Effect of Intermittent Normobaric Hypoxia on Kinetic Properties of Mitochondrial Enzymes

L. D. Lukyanova, A. M. Dudchenko, T. A. Tsybina, E. L. Germanova, E. N. Tkachuk*, and I. V. Erenburg*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 12, pp. 644-651, December, 2007
Original article submitted December 19, 2006

We studied the effect of intermittent normobaric hypoxia on the formation of adaptive signs and state of mitochondrial enzymes in the cerebral cortex of rats with different resistance to hypoxia. Kinetic parameters for mitochondrial enzymes in the substrate region of the respiratory chain of the cerebral cortex underwent various changes in low resistant and highly resistant rats over the first 2 h after 1-h intermittent normobaric hypoxia. Low resistant animals were characterized by more effective functioning of rotenone-sensitive NADH-cytochrome C reductase and succinate-cytochrome C reductase under conditions of increased reduction status of the cell. These features correlated with the increase in the general resistance of animals. Significant changes in kinetic properties of mitochondrial enzymes and signs of the development of resistance were not found in highly resistant rats. Reciprocal relations between mitochondrial enzyme complexes in the substrate region of the respiratory chain probably play a role of the signal regulatory mechanism, which mediates tissue-specific and general resistance of rats under conditions of intermittent normobaric hypoxia. These effects did not depend on oxygenation of the inhaled gas mixture during the inter-hypoxic period.

Key Words: *intermittent normobaric hypoxia; kinetic parameters; mitochondrial enzyme complexes; signal function; adaptation*

Much attention is now paid to nondrug improvement of the nonspecific resistance by intermittent normobaric hypoxia (INH). There is a large body of evidence that this treatment improves various functional parameters under normal and pathological conditions. Hence, this method can be used for preventive and therapeutic purposes. However, the molecular mechanisms underlying the action of INH are poorly understood. The mitochondrial respiratory chain serves as a target for hypoxia. Under conditions of rapid and sharp decrease in oxygen supply to cells, this structure is involved in the regulation of oxygen homeostasis. The mitochondrial respiratory chain plays a signal role and modulates oxygen consumption and rate of oxygen release from the extracellular medium to mitochondria. Opposite changes in activity of mitochondrial enzyme complexes I and II were found at the early stage of hypoxia [1,4-7,10-12]. They provide the maintenance of electron transport function in the cytochrome region of the respiratory chain. It remains unclear whether this mechanism is active during INH.

This work was designed to study the effect of INH on the resistance of rats and kinetic properties of mitochondrial enzymes in the cerebral cortex. This tissue is most sensitive to hypoxia. We evaluated the role of oxygenation in respiration during the inter-hypoxic period. The role of bioenergetic mechanisms in the development of resistance was

Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences; *Laboratory of Clinical Researches, Hypoxia Medical Academia, Moscow

0007-4888/07/1446-0795

studied in animals with different resistance to hypoxia.

MATERIALS AND METHODS

Experiments were performed on male rats with different initial resistance to hypoxia. The resistance of experimental animals to acute hypobaric hypoxia was evaluated in an altitude chamber 1 month before the start of study. The animals were elevated to a critical altitude of 11,500 m in an altitude chamber. We measured the lifetime of rats (LT_1) from the moment of reaching the critical altitude to the second agonal breath. There were animals with low (LT_1 ≤3 min), intermediate (LT_1 5-6 min), and high resistance (LT_1 ≥10 min). These animals were divided into experimental groups to study the effect of INH on the general resistance of rats and kinetic parameters of mitochondrial enzymes.

INH training included intermittent passive inhalation of a hypoxic mixture with 12-8% O_2 and atmospheric air (20% O_2, INH/O_{20}, 5-10 min) or gas mixture with 30% O_2 (INH/O_{30}, 3-5 min) at constant pressure. This daily treatment was performed 6-7 times a day (training session) for a total time of 1 h.

