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BIOCHEMISTRY

The Drug Hypoxen: A New Inhibitor of Mitochondrial Respiration and Dehydrogenases

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Abstract—The effect of hypoxen on the oxygen consumption and activity of dehydrogenases in rat liver mitochondria has been studied. The addition of hypoxen to mitochondria caused a reduction of the rate of phosphorylating and uncoupling respiration. The minimal effective concentration of hypoxen was 15 μg/ml with succinate, 60 μg/ml with pyruvate or palmitoylcarnitine, and 120 μg/ml with glutamate as the substrates. The activities of malate, glutamate, and succinate dehydrogenases in mitochondria were significantly decreased by the effect of hypoxen.

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The sodium salt of [poly-(2,5-dihydroxyphenylen)]-4-thiosulfonic acid or hypoxen is a synthetic drug which is effective in treatment of acute myocardial infarction (Semigolovski, 1998), hemorrhage (Smirnov, Krivoruchko, 1998), burn wounds (Kochetigov, Makeev, 1992), animal models of pancreatitis (Tolstoi et al., 2001), viral (Eropkin et al., 2007), and other diseases (Smirnov, Kuz'mich, 2001). In spite of the widely described positive effect of hypoxen, the mechanism of its action is still unknown. According to the manufacturer's instructions, hypoxen regulates intercellular metabolism and affects the mitochondrial respiratory chain carrying electrons from reduced nicotinamide-adenine dinucleotide (NADP) and succinate to complex III by passing NADP-dehydrogenase (EC 1.6.5.3) complex I and succinate dehydrogenase (EC 1.3.5.1) complex II. However, according to the literature data, study of the effect of hypoxen on mitochondrial respiration and the activity of mitochondrial dehydrogenases has not been undertaken.

The aim of this work was investigation of the influence of various hypoxen concentrations on oxygen consumption in succinate, glutamate, pyruvate, and palmitoylcarnitine oxidation and on the activities of malate dehydrogenase (MDH, EC 1.1.1.37), glutamate dehydrogenase (GDH, EC 1.4.1.3), and succinate dehydrogenase (SDH, EC 1.3.99.1) in isolated rat liver mitochondria.

MATERIAL AND METHODS

Rats of Wistar breed with body weight 200–250 g were used for this study and kept in a vivarium at normal illumination and free access to water and food.

The experimental procedures were approved by the Service of Laboratory Animal Care at the Institute of Theoretical and Experimental Biology, Russian Academy of Sciences, and carried out according to the rules of the European Association on animal experiments.

The pharmaceutical drug hypoxen (Oliphen, Moscow) was purchased in a pharmacy. Other chemical preparations and enzymes were acquired from the Sigma-Aldrich Co. (United States).

All preparative procedures were carried out at the temperature 2–4°C. Mitochondria were isolated from the anterior lobe of the liver by the commonly used method of differential centrifugation (Kosenko, 1981). The medium for isolation contained 0.21 M mannitol, 0.07 M sucrose, and 5 mM tris, pH 7.4; for homogenization, to this medium was added ethylene glycol tetraacetate (EGTA) until the final concentration reached 1 mM and 0.5 mg/ml of albumin. The homogenate was centrifuged at 800 g for 10 min then supernatant repeatedly centrifuged at 3300 g for 10 min. The obtained sediment was washed three times and suspended in the isolation medium. The mitochondrial protein was determined by the Lowry method (Lowry et al., 1951).

