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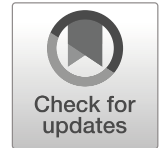

Effect of hypothermia on the functional activity of liver mitochondria of grass snake (*Natrix natrix*): inhibition of succinate-fueled respiration and K^+ transport, ROS-induced activation of mitochondrial permeability transition

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Abstract

The article considers the comparative analysis of the functional activity of mitochondria isolated from the liver of grass snakes, *Natrix natrix* (Linnaeus, 1758) that were kept at different temperatures (23–26 °C and 4-5 °C). It was found that liver mitochondria of hypothermia-exposed grass snakes are characterized by weak coupling of oxidative phosphorylation as compared to mitochondria of active animals which is caused by inhibition of succinate-fuelled respiration in ADP-stimulated state, as well as by activation of basal non-phosphorylating rate. Inhibition of mitochondrial respiration in hibernating animals is associated with a decrease in the activity of the respiratory chain complexes of organelles. A significant decrease in the rate of K^+ transport in the liver mitochondria of hibernating animals has been established. Under these conditions, a decrease in the calcium capacity of the organelles was also revealed, which indicates a decrease in the resistance of the mitochondria of hibernating animals to the induction of the Ca^{2+}-dependent mitochondrial pore. All these changes in the functional activity of mitochondria are observed on the background of increasing H_2O_2 production as well as increasing the proportion of polyunsaturated fatty acids in phospholipid composition of mitochondrial membranes, which are the targets of reactive oxygen species. It can lead to increased formation of lipid peroxides and activation of destructive processes associated with the induction of Ca^{2+}-dependent mitochondrial pore.

Keywords Grass snake · Liver mitochondria · Hypothermia · Hibernation · Ca^{2+}-dependent mitochondrial pore · Potassium ions · Fatty acids · Reactive oxygen species

Abbreviations

CsA	cyclosporin A
DNP	2,4-dinitrophenol
MCU	mitochondrial calcium uniporter
MPT	mitochondrial permeability transition
ROS	reactive oxygen species

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Introduction

The resistance of any organism to extreme environmental conditions is based on its ability to withstand the effects of external factors with minimal damage to life, which ultimately leads to the survival of the species. One of the ways for animals to adapt to changing environmental conditions is to reduce the metabolic rate, which includes estivation, diapause, daytime torpor, hibernation, etc. Hibernating animals are able to withstand a decrease in ambient temperature for a long time, or, conversely, extremely high temperatures. In hibernating mammals, heart rate slows down, blood pressure and oxygen absorption decrease (Carey et al. 2003; Geiser 2004; Storey and Storey 2010; Hadj-Moussa et al. 2018; Andrews 2019). The animal's organism switches from carbohydrate metabolism to enhanced metabolism of stored lipids (Andrews 2004;

Williams et al. 2011; Hadj-Moussa et al. 2018). Many changes occur as the animal goes into hibernation, including reversible phosphorylation of proteins, differential expression of certain genes, as well as changes in the level of non-coding RNA (Storey and Storey 2004; Williams et al. 2011; Lyons et al. 2013; D'Alessandro et al. 2017; Andrews 2019).

It is believed that under the conditions of hibernation key changes occur at the level of mitochondria (Gellerich et al. 2004; Staples 2016). Significant inhibition of oxygen consumption and oxidative phosphorylation is observed in liver mitochondria and, to a lesser extent, in mitochondria of other tissues of hibernating animals (Barger et al. 2003; Brown et al. 2012; Staples 2016). Inhibition develops rapidly as the animal falls into a hibernation even at relatively high body temperature (30–37 °C) and is reversed when it comes out of hibernation (Chung et al. 2011; Staples 2016). In vitro, low oxygen consumption rate in isolated mitochondria is preserved despite of temperature increase in the polarographic cell (usually 26 °C or 37 °C). It proves that a change in the functioning of liver mitochondria during natural hibernation is an active regulated process, rather than passive consequence of the low body temperature. There are different points of view on the mechanisms for the implementation of such inhibition. It is assumed that it may be caused by the inhibition of respiratory chain complexes, Krebs cycle enzyme systems or substrate-transporting proteins (Staples 2016). Cellular processes that require energy, such as transmembrane ion transport, transcription and translation, including those occurring in mitochondria, are also significantly suppressed in hibernating animals (Fedotcheva et al. 1985; Brustovetsky et al. 1992; MacDonald and Storey 1999; van Breukelen and Martin 2002). In addition, in animals it is known that a decrease in body temperature is accompanied by an increase in the level of unsaturated fatty acids in the composition of membrane lipids, which makes it possible to preserve the basic metabolic functions of organs and tissues (Aloia 1980; Carey et al. 2003; Ruf and Arnold 2008).

Representatives of all classes of poikilothermic vertebrates, with rare exceptions (for example, fish), are characterized by a much lower intensity of oxidative metabolism compared to mammals and birds (Ivanov 2008). In general, hypobiosis in poikilothermic organisms proceeds against the background of a decrease in the level of glucose and an increase in the level of metabolic products of lipids, proteins and transaminases in the blood (Ivanov 2008). Apparently, these changes help poikilotherms adapt/survive (to) periods of hypoxia and anoxia for a long period. It should be noted that the physiological mechanisms of adaptation of poikilothermic vertebrates related to the functioning of mitochondria are poorly studied. Most of the works dealing with hypoxia/anoxia-resistant animals are focused on the mechanisms of biochemical adaptation (glycolysis) (Lutz and Nilsson 1997; Savina et al. 2009). At the same time, the effects of hypoxia/anoxia on the processes of oxidative phosphorylation and the activity of the respiratory complexes, the transport of ions through the mitochondrial membrane or the parameters of formation of mitochondrial pores are either not examined at all or sporadic and non-systemized. In this regard, the study of the processes of mitochondrial ion transport regulation and Ca^{2+}-dependent pore opening in the mitochondria of poikilothermic animals can contribute to the establishment of new adaptation mechanisms and clarify the general patterns of functioning of living systems in extreme environmental conditions.

