the sensitivity of respiration of liver pieces of cold-adapted rats to amytal by enhanced exogenous NADH oxidation in mitochondria and/or microsomes [3]. According to Skulachev, the mechanism of the cold-adaptation-induced activation of the external pathway is in the partial desorption of cytochrome *c* pool from the inner mitochondrial membrane into intermembrane space. As a result, this cytochrome *c* can form a link between the NADH-cytochrome b_5 reductase of the outer membrane and the cytochrome oxidase of the inner mitochondrial membrane [4].

Later we demonstrated that a short cold exposure of cold-adapted rats results in the activation of amytal- and antimycin-A resistant (but mersalyl- and cyanide-sensitive) pathway of exogenous NADH oxidation [5]. To obtain this effect a long-time cold adaptation (about a month) and some special conditions (exposure of 120–150 g rats to cold for 6–8 h daily and keeping the animal before decapitation at about 29°C and then for 15 min at 2–4°C) described in Refs. 5 and 6 were needed.

Yet at these conditions the rates of exogenous NADH oxidation in different series of experiments varied, suggesting the influence of some unknown agent.

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

In this connection, data should be mentioned on the role of thyroid hormones in the long-term adaptation to cold, and the potentiation of catecholamine effect (for a review, see Ref. 7), and on thyroxine-induced mitochondrial swelling [8,9]. The swelling of mitochondria in a saline medium was found to increase the rate of external NADH oxidaton [6,10]. A suggestion about the activation of the external pathway of NADH oxidation under hyperthyroidism was published earlier [9].

In this paper we have shown that the thyroid gland preparation treatment decreases the adaptation time required for pronounced external pathway activation upon cold exposure and that a 10-day treatmentof the rat with thyroid gland preparation per se increases exogenous NADH oxidation.

Methods

Mitochondria were isolated and suspended in a medium containing 0.3 M sucrose, 5 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5. The liver was homogenised manually with a teflon pestle (the medium-to-tissue ratio was 8 : 10. The first centrifugation was for 10 min at $800 \times g$, the second for 10 min at $12\,000 \times g$. Experiments were also performed with mitochondria sedimented from the supernatant by centrifugation for 10 min at $4000 \times g$; the pellet was resuspended in the isolation medium and then centrifuged for 10 min at $12\,000 \times g$. This mitochondrial fraction is referred to in the text as 'washed' mitochondria. The mitochondrial sediment isolated from 5 g of the liver was suspended in 0.4 ml of the isolation medium, the final mitochondrial suspension contained about 80–100 mg protein/ml.

Oxygen consumption was measured in a Clark-type oxygen electrode and a LP-60 polarograph at 37°C in a 1 ml chamber. An isotonic incubation mixture containing 120 mM KCl/20 mM NaCl/4 mM KH_2PO_4/4 mM glutamate/1 mM malate (pH 7.0 or 7.4) and an hypotonic incubation mixture containing 70 mM KCl/5 mM KH_2PO_4 (pH 7.0 or 7.4) were used. The polarographic cell contained 2–3 mg of mitochondrial protein per ml. Protein was determined by the Biuret method.

Female white rats (120–150 g) were studied. To induce hyperthyroidism, thyroid gland preparation was added to bread (300 mg per 100 g of rat weight) [11]. The content of thyroxine and 3,5,3'-triiodothyronine in the thyreoidine (Thyroid gland preparation) varied around 0.04% and 0.015% respectively [12]. The control group was kept at 22–26°C. The cold-treated rats were exposed for 8–10 h daily at 2–4°C and at 22–26°C for the rest of the day.

Glutamate, amytal, Hepes (from Serva) palmitic and oleic acids, the V fraction of bovine serum albumin (essentially fatty-acid-free), antimycin A (from Sigma), NADH (from Reanal), polyvinylpyrrolidone, M_r 44000 (from BDH) were used. 2,4 dinitrophenol and sucrose were crystallized in ethanol. Palmitic and oleic acids were dissolved in ethanol.

