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Functioning of the mitochondrial ATP-dependent potassium channel in rats varying in their resistance to hypoxia. Involvement of the channel in the process of animal's adaptation to hypoxia

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Abstract The mechanism of tissue protection from ischemic damage by activation of the mitochondrial ATP-dependent K^+ channel ($mitoK_{ATP}$) remains unexplored. In this work, we have measured, using various approaches, the ATP-dependent mitochondrial K^+ transport in rats that differed in their resistance to hypoxia. The transport was found to be faster in the hypoxia-resistant rats as compared to that in the hypoxia-sensitive animals. Adaptation of animals to the intermittent normobaric hypoxia increased the rate of transport. At the same time, the intramitochondrial concentration of K^+ in the hypoxia-sensitive rats was higher than that in the resistant and adapted animals. This indicates that adaptation to hypoxia stimulates not only the influx of potassium into mitochondria, but also K^+/H^+ exchange. When $mitoK_{ATP}$ was blocked, the rate of the mitochondrial H_2O_2 production was found to be significantly higher in the hypoxia-resistant rats than that in the hypoxia-sensitive animals. The natural flavonoid-containing adaptogen Extralife, which has an evident antihypoxic effect, increased the rate of the mitochondrial ATP-dependent K^+ transport in vitro and increased the *in vivo* tolerance of hypoxia-sensitive rats to acute hypoxia 5-fold. The involvement of the mitochondrial K^+ transport in the mechanism of cell adaptation to hypoxia is discussed.

Keywords Mitochondrial ATP-dependent K^+ channel · K^+/H^+ exchange · ROS · Hypoxia · Adaptation · Flavonoid-containing adaptogen

Abbreviations

$mitoK_{ATP}$	mitochondrial ATP-dependent K^+ channel
HR rats	hypoxia-resistant rats
HS rats	hypoxia-sensitive rats
INH	intermittent normobaric hypoxia
5-HD	5-hydroxydecanoate
ST	survival time
ROS	reactive oxygen species
BSA	bovine serum albumin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DNP	2,4-dinitrophenol

Introduction

It is generally accepted that the cytoplasmic and mitochondrial membranes contain ATP-dependent potassium channels ($cytoK_{ATP}$ and $mitoK_{ATP}$), which play an important role in the protection of heart and brain from ischemia (Garlid et al. 1997; O'Rourke 2004). These channels have a number of common, as well as specific, activators and inhibitors. The inhibitory analysis showed that the anti-arrhythmic effect of some pharmacological activators is mediated by both channels (primarily by $cytoK_{ATP}$), while

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the anti-ischemic effect mainly depends on mitoK$_{ATP}$ (Tsai et al. 2002; Krylova et al. 2006).

It has been shown that the cardioprotective effect of long-term intermittent hypoxia requires the opening of the ATP-dependent K^+ channels and it seems to be mitoK$_{ATP}$, rather than cytoK$_{ATP}$ that is involved in the protective mechanism (Asemu et al. 1999; Eells et al. 2003; Neckar et al. 2002; Kolàř and Oštàdal 2004). It has also been found that the opening of mitoK$_{ATP}$ facilitates the removal of Ca^{2+} overload during ischemia/reperfusion or long-term intermittent hypoxia, which may underlie the mechanism of cardioprotection (Zhu et al. 2003).

In spite of all the facts indicating participation of mitoK$_{ATP}$ in the protection of tissues from ischemic damage, the mechanism of its involvement in cardioprotection remains to a large extent unexplored. In particular, nothing is known about the role that may be played by mitoK$_{ATP}$ in providing individual variability in the resistance of animals to hypoxia. The extent of resistance, meanwhile, is one of the key factors that determine the development of early and delayed adaptive mechanisms in an individual (Lukyanova 2004; Lukyanova et al. 2009a, b).

Discovering the regulatory role of mitoK$_{ATP}$ in the physiology of the animal and human organism has highlighted the importance of search for pharmacological activators of cyto- and mitoK$_{ATP}$, which would possess cardioprotective abilities. This goal has been given much attention (Garlid et al. 1997; Mironova et al. 1999; Grigoriev et al. 1999). Recently, we have found natural activators of mitoK$_{ATP}$, whose metabolic precursors exert a cardioprotective effect (Mironova et al. 2004; Krylova et al. 2006).

There is still much to be done. The search for the modulators of mitoK$_{ATP}$ activity has not finished, and it would be especially important to search for them among already known natural adaptogens. Thus flavonoid reported to have antihypoxic, adaptogenic and antioxidant abilities may be involved in such a regulation (Lukyanova et al. 2007, Mironova et al. 2008).

Using three different methodical approaches, we show that in the hypoxia-resistant (HR) rats, the rate of the ATP-dependent K^+ influx into the heart and liver mitochondria is significantly higher comparatively to that in the hypoxia-sensitive (HS) animals. The adaptation of animals by the method of intermittent normobaric hypoxia (INH) increases the rate of K^+ influx, whereas the concentration of K^+ in the mitochondrial matrix drops.