This work considers a comparative study of the functional state of liver mitochondria of grass snake *Natrix natrix* (Linnaeus, 1758) depending on the physiological state of the animal (active and hibernation induced by hypothermia). It is one of the widely spread snake species of the Northern Eurasia. Depending on the latitude, this species of snakes can hibernate from October–November to March–April. For wintering, grass snake chooses underground shelters where the temperature does not fall below 0 °C. The wide geographical distribution makes this animal a good model for studying the molecular mechanisms of adaptation of poikilothermic animals to the conditions of low temperatures and hibernation, including at the level of mitochondria. In this paper, we have shown for the first time that under hypothermia conditions liver mitochondria of grass snake are characterized by low efficiency of oxidative phosphorylation in the case of succinate-fueled respiration, a decrease in the activity of the respiratory chain complexes and a reduction in the rate of transmembrane transport of potassium ions. In addition, it was found that under hypothermia conditions there is a decrease in the calcium capacity of the liver mitochondria of these animals, which leads to a reduction in their resistance to the induction of mitochondrial Ca^{2+}-dependent cyclosporin A-sensitive pore. It was assumed that this phenomenon may be caused by a change in the fatty acid composition of the lipids of mitochondrial membranes, by an increase in the proportion of polyunsaturated fatty acids in their structure, which are the target of reactive oxygen species.

Materials and methods

Experimental animals and chemicals

Mature males of the grass snake, *Natrix natrix* (Linnaeus, 1758) weighing 80–130 g were caught in the Pine Grove forest park in the south-eastern part of Yoshkar-Ola (Russia) in July and August. The study was carried out in accordance with the European Convention for the Protection of Vertebrates used for experimental and other purposes (1986) and the principles of the Helsinki Declaration (2000). All the experimental protocols were

approved by the Mari State University Ethics Committee (Yoshkar-Ola, Russia).

The captured animals were kept for a week in the terraria (80x50x30 cm) with coconut soil. In the containers openings for ventilation were made. Heating and light was provided by an electric lamp of 60 W. The temperature in containers varied between 23 and 26 °C, which is the optimal conditions for this species (Skoczylas 1970). Each terrarium was equipped with a small plastic water container. Every day the terrarium was sprayed using water pulverizer for maintenance of humidity. After one week, the animals were divided into two groups. The first group of animals ($n = 6$) kept at the optimum temperature. The second group of animals ($n = 6$) were placed in small plastic containers (30 L) with ventilation in a laboratory refrigerator (POZIS Paracels, Russia) at a temperature of 4-5 °C for two weeks. The snakes under hibernation conditions had pulverized every two days. After two weeks, the animals were sacrificed by the decapitation method.

All chemicals were purchased from Sigma-Aldrich (USA).

Isolation of mitochondria

Mitochondria from the liver of animals were isolated by the conventional method of differential centrifugation (Samartsev et al. 1997). The isolation medium contained 250 mM sucrose, 1 mM EGTA, and 5 mM Hepes-KOH buffer (pH 7.4). During the experiment, the suspension of mitochondria (50–70 mg of mitochondrial protein in 1 mL) was stored on ice in a narrow plastic tube.

Mitochondrial respiration and phosphorylation

The oxygen consumption rate of mitochondria was monitored at 25 °C with a Clark oxygen electrode in a 1-mL thermostatic closed cuvette under magnetic stirring. Mitochondria (1.3 mg of protein/mL) were added to an incubation medium containing 200 mM sucrose, 20 mM KCl, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM KH_2PO_4, and 10 mM HEPES-KOH buffer (pH 7.4). The concentrations of substrates were as follows: 2.5 mM potassium malate, 2.5 mM potassium glutamate, 5 mM potassium succinate, 0.2 mM ADP, and 2 μM rotenone. Estimated were the mitochondrial respiration in resting state (i.e., basal mitochondrial respiration in the presence of exogenous substrates), in state 3 (exogenous substrates plus ADP), in state 4 (after ADP exhaustion) (Chance and Williams 1955). The oxygen consumption rates are presented as nmol O_2*min^{-1}* mg^{-1} of mitochondrial protein. Respiratory control ratio (RCR), i.e. the ratio of respiratory rate in state 3 to that in state 4. It should be noted that the respiration rate of liver mitochondria in state 4 was evaluated in the presence of oligomycin, since the liver mitochondria of these animals are characterized by a high level of ATPase activity (not shown).

Measuring activity of complexes of the mitochondrial electron transport chain (ETC)

Activity of ETC complexes of grass snake liver mitochondria was evaluated spectrophotometrically according to the protocol (Spinazzi et al. 2012) using a plate reader Multiskan GO (Thermo). To disrupt mitochondrial membranes and make respiratory chain complexes accessible for the assay (as well as to maximize their enzymatic activity), isolated liver mitochondria (10–15 mg mitochondrial protein per mL) were subjected to three cycles of freezing/thawing at −20/+30 °C in a hypotonic buffer, containing 10 mM Tris-HCl, pH 7.6. The composition of the buffers used for the analysis of activity of individual complexes is given in (Spinazzi et al. 2012). The activity of complex I was estimated at 25 °C by the efficiency of oxidation of added NADH by the suspension of disrupted mitochondria, which was followed by the decrease of absorbance at 340 nm. The activity of complex II was evaluated at 37 °C (in order to fully activate the enzyme) by the efficiency of reduction of 2,6-dicholrophenol indophenol (sodium salt) by the suspension of disrupted mitochondria in the presence of succinate, which was followed by the decrease of absorbance at 600 nm. The activity of complex III was measured at 25 °C by the efficiency of reduction of added cytochrome *c* by the suspension of disrupted mitochondria, which was followed by the decrease of absorbance at 550 nm. The activity of complex IV was estimated at 25 °C by the efficiency of oxidation of added cytochrome c (preliminary reduced by the suspension of disrupted mitochondria as described in (Spinazzi et al. 2012)), which was followed by the decrease of absorbance at 550 nm. The activities of the respiratory chain complexes were registered within 2–3 min after the beginning of the redox reaction (nmol*min^{-1}*mg^{-1} protein).