The effect of INH on adaptation of low resistant, medium resistant, and highly resistant rats was evaluated 2 and 24 h after the 1st training session, as well as 1 day after the 8th, 15th, and 21st training sessions. The animals were repeatedly mounted to the critical altitude in a pressure chamber. LT_2 was measured. LT_2/LT_1 ratio (adaptation coefficient) was expressed in percent. The LT_2/LT_1 ratio was compared in treated and control animals (no training under INH conditions).

Kinetic parameters (K_m and V_{max}) of mitochondrial enzymes in the cerebral cortex of low and highly resistant rats (rotenone-sensitive NADH-cytochrome C reductase [8], succinate-cytochrome C reductase [9], and cytochrome C oxidase [1]) were measured 2 h and 1 day after the 1st training session under conditions of INH/O_{20} or INH/O_{30}. Otherwise, these parameters were evaluated 1 day after the course of training with 8, 15, or 21 training sessions. The animals were decapitated. The brain was removed and placed in cold homogenization medium of 5 mM Tris-HCl, 1 mM EDTA, and 0.32 M sucrose. The cortex was separated, weighted, and homogenized at the temperature of ice melting. The homogenate was stored at -20°C.

To study kinetic parameters of rotenone-sensitive NADH-cytochrome C reductase (complexes I+III), the concentration of NADH (substrate) varied from 1.5 to 80 μM. We used 0.1 M phosphate buffer (pH 7.4) with 12 mM sodium azide, 9.8 mM nicotinamide, and 76 mM oxidized cytochrome C. The rate of NADH-cytochrome C reductase was calculated as the difference between reaction rates in the absence and presence of 19.5 μM rotenone at this concentration of the substrate.

To study kinetic parameters of succinate-cytochrome C reductase (complexes II+III), the concentration of sodium succinate (substrate) varied from 0.5 to 11 mM. We used 0.1 M phosphate buffer (pH 7.4) with 12 mM sodium azide and 76 mM cytochrome C.

To study kinetic parameters of cytochrome C oxidase (complexes IV), the concentration of ascorbate-reduced cytochrome C (95-97%, substrate) in 0.1 M phosphate buffer (pH 7.4) varied from 3.5 to 80 μM.

Kinetic studies were performed on a Specord M 400 spectrophotometer at 37°C. The reaction was induced by addition of 2% brain homogenate (0.2% brain homogenate in study of cytochrome C oxidase activity) to the incubation medium. The reaction was monitored by measuring changes in absorption at 550 nm (oxidation or reduction of cytochrome C).

K_m and V_{max} were calculated using Lineweaver—Burk plot by the method of least squares (MS Excel software).

The weight of stress marker organs (thymus, adrenal glands, and spleen) was measured 2 h and 1 day after the 1st training session to estimate the contribution of the stress component to INH-induced changes.

The results were analyzed by Student's *t* test.

RESULTS

The resistance of low resistant rats increased 2 h after the 1st training session with INH/O_{20}. This effect was preserved on day 1 (preconditioning), progressively increased during further training, and reached maximum after 8-15 training sessions. The resistance of low resistant rats decreased after the 21st training session (Fig. 1). However, the resistance of low resistant rats increased 1 day after the 1st training session with of INH/O_{30} and progressively increased during the follow-up period (despite the course of training).

Training with INH/O_{20} or INH/O_{30} had little effect on the resistance of highly resistant rats. The sensitivity of several animals to acute hypobaric hypoxia even increased under these conditions, which reflects a decrease in the resistance of these rats (Fig. 1). Our results are consistent with published data that highly resistant rats do not adapt to