It has also been demonstrated that the herbal antihypoxic flavonoid-containing adaptogen Extralife activates the ATP-dependent K^+ transport in isolated mitochondria and, when introduced intraperitoneally, significantly prolongs the time of animal survival under hypoxia. Both effects have been found to be canceled by 5-hydroxydecanoate (5-HD), a specific inhibitor of mitoK$_{ATP}$.

Materials and methods

Chemicals All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, U.S.A.

Testing animals for the ability to endure acute hypobaric hypoxia

The experiments were performed on male Wistar rats (180–200 g), which were selected on the basis of their resistance to hypoxia (hypoxia-resistant and hypoxia-sensitive animals). The resistance was determined 3–4 weeks before the experiments using a special testing procedure (Berezovsky 1978; Lukyanova et al. 2009a, b). In the procedure, animals were placed into a pressure chamber and tested for their ability to endure a critical life-incompatible altitude (11.5 km). The parameter registered was the survival time (ST)—from the moment of reaching the critical altitude to the moment of pathological breathing (second agonal breath). This parameter characterizes viability of animals under extreme hypoxic conditions and reflects the ability to fully mobilize the nonspecific protective functions of the organism—those that are responsible for the organism survival (mainly, the functions of respiratory centers and cardiovascular system)—in the sublethal period. After registering ST, the pressure in the chamber was normalized, and the animals restored the normal posture and locomotor activity within 4–6 min.

After testing, the animals were ranked and those with low and high ST values were selected in two experimental groups: the group of HS animals, whose ST did not exceed 2 min; and the group of HR ones, which had an ST value of 10 min or more. Each group amounted to approximately 25% of the total number of tested animals.

Adaptation of HS animals to hypoxic conditions

A month after the hypoxia resistance test, HS rats were subjected to a course of intermittent normobaric hypoxia (INH) in the regime of preconditioning. The daily 1-h INH training session included 6–7 alternating periods of passive breathing in the hypoxic (10% O_2; 5 min) and normal (20% O_2; 3 min) atmosphere at constant pressure. The course lasted 3 weeks (5 sessions a week), which is defined here as long-term adaptation to INH in the regime of preconditioning.

Examining the effect of extralife on mitoK$_{ATP}$ channels

The effect of the Extralife preparation (a water-soluble flavonoid-containing frozen-dried extract from *Phentaphylloides fruticosa*), which possesses adaptogenic, antihypoxic

and antioxidant features (Lukyanova et al. 2007), was studied in the experiments *in vivo* and in vitro.

In the experiments *in vivo*, we examined the role of mitoK$_{ATP}$ in the adaptation of the Extralife-treated animals to hypoxia. The preparation was administered intraperitoneally at the dose of 20 mg/kg 30 min before placing the animal in the pressure chamber. Then, after lifting to the critical height, the animal was tested for ST. In another series of experiments, the preparation was introduced together with the mitoK$_{ATP}$ inhibitor 5HD (5 mg/kg).

Isolation of mitochondria

Mitochondria were isolated from the liver and heart of Wistar rats by the standard differential-centrifugation technique (Belosludtsev et al. 2005).

In the case of liver mitochondria, the isolation medium contained 210 mM *D*-mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES/NaOH (pH 7.4); the washing medium was the same except that EDTA was replaced by 0.05 mM EGTA. Finally, the mitochondrial pellet was resuspended in the EDTA/EGTA-free medium.

In the case of heart mitochondria, the isolation medium contained 210 mM *D*-mannitol, 70 mM sucrose, 2 mM EGTA, 1% BSA and 10 mM HEPES/NaOH (pH 7.4). The washing/resuspending medium was the same minus EGTA and BSA.

The final suspension of mitochondria contained 80–100 (liver) or 35–50 (heart) mg protein/ml.

ATP-inhibitable K^+ transport

The ATP-inhibitable transport of K^+ in mitochondria was estimated by (1) energy-dependent uptake of K^+ by mitochondria and (2) K^+ efflux from mitochondria induced by the uncoupler (DNP).

The energy-dependent K^+ uptake was followed spectrophotometrically, by the swelling of mitochondria (Jaburek et al. 1998). The medium contained 50 mM KCl, 5 mM Na_2HPO_4, 0.1 mM EGTA, 5 mM HEPES/KOH (pH 7.2), 5 μM cytochrome *c*, 1 μg/ml oligomycin, 5 mM succinate and 2 μM rotenone or substrates reducing complex I (4 mM glutamate+1 mM malate). The concentration of mitochondrial protein was 0.2 mg/ml. ATP-Mg^{2+} was added into the medium before mitochondria. The rate of mitochondrial swelling was followed as a decrease in absorbance at 520 nm (ΔA_{520})/min per mg of mitochondrial protein using a Shimadzu spectrophotometer at constant stirring and thermostating (26 °C).