Results

The addition of NADH to rat-liver mitochondria, incubated in the presence of amital and antimycin A, does not change the rate of oxygen consumption in the control group. 5-day-cold-adaptation and a subsequent short-term exposure to cold before decapitation result in the appearance of measurable oxidation of added NADH via external amytal- and antimycin-resistant pathway in liver mitochondria (Table I).

The rate of NADH oxidation via the external pathway was about 1,7-times higher than that in control group, if the animals were kept for 8 to 10 h daily at 2–4°C for 4–5 days (and at 22–26°C for the rest of the day) and treated with thyreoidine (300 mg per 100 g of the weight); and if at least 3 h before decapitation, the rats were kept at 26°C. The exposure of a rat of this group for 15 min at 4°C before decapitation results in a further increase (1.5-fold) in the rate of exogenous NADH oxidation (Table I).

The activation of external pathway of NADH oxidation was observed if the rats were administered thyreoidine for 10 days and all this time kept at 22–26°C. In this case the thyreoidine treatment results in an increase of the respiration rate in the presence of glutamate and malate and also upon addition of 25 μM 2.4 dinitrophenol. The NADH oxidation rate via the external pathway increased

TABLE I

EFFECT OF 5 DAYS THYROID-GLAND-PREPARTION- AND COLD TREATMENT AND 15 MIN COLD EXPOSURE BEFORE DECAPITATION ON THE RESPIRATION OF LIVER MITOCHONDRIA

Incubation mixture: 70 mM KCl/5 mM potassium phosphate/4 mM glutamate/1 mM malate (pH 7.4). Additions: $2 \cdot 10^{-5}$ M dinitrophenol/2 mM amytal/$1.8 \cdot 10^{-6}$ M antimycin A/1 mM NADH. 'Control group': animals were kept at 24–26°C. 'Cold-treated → 3 h at 26°C → 15 min at 4°C' group: animals were exposed for 8–10 h at 2–4°C daily and at 24–26°C for the rest of the day; 3 h before decapitation they were kept at 26°C and then placed in cold (4°C) for 15 min. 'Thyreoidine + cold-treated → 3 h at 24°C' group: the animals were exposed for 8–10 h at 2–4°C daily and at 24–26°C for the rest of the day and received thyroid gland preparation 200 mg per 100 g weight daily; 3 h before decapitation they were kept at 24–26°C. 'Thyroid gland preparation + cold-treated → 3 h at 24°C → 15 min at 4°C' group: the animals were exposed for 8–10 h at 2–4°C daily and at 24–26°C for the rest of the day and received thyroid gland preparation 300 mg per 100 g weight daily; 3 h before decapitation they were kept at 24–26°C and then placed in cold (4°C) for 15 min. The number of animals is given in parentheses.

Animals	Oxygen consumption (nmol O_2 per mg protein per min)			
		DNP	DNP, amytal, antimycin A	DNP, amytal, antimycin A, NADH
Control	9.1 ± 0.8 (8)	41.0 ± 3.2 (8)	2.7 ± 0.3 (8)	3.0 ± 0.2 (8)
Cold-treated → 3 h at 26°C → 15 min at 4°C	10.1 ± 1.1 (6)	40.1 ± 0.8 (6)	2.2 ± 0.3 (6)	4.2 ± 0.3 (6)
Thyreoidine + cold-treated → 3 h at 26°C	15.0 ± 1.1 (7)	50.6 ± 5.3 (7)	4.4 ± 0.5 (7)	5.2 ± 0.6 (7)
Thyreoidine + cold-treated → 3 h at 26°C → 15 min at 4°C	18.5 ± 1.9 (8)	56.6 ± 5.7 (8)	4.6 ± 0.9 (8)	8.2 ± 0.7 (8)

most of all; its ratio to the uncoupal respiration rate grew approx. 2-fold (in 2.2 ± 0.2 (10)).

Subsequently, we compared the control group with the other in which the rats were cold-adapted and administered thyroid gland preparation (for 5 days) and subjected to a 15 min cold exposure

TABLE II

THE ROLE OF THE ION COMPOSITON OF AN INCUBATION MIXTURE FOR THE THYROID-GLAND-PREPARATION AND COLD-TREATMENT-INDUCED ACTIVATION OF EXTERNAL NADH OXIDATION

Animal groups as in Table I. All the samples contain 4 mM glutamate/1 mM malate, (pH 7.0). Additions: $2 \cdot 10^{-5}$ M dinitrophenol (DNP)/2 mM amytal/$1.8 \cdot 10^{-6}$ M antimycin A/1 mM NADH. In parentheses is given the number of animals.

