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Respiratory Characteristics of Rat Liver Mitochondria Depend on the Duration of Moderate Hypothermia

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The development of pathological or compensatory-and-adaptive reactions in homoeothermic animals during various periods of hypothermia can be caused by shifts in the respiratory functions of the mitochondria. Short-term hypothermia promoted an increase in the rates of the glutamate- and succinate-dependent respiration of mitochondria. Phosphorylation rate increased as well, while oxidative phosphorylation coefficient (P/O), respiratory control, and 2,4-DNP sensitivity depended. Changes in respiratory characteristics in the dynamics of prolonged hypothermia depends on the type of substrate. Prolongation of hypothermia to 1 h was associated with further intensification of succinate-dependent respiration, decrease in P/O and respiratory control, while prolongation of hypothermia to 3 h led to their normalization. The majority of respiratory characteristics of glutamate-dependent respiration did not change under these conditions and their levels were the same as during short-term hypothermia.

Key Words: *moderate hypothermia; rats; liver; mitochondria; respiratory characteristics*

Therapeutic hypothermia is widely used in practical medicine for prevention of the risks of hypoxic, ischemic, and reperfusion damage to organs and tissues and for their protection [13,14]. The protective effects of hypothermia are mainly explained by deceleration of metabolism under conditions of low body temperature, due to lower tissue need in oxygen and glucose [12]. Moderate prolonged hypothermia is most often used for this purpose [10]. However, along with positive effects, moderate hypothermia can cause numerous untoward shifts limiting its use. Hypothermia leads to metabolic acidosis, increases plasma viscosity, impairs erythrocyte deformability and impedes their passage through microcapillaries, and increases hemoglobin affinity for oxygen. Oxygen deficit in tissues resulting from these processes can cause generation of active oxygen and nitrogen species followed by the development of oxidative stress [13,15].

We have previously shown [5] that the intensity of oxidative modification of proteins and LPO in rat blood depend on the duration of hypothermia: short-term exposure leads to their intensification, while long-term (3 h) exposure leads to their normalization. The mechanisms underlying these rapid (within just 3 h) and dramatic shifts in the blood levels of intermediates of free radical oxidation remain not quite clear.

As we know, the main sources of ROS in the cell are electron-transporting chains of mitochondria [1], and hence, studies of their functional status seem to be the best approach to thorough studies of the mechanisms of ROS generation and utilization during various periods of hypothermic exposure. Respiratory characteristics of mitochondria, such as respiration rate, oxidative phosphorylation coefficient (P/O), respiratory control, and sensitivity to protonophores, are sensitive indicators of these shifts.

We study the relationship between the respiratory parameters of rat liver mitochondria and the duration of moderate hypothermia.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats (200-220 g; n=32) with due consideration of Order

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No. 199n of the Ministry of Health of Russia (On Approval of Rules for Good Laboratory Practice; April 1, 2016). Hypothermia was induced by external cooling of animals in Plexiglas boxes with a shell through which cold (5°C) water was circulating. Body temperature was reduced to 30°C over 30 min at a velocity of 0.28°C/min (short-term hypothermia) and was maintained at this level during 1 and 3 h (prolonged moderate hypothermia).

Mitochondria were isolated by differential centrifugation in a sucrose density gradient [7]. The isolation medium contained 0.25 M sucrose, 5 mM HEPES, 0.5 mM EDTA, and 0.1% BSA (pH 7.4).

Polarographic registration of respiration was carried out on an Expert 001 analyzer with Clarke's electrode. The incubation medium contained 0.32 M sucrose, 3 mM HEPES, 0.25 mM EDTA, 1 mM $MgCl_2$, and 13 mM KCl (pH 7.2). Succinate-dependent respiration was studied in incubation medium with 1.5 μM rotenone (Sigma), inhibitor of complex I of electron transport chain (ETC). The initial rate of mitochondrial respiration (V1) was recorded after addition of mitochondrion suspension (0.5 mg protein/ml) to the incubation medium. Then, respiration rates in different metabolic states (after Chance) were then registered: V2 — respiration rate in metabolic state 2 (after addition of substrates: 3 mM potassium succinate or 5 mM potassium glutamate); V3 — respiration rate in metabolic state 3 (after addition of 100 μM ADP and 1 mM KH_2PO_4); V4 — respiration rate in state 4 (after the added ADP was over); and V5 — respiration rate in state 5 (after addition of uncoupler 2,4-dinitrophenol (2,4-DNP), 100 μM).