The ATP-dependent DNP-induced efflux of K^+ from mitochondria was measured with a K^+-selective electrode (Baranova et al. 2000) in a 1-ml cell at constant stirring and thermostating (26 °C). The medium contained 170 mM sucrose, 80 mM D-mannitol, 5 mM Na_2HPO_4, 10 mM Tris-HCl (pH 7.4) and 1 μg/ml oligomycin; the concentration of mitochondrial protein was 1–1.2 mg/ml. ATP-Mg^{2+} was added into the medium before mitochondria. The rate of K^+ efflux was expressed as the change of K^+ concentration in the medium ($\Delta[K^+]$)/min per mg of mitochondrial protein.

Estimation of K^+ quantity in mitochondria

The mitochondrial K^+ was assayed with a K^+-selective electrode after the addition of 0.05% Triton X-100 to the suspension of mitochondria (stirring; thermostating, 26 °C; cell volume, 1 ml; concentration of mitochondrial protein, 1–1.5 mg/ml).). The medium contained 170 mM sucrose, 80 mM D-mannitol, 5 mM Na_2HPO_4, 10 mM Tris-HCl (pH 7.4); the concentration of mitochondrial protein was 1–1.2 mg/ml.

Assay for H_2O_2 production in mitochondria

The production of H_2O_2 in mitochondria was determined with the fluorescent dye Amplex Red (Molecular Probes, Inc.) using a horseradish peroxidase system (Ferranti et al. 2003). The method is based on the change of fluorescence of the oxidized Amplex Red product resorufin (excitation, 563 nm; emission, 587 nm). The assay medium contained 120 mM KCI, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 10 mM HEPES/KOH (pH 7.3), 1 mM EGTA, 2 μM oligomycin and 0.1% BSA. Just before measurements, the medium was supplemented with peroxidase (1 unit/ml), Amplex Red (10 μM), mitochondria (0.16–0.25 mg of protein/ml) and rotenone (2 μM). The reaction was launched with respiratory substrates (0.25 mM malate+0.25 mM glutamate). The measurements were carried out using a GARY Eclipse spectrofluorimeter (Varian Inc., USA) at constant stirring and thermostating (36 °C). The kinetics of H_2O_2 production was recorded for 3 min. The quantity of H_2O_2 formed was calculated against a calibration curve obtained with a standard H_2O_2 solution (10 μM), whose concentration was adjusted spectrophotometrically.

Results

Examining the systems of mitochondrial ATP-dependent K^+ transport in rats with different tolerance to acute hypobaric hypoxia

The rate of the ATP-dependent swelling was found to be 50–75% higher in the HR rats than in the HS animals, which is especially evident using NADH-dependent respiratory substrates (Table 1).

Similar results were obtained on another system: the uncoupler-induced efflux of K^+ from mitochondria, which

Table 1 ATP-dependent K^+ uptake by heart and liver mitochondria in the HR and HS rats, as well as HS animals adapted to hypoxia with a course of INH therapy ($p<0.05$)

Tissue	Resistance of rats to hypoxia	Glutamate (4 mM) + malate (1 mM)		Succinate (5 mM)	
		A_{540}/min per mg protein	% of the level in HS rats	A_{540}/min per mg protein	% of the level in HS rats
Heart	Sensitive	0.230±0.03		0.202±0.02	
	Resistant	0.364±0.02	158	0.230±0.02	113
	Adapted	0.349±0.04	151	0.272±0.03	133
Liver	Sensitive	0.128±0.01		0.126±0.01	
	Resistant	0.225±0.02	175	0.134±0.02	105.7
	Adapted	0.208±0.01	162	0.212±0.02	168

we recorded using an ion-selective valinomycin-based electrode. Earlier we found that the efflux—down the concentration gradient after uncoupler-triggered collapse of $\Delta\psi$—is inhibited by ATP. Therefore, we supposed that under these conditions, K^+ effluxes from mitochondria through the mitoK_{ATP} channel (Baranova et al. 2000). As follows from Table 2, HR rats show significantly higher rates of ATP-dependent K^+ efflux than HS ones, with the relative difference being close to that recorded for energy-dependent mitochondrial swelling.

It should be noted that both types of animals reveal evident tissue-specific differences in the activity of mito-K_{ATP}. We have found that the heart mitochondria swell 1.5 times faster than the liver ones (Table 1). The rate of DNP-induced K^+ efflux from the heart mitochondria is also 1.5 times higher (Table 2).

Tissue-specific differences have also been revealed for the constant of the channel inhibition by ATP ($K_{1/2}$). In the heart mitochondria, $K_{1/2}$ is substantially lower than that in the liver organelles (Table 3). As seen from the table, the value of $K_{1/2}$ in the HS animals is twice as large as that in the HR ones.

The assay of mitochondrial K^+ shows that in the HR rats, the capacity of mitochondria for K^+ is about 25% lower than in the HS animals (Fig. 1). The data obtained indirectly indicate that the activation of the K^+ influx into mitochondria is accompanied by the accelerated efflux of the ion, probably due to activation of the electroneutral K^+/H^+ exchange, which was predicted earlier (Garlid and Paucek 2003).