Oxygen consumption was registered using an entirely automated two-chamber oxygraph Oxygraph-2K (Oroboros, Austria) equipped with magnetic shaker. The incubation of mitochondria (1 mg of mitochondrial protein per 1 ml) in each experiment was carried out either in 2 ml sucrose incubation medium (containing 0.2 M sucrose, 10 mM KCl, 5 mM KH_2PO_4, and10 mM tris-buffer at pH 7.4) or in 2 ml of KCl-medium (containing 120 mM KCl, 0.1 mM EGTA, 2.5 mM KH_2PO_4, 2.5 mM $MgCl_2$, and 10 mM Hepes-buffer at pH 7.4). In the cuvette section the temperature 25 ± 0.1°C was maintained. Hypoxen was added to the incubation medium until the final con-

centration 0.3–120 μg/ml directly before analysis; each analysis was begun with the substrate addition. Substrates and reagents were added until the following concentrations: 5 mM succinate and 1 μM rotenone; 5 mM pyruvate and 2.5 mM malate; 5 mM glutamate and 2.5 mM malate; 20 μM palmitoylcarnitine and 2.5 mM malate; 200 μM adenosine diphosphate (ADP); 15 μM 2,4-dinitrophenol (DNP) (with pryvate plus malate as substrates) or 50 μm DNP (with pyruvate plus malate as substrates) or 50 μm DNP (with other substrates).

State 2 respiration (before the addition of ADP, V_2), state 3 respiration (simulated by ADP, V_3), state 4 respiration (after cessation of ADP phosphorylation, V_4), uncoupled respiration (DPN-stimulated, V_u), respiratory control index (RCI = state 3/state 4 ratio), and the ADP/O ratio (the ADP phosphorylated/oxygen consumed ratio) were calculated by the internal Oxygraph-2K program, Oroboros software Datlab 4.1.

The activities of SDH, NAD-dependent MDH, and NADP (nicotinamide adenine dinucleotide phosphate)-dependent GDH were determined by the methods preliminarily described by Kosenko (Kosenko et al., 1997). Hypoxen (up to the final concentration 60 μg/ml) was added to the incubation medium directly before the addition of mitochondria. The speeds of absorption alteration of the products of enzymatic reactions were registered using a spectrophotometer at 420 nm (ferrocyanide) and 340 nm (NADP and NADPH).

Activity of mitochondrial enzymes GDH and MDH were also measured in some experiments on a culture of bud fibroblasts of the Chinese hamster VNK-21. The cells (in concentration 10^5 cells/ml) were pre-incubated in the presence of various concentrations of hypoxen (from 1 until 30 μg/ml) at 25°C for 30 min and then lysated, and the activity of enzymes was measured in lysates as was already mentioned above. It was established preliminarily that hypoxen in the concentration 5 μg/ml effected the activities of enzymes in this cell culture.

Statistical analysis was carried out using the program GraphPad Prism version 4.0 for Windows (GraphPad Software, United States). The results were expressed as the average and standard deviation of the average. The difference between pairs within the groups was analyzed with Students' test.

RESULTS

During oxidation of succinate in mitochondria, the speeds of V_3 and V_u (figure), V_2, and V_4 values of CRC and ADP/O (not shown) did not change in the presence of hypoxen in concentrations from 0.3 to 10 μg/ml.

Hypoxen in concentrations of 15 and 30 μg/ml significantly decreased the respiration speeds V_3 and V_u (state 3 and the uncoupled state) (figure) at the other constant indexes of oxidative phosphorylation.

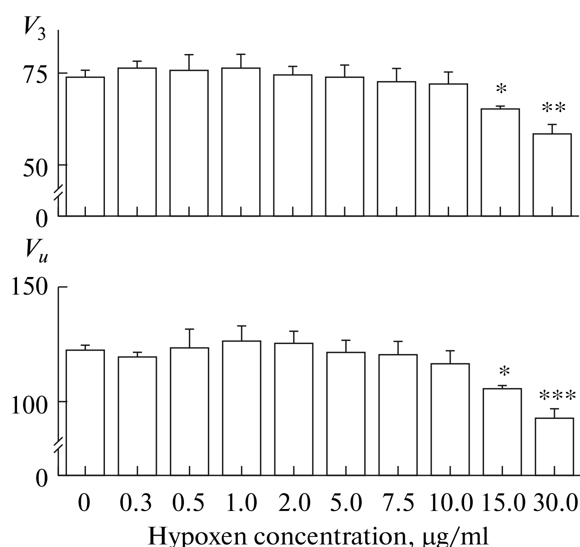

Figure. The speeds of phosphorylating (V_3) and uncoupled (V_u) succinate oxidation in mitochondria at various concentrations of hypoxen (incubation in the medium with sucrose). On the Y-axis is speeds of respiration, ng-at O/min on 1 mg of mitochondrial protein. For every hypoxen concentration, the averages and their standard mistakes within 14–15 experiments have been shown. $^{**}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ in comparison with the control without hypoxen.