Animals	Oxygen consumption (nmol O_2 per mg^{-1} protein per min)							
	70 mM KCl/5 mM potassium phosphate				120 mM KCl/20 mM NaCl/4 mM potassium phosphate			
	–	DNP	DNP, amytal, antimycin A	DNP, amytal, antimycin A, NADH	–	DNP	DNP, amytal, antimycin A	DNP, amytal, antimycin A, NADH
Control	12.7 ± 0.7 (6)	57.8 ± 5.7 (6)	2.7 ± 0.3 (6)	3.5 ± 0.4 (6)	10.1 ± 1.2 (6)	47.8 ± 3.8 (6)	2.0 ± 0.3 (6)	2.6 ± 0.2 (6)
Thyreoidine + cold-treates → 3 h at 26°C → 15 min at 4°C	23.7 ± 2.2 (6)	50.7 ± 8.8 (6)	2.1 ± 0.4 (6)	11.3 ± 0.6 (6)	20.1 ± 2.3 (6)	54.1 ± 7.1 (6)	3.3 ± 0.9 (6)	6.1 ± 0.4 (6)

before decapitation by varying the incubation mixture. When mitochondria were incubated in an isotonic mixture, the rate of NADH oxidation via the external pathway decreased but the difference between the groups was still pronounced (Table II). With the pH of the hypotonic medium down to 7.0, the rate of NADH oxidation increased about 1.5-fold in the mitochondria of the experimental group, while the increase was less in the control group (Table II).

The addition of 0.5–2 mM Mg^{2+} was found to decrease the NADH oxidation rate (Table III). In this case too, the difference between exogenous NADH oxidation rates in the compared groups remained significant.

A comparison of the data given in Table I and II also indicates that oxygen consumption rates in the presence of amytal and antimycin A are independent of NADH oxidation rates. This conclusion was corroborated in experiments upon addition of fatty acids.

20 μM palmitate of 20 μM oleate increased the external pathway of NADH oxidation approx. 1.5-times in the mitochondria of the experimental and control groups but caused a decrease in mitochondrial respiration in the presence of amytal and antimycin. The effect of fatty acids was the same or more pronounced in experiments with 'washed' mitochondria (Fig. 1).

Albumin (70 mg/ml) increased the rate of

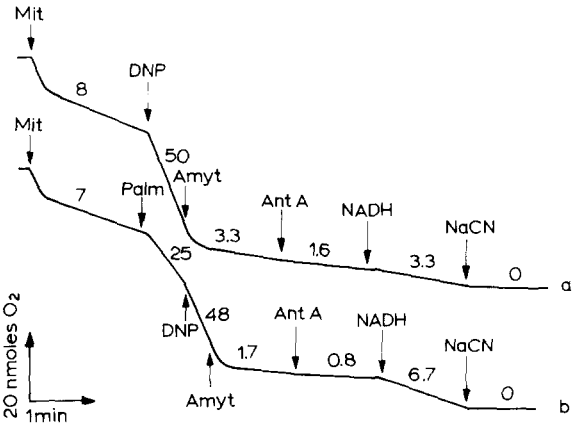

Fig. 1. The effect of palmitate on the respiration of 'washed' liver mitochondria of the control group. For mitochondria washing conditions, see Methods. The incubation mixture contained 70 mM KCl/5 mM potassium phosphate/4 mM glutamate/1 mM malate (pH 7.4); 37°C. At specified times indicated by arrows the mitochondria (Mit), 20 μM palmitate (palm) $2 \cdot 10^{-5}$ M dinitrophenol (DNP), 2 mM amytal (Amyt), $1.8 \cdot 10^{-6}$ antimycin A (Ant A), 1 mM NADH (NADH) and 1 mM NaCN (NaCN) were added. Figures above the polarograms are oxygen consumption rates in nmoles O_2 per mg protein per min.