The results were processed by ANOVA unifactorial analysis of dispersions using Statistica 8.0 software (StatSoft, Inc.). The differences between compared groups were considered to be significant ($p<0.05$) if the estimated empirical values of F (Fisher's test) surpassed the critical values. The data were presented as the $M\pm m$.

RESULTS

The respiratory characteristics of the rat liver mitochondria in health and under conditions of moderate hypothermia of different duration were studied. The respiratory rate of isolated mitochondria on endogenous substrates (V1) was very low (Fig. 1). Addition of glutamate into the wells stimulated the respiration (V2). Addition of ADP promoted activation of mitochondrial respiration still more (V3). The added ADP was spent after some time, which led to inhibition of electron transport and a decrease in the respiration rate (V4). Addition of protonophore 2,4-DNP to the wells increased respiratory rate in comparison with the rate of non-phosphorylating respiration (V5).

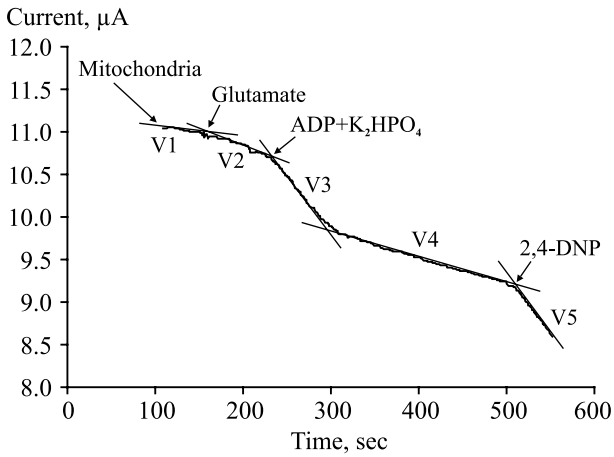

Fig. 1. Polarographic curve of mitochondrial respiration of control rat liver (substrate:potassium glutamate). Linear segments are shown, the slopes of which were used to calculate the mitochondrial respiration rates in different metabolic states.

A decrease in rat body temperature to 30°C led to a 2.9-fold increase in V1 (Table 1). Prolongation of moderate hypothermia to 1 h promoted further increase in V1, while after 3 h the value somewhat decreased in comparison with short-term hypothermia.

The intensity of hypothermia effect on mitochondrial respiration depended on the oxidation substrate: succinate (ETC complex II substrate) or glutamate (ETC complex I substrate). Study of the rate of succinate-dependent respiration showed that short-term hypothermia stimulated the rate of O_2 consumption by 34% at V2. Prolongation of hypothermia to 1 h promoted further stimulation of V2. Prolongation of hypothermia to 3 h led to a significant (by 37.9%) decrease in the respiratory rate in comparison with 1-h hypothermia.

Addition of ADP to rat liver mitochondria stimulated respiration, V3 in rats with short-term hypothermia surpassing the control values by 22.1%. Increasing the duration of hypothermia to 1 h led to further intensification of phosphorylating respiration, its rate decreasing after 3 h of hypothermic exposure.

The initial stages of hypothermia stimulated non-phosphorylating respiration of the rat mitochondria at V4, increasing it by 32.4%. This stimulation was most intensive after 1-h hypothermia and decreased significantly after 3 h. Registration of mitochondrial respiration rate at V5 (after addition of respiration uncoupler 2,4-DNP into incubation well) revealed a trend to respiration stimulation at the initial stages of hypothermia, reaching a level 2-fold higher than the control within 1 h of hypothermia prolongation. Further prolongation of hypothermia (to 3 h) promoted a decrease in V5 to the level of short-term hypothermia.

Study of the rates of glutamate-dependent respiration showed that V2 increased by 2.17 times in

short-term hypothermia. Prolongation of hypothermia to 1 h caused no further stimulation of respiration. V2 values did not normalize in 3-h hypothermia and their level remained 2.23-fold higher than normally. Changes in V3 and V4 at various stages of moderate hypothermia were similar: short-term hypothermia significantly increased their values, while prolonged exposure caused in fact no further effects. Registration of mitochondrial respiration after addition of 2,4-DNP demonstrated a significant stimulatory effect of short-term hypothermia: V5 surpassed the control values 2-fold. Prolongation of hypothermia to 1 h promoted further stimulation of uncoupled respiration: its rate by 23.8% surpassed that in short-term hypothermia, while after 3-h hypothermia the value reduced to that in short-term hypothermia.