The results shown in the figure were obtained after incubation of mitochondria in a sucrose medium that is widely used for registration of mitochondrial respiration. However, the closest to physiological conditions is the KCl-medium in our opinion; therefore, the effect of hypoxen on succinate oxidation in this medium was checked. It appeared that hypoxen in low concentrations (0.3–10 μg/ml) did not affect mitochondrial respiration in these conditions that are close to physiological. The higher concentrations of hypoxen exerted a dose-dependent inhibitory effect on succinate oxidation in mitochondria inhibited in the KCl-medium (Table 1). Thus, in the presence of 60 μg/ml of hypoxen, the speed V_3 and V_u and CRC decreased by 39% ($p < 0.0001$), 47% ($p < 0.0001$), and 49% ($p = 0.0033$), respectively, in comparison with the control values. However, the speed V_4 increased by 20% ($p < 0.001$) indicating on uncoupled hypoxen effect.

The hypoxen provided another effect on oxidation of other substrates studied. The hypoxen in concentrations lower than 60 μg/ml did not effect all indexes of mitochondrial respiration (V_2–V_4, V_u, CRC, and ADP/O) (not shown) in the KCl-medium during the oxidation of glutamate and malate, palmitoylcarnitine and malate, and piruvate and malate. The indexes of oxidation of these substrates depending on the concentration of hypoxen are shown in Table 2.

Table 1. The influence of hypoxen on the respiration speed in rat liver mitochondria during succinate oxidation

Respiration index, ng-at O/min on 1 mg of protein	Hypoxen concentration, mg/ml				
	0 (14)	15 (5)	30 (4)	60 (4)	120 (4)
V_3	105.3 ± 3.8	85.7 ± 4.6*	73.5 ± 4.6**	64.5 ± 2.6***	64.5 ± 1.6***
V_4	20.6 ± 0.3	23.8 ± 2.5*	28.2 ± 0.7***	24.9 ± 1.4***	23.1 ± 1**
V_u	139.5 ± 3	108.8 ± 2.4***	93.5 ± 1.6***	74.3 ± 3.1***	81.5 ± 4.9***
CDC	5.12 ± 0.26	3.98 ± 0.34*	2.62 ± 0.19***	3.32 ± 0.4**	2.75 ± 0.2***

Note: The number of experiments is indicated in brackets. M ± m for Tables 1–4. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ in comparison with the control without hypoxen for Tables 1–3

Table 2. Influence of hypoxen on respiration speed in mitochondria during palmitoylcarnitine and malate, glutamate and malate, and piruvate and malate oxidation

Substrate	Respiration index, ng-at O/min on 1 mg of protein	Hypoxen concentration, mg/ml		
		0	60	120
Palmitoylcarnitine + malate	V_3	49.4 ± 1.9	41.2 ± 1.4**	36.7 ± 2.2**
	V_4	15.5 ± 0.5	15.6 ± 0.3	20.7 ± 0.7***
	V_u	64.5 ± 3.3	52.1 ± 7.4	51.9 ± 1.4*
	CDC	3.28 ± 0.2	2.6 ± 0.15*	1.91 ± 0.19**
Glutamate+malate	V_3	72.7 ± 1.9	68 ± 2.1	57.6 ± 2.1***
	V_4	10.4 ± 0.8	10.8 ± 0.4	15 ± 0.7**
	V_u	97.2 ± 3.8	90.7 ± 3.3	80.3 ± 4.9*
	CDC	7.2 ± 0.64	6.3 ± 0.3	4.2 ± 0.44*
Piruvate +malate	V_3	26.6 ± 1	26 ± 1.1	24.9 ± 1.1
	V_4	9.1 ± 0.2	11.8 ± 0.9***	13.8 ± 0.5***
	V_u	32.3 ± 1.3	29.9 ± 3	30.7 ± 0.8
	CDC	2.97 ± 0.14	2.23 ± 0.2*	1.93 ± 0.16**

Note: The number of experiments for each hypoxen concentration was 4–12.