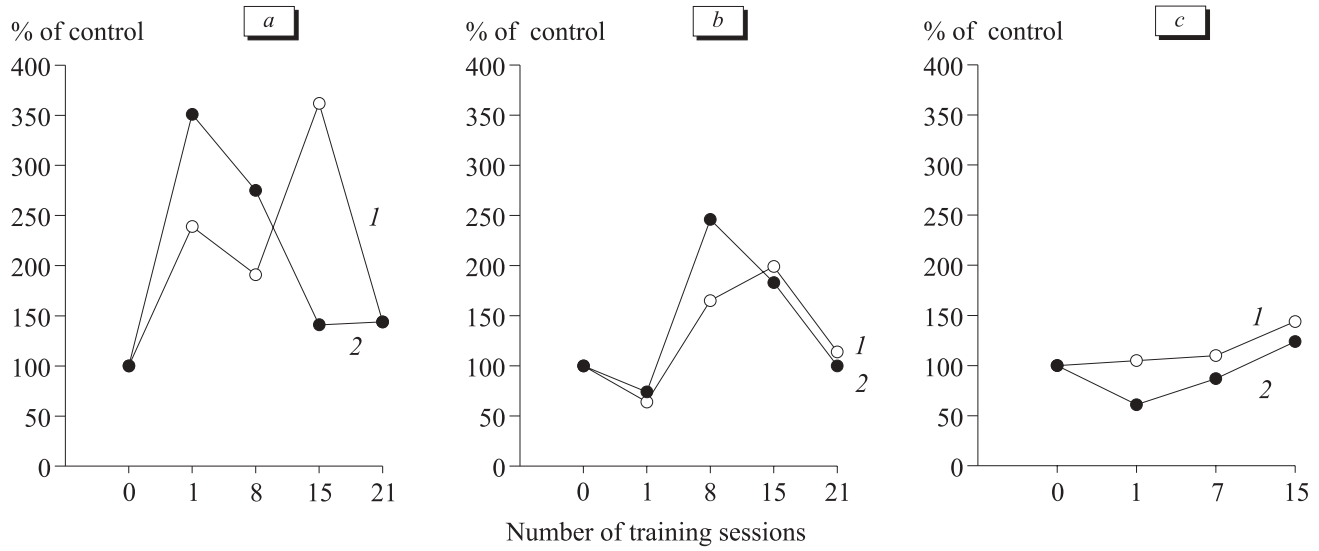

Fig. 1. Effect of duration of the training course with INH/O_{20} (*1*) and INH/O_{30} (*2*) on the resistance of rats to the critical height in an altitude chamber on day 1 after training. Low resistant (LT=1-2 min), medium resistant (LT=5 min), and highly resistant rats (LT more than 10 min).

hypoxia [7]. Medium resistant animals were intermediate between low resistant and highly resistant rats. The effect of preconditioning was not observed after the 1st training session with INH/O_{20} or INH/O_{30}. The resistance of these animals decreased after 1 day. However, the resistance of these rats slightly increased in the follow-up period. These changes in medium resistant animals were less pronounced than in low resistant rats. Similarly to low resistant rats, the resistance of medium resistant animals increased most significantly after 8-15 training sessions. At the same time, the resistance of these rats decreased in the follow-up period (Fig. 1).

Our results indicate that various regimens of INH contribute to the resistance of rats to hypoxia. The degree of these changes depends on the genotype of animas and decreases in the following order: low resistant rats>medium resistant rats>highly resistant rats. Low resistant rats exhibited only slight resistance or did not become resistant. Oxygenation of the inhaled gas mixture during the inter-hypoxic period modulated the development of resistance only in low resistant rats. High oxygenation of the inhaled gas mixture (30% O_2 *vs*. atmospheric air) during the inter-hypoxic period potentiated the influence of preconditioning on day 1 (as compared to INH/O_{20}), but had a negative effect on the development of resistance over the next 2 weeks.

Single training session with various types of INH had different effects on the weight of stress marker organs in low resistant and highly resistant animals. The weight of organs in low resistant rats increased over the 1st day after INH/O_{20}, which reflects activation of the sympathoadrenal system under these conditions. However, INH/O_{30} had little effect on animals of this group. The weight of the thymus, adrenal glands and, to a lesser extent, of the spleen in highly resistant rats decreased under both conditions of training. Hence, highly resistant rats demonstrated stress signs typical of exhaustion stage.