The data of polarographic registration of respiration were used to calculate the following respiratory characteristics: oxidative phosphorylation coefficient (P/O); proportion of ADP concentration (nmol) to quantity of O_2 consumed over the period of phosphorylating respiration; respiratory control (RC) after Lardi; V3/V2 proportion, characterizing the capacity of mitochondria to react to ADP addition by acceleration of respiration; RC after Chance; V3/V4 ratio, indicating the integrity of mitochondrial structure and also a characteristic of the inhibitory effect of ATP, formed over the period of respiration, on the electron transfer via ETC; V5/V4 ratio, indicator of mitochondrial sensitivity to 2,4-DNP; V2/V4 ratio, characterizing the capacity of mitochondrial membranes to retain their energy potential; and phosphorylation velocity (V_{phosph}).

Study of succinate-dependent respiration showed that short-term hypothermia and its prolongation to 1 h promoted an increase in V_{phosph} (Fig. 2, *a*). This was paralleled by a decrease in P/O, which reached the minimum values in 1-h hypothermia (Fig. 2, *b*). Analysis of RC after Lardi and Chance showed a decrease in these parameters in short-term hypothermia, the decrease reaching 20.2 and 23.6% of control level, respectively, in 1-h hypothermia (Fig. 2, *c*, *d*). Prolongation of hypothermia to 3 h promoted an increase in RC to the values close to the control. Interestingly, V2/V4 did not differ from the control during hypothermia irrespective of its duration (Fig. 2, *e*). This fact indicated that the mitochondria of hypothermic animals, despite significant changes in the function of the respiratory chain, retained their own energy potential. At the initial stages of moderate hypothermia, V5/V4 characterizing the sensitivity of mitochondria to 2,4-DNP decreased (Fig. 2). Increasing the duration of hypothermia to 1 h promoted an increase in this parameter, while 3-h hypothermia led to its complete normalization.

Study of mitochondrial glutamate-dependent respiration showed that V_{phosph} increased significantly (by 70.4%) under conditions of short-term hypothermia and remained high in 1- and 3-h hypothermia (Fig. 2, *a*). The P/O proportion decreased at the initial stage of hypothermia and its prolongation to 1 h and normalized after Increasing the duration of the exposure to 3 h (Fig. 2, *b*). Short-term hypothermia slightly reduced the RC: by 12.8% after Lardi and by 15.6% after Chance (Fig. 2, *c*, *d*). Increasing the duration of hypothermia to 1 and 3 h promoted a more pronounced decrease in RC according to Lardi (by 27.1%), while RC according to Chance reached the control level. The mitochondria under these conditions retained their energy potential. Short-term hypothermia decreased the V5/V4 proportion, while its prolongation to 3 h normalized it (Fig. 2, *f*).

TABLE 1. Respiration Rates of Rat Liver Mitochondria (nmol O_2/min/mg) in Moderate Hypothermia of Different Duration (*M*±*m*, *n*=8)

Group	V1	V2	V3	V4	V5
Substrate — succinate					
Control	4.00±0.33	15.10±0.90	51.04±4.80	15.80±1.70	47.21±4.10
Hypothermia 30 min	11.6±0.27*	20.37±1.10*	62.33±4.37	20.95±1.40	53.90±1.73
Hypothermia 1 h	12.45±0.57*	31.64±1.70*^{o}	84.43±4.90*^{o}	34.10±2.44*^{o}	95.88±3.94*^{o}
Hypothermia 3 h	9.45±0.70*$^{o+}$	19.67±1.70*$^{+}$	59.6±3.80^{+}	18.62±1.83^{+}	57.29±2.90^{+}
Substrate — glutamate					
Control	4.14±0.14	7.17±0.36	27.58± 0.99	7.93±0.40	15.90±0.95
Hypothermia 30 min	12.60±0.50*	15.6±0.19*	52.27±2.54*	17.84±1.34*	33.60±2.54*
Hypothermia 1 h	13.56±0.60*	19.26±1.80*	54.54±3.90*	18.05±1.28*	37.03±3.10*
Hypothermia 3 h	12.19±0.87*	16.05±0.96*	47.11±1.65*	15.42±0.57*	30.50±2.20*

Note. $p<0.05$ in comparison with *control, ^{o}hypothermia 30 min, $^{+}$hypothermia 1 h.