Determination of mitochondrial K^+ transport

The mitochondrial energy-dependent K^+ transport was estimated by uptake of K^+ by mitochondria and K^+ efflux from mitochondria induced by the uncoupler 2,4-dinitrophenol (DNP) (Baranova et al. 2000). The energy-dependent K^+ uptake was assessed by the decrease of A_{520}, which reflects swelling of mitochondria (Dubinin et al. 2017). The measurements were carried out in a thermostated cuvette (25 °C) using a spectrophotometer KFK-0-01 («ZOMZ», Russia). The medium contained 50 mM KCl, 5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM Mops-Tris, pH 7.4, 1 μg/mL oligomycin and 2 μM rotenone. The concentration of mitochondrial protein was 0.2 mg/mL. The reaction was started by adding the respiration substrate (5 mM succinate), after preincubation of mitochondria in the reaction medium for 2 min. The rate of mitochondrial swelling was calculated as the change of A_{520} within 1 min after the reaction start (ΔA_{520}*min^{-1}*mg^{-1} of mitochondrial protein).

2. The uncoupler-induced K^+ efflux was measured with an ion-selective electrode (Baranova et al. 2000). The rate of K^+ efflux from mitochondria was calculated as the change in the outer K^+ concentration per min after the addition of DNP (nmol K^{+}* min^{-1}* mg^{-1} mitochondrial protein). The incubation medium contained 180 mM sucrose, 70 mM mannitol, 5 mM NaH_2PO_4, 1 μg/mL oligomycin and 10 mM Tris/HCl, pH 7.4. The protein concentration was 1.5 mg/mL, the concentration of DNP was 50 μM.

Mitochondrial calcium retention capacity

The concentration of Ca^{2+} in the reaction medium (external [Ca^{2+}]) was measured with an ion-selective electrode (Dubinin et al. 2016). The reaction medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 1 mM KH_2PO_4, 10 μM EGTA, 1 μM rotenone, and 10 mM HEPES-KOH, pH 7.4. The measurements were carried out in in a stirred cuvette at room temperature (~22 °C). The concentration of mitochondrial protein was 1 mg/mL. After several additions, external [Ca^{2+}] increased, indicating a massive release of the ion from the organelles due to the opening of the mitochondrial permeability transition pore (MPT pore) in the inner mitochondrial membrane. The amount of Ca^{2+} released upon permeability transition (defined as Ca^{2+} capacity) was used as a measure of the MPT pore opening probability. The rate of Ca^{2+} uptake (nmol Ca^{2+}* min^{-1}* mg^{-1} mitochondrial protein) was determined by the rate of 100 μM Ca^{2+} absorption by liver mitochondria in the presence of 1 μM CsA using a calcium-selective electrode. The concentration of mitochondrial protein also was 1 mg/mL.

Production of H_2O_2 by liver mitochondria

The rate of H_2O_2 production by the suspension of grass snake liver mitochondria was measured with the fluorescent indicator Amplex Red (excitation wavelength, 560 nm; emission wavelength, 590 nm) using Fluorat-02-Panorama spectrofluorometer (Lumex Instruments, Russia) (Dubinin et al. 2018). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 1 mM KH_2PO_4, 10 μM EGTA, 1 μM rotenone, and 10 mM HEPES-KOH, pH 7.4. At the beginning of measurements, horseradish peroxidase (1 U/mL), superoxide dismutase (4 U/mL) and 10 μM Amplex Red were added to the incubation medium. The concentration of mitochondrial protein in the cuvette was 0.15 mg/mL.

The amount of the resulting hydrogen peroxide was calculated from the calibration curve. A standard hydrogen peroxide solution was prepared on the day of experiment; its concentration was determined using the molar absorption coefficient $E_{240} = 43.6\ M^{-1}*cm^{-1}$.

Determination of fatty acid content and composition in mitochondrial membranes

The content of fatty acids in the phospholipid composition of mitochondrial membranes was determined using the method of gas chromatography. Extraction of lipids from mitochondria was performed according to the modified method of Bligh and Dyer (1959). Mitochondria (2 mg/mL) were placed in a centrifuge tube, then 200 μL of water and 900 μL of chloroform/methanol mixture (2:1, *v*/v) were added. The mixture was kept at room temperature for 30 min and was occasionally shaked up. Heptadecanoic acid (C17:0, 15 μg/mL) in hexane was added to each sample as an internal standard. Chloroform and water/methanol layers were separated by centrifugation at 10,000 g for 10 min at 4 °C. The lower layer of the extract containing the lipid fraction was collected and evaporated to dryness in a stream of argon at 25 °C. The obtained fractions were methylated with one volume of 5% sulfuric acid/methanol (v/v) at 100 °C for 3 h. The reaction was stopped by the addition of three volumes of 5% K_2CO_3 (v/v). Methyl ethers were extracted in four volumes of hexane and then evaporated in a stream of nitrogen to a volume of 100 μL. Samples were analyzed on a Chromatek-Crystal 5000 gas chromatography system (Chromatek, Russia). The following indexes for each group of animals were calculated on the basis of the obtained chromatograms: TFA, the total amount of fatty acids; SFA, sum of saturated fatty acids; PUFA, sum of polyunsaturated fatty acids; the unsaturation index (UI) calculated by multiplying the amount of fatty acid by the number of double bonds in its structure.

Statistical analysis

The data were analyzed using the GraphPad Prism 5 and Excel softwares and were presented as means ± SEM of three to six experiments. Significant differences between data points were determined by a two-tailed *t* test.