The maximal speeds (V_3 and V_u) of palmitoylcarnitine oxidation significantly decreased at hypoxen concentrations of 60 and 120 μg/ml, while hypoxen at a concentration of 120 μg/ml provided a weak influence on glutamate oxidation. The hypoxen in concentrations of 60 and 120 μg/ml did not effect the speeds V_3 and V_u in piruvate oxidation; however, the speed V_4 increased by 30 and 55% ($p < 0.001$) and CRC decreased by 21 and 40% ($p < 0.05$), respectively.

The influence of hypoxen on the activity of dehydrogenases in mitochondria is shown in Table 3. The activity of MDH, GDH, and SDH decreased by 32-40% ($p < 0.05$) in the presence of hypoxen at the concentration 60 μg/ml.

Pre-incubation cells VNK-21 with hypoxen in concentrations from 1 to 30 μg/ml resulted in dose-dependent decrease of the activity of mitochondrial enzymes MDH and GDH. The activity of MDH decreased 2.1 times and that of GDH decreased more than 3 times at a hypoxen concentration of 30 μg/ml (Table 4). This allows us to affirm that the effect of hypoxen on mitochondrial enzymes does not have cell specificity and can have physiological meaning. It follows that hypoxen in physiologically significant concentrations has an effect on enzymes of the mitochon-

Table 3. The influence of hypoxen on activities of MDH, GDH, and SDH in isolated rat liver mitochondria

Hypoxen concentration, mg/ml	Enzyme activity, ng-at on 1 mg of mitochondrial protein (% of control)		
	MDH	GDH	SDH
0	417 ± 43	88.3 ± 11.7	6.17 ± 0.5
60	282 ± 13* (68%)	51.7 ± 5* (60%)	4 ±0.33* (65%)

Note: The number of experiments for each hypoxen concentration was 4 (for Tables 3, 4).

drial respiratory chain and this effect does not have cell specificity.

DISCUSSION

The influence of various concentrations of hypoxen on mitochondrial respiration was seen in four types of substrate oxidation: piruvate and glutamate, returning reduction equivalent on NAD-dependent dehydrogenases; succinate, delivering electrons on FAD (flavine adenine dinucleotide)-dependent SDH; and palmitoylcarnitine, the donor of reduction equivalent subsequent on FAD-dependent acyl-CoA dehydrogenase (EC 1.1.1.35). In addition, the influence of various concentrations of hypoxen on the activity of mitochondrial NAD(P)-dependent GDH and NDH and FAD-dependent SDH has been studied.

Though the pharmokinetics of hypoxen has still not been studied, the probable concentrations of the preparation in the patients' blood can be evaluated by calculation. In clinical practice, hypoxen is injected intravenous at a dose of 250 mg three times per day over the course of 1–3 weeks. In these conditions the stationary concentration of hypoxen in the blood can reach 30 μg/ml. Therefore, we have applied hypoxen in the diapason of concentrations from 0.3 to 120 μg/ml.

We have shown that hypoxen effects the oxidation energy exchange in intact rat liver mitochondria. The minimal effective hypoxen concentration for succinate oxidation was 15 μg/ml. At this concentration and higher, hypoxen caused dose-dependent inhibition of succinate oxidation in the states of phosphorylating and uncoupled respiration. Hypoxen was less influential on the oxidation of other substrates. The indexes of mitochondrial respiration with palmitoylcarnitine or piruvate as the substrates changed only at the hypoxen concentration 60 μg/ml and that with glutamate, only at 120 μg/ml.