Study of kinetic parameters of mitochondrial enzymes showed that basal K_m and V_{max} for succinate-cytochrome C reductase and cytochrome C oxidase do not differ in the cerebral cortex of low resistant and highly resistant rats. However, K_m and V_{max} for rotenone-sensitive NADH-cytochrome C reductase in the brain of low resistant animals were much lower than in highly resistant rats (Table 1). These results are consistent with published data and provide support for our hypothesis [1]. We hypothesized that mitochondrial NADH-cytochrome C reductase in the brain of highly resistant rats has a more effective stoichiometric regulation at high concentration of NADH compared to low resistant animals. Hence, this enzyme in the brain of highly resistant rats is characterized by high functional activity in a wide range of NADH concentrations. These concentrations saturate similar enzyme complex in the brain of low resistant rats. Excessive amounts of reduced equivalents are accumulated in cells under conditions of hypoxia. Therefore, higher function of the mitochondrial respiratory chain in the brain of highly resistant rats under these conditions provides greater resistance of animals to oxygen deficiency (compared to low resistant rat).

The response and variations in kinetic parameters of mitochondrial enzymes in the cerebral cortex differed in low resistant and highly resistant rats during training with INH. The following changes were observed in the brain of low resistant rats

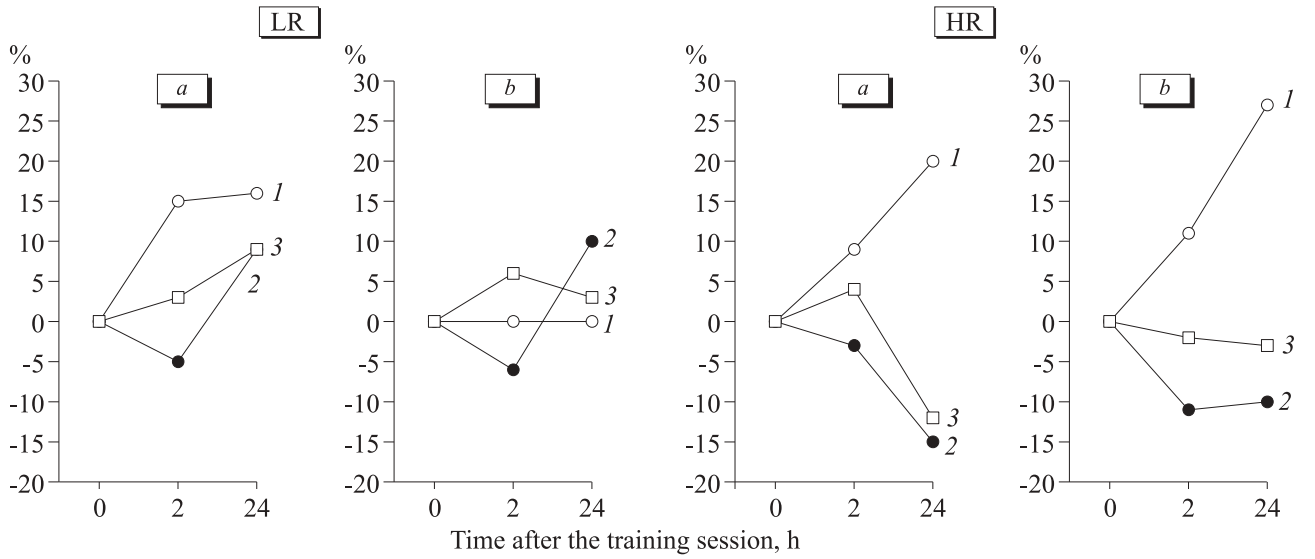

Fig. 2. Weight of stress-marker organs in highly resistant (HR) and low resistant (LR) rats 2 and 24 h after the 1st training session with INH/O_{20} (*a*) and INH/O_{30} (*b*): thymus (*1*), adrenal glands (*2*), and spleen (*3*).