Results

Hypothermia results in inhibition of succinate-fueled respiration in grass snake

The effect of hypothermia on the functional state of grass snake liver mitochondria was evaluated by the rate of mitochondrial respiration with glutamate/malate (substrates of complex I of the respiratory chain) or succinate (a substrate of complex II) in the presence of rotenone. As seen from Table 1 hypothermia had no effect on glutamate/malate driven respiration in all the functional states. In the case of succinate-

Table 1 The main bioenergetic parameters of grass snake liver mitochondria

Animals	Basal respiration	State 3	State 4	RCR
	nmol O_2*min^{-1}* mg^{-1} protein			
Glutamate/ malate				
Active (*n* = 3)	3.7 ± 0.4	7.1 ± 0.5	2.9 ± 0.2	2.5 ± 0.1
Hypothermia (n = 3)	4.1 ± 0.6	7.4 ± 1.1	3.0 ± 0.4	2.4 ± 0.1
Succinate/ rotenone				
Active (n = 6)	5.4 ± 0.4	15.9 ± 0.6	1.3 ± 0.1	12.1 ± 0.8
Hypothermia (n = 6)	5.0 ± 0.2	11.4 ± 1.0*	4.1 ± 0.3*	2.9 ± 0.3*

Medium composition: 200 mM sucrose, 20 mM KCl, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM KH_2PO_4, and 10 mM HEPES-KOH buffer (pH 7.4) Respiration of mitochondria was fueled by 2.5 mM glutamate and 2.5 mM malate or 5 mM succinate. Respiration of mitochondria in the state 3 was initiated by 200 μM ADP. Data are presented as the mean ± S.E.M.; n—number of experimental animals

* Differences between active and hypothermia-exposed animals were statistically significant ($p < 0.01$)

fueled respiration hypothermia does not affect the respiration of mitochondria in the main metabolic state (V_2). But at the same time, there is a suppression of mitochondrial respiration in the ADP-stimulated (V_3) state in hypothermia-exposed animals. It is necessary to note an increase in the respiration rate of the mitochondria of these animals in the state V_4, possibly due to a higher activity of fatty acid-uncoupled respiration. In the presence of 0.5 mg/mL BSA which capable of binding free fatty acids, this parameter was reduced by 30% (not shown). Due to the changes in respiration rates in states V_3 and V_4, the parameter of respiratory control in liver mitochondria of hypothermia-exposed grass snakes was significantly reduced.

In addition, we have investigated the effect of hypothermia on the activity of the complexes of the mitochondrial respiratory chain of the studied animals. Table 2 shows that hypothermia results in inhibition of the respiratory complexes.

Hypothermia results in inhibition of K^+ transport in grass snake

In this work, we also assessed the effect of hypothermia on mitochondrial K^+ transport by measuring two parameters: the energy-dependent K^+ influx into mitochondria and the DNP-induced K^+ efflux from the organelles.

The first parameter, which was determined by the swelling of mitochondria in a K^+ medium after the addition of the substrate, reflects the flux of K^+ via the mitoKATP channel (Dubinin et al. 2017). We have found that the activity of K^+ uniport in grass snake liver mitochondria is suppressed by ~ 45% under hypothermia conditions (Fig. 1a, b).

Similar results were obtained when another K^+ transport parameter, the DNP-induced K^+ efflux, was measured (Fig. 1c). As it can be seen in the figure, hypothermia decreased the rate of K^+ efflux from mitochondria under those conditions by more than 80%.

Hypothermia leads to a decrease in the mitochondrial calcium capacity in grass snake

Calcium capacity of mitochondria is one of the indicators that determine the resistance of cells to death. This parameter determines the efficiency of pore induction in these organelles and represents the maximum amount of Ca^{2+} that can be accumulated in the matrix without subsequent induction of permeability transition (Rasola and Bernardi 2011; Dubinin et al. 2016). Figure 2a, b shows that the calcium capacity of liver mitochondria in active grass snakes is 2.5–3 times higher than in hypothermia-exposed animals. At the same time, in the presence of the mitochondrial pore inhibitor CsA, the calcium capacity of the mitochondria of the studied groups of animals

Table 2 Effect of hypothermia on the activity of complexes of the mitochondrial respiratory chain

Animals	Values in nmol*min^{-1}*mg^{-1}			
	I	II	III	IV
Active (n = 6)	6.7 ± 0.3	25.6 ± 1.3	90.0 ± 3.2	107.7 ± 3.9
Hypothermia (n = 6)	4.8 ± 0.5*	14.8 ± 1.8**	50.4 ± 4.2**	80.2 ± 2.2**

The experimental conditions are described in Materials and methods. The results are presented as means ± SEM. Differences between active and hypothermia-exposed animals were statistically significant (*$p < 0.05$; **$p < 0.01$)