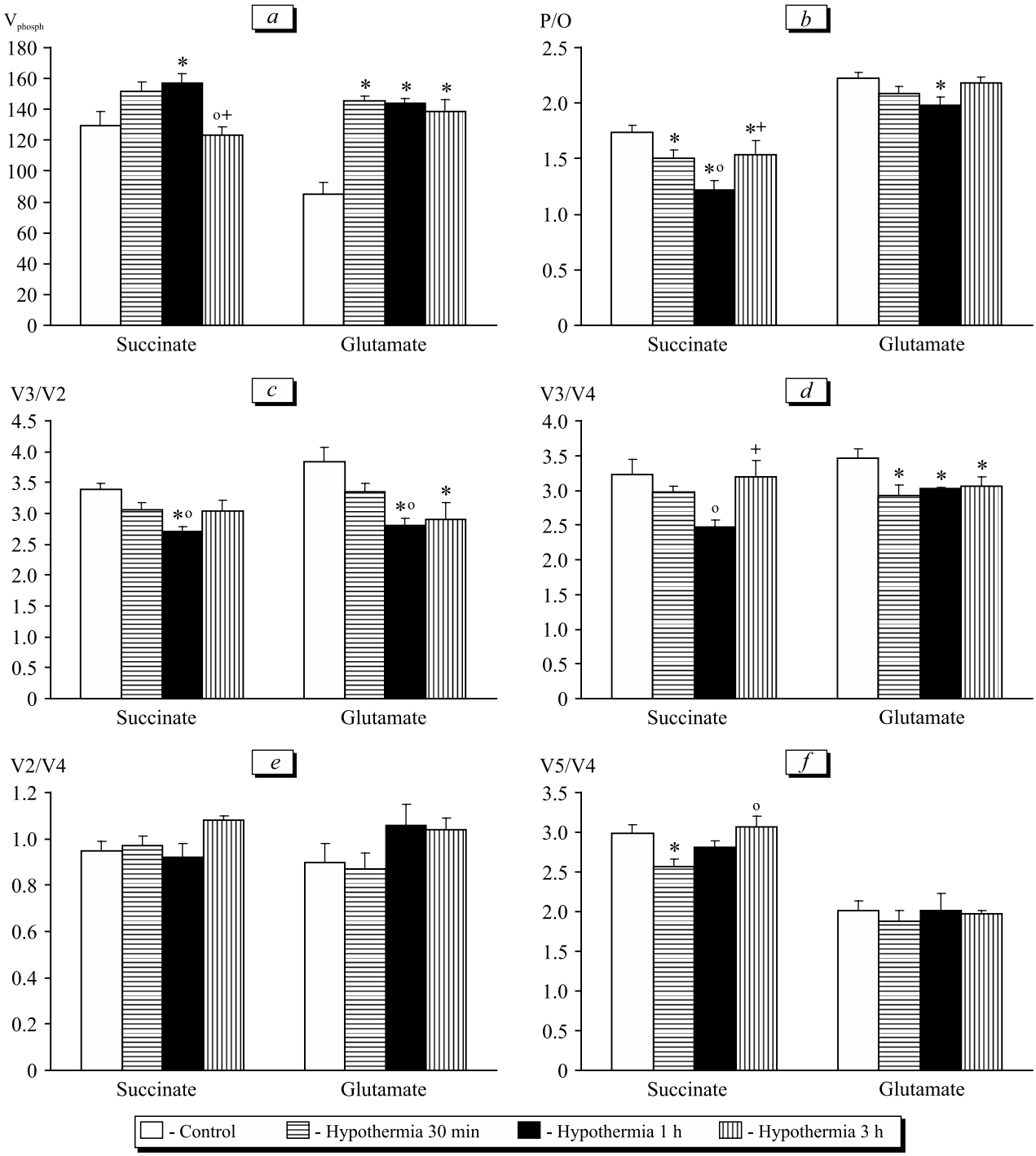

Fig. 2. Respiratory characteristics of rat liver mitochondria during moderate hypothermia of different duration. $p \leq 0.05$ in comparison with *control, °hypothermia 30 min, +hypothermia 1 h.