2 h after single exposure to both regimens of INH (Table 1, Fig. 2):

1) significant increase in K_m and V_{max} for rotenone-sensitive NADH-cytochrome C reductase (complexes I+III), which approach the values in control highly resistant rats. These changes persisted 1 day after treatment;

2) significant decrease in K_m for succinate-cytochrome C reductase (complexes II+III), which reflects the increase in enzyme affinity for the substrate (succinate);

3) no significant changes in K_m and V_{max} for cytochrome C oxidase during this period (complex IV).

Simultaneous increase in K_m and V_{max} for rotenone-sensitive NADH-cytochrome C reductase in the brain of low resistant rats persisted for a long time. These values progressively increased in the follow-up period and reached maximum after 8-15 training sessions (Fig. 2, Table 2), which coincided with significant changes in the resistance of animals. K_m and V_{max} for NADH-cytochrome C reductase returned to the basal level after 21 training sessions, which correlated with the decrease in the resistance of animals to hypoxia. These data show that INH is followed by rapid changes in kinetic parameters of mitochondrial complex I+III in the brain of low resistant rats. These changes are directed towards optimization (improvement of the effectiveness) of respiratory chain function under conditions of increased reduction status of respiratory carriers. The dynamics of this process is similar to variations in the general resistance of animals.

The decrease in K_m and V_{max} for succinate-cytochrome C reductase reflected an increase in enzyme affinity for the substrate (succinate). These opposite changes were most significant 2 h after the 1st training session with INH, but became less pronounced in the follow-up period. Intracellular succinate concentration decreases in the early stage of hypoxia, which is associated with high rate of succinate oxidation. In this stage, the observed changes allow the enzyme to maintain high activity of the succinate oxidase pathway of oxidation [2-3]. Variations in kinetic properties of the substrate regions in the respiratory chain are directed toward electron supply to the cytochrome region, maintenance of cytochrome C oxidase activity, and preservation of energy synthesis in mitochondria. Our conclusion was also supported by the absence of significant changes in kinetic parameters of cytochrome C oxidase during these periods (Table 2).

INH contributes to reciprocal changes in activity of the substrate region in the respiratory chain of the cerebral cortex in low resistant rats. The increase in parameters for one enzyme complex is accompanied by a decrease in the corresponding parameters for another complex. These changes were observed under conditions of INH/O_{20} and INH/O_{30}. Hence, changes in oxygenation during the interhypoxic period had little effect on these relations.

It should be emphasized that K_m for cytochrome C oxidase in the brain of low resistant rats significantly decreased by the end of INH training, which reflects an increase in enzyme affinity for the substrate (Fig. 3, Table 2). Mitochondria lose a part of cytochrome C during hypoxia. Hence, the increase in affinity is of considerable physiological

TABLE 1. K_m and V_{max} for Mitochondrial Enzyme Complexes on Day 1 after Single Training Session with INH (*n*=5-10; *M*±*m*)