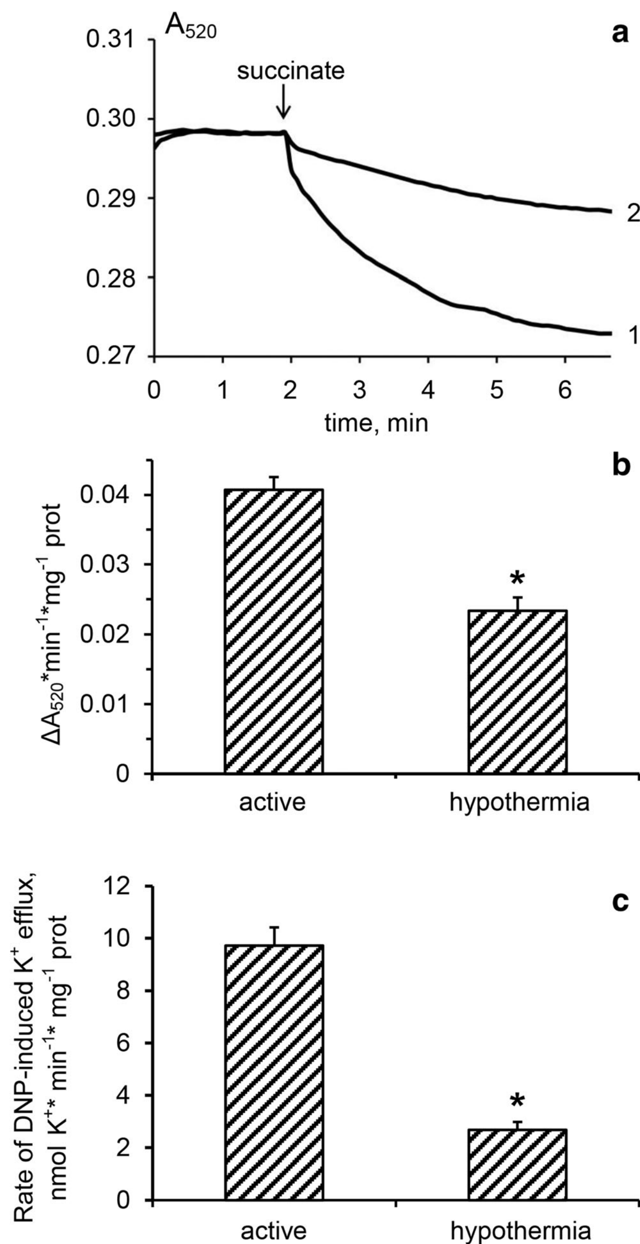

Fig. 1 Effect of hypothermia on the transport of K^+ across the inner mitochondrial membrane. **a** Energy-dependent swelling of liver mitochondria (0.2 mg/mL) isolated from active grass snakes (curve 1) and hypothermia-exposed animals (curve 2), swelling was induced by the addition of 5 mM succinate to the medium containing 50 mM KCl, 5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM Mops/tris, pH 7.4, 1 μg/mL oligomycin and 2 μM rotenone. **b** Dependence of the rate of energy dependent swelling of grass snake liver mitochondria on the metabolic state of the animals. The results are presented as mean values ± SEM ($n = 6$). **c** The effect of hypothermia on the rate of DNP-dependent efflux of K^+ from grass snake liver mitochondria. The incubation medium contained 180 mM sucrose, 70 mM mannitol, 5 mM NaH_2PO_4, 1 μg/mL oligomycin and 10 mM Tris/HCl, pH 7.4. The protein concentration was 1.5 mg/mL, the concentration of DNP was 50 μM. The results are presented as mean values ± SEM (n = 6). * Differences between active and hypothermia-exposed animals were statistically significant ($p < 0.01$)

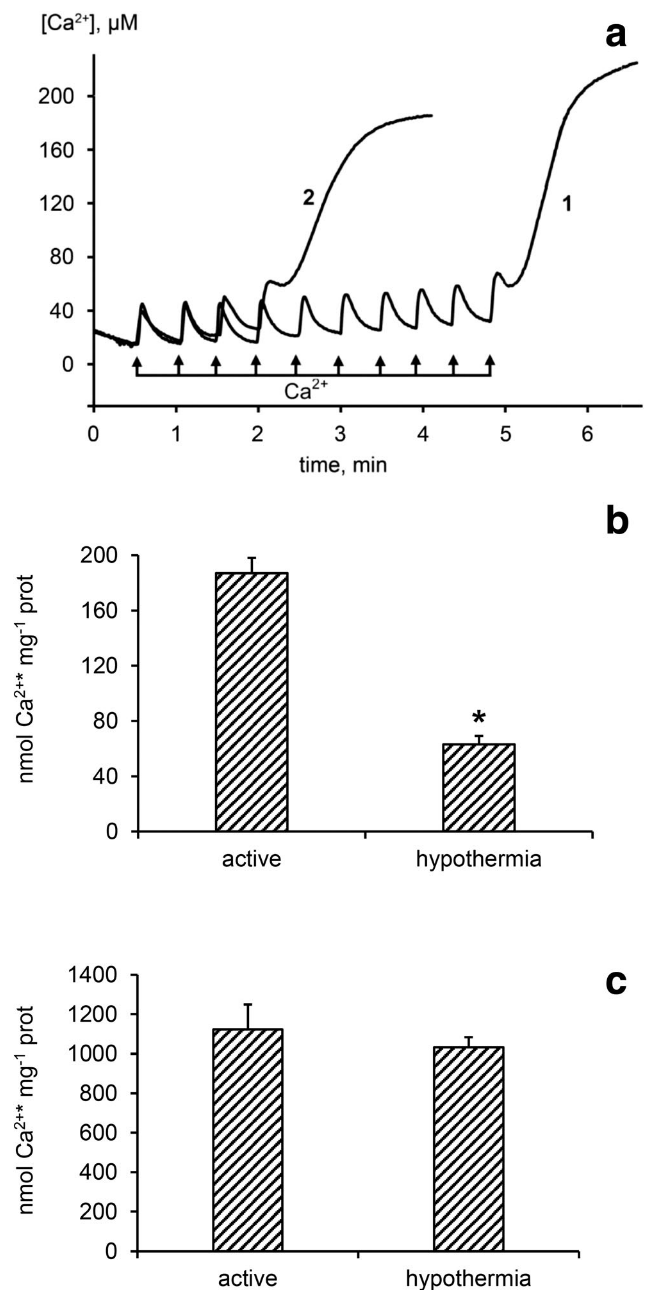

Fig. 2 Effect of hypothermia on the opening of MPT pore in liver mitochondria. **a** Retention of Ca^{2+} by mitochondria isolated from active (curve 1) and hypothermia-exposed animals (curve 2). Additions: 20 nmol $CaCl_2$ per 1 mg of protein (Ca^{2+}). **b** Dependence of the liver mitochondrial Ca^{2+} capacity on the metabolic state of the animals in the absence and presence (**c**) of 1 μM CsA. The results are presented as mean values ± SEM (n = 6). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 1 mM KH_2PO_4, 10 μM EGTA, 1 μM rotenone, and 10 mM HEPES-KOH, pH 7.4. The concentration of mitochondrial protein was 1.0 mg/mL. * Differences between active and hypothermia-exposed animals were statistically significant ($p < 0.01$)

is almost the same (Fig. 2c). It should also be noted that, unlike K^+ uniport, the rate of Ca^{2+} uptake did not undergo significant changes in hypothermia conditions (Fig. 3).