The supposition about the activation of K^+ efflux in response to its accelerated influx has been confirmed in our experiments with prolonged, 30-min registration of mitochondrial swelling (usually, the measurements take 1.5–3 min). Within a 30-min period, we observed two waves of swelling/contraction oscillations (Fig. 2). The results obtained are in agreement with the literature data, which show that the valinomycin-induced energy-dependent K^+ influx into mitochondria is accompanied by the subsequent activation of the electroneutral efflux of the ion in exchange for a proton, this maintaining the volume of mitochondria at a certain level (Schönfeld et al. 2003). This recycling of K^+ has been found to be inhibited by ATP·Mg^{2+}, probably, due to inhibition of the system of K^+ entry (Fig. 2).

MitoK_{ATP}-controlled generation of H_2O_2 by mitochondria in the hypoxia-resistant and hypoxia-sensitive rats

It is known that mitoK_{ATP} controls the rate of H_2O_2 production in mitochondria. The addition of ATP to mitochondria will inhibit the channel and increase the rate of H_2O_2 generation, which can be partially cancelled by a channel activator (Ferranti et al. 2003). Our experiments have shown that in the HR rats, the rate of mitochondrial H_2O_2 production in the presence of NAD-dependent substrates almost triples after the addition of ATP, whereas in the HS animals, it increases only by a factor of 1.5 (Fig. 3a). In the liver mitochondria, H_2O_2 generation also grows after the addition of ATP, although the difference between HR and HS rats is less pronounced.

Table 2 DNP-induced K^+ efflux from heart and liver mitochondria in the HR and HS rats, as well as HS animals adapted to hypoxia with a course of INH therapy

Resistance of rats to hypoxia	Liver mitochondria		Heart mitochondria	
	μM/min per mg protein	% of the level in HS rats	μM/min per mg protein	% of the level in HS rats
Sensitive	38.17±3.21		51.1±5.35	
Resistant	66.67±0.82**	175	96.6±7.41**	189
Adapted	59.3±6.04*	155	81.2±5.31**	159

* $p<0.05$; ** $p<0.01$

Table 3 ATP-induced inhibition of energy-dependent K^+ influx ($K_{1/2}$, μM) into liver and heart mitochondria in the HR and HS rats

	Resistance of rats to hypoxia	
Tissue	Resistant	Sensitive
Liver	43.1±2.2	79.8±7.1
Heart	6.3±1.2	14.7±2.1

A similar ATP-induced increase in the rate of H_2O_2 production, both in heart and liver mitochondria, is observed in the presence of endogenous substrates (Fig. 3b). With the NAD-dependent substrates (glutamate + malate), the rates of H_2O_2 generation were higher.

Parameters of K^+ exchange in the mitochondria of hypoxia-sensitive rats adapted to low-oxygen conditions

Earlier we found that a prolonged adaptation of animals to various forms of hypoxia would increase their hypoxic tolerance, the effect being more pronounced in the HS individuals (Lukyanova 2004; Lukyanova et al. 2009a, b). It was shown that the less resistant animal is the higher is its ability to adapt.

In our experiments, the HS rats that received a course of INH had their resistance to hypoxia increased, whereas the INH-treated HR animals demonstrated a decreased resistance during the first week of training, and only later did we observe a slight positive effect (Fig. 4). Therefore, only HS rats were used in the "hypoxic adaptation" experiments in the present work.

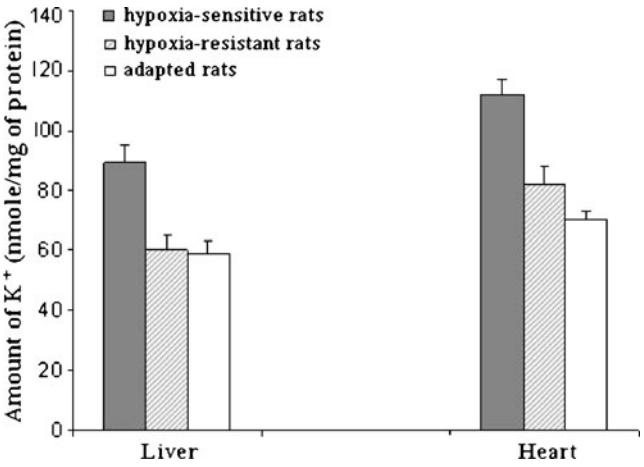

Fig. 1 Quantity of K^+ in the heart and liver mitochondria of the HR and HS rats, as well as HS animals adapted to hypoxia with a course of INH therapy. The medium contained 170 mM sucrose, 80 mM D-mannitol, 5 mM Na_2HPO_4, 10 mM Tris-HCl (pH 7.4) and 1 μg/ml oligomycin; the concentration of mitochondrial protein was 1–1.2 mg/ml. The quantity of K^+ in mitochondria was calculated from the change of K^+ concentration in the medium after disrupting mitochondria with Triton X-100