Time from hypoxia to decapitation	Low resistant			Highly resistant		
	control	INH/O_{30}	INH/O_{20}	control	INH/O_{30}	INH/O_{20}
K_m for rotenone-sensitive NADH-cytochrome C reductase ([NADH], μM)						
2 h	17.7±3.5	30.6±5.2	23.2±3.3	25.2±4.0	22.0±2.5	21.1±2.2
1 day	17.7±3.5	31.7±3.0	27.5±3.5	25.2±4.0	31.7±2.7	20.7±1.9
K_m for succinate-cytochrome C reductase ([succinate], mM)						
2 h	1.31±0.06	1.13±0.05	0.89±0.03	1.33±0.03	1.33±0.09	1.45±0.03
1 day	1.31±0.06	1.31±0.05	1.23±0.09	1.33±0.03	1.33±0.05	1.36±0.05
K_m for cytochrome C oxidase ([reduced cytochrome C], mM)						
2 h	45.2±1.8	56.5±3.4	47.1±2.2	40.4±3.1	44.7±1.8	40.4±1.2
1 day	45.2±1.8	47.0±3.9	52.5±2.6	40.4±3.1	42.0±4.2	65.2±4.0
V_{max} for rotenone-sensitive NADH-cytochrome C reductase (μM cytochrome C/mg tissue/min)						
2 h	5.85±0.45	7.05±0.76	6.81±0.48	6.62±0.52	7.32±0.59	5.88±0.75
1 day	5.85±0.45	7.11±0.57	5.86±0.68	6.62±0.52	8.68±0.59	5.83±0.12
V_{max} for succinate-cytochrome C reductase (μM cytochrome C/mg tissue/min)						
2 h	8.56±0.36	8.62±0.18	8.49±0.08	9.28±0.19	9.57±0.33	9.10±0.18
1 day	8.56±0.36	8.30±0.14	8.73±0.13	9.28±0.19	9.71±0.26	9.72±0.19
V_{max} for cytochrome C oxidase (μM cytochrome C/mg tissue/min)						
2 h	149.6±4.3	157.1±8.8	150.9±8.5	104.0±16.3	124.9±13.4	101.0±7.8
1 day	149.6±4.3	128.8±8.5	134.4±3.4	104.0±16.3	75.8±5.0	165.3±14.6

significance. The enzyme may function at low concentrations of reduced cytochrome C in low resistant animals after adaptation to hypoxia. Cytochrome oxidase gain new kinetic properties under these conditions, which contributes to higher functional activity of the enzyme during the impairment of oxygen supply to cells.

Changes in kinetic characteristics of enzymes are probably related to the formation of isoforms with new properties. For example, enzyme complex I is a flavin adenine mononucleotide-containing protein that includes several tens of subunits. Some of them are translated from the mitochondrial genome and others are translated from the nuclear genome. This multipeptide nature of the enzyme probably contributes to the appearance of isoforms with new kinetic characteristics during prolonged hypoxia. It remains unclear whether 2-h period can provide expression of new enzyme isoforms, or variations in kinetic characteristics are mediated by other regulatory mechanisms.

K_m for rotenone-sensitive NADH-cytochrome C reductase in the brain of highly resistant (non-adapted) rats did not increase, but even decreased by 40% 4 h after single training session with INH. This parameter returned to normal in the follow-up period (Table 1). Kinetic parameters of succinate-cytochrome C reductase remained unchanged under these conditions. These data are consistent with the results of our previous experiments. Regulatory differences in properties of mitochondrial complexes I and II in the brain of low resistant and highly resistant rats were revealed during hypoxia and ischemia [1,4-6,10]. The first response of low resistant animals to hypoxia is inhibition of complex I and activation of complex II in the brain. By contrast, activation of complex I in the brain of highly resistant animals is not accompanied by changes in complex II activity. We hypothesized that the succinate oxidase oxidation pathway serves as a signal mechanism, whose involvement in function of the respiratory chain determines the course of adaptive processes.

TABLE 2. K_m and V_{max} for Mitochondrial Enzyme Complexes in Low Resistant Rats after Training Course with INH (*n*=5-10; *M*±*m*)