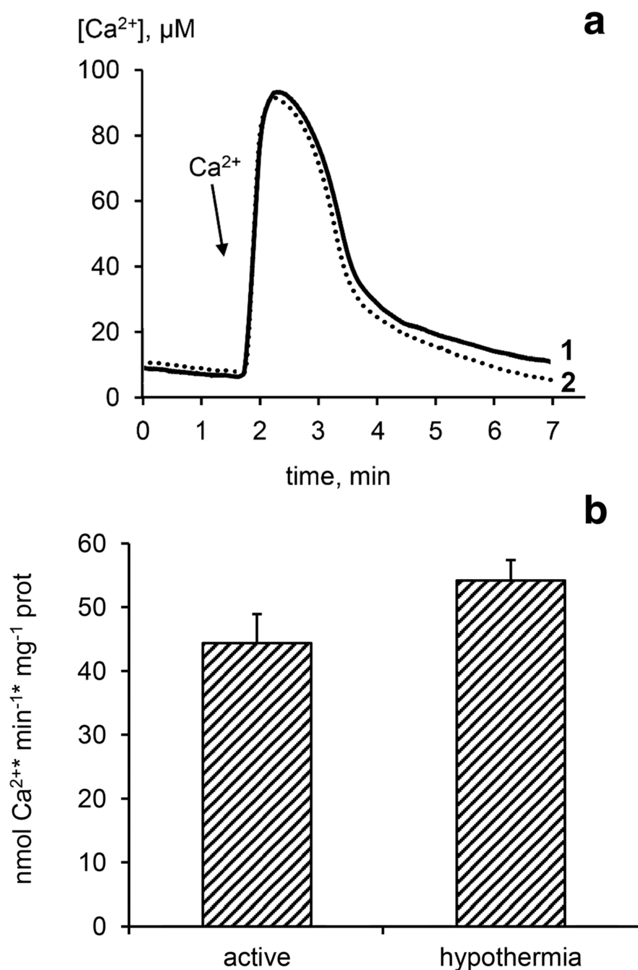

Fig. 3 Effect of hypothermia on the Ca^{2+} uptake by liver mitochondria. **a** Retention of Ca^{2+} by mitochondria isolated from active (curve 1) and hypothermia-exposed animals (curve 2) in the presence of 1 μM CsA. Additions: 100 nmol $CaCl_2$ per 1 mg of protein (Ca^{2+}). **b** Dependence of the Ca^{2+} uptake rate on the metabolic state of the animals. The results are presented as mean values ± SEM (n = 6). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 1 mM KH_2PO_4, 10 μM EGTA, 1 μM rotenone, and 10 mM HEPES-KOH, pH 7.4. The concentration of mitochondrial protein was 1.0 mg/mL

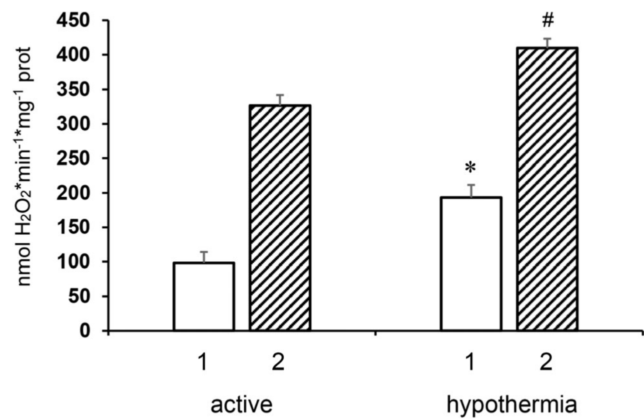

Fig. 4 Effect of hypothermia on H_2O_2 production rate in isolated mitochondria from active grass snakes and hypothermia-exposed animals before (1) and after the addition of 100 μM Ca^{2+} (2). The experimental conditions are listed in "Materials and Methods". The data represent mean ± SEM (n = 6). #, * Differences between control (mitochondria of active animals before and after the addition of Ca^{2+}, respectively) and experiment (hypothermia-exposed) groups were statistically significant ($p < 0.01$)

Hypothermia induces an increase in H_2O_2 production in grass snake

It is known that cold stress induces an increase in the production of reactive oxygen species by mammalian mitochondria. In this work, we examined the effect of hypothermia on the rate of H_2O_2 production by grass snake liver mitochondria. Figure 4 shows that hypothermia increases the H_2O_2 production in liver mitochondria of these poikilothermic animals. In this case, the subsequent addition of Ca^{2+} stimulates the production of hydrogen peroxide in these organelles, while the rate of H_2O_2 production is also higher in the liver mitochondria of hibernating animals (Fig. 4).

Hypothermia is accompanied by an increase in the content of unsaturated fatty acids in the lipid composition of liver mitochondrial membranes

One of the mechanisms of animal adaptation to hypothermia conditions is a change in the fatty acid composition of membrane lipids. Using gas chromatography, we analyzed the qualitative and quantitative content of fatty acids in the composition of phospholipids of mitochondrial membranes isolated from the liver of the studied groups of animals.

Table 3 presents data describing differences in the composition of fatty acids in the liver mitochondria of active grass snakes and hibernating animals. There is an increase in the total amount of fatty acids (TFA) in the composition of membrane phospholipids of liver mitochondria isolated from hypothermia-exposed grass snakes compared to active ones. A significant increase in the amount of polyunsaturated fatty acids (PUFA), especially linoleic (18:2) and arachidonic (20:4) is observed. It leads to a significant increase in the unsaturation index (UI) of phospholipid membranes in liver mitochondria of hypothermia-exposed grass snakes.