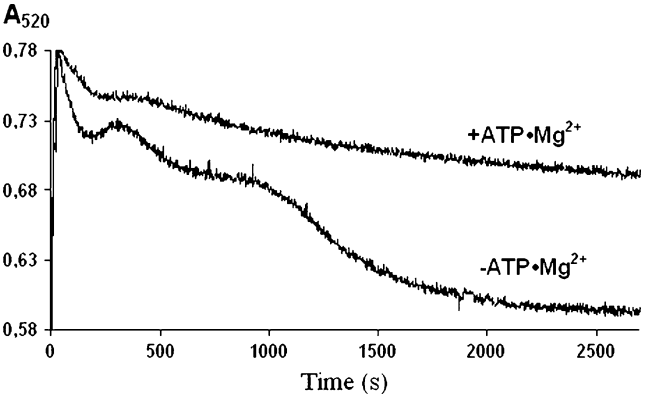

Fig. 2 Oscillations of the rat liver mitochondrial volume. The medium contained 50 mM KCl, 5 mM Na_2HPO_4, 0.1 mM EGTA, 5 mM HEPES/KOH (pH 7.2), 5 μM cytochrome *c*, 1 μg/ml oligomycin, 5 mM succinate and 2 μM rotenone. The concentration of mitochondrial protein was 0.2 mg/ml, ATP-Mg^{2+} - 500 μM

A 3-week adaptation of HS rats to hypoxia by the INH method has been shown to lower the quantity of K^+ in the heart and liver mitochondria significantly, i.e. by 30% (Fig. 1). The rate of energy-dependent mitochondrial swelling in the presence of NAD-dependent substrates increases too—up to the level observed in the HR animals (Table 1). The effect is substantially smaller if respiration of mitochondria is supported with succinate.

The adaptation of HR rats to hypoxia also increases the rate of the DNP-dependent K^+ transport (Table 2). This effect has been demonstrated both in the heart and liver mitochondria.

Effect of extralife on the transport of K^+ in mitochondria and survival of animals under hypoxic conditions

In this work, we have examined the effects of Extralife, a water extract from the flowers of *Phentaphylloides fruticosa* containing a large amount of flavonoids, including quercetin, and having a wide spectrum of biological effects: antioxidant, antihypoxic and adaptogenic (Lukyanova et al. 2007). Earlier we showed that this preparation accelerates and enhances the formation of the organism tolerance to acute hypoxia (Lukyanova et al. 2007, Mironova et al. 2008).

The experiments were conducted under both in vitro and *in vivo* conditions. As can be seen from Fig. 5, low concentrations of Extralife increased the rate of energy-dependent mitochondrial swelling by a factor of 2–3.

The effect of Extralife on mitoK_{ATP} was tissue-specific: in the liver mitochondria it was weaker than in the heart organelles, although its concentration range was approximately the same (Fig. 5). This fact may indicate the channel

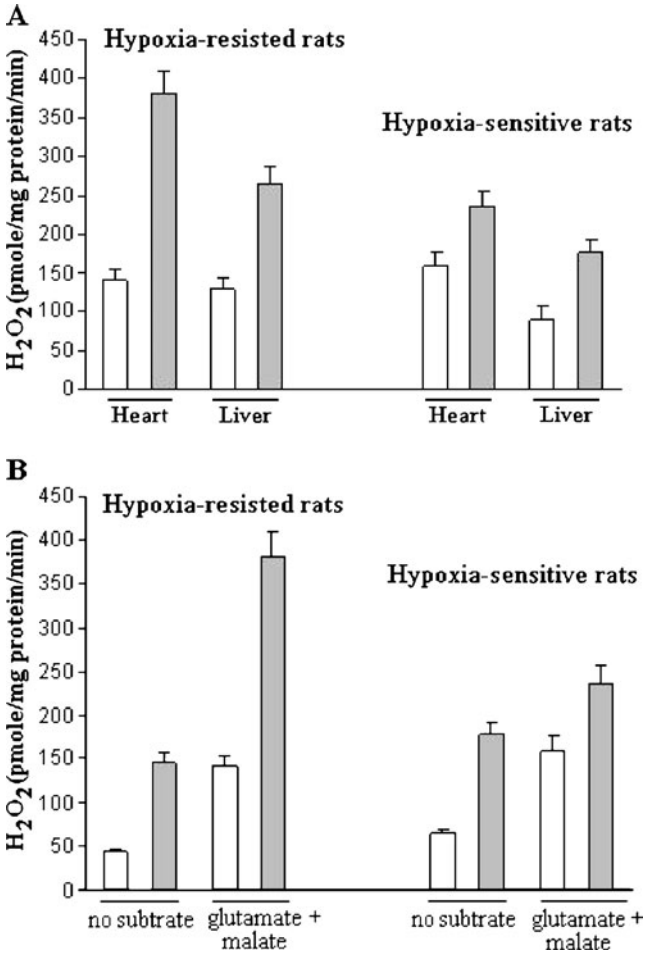

Fig. 3 H_2O_2 production by heart and liver mitochondria (**a**) and substrate dependence of H_2O_2 production in heart mitochondria (**b**) of animal with different resistance to hypoxia. The assay medium contained 120 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 10 mM HEPES/KOH (pH 7.3), 1 mM EGTA, 2 μM oligomicin and 0.1% BSA. Right before measurements, the medium was supplemented with peroxidase (1 unit/ml), Amplex Red (10 μM), mitochondria (0.16–0.25 mg of protein/ml). The reaction was launched with respiratory substrates (0.25 mM malate+0.25 mM glutamate). *White columns*—without ATP; *grey columns*—with 200 μM ATP