Hence, short-term hypothermia promoted significant stimulation of mitochondrial respiration, more manifest for glutamate-dependent respiration. This fact suggested that the most significant changes at the initial stages of hypothermia involved the work of the mitochondrial respiratory chain complex I — NADH-coenzyme Q-oxidoreductase. As respiration was stimulated irrespective of the metabolic states of the mitochondria, other factors also contributed to this stimulation, for example: increase in ATP-synthetase activity (due to which, presumably, V3 and phosphorylation rates increased); higher permeability of mitochondrial membranes for protons (due to which the rate of non-phosphorylating respiration increased,

while RC, P/O, and sensitivity to 2,4-DNP decreased); lesser viscosity of the mitochondrial inner membrane, promoting greater mobility of ubiquinone in the lipid matrix (due to which, presumably, V5 increased). Changed membrane viscosity could be caused by accumulation of lipid peroxides in it. In a previous study we demonstrated that short-term hypothermia and its prolongation to 1 h led to an increase in the levels of conjugated dienes, lipid hydroperoxides, and malondialdehyde in the mitochondria [11]. On the other hand, the viscosity of mitochondrial membranes could decrease because of their changed fatty acid composition. In a previous study [2] short-term hypothermia resulted in a decrease in saturation of synaptic membranes by summary lipids in the rat brain. However, we do not know whether this phenomenon could be true for liver cells.

Interestingly that the period needed to reach the hypothermic state was rather short for the development of significant changes in the physical characteristics of the lipid bilayer. That is why an increase in the passive permeability of the mitochondrial membrane, promoting partial dissociation of respiration and phosphorylation, seemed to be the most probable cause of respiration stimulation in short-term hypothermia. At the initial stages, hypothermia was associated with a stress reaction, involving activation of the hypothalamic—pituitary—adrenal system and triggering the mechanisms aimed at thermoproduction increase [6]. As a result, metabolism was intensified, as was glycogen and lipid degradation. Activation of lipolysis led to an increase in the concentration of free fatty acids, which caused dissociation of oxidative phosphorylation by the protonophore mechanism with involvement of the internal membrane transporter proteins — ADP/ATP and aspartate/glutamate antiporters [8]. Stimulation of mitochondrial respiration could be also caused by thyroid hormones (their concentrations increasing significantly in short-term hypothermia [6]) and uncoupler proteins UCP-2 and UCP-3 (detected in low concentrations in various tissues of homoeothermic animals [4].

Interestingly, prolongation of hypothermia to 1 h promoted further intensification of succinate-dependent respiration, many characteristics of glutamate-dependent respiration of the mitochondria persisting at the level of short-term hypothermia. These facts could indicate activation of the ETC complex II — succinate:ubiquinone oxidoreductase at this stage of hypothermia. It was shown that disorders in oxygen homeostasis in the mitochondria led to transfer from oxidation of NAD-dependent substrates in the respiratory chain to succinate oxidation, due to which the energy-producing function of the mitochondria was supported [3]. Hence, intensification of succinate-dependent respiration could be aimed at compensation for hypoxia developing in liver cells over 1 h of hypothermia.

It was hypothesized that the succinate dehydrogenase pathway worked as an urgent compensatory mechanism under conditions of acute hypoxic exposure and was not involved in the formation of long-term mechanisms of adaptation [3]. This hypothesis was in line with the results of our study. Many parameters of succinate-dependent respiration normalized after 3-h hypothermia, in contrast to the parameters of glutamate-dependent respiration, which were the same as in short-term hypothermia. Presumably, the respiratory complex I played the key role in prevention of mitochondrial dysfunction under conditions of a lasting exposure to low temperature. Importantly that complex I was the most effective of the factors involved in generation of superoxide radical in the mitochondria [1]. High levels of glutamate-dependent respiration and low levels of RC in the rat liver mitochondria, detected during all periods of moderate hypothermia, indicated more intense proton conduction at the level of complex I, which, in accordance with Skulachev's hypothesis [9], was one of approaches to suppression of ROS production in the mitochondria. In a previous study we showed that prolongation of moderate hypothermia to 3 h reduced the intensity of free radical processes in the rat liver mitochondria [11].

Hence, the data of our study of the respiratory parameters suggest reprogramming of the work of electron-transporter chains in the mitochondria during prolongation of hypothermia, which are paralleled by the development of compensatory and adaptive reactions at the levels of complexes I and II, aimed at reduction of ROS generation and retention of the energy-producing function of the mitochondria.

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