Discussion

Under the conditions of hibernation, the majority of the intracellular processes in the organism of animals are aimed at suppressing metabolic processes, i.e. there is a transition into hypobiotic state, which provides an increase in body resistance and economy of metabolic resources. It is believed that during hibernation, key changes occur at the level of mitochondria (Staples 2016). Primarily, these processes are

Table 3 Changes in fatty acid composition of mitochondria in response to hypothermia (μg per mg of mitochondrial protein)

Fatty acids	Animals	
	Active (n = 6)	Hypothermia (n = 6)
14:1	ND	0.19 ± 0.01
16:0	14.71 ± 1.87	16.97 ± 1.62
16:1	ND	0.42 ± 0.08
18:0	10.54 ± 1.34	12.21 ± 1.37
18:1	16.47 ± 1.78	18.56 ± 2.34
18:2	2.30 ± 1.02	9.36 ± 1.55*
18:3	ND	2.10 ± 0.57
20:0	0.87 ± 0.28	0.89 ± 0.15
20:2	4.03 ± 0.88	3.19 ± 0.89
20:3	0.59 ± 0.22	0.71 ± 0.14
20:4	4.63 ± 1.49	10.30 ± 1.55*
22:0	0.96 ± 0.19	0.50 ± 0.02
22:2	0.18 ± 0.02	2.32 ± 0.11*
24:0	2.85 ± 0.24	2.03 ± 0.29
24:1	3.54 ± 1.40	1.10 ± 0.15
22:6	2.05 ± 0.07	2.88 ± 0.45
TFA	57.78 ± 2.75	82.92 ± 5.40*
SFA	27.55 ± 2.67	32.31 ± 1.91
PUFA	11.11 ± 3.16	28.32 ± 2.25*
UI	54.72 ± 8.34	111.26 ± 8.87*

The experimental conditions are described in Materials and Methods. The results are presented as means ± SEM

ND, not determined; *TFA*, total fatty acids; *SFA*, sum of saturated fatty acids; *PUFA*, sum of polyunsaturated fatty acids; *UI*, unsaturation index defined in the text

*Differences between active and hypothermia-exposed animals were statistically significant ($p < 0.01$)

associated with inhibition of oxygen consumption by these organelles (Barger et al. 2003; Brown et al. 2012). Indeed, similar processes are also observed in the grass snake. Table 1 shows that in the case of hypothermia the coupling of oxidative phosphorylation in the succinate-fueled grass snake liver mitochondria is significantly lower — the respiratory control coefficient of the mitochondria of these animals is reduced by more than 4 times compared with the liver mitochondria of active snakes. In this case, a decrease in the level of coupling of these processes is caused by both a decrease in the rate of oxygen consumption in state 3 (ADP-stimulated) and an increase in the respiration rate in state 4, when the entire ADP was consumed in the process of ATP synthesis. It can be assumed that in this case the increase in the respiration rate in state 4 is partly caused by the general increase in the content of fatty acids in the mitochondrial membranes of hibernating animals (some of which are presumably free) (Table 3) - natural uncouplers of oxidative phosphorylation (Samartsev 2000).

A decrease in the respiratory rate of mitochondria in the liver of hibernating animals in state 3 may be caused by the inhibition of the complexes of the mitochondrial respiratory chain (Staples 2016). In fact, as it can be seen from Table 2, hypothermia results in a decrease in the activity of the respiratory chain complexes (I, II, III and IV). It has previously been shown that in red-eared slider turtles (Galli et al. 2013) and common frog (St-Pierre et al. 2000), F_1F_O-ATPase (complex V) is also inhibited under hibernation conditions. In our case, it should be noted that during hypothermia there is a significant suppression of succinate, but not glutamate/malate driven respiration in liver mitochondria of grass snake. It is worth noting that the level of oxygen consumption by liver mitochondria oxidizing NAD-dependent substrates is extremely low (especially in state 3). Possibly, as a result of this we were unable to register the inhibition of respiration of hibernating animal mitochondria oxidizing this type of substrate. The observed picture is compliant with the data on hibernating mammals. According to these data, suppression of liver mitochondrial state 3 respiration during hypothermia is primarily caused by the inhibition of succinate oxidation (Fedotcheva et al. 1985; Staples 2016). However, in some cases in mammals and other vertebrates, suppression of liver mitochondrial state 3 respiration during hibernation has also been observed with several NAD-linked substrates, including pyruvate, glutamate and β-hydroxybutyrate (Brustovetsky et al. 1990; Muleme et al. 2006; Komelina et al. 2015). Indeed, succinate dehydrogenase activity is significantly inhibited during hibernation, probably by oxaloacetate (Fedotcheva et al. 1985; Armstrong and Staples 2010; Staples 2016). Excess oxaloacetate may occur due to inhibition of the initial stages of the TCA cycle, in particular, citrate synthase, as shown for red-eared slider turtles (Pamenter et al. 2016) and common frog (Boutilier and St-Pierre 2002), as well as inhibition of the pyruvate dehydrogenase complex (Boutilier and St-Pierre 2002) - in this case less acetyl-CoA is formed and, therefore, less oxaloacetate will react with it in the citrate-synthase reaction.

As it was mentioned in the introduction, transmembrane transport of ions and, in particular, potassium ions in mitochondria is also significantly suppressed in hibernating mammals (Fedotcheva et al. 1985; Brustovetsky et al. 1992) and, apparently, due to the development of hypoxia (Mironova et al. 2010; Akopova 2018) It is believed that K^+ homeostasis maintains osmotic stability of mitochondria during hypothermia and related hypoxia (Brierley and Yung 1980; Mironova et al. 2010; Akopova 2018). Therefore, possible changes in the transport of K^+, associated with the modifications in the composition and structure of mitochondrial membranes during hibernation (Aloia 1980; Mironova et al. 2010) can play an important role in changing the physiological state of hibernating animal.