having a different functional load in the liver and heart. The specific inhibitor of mitoK$_{ATP}$ 5HD removed the effects of Extralife, almost to the control level (Fig. 5c)

As we suggest, activation of the ATP-dependent K^+ transport in mitochondria is necessary to launch the fast-response adaptive mechanisms of the organism under extreme conditions, including hypoxia. If so, then activation of mitoK$_{ATP}$ should increase the organism tolerance to extreme conditions, whereas suppression of the channel should have the opposite effect. In this connection, we have conducted a series of *in vivo* experiments—to test if mitoK$_{ATP}$ has relevance to the adaptation of animals to hypoxia.

In these experiments, the preparations (Extralife and 5HD) were administered to rats intraperitoneally, and the

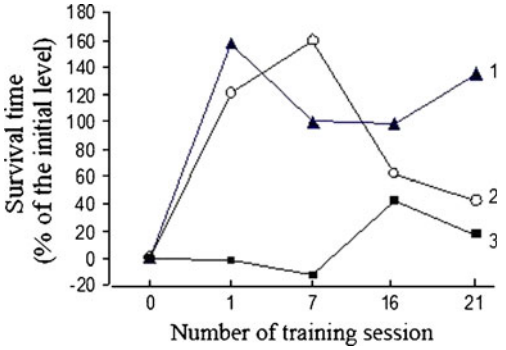

Fig. 4 Effect of INH training on the survival time of rats with different resistance to hypoxia. Highly sensitive to hypoxia (*trace 1*), average sensitive (*trace 2*) and highly resistant (*trace 3*) to hypoxia animals

animals were lifted in a pressure chamber to the critical height of 11.5 km. The experiments showed that under extreme hypoxic conditions, Extralife increased ST by a factor of 5 and the time of motional activity loss by a factor of 3, whereas the inhibitor of mitoK$_{ATP}$ 5HD removed this effects (Fig. 6).

Discussion

In order to understand the mechanism of the organism adaptation to hypoxia and the role of mitoK$_{ATP}$ in the process, we have examined the mitochondrial ATP-dependent transport of K^+ in the rats with genetically different resistance to hypoxia, as well as animals experimentally adapted to hypoxia. As considered now, the mitochondrial ATP-dependent K^+ transport is mediated by mitoK$_{ATP}$ (Jaburek et al. 1998).

It is known that individuals of an animal population differ in their tolerance to oxygen deficiency (Berezovsky 1978). As shown earlier, animals that belong to two opposite, in relation to hypoxia tolerance, types (HR and HS) would have essentially different "functional-metabolic" profiles. The profiles differ by the effectiveness of the organism's energy support, by central nervous system regulation, by neuro-humoral, stress-activating and stress-limiting systems, by oxygen-transport function of blood and also by the state of membranes and receptor apparatus (Berezovsky 1978; Lukyanova et al. 1993).

The results obtained in the present work allow us to suppose that the activity of mitoK$_{ATP}$ is genetically determined. In the rats genetically resistant to hypoxia, the rate of ATP-dependent K^+ influx into mitochondria is approximately 1.5–2 times higher than that in the hypoxia-sensitive animals (Table 1). In addition, HR rats have a lower value of $K_{1/2}$ for ATP (Table 3), which might mean differences in the structure of mitoK$_{ATP}$ in the HR and HS animals. The two types of animals also differ by the quantity of K^+ in the mitochondria (Fig. 1).