SDH participates in mitochondrial oxidation of succinate in the presence of rotenone, but complex I is not involved. The the significant decrease of maximal activity of SDH in mitochondria affected by hypoxen found by us can result in its inhibitory effect on mitochondrial respiration during succinate oxidation.

The activities of GDH and MDH in mitochondria measured in optimal conditions significantly decreased with the addition of hypoxen in spite of the lack of its influence on mitochondrial respiration during glutamate and malate oxidation. These data indicate that, although hypoxen is an inhibitor of GDH and MDH, the activity of these enzymes does not constrain the mitochondrial glutamate oxidation.

The functional analysis of piruvate dehydrogenase (PDH, EC 1.2.4.1) is based on determination of the speed of uncoupled mitochondrial respiration with piruvate and malate as the substrates. The product of the reaction catalyzed by PDH is acetyl-CoA, but for creation of citrate and further oxidation in the citrate cycle, the condensation of acetyl-CoA with oxaloacetate created in a reaction catalyzed by MDH is required. The real activity of PDH can be calculated as half of the total speed of piruvate oxidation with malate (U. Rasmussen and H. Rasmussen, 2000) because the total speed of creation of two molecules of NADH is created by similar speeds of NADH creation in reactions catalyzed by PDH and MDH. In our experiment the speed of piruvate and malate oxidation did not change in the presence of the mentioned

Table 4. The influence of hypoxen on activity of mitochondrial enzymes MDH and GDH in the cell culture BNK-21

Hypoxen concentration, mg/ml	Enzyme activity pcat on 10^5 cells	
	MDH	GDH
0	212 ± 10	177 ± 5
1	217 ± 10	178 ± 8
5	163 ± 4*	80 ± 9**
15	142 ± 3**	58 ± 2**
30	100 ± 2**	48 ± 1**

Note: * $p < 0.005$, ** $p < 0.001$ in comparison with the control.

hypoxen concentrations that mean hypoxen did not change the real activity of PDH.

Two dehydrogenases of beta-oxidation of fatty acids and MDH are involved in mitochondrial respiration in the presence of palmitoylcarnitine and malate. Malate oxidation is controlled by acetyl-CoA entry from the beta-oxidation cycle; i.e., the activity of these three enzymes is equal. According to the data of U. Rasmussen and H. Rasmussen (U. Rasmussen and H. Rasmussen, 2000), the speed of beta-oxidation is the third part of the total speed of mitochondrial oxidation during palmitoylcarnitine oxidation. As is shown in the present work, hypoxen inhibits the palmitoylcarnitine oxidation in state 3 of respiration and, thus, decreases the speed of beta-oxidation (as the speed of the reaction catalyzed by MDH).

Thus, the decrease in the speed of phosphorylating and uncoupled mitochondrial respiration by hypoxen is not provided by the influence of an inhibitor on the respiratory chain but by the effect on mitochondrial dehydrogenases: it decreases the maximal and real activity of SDH, MDH, and GDH, as well as the real activity of beta-oxidation of fatty acids.

Hypoxen shows a moderate uncoupling effect on oxidation of each substrate in mitochondria under incubation in the KCl-medium that is expressed in a CDC decrease; however, this effect has not been observed ina sucrose medium. This fact is still not explained and requires additional studies.

CONCLUSION

Hypoxen causes alterations in the energy exchange of mitochondria. The inhibitory effect of hypoxen decreases along the row: succinate ≥ palmitoylcarnitine ≥ piruvate ≥ glutamate in the oxidation of various substrates. Hypoxen decreases the activities of SDH, GDH, and MDH in mitochondria. Thus, the results of the present study evidence that hypoxen does not cross complex I of the respiratory chain and inhibits complex II. The data provide the first and direct proof that hypoxen is an inhibitor of the respiratory chain and dehydrogenases of mitochondria.

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