Number of training sessions	Control	INH/O_{30}	INH/O_{20}
K_m for rotenone-sensitive NADH-cytochrome C reductase ([NADH], μM)			
1	17.7±3.5	31.7±3.0	27.5±3.5
8	18.6±2.2	26.8±3.4	32.4±6.4
15	18.6±2.2	34.7±6.4	31.7±6.6
21	18.6±2.2	23.6±2.5	22.6±3.1
K_m for succinate-cytochrome C reductase ([succinate], mM)			
1	1.31±0.06	1.31±0.05	1.23±0.09
8	0.88±0.04	0.73±0.02	0.74±0.03
15	0.88±0.04	0.73±0.03	0.79±0.07
21	0.88±0.04	0.76±0.04	0.76±0.04
K_m for cytochrome C oxidase ([reduced cytochrome C], mM)			
1	45.2±1.8	47.0±3.9	52.5±2.6
8	51.7±2.7	53.2±6.0	60.2±3.7
15	51.7±2.7	53.2±5.4	52.2±1.8
21	51.7±2.7	39.2±2.5	37.7±1.5
V_{max} for rotenone-sensitive NADH-cytochrome C reductase (μM cytochrome C/mg tissue/min)			
1	5.85±0.45	7.11±0.57	5.86±0.68
8	6.54±0.49	6.64±0.80	8.54±1.29
15	6.54±0.49	10.46±1.97	8.21±1.02
21	6.54±0.49	8.23±0.68	6.95±0.92
V_{max} for succinate-cytochrome C reductase (μM cytochrome C/mg tissue/min)			
1	8.56±0.36	8.30±0.14	8.73±0.13
8	2.85±0.19	2.17±0.06	2.14±0.06
15	2.85±0.19	2.51±0.11	2.66±0.15
21	2.85±0.19	2.68±0.09	2.69±0.21
V_{max} for cytochrome C oxidase (μM cytochrome C/mg tissue/min)			
1	149.6±4.3	128.8±8.5	134.4±3.4
8	144.3±8.0	118.1±16.5	141.7±9.4
15	144.3±8.0	120.8±5.8	134.3±9.1
21	144.3±8.0	109.3±4.2	109.4±4.6

Our results indicate that the bioenergetic mechanisms for INH are similar to those for other types of hypoxia. These data provide support to our hypothesis that mitochondrial complexes play a role in the response to various types of hypoxia. Moreover, they are closely related to the development of resistance.

This study allowed us to make the following conclusions.

INH increases general resistance of animals. These changes are most pronounced in low resistant animals after 8-15 training sessions. The effect becomes less significant with increasing the initial resistance and is not observed in highly resistant rats. INH causes a strain of the sympathoadrenal system in highly resistant rats.

Kinetic parameters for mitochondrial enzymes in the substrate region of the respiratory chain of the cerebral cortex underwent various changes in low resistant and highly resistant rats over the first 2 h after INH. Low resistant animals were characterized by higher functional activity of rotenone-sensitive NADH-cytochrome C reductase and succinate-cyto-