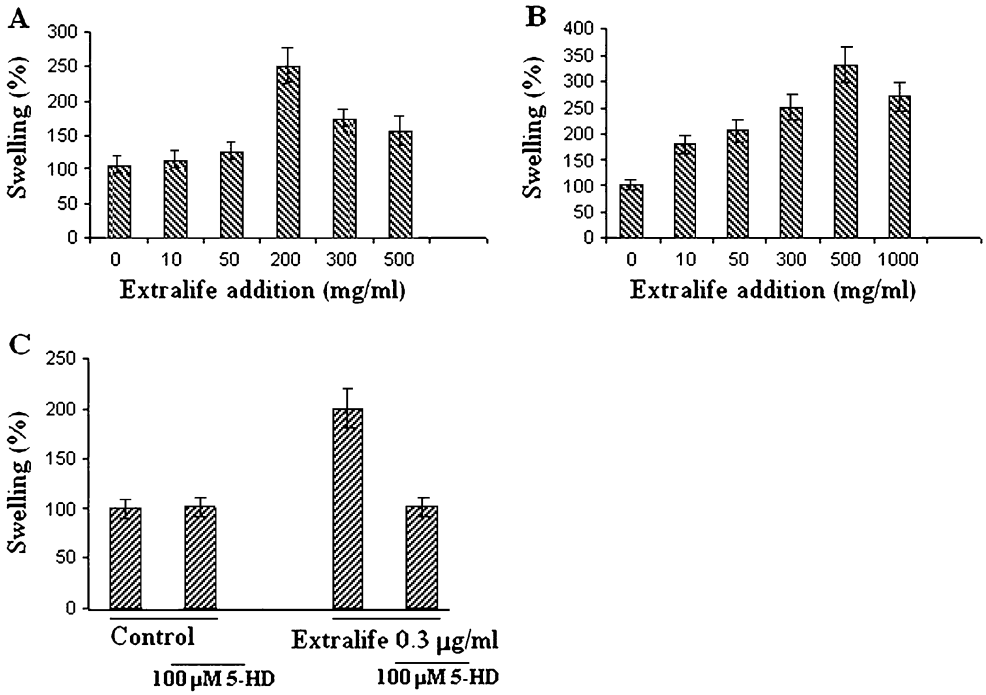

Fig. 5 Effect of Extralife on the energy-dependent swelling of liver (**a**) and heart (**b**, **c**) mitochondria and influence of the mitoK$_{ATP}$ inhibitor 5HD on the effect of Extralife (**c**). The reaction medium was the same as in Fig. 2

The higher level of mitoK$_{ATP}$ activity in the HR rats is also indicated by the different rate of mitoK$_{ATP}$-dependent H_2O_2 generation in the mitochondria of these two animal types. According to the literature data, the addition of ATP to mitochondria is accompanied by an increase in H_2O_2 production (Ferranti et al. 2003). The authors of this publication, who experimented with specific mitoK$_{ATP}$ modulators, came to the conclusion that the effect of ATP is realized through the closure of mitoK$_{ATP}$ and that the channel controls the rate of the H_2O_2 production in mitochondria. As follows from Fig. 4, the rate of the ATP-dependent H_2O_2 accumulation in the heart mitochondria of the HR animals is twice the rate of this process in the HS rats. These results are in a good agreement with the above data on the activation of the mitochondrial ATP-dependent K^+ transport in the HR animals.

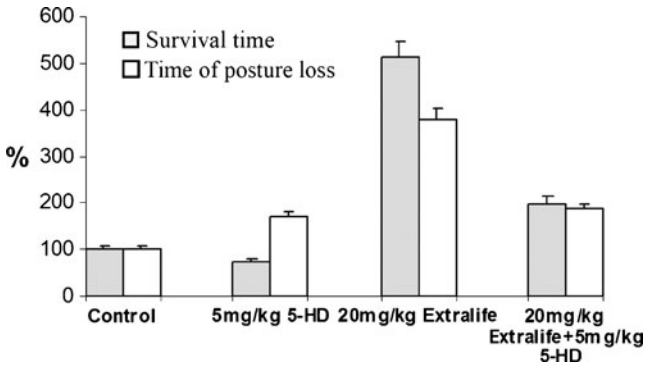

Fig. 6 Effect Extralife on the parameters of the organism resistance to hypoxia (ST and the time of posture loss after lifting to the critical altitude) and elimination of the effect by inhibitor of mitoK$_{ATP}$ -5HD. Experimental conditions see in "Materials and Methods")

It should be noted that the difference in the rates of the mitochondrial energy-dependent K^+ transport in the HR and HS rats is more evident in the case of NAD-dependent substrates, as compared to succinate (Table 1). This indicates a direct correlation between the activities of mitoK$_{ATP}$ and complex I (but not complex II), which agrees with the literature data (Portenhauser et al. 1971).

Another important finding of this work is the results showing tissue specificity of mitoK$_{ATP}$. Regardless of the method used and the degree of animal tolerance to hypoxia, the ATP-dependent transport of K^+ and the rate of ATP-dependent H_2O_2 production were shown to be higher in the heart mitochondria than in the liver ones (Tables 1 and 2; Fig. 3), which agrees with literature data (Ferranti et al. 2003). The values of $K_{1/2}$ for ATP in the heart and liver mitochondria were also different: in the heart they were substantially lower than in the liver (Table 3). These differences indicate that the activity of mitoK$_{ATP}$ in the heart mitochondria is higher than that in the liver organelles and might also mean tissue-specific differences in the regulation of the channel.

In the experiments with gradual adaptation of animals to hypoxia, we found that the effectiveness of adaptation depended on the initial hypoxic resistance. HS animals were more susceptible to adaptation (Fig. 4). At the same time, HR animals almost did not improve their parameters upon hypoxic training. Therefore, only HS animals were used in the work.