As it can be seen from Fig. 1, hypothermia also causes significant suppression of K^+ transport in grass snake liver mitochondria. The inhibitory effect has been demonstrated on two models: the model of energy-dependent K^+ influx and the model of DNP-induced K^+ efflux which are mediated by mitoKATP. The obtained data indicate that in case of succinate-fueled respiration, hypothermia leads to a decrease in both the energy-dependent K^+ influx into matrix and the passive efflux of K^+ from mitochondria. It is compliant with the data obtained earlier on the liver mitochondria of hibernating ground squirrels (Fedotcheva et al. 1985; Brustovetsky et al. 1992).

It should be noted that hypothermia does not affect the other ion transporting system of grass snake mitochondria - Ca^{2+} uniporter. In contrast to the K^+ transport, the rate of Ca^{2+} absorption by the liver mitochondria in active and hibernating snakes remains almost unchanged (Fig. 3). This distinguishes these reptiles from hibernating mammals. It is believed that the latter are characterized by a decrease in the transport of K^+ and Ca^{2+} ions due to a decrease in $\Delta\Psi$ in the mitochondria of hibernating animals. (Brustovetsky et al. 1992). In our experiments, we did not observe a noticeable change in the membrane potential among the experimental groups of animals (not shown). At the same time, it can be assumed that, under the conditions of hibernation, ion transport in liver mitochondria can be affected by a change in the expression of the main ion transporting proteins and their subunits, as shown in the case of the plasma membrane Na^+K^+ -ATPase (MacDonald and Storey 1999) and Ca^{2+}-ATPase of sarco(endo)plasmic reticulum (Malysheva et al. 2001). It can be assumed that hypothermia inhibits the expression and decrease in the amount of the mitoKATP channel protein, but the number of MCU in the mitochondria does not change.

One of the parameters reflecting mitochondrial integrity is the calcium-loading capacity. It is known that disturbances of Ca^{2+} homeostasis and an increase in the concentration of this ion in the cytoplasm of the cell occur under hypothermia conditions (Boutilier 2001; Rauen and de Groot 2004). Mitochondria, as one of the major calcium depot in the cell, are able to accumulate some amount of this ion in the matrix space. At the same time, the critical concentrations of this ion in the matrix of organelles cause the induction of the mitochondrial permeability transition (MPT pore opening). Appearance of this pore leads to the impairment of the energy-transducing functions of mitochondria (e.g., ATP synthesis), swelling of mitochondrial matrix, rupture of outer membrane and the release of cytochrome *c* and other pro-apoptotic proteins (Rasola and Bernardi 2011). It was previously shown that mitochondria of hibernating ground squirrels have a lower calcium capacity compared to active animals (Ballinger et al. 2017). It was found that liver mitochondria of hypothermia-exposed grass snakes also have a significantly lower calcium capacity compared with liver mitochondria of active animals (Fig. 2a-b). In other words, much lower concentrations of Ca^{2+} are able to induce pore opening in the liver mitochondria of hibernating grass snakes compared to active ones. At the same time, it should be noted that the pore inhibitor CsA increased the calcium capacity of both groups of animals to almost the same level (Fig. 2c).

It can be suggested that the low resistance of liver mitochondria of hibernating snakes to induction of Ca^{2+}-dependent pore compared with active animals is caused by an increase in H_2O_2 production in these organelles (Fig. 4). It is known that enhancing the production of reactive oxygen species in mammalian mitochondria leads to a decrease in the threshold Ca^{2+} concentration and favors the induction of the mitochondrial pore (Zavodnik et al. 2013; Dubinin et al. 2016). Therefore, it can be assumed that a similar pattern is also observed in the case of poikilothermic animals.

What is the reason for the increase in H_2O_2 production in liver mitochondria of hypothermia-exposed grass snakes? On the one hand, it may be caused by the suppression of the activity of the complexes of the mitochondrial respiratory chain of these animals. On the other hand, it is known that, depending on the conditions, fatty acids can also cause an increase in the production of ROS by mitochondria (Schönfeld and Wojtczak 2007, 2008).

Also, one of the reasons for the destruction of membranes, associated with the induction of the mitochondrial pore, may be a change in the fatty acid composition of the phospholipids forming these membranes. It is known that in hibernating homoeothermic animals as well as in poikilothermic ones, a decrease in body temperature is accompanied by an increase in the level of polyunsaturated fatty acids in the composition of lipid membranes (Aloia 1980; Carey et al. 2003; Ruf and Arnold 2008). Such polyunsaturated fatty acids play an important role in maintaining the fluidity of membranes upon lowering of the body temperature (Munro and Thomas 2004; Ruf and Arnold 2008). The increase in their number in the composition of membrane phospholipids leads to a decrease in the viscosity of the membranes and ensures the normal functioning of membrane-bound proteins under conditions of low temperature. However, it is necessary to take into account that the double bonds of unsaturated fatty acids are the targets of ROS. For this reason, the entering of ground squirrels into hibernation is accompanied by both an increase in lipid unsaturation and an increase in the content of the primary products of lipid peroxidation - diene conjugates (Carey et al. 2003).

We found similar changes in the case of grass snake liver mitochondria — hypothermia results in a substantial increase in the amount of polyunsaturated fatty acids (PUFA), especially linoleic (18: 2) and arachidonic (20: 4) (Table 3). These changes lead to a significant increase in the unsaturation index (UI) of phospholipid membranes in liver mitochondria of hypothermia-exposed grass snakes. It can lead to increased

formation of lipid peroxides and activation of destructive processes associated, inter alia, with the induction of Ca^{2+}-dependent mitochondrial pore.

In general, it should be noted that hypothermia in liver mitochondria of grass snake leads to functional changes similar to hibernating mammals: 1) suppression of succinate-fuelled respiration; 2) decrease in the rate of mitochondria energy-dependent K^+ transport; 3) reduction in resistance to Ca^{2+}-dependent pore induction; 4) H_2O_2 production enhancement; 5) increase in the unsaturation index (UI) of membrane phospholipids. All of this indicates the presence of similar mechanisms of adaptation of poikilothermic and homoeothermic animals to the conditions of hypothermia and survival under adverse environmental conditions.

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