NADH oxidation in experiments with isotonic incubation mixture containing 1.5 mM Mg^{2+} (Fig. 2). The same effect is produced by 70 mg/ml polyvinylpyrrolidone (the data are not shown). If

TABLE III

THE EFFECT OF Mg^{2+} ON THE THYROID-GLAND-PREPARATION- AND COLD-TREATMENT ACTIVATION OF EXTERNAL PATHWAY OF NADH OXIDATION

Animal groups as in Table I. Incubation mixture: 70 mM KCl/5 mM potassium phosphate/4 mM glutamate/1 mM malata (pH 7.0). Additions: $2 \cdot 10^{-5}$ M dinitrophenol (DNP/2 mM amytal/$1.8 \cdot 10^{-6}$ M antimycin A/1 mM NADH. Results are expressed as a mean of five experiments ± S.E.M.

Animals	Oxygen consumption (nmol O_2 per min per mg protein)					
	Without $MgCl_2$		0.5 mM $MgCl_2$		2 mM $MgCl_2$	
	DNP	DNP + amytal + + antimycin A + NAD	DNP	DNP + amytal + + antimycin A + NADH	DNP	DNP + amytal + + antimycin A + NADH
Control	45.1 ± 2.0	3.8 ± 2.2	45.3 ± 2.4	2.9 ± 0.3	49.1 ± 2.2	2.3 ± 0.2
Thyreoidine + cold-treated → 3 h at 26°C → 15 min at 4°C	53.2 ± 5.2	11.8 ± 1.2	53.3 ± 6.9	8.6 ± 1.9	60.3 ± 0.7	6.8 ± 0.9

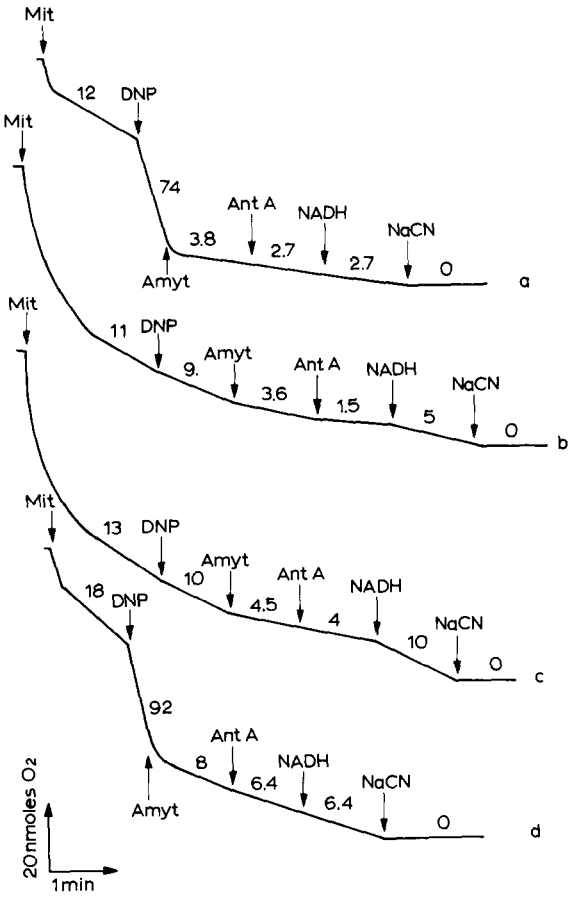

Fig. 2. The effect of bovine serum albumin on the respiration of 'washed' rat liver mitochondrial of control (a, b) and thyroid-gland-preparation and cold-treated rats (c, d). For mitochondria washing conditons, see Methods. Thyroid-gland-preparation and cold-treatment as in Table I. The incubation mixture contained 120 mM KCl/30 mM NaCl/4 mM potassium phosphate/4 mM glutamate/1 mM malate/1.5 mM $MgCl_2$ (pH 7.0, 37°C). At specified times indicated by an arrow the miltochondria (M.t), $2 \cdot 10^{-5}$ M DNP (DNP), 2 mM amytal (Amyt), $1.8 \cdot 10^{-6}$ M antimycin A (Ant A), 1 mM NADH (NADH) and 1 mM NaCN (NaCN) were added; (a) and (d), without albumin, (b) and (c), incubation mixture contained 70 mg/ml albumin. Figures above the polarogram are oxygen consumption rates in nmol O_2 per mg protein per min.

polyvinylpyrrolidone was added after 0.5 mM EGTA, it has a diminished effect.