It has been established that prolonged adaptation of HS rats by the INH method changes the activity of mitoK$_{ATP}$ in these animals, which manifests itself in the increase of the rate of mitochondrial ATP-dependent K^+ transport almost to the level observed in the HR animals (Tables 1 and 2). At the same time, the quantity of K^+ in the heart and liver mitochondria of the adapted animals did not grow. It even decreased if compared to the initial level of this parameter in the HS rats (Fig. 1).

From the data obtained (Table 1, Figs. 1 and 2) we can conclude that the activation of the K^+ influx in the process of animal's adaptation to hypoxia is compensated for by the acceleration of the ion efflux, probably, due to the activation of the K^+/H^+ exchange, as predicted earlier (Garlid and Paucek 2003). This agrees with the previous reports, in which the energy-dependent influx of K^+ into mitochondria was shown to be accompanied by a rapid activation of the electroneutral efflux of the ion in exchange for a proton (Schönfeld et al. 2003; Shalbuyeva et al. 2006).

As follows from the results of this work (Figs. 1 and 2), not only mitoK$_{ATP}$ but the K^+/H^+ exchange system as well is involved in the mechanisms that underlie gradual adaptation of the organism to hypoxia. The adaptation is accompanied by an enhanced recycling of K^+ in mitochondria and leads to a so-called "mild" uncoupling, which is known to decrease the rate of the production of reactive oxygen species (ROS) in mitochondria (Korshunov et al. 1997; Ferranti et al. 2003).

It is known that prolonged myocardial ischemia and, especially, the following reperfusion are accompanied by an anomalously high ROS production (Vanden Hoek et al. 1998), which is the main factor of cell damage (Akao et al. 2003). The activation of mitoK$_{ATP}$ by means of pharmacological modulators of the channel introduced into the organism would decrease ROS production (Neckar et al. 2005). The decreased ROS production, due to activation of K^+ and, perhaps, Ca^{2+} (Belosludtsev et al. 2009) cycles, may underlie the mechanism of the organism adaptation to hypoxia and the cardioprotective potency of mitoK$_{ATP}$ activators.

This conclusion was confirmed in our in vitro and *in vivo* experiments with the preparation Extralife. Its ability to launch mechanisms of the organism's fast adaptation to hypoxia was confirmed in the present study. A prophylactic one-time introduction of the preparation increased the survival time of rats at the critical height in the pressure chamber by a factor of 5 (Fig. 6). Apparently, this fast protective effect of Extralife relates to the activation of mitoK$_{ATP}$—as it is largely removed by the specific inhibitor of the channel 5HD (Fig. 5).

In the experiments in vitro, a low concentration of Extralife increased the rate of ATP-dependent K^+ efflux into mitochondria, whereas 5HD—when introduced together with Extralife—inhibited this effect (Fig. 5).

Thus, the flavonoid-containing preparation Extralife, which has antihypoxic properties, is able to activate mitoK$_{ATP}$, with the effect developing under both in vitro and *in vivo* conditions. Therefore, activation of mitoK$_{ATP}$ is necessary for the organism to develop the mechanisms of fast adaptation to hypoxia.

On the basis of the results obtained and literature data, we suggest the following mechanism of mitoK$_{ATP}$ participation in the gradual INH-based adaptation of the organism to hypoxia. The decrease of oxygen concentration during transient hypoxia inhibits the respiratory chain, which will be accompanied by the temporary enhanced ROS production in mitochondria. It is known that low concentrations of peroxide radicals activate mitoK$_{ATP}$ (Costa et al. 2006), as well as protein kinase (Vanden Hoek et al. 1998). There are observations that mitoK$_{ATP}$ can be activated by protein kinase (Sato et al. 1998). A long hypoxic training would lead not only to the activation of mitoK$_{ATP}$ but to the stimulation of its expression: The introduction of pharmacological mitoK$_{ATP}$ activators into the organism was shown to increase the expression of the channel protein (Lu and Halvorsen 1997). Activation of the ATP-dependent K^+ influx into mitochondria would result in the stimulation of its efflux. This enhanced K^+ recycling in mitochondria will create a situation of "mild" uncoupling, which, in the long run, would lower the massive, abnormal ROS production usually observed under long-term hypoxia.

It is well known that the increased production of ROS is one of the main damaging factors in the cell. Their accumulation leads to the oxidation of membrane lipids and the opening of the mitochondrial CsA-sensitive pore, which is followed by the lysis of mitochondria and necrosis of tissues (Li et al. 2004).

The decrease in ROS production upon activation of K^+ and, possibly, Ca^{2+} cycles (Belosludtsev et al. 2009) may underlie the mechanism of the organism adaptation to hypoxia, as well as the cardioprotective ability of mitoK$_{ATP}$ activators. The role of mitoK$_{ATP}$ in the prevention of ROS accumulation in the mitochondrial membrane makes the channel physiologically relevant—not only in regard to ischemia but to other pathological states as well.

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