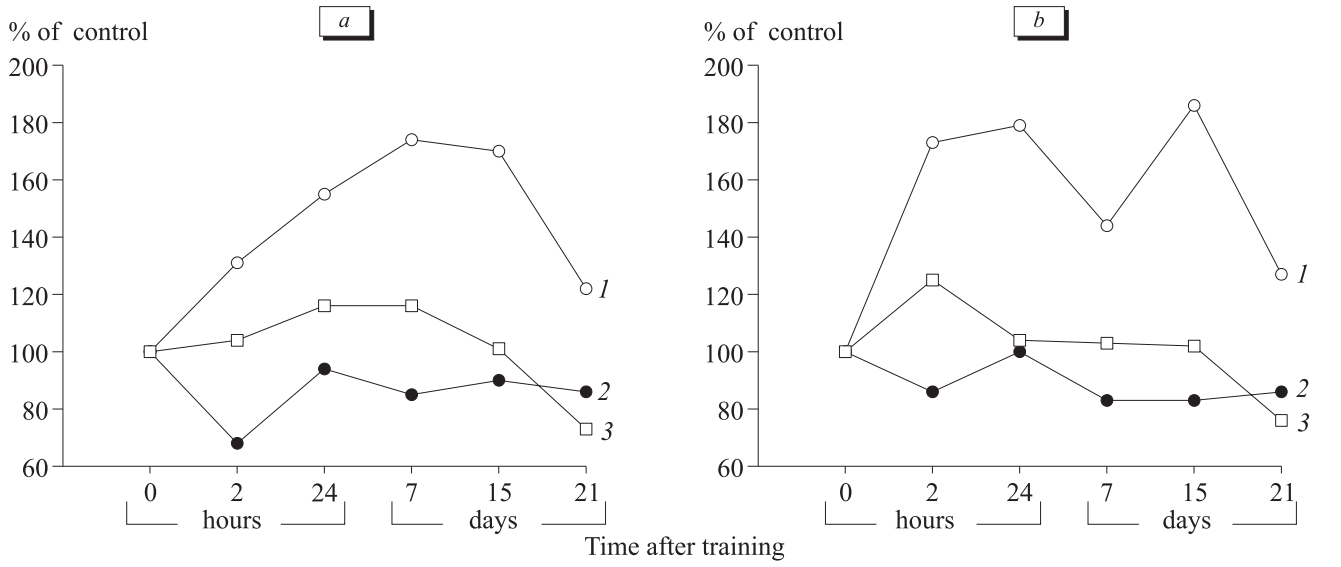

Fig. 3. Dependence of K_m for mitochondrial enzymes on the duration of training course with INH/O_{20} (*a*) and INH/O_{30} (*b*). K_m for NADH-cytochrome C reductase (*1*), succinate-cytochrome C reductase (*2*), and cytochrome C oxidase (*3*).

chrome C reductase under conditions of increased reduction status of the cell. These features correlated with an increase in general resistance of animals. Significant changes in kinetic properties of mitochondrial enzymes and signs for the development of resistance were not found in highly resistant rats.

Reciprocal relations between mitochondrial enzyme complexes in the substrate region of the respiratory chain probably play a role of the signal regulatory mechanism, which mediates tissue-specific and general resistance of rats under conditions of INH (similarly to other types of hypoxia).

INH/O_{20} and INH/O_{30} have similar effect on the test parameters.

REFERENCES

1. A. M. Dudchenko, G. N. Chernobaeva, V. V. Belousova, *et al.*, *Byull. Eksp. Biol. Med.*, **115**, No. 3, 251-254 (1993).
2. M. N. Kondrashova, *Mitochondria, Cells, and Intracellular Signaling* [in Russian], Pushchino (2000), pp. 71-74.
3. M. N. Kondrashova, *Reception and Intracellular Signaling* [in Russian], Pushchino (2005), pp. 249-253.
4. L. D. Lukyanova, *Byull. Eksp. Biol. Med.*, **124**, No. 9, 244-254 (1997).
5. L. D. Lukyanova, *Fiziol. Ukrain. Zh.*, **49**, No. 3, 17-35 (2003).
6. L. D. Lukyanova, *Problems of Hypoxia: Molecular, Physiological, and Clinical Aspects* [in Russian], Eds. L. D. Lukyanova and I. B. Ushakov, Moscow (2004), pp. 5-31.
7. L. D. Lukyanova, *Ibid.*, pp. 156-169.
8. C. L. Hoppel and C. Cooper, *Arch. Biochim. Biophys.*, **135**, 173-185 (1969).
9. D. Howard, *Methods in Enzymology*, Eds. R. W. Estabrook and M. E. Pullman (1967), Vol. 10, 213-217.
10. L. D. Lukyanova, *Adaptation Biology and Medicine*, Eds. B. K. Sharma *et al.* (1997), Vol. 1, pp. 236-250.
11. L. D. Lukyanova, *Ibid.*, Eds. J. Moravec *et al.* (2002), Vol. 3, pp. 289-303.
12. L. D. Lukyanova, *Ibid.*, Eds. A. Hargens *et al.* (2004), Vol. 4, pp. 11-22.