Discussion

Table I indicates that if the rats, simultaneously with cold exposure, are given thyroid gland preparation, then in just 4–5 days, a 15-min-exposure to cold activates the external pathway of NADH oxidation. The same activation is observed if thyroid gland preparation is administered for a longer time and the animals are kept at room temperature.

If mitochondria are incubated in conditions closer to intracellular (an isotonic medium with Mg^{2+}), the external pathway is reduced appreciably (Fig. 2d). Yet the properties of isolated and cell mitochondria may differ greatly. Thus the mitochondrial intermembrane space is considerably less (in relation to the matrix volume) within the cell than in isolated mitochondria [13], while the decreasing distance between the mitochondrial membranes enhances the external pathway [14]. The external pathway increase in the presence of 70 mg/ml albumin or 70 mg/ml polyvinylpyrrolidone appears to be associated with this effect. The decrease in uncoupled respiration in this case (Fig. 2 b and c) may be due to a higher oncotic pressure in the incubation mixture. However, the EGTA effect in difficult to explain in this case. The mitochondrial ultrastructure in the presence of EGTA and polyvinylpyrrolidone is now under study.

It is noteworthy that the conditions for external pathway activation [6] are nearly identical with those under which a large Ca^{2+} efflux from liver mitochondria is observed [15,16]. Both processes are activated by phosphate, pH decrease and some other factors, inducing mitochondrial swelling; and they are inhibited by local anaesthetics, Mg^{2+}, etc. These effects are linked to the activation or inhibition of mitochondrial phospholipase A_2 [6,15,16]. A parallel increase in mitochondrial phospholipase activity in tissue and exogenous NADH oxidation in isolated mitochondria is shown for cold-treated rats by measuring the cardiolipin lysoforms in the liver pieces [22]. There is evidence on a phospholipase A_2 activity increase during hyperthyreosis [17]. This effect can be anticipated during a changes in the mitochondrial phospholipid composition.

The energy coupling of liver mitochondria is believed to have a relatively low sensitivity to fatty acids [18]. However, this holds true only for incubation in conditions under low phospholipase A_2 activity. In other cases (for example, when

mitochondria are incubated in a hypotonic saline medium with $8 \cdot 10^{-6}$ M $CaCl_2$) even as much as $5 \cdot 10^{-6}$ M oleate induces an increase in the respiration rate in the presence of glutamate and malate or exogenous NADH [6]. The present investigation demonstrates that a similar effect is produced upon $2 \cdot 10^{-5}$ M oleate or $2 \cdot 10^{-5}$ M palmitate addition (Fig. 1).

Apart from the uncoupling effect [19] fatty acids can increase the permeability of the inner mitochondrial membrane for potassium [20]. A low concentration of fatty acids induced a mitochondrial swelling due to the activation of phospholipase A_2 [21]. There is evidence that phospholipase A_2 is responsable for the mitochondrial swelling which ativates the external pathway of NADH oxidation [6,10].

These data suggest that in our experimental conditions, the addition of palmitic or oleic acids results in the activation of phospholipase A_2 and that this activation is responsible both for the stimulation of respiration (an increase in the ion conductivity of the inner mitochondrial membrane) and for the increase of external NADH oxidation (induced by mitochondrial swelling).

It should be noted that the study of energy coupling and swelling of isolated mitochondria may yield only a set of possible characteristics for these mitochondria in tissue as isolation procedures may have a pronounced effect [23]. One may speculate that activated external NADH oxidation (which bypasses the first and second energy-coupling sites of the respiratory chain) is involved in the production of extra heat under an abrupt decrease in the ambient temperature.

In conclusion, an increase in exogenous NADH oxidation in liver mitochondria was demonstrated under a combined thyroid gland preparation and cold treatment of rats. This system may be of use as a model for studying the mitochondrial external NADH oxidation pathway in the liver cell.

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