Kinetic Characteristics of Pyridine Nucleotide Reduction as an Indicator of Energy Production

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Changes in NADH fluorescence in brain mitochondria of rats exposed to hypercapnic hypoxia were studied. The status of the succinate oxidation system determines the level and kinetic characteristics of pyridine nucleotide reduction. The kinetic characteristics of energy production are more susceptible to hypoxia than changes in NADH level.

Key Words: *brain mitochondria; NADH; hypoxia; transamination; succinate oxidation*

The state of the energy production system is evaluated by the characteristics suggested by B. Chance [5]. We consider that such an approach provides insufficient information about the system in general and does not allow a comparison between the contribution of the fast and slow metabolic cluster [1,2] to the maintenance of energy homeostasis. We investigated the level of NADH fluorescence in brain mitochondria (MC) of rats exposed to hypercapnic hypoxia. Special attention was paid to the relationship between the rate of reduction of pyridine nucleotide (PN) oxidized with ADP and the oxidation substrate and functional state of the organism.

MATERIALS AND METHODS

Male Wistar rats aged 2 months (200-250 g) from Rassvet Breeding Center (Tomsk) were used. During 2-week acclimatization and the experiment the rats were kept in standard plastic cages (no more than 15 per cage) at 20-25°C, humidity no more than 50%, outflow/inflow 8:10 air exchange, and day-night light regimen. Hypercapnic hypoxia was modeled by placing the animal for 2 h into a sealed 5-liter vessel. The brain MC oxidation system was evaluated fluorimically by the level of PN reduction (PNR) on a Hitachi M-850 spectrofluorimeter (λ_{ex}=355 nm and λ_{em}= 450 nm) [7]. The oxidation substrates were succinic acid (0.5 mM), malate-glutamate mixture (3 mM each), their combination with SDH inhibitor malonate (2 mM) or aminotransferase inhibitor aminooxyacetic acid (0.5 mM). PNR was studied during oxidation of endogenous substrates (arbitrary metabolic state *2*), denoted as the "silent" status *2* (*2S*), and the PNR was consequently denoted as F_{2S} (Fig. 1). PNR changes were studied after addition of 50 μM ADP to the medium (transition from the *2S* metabolic status to active phosphorylation *2AP* and then to a resting status *2R*, i.e., the $F_{2S}\rightarrow F_{2AP}\rightarrow F_{2R}$ transitions were examined). Effects of oxidation substrate (transition from *2R* to *4S*, i.e. $F_{2R}\rightarrow F_{4S}$) and of subsequent addition of ADP during utilization of exogenous substrates (metabolic states *4S, 3, 4R*, i.e., $F_{4S}\rightarrow F_{3}\rightarrow F_{4R}$ transformations) were examined. The time of *2S* to *2R* transition (Tr_2) and *4S* to *4R* transition (Tr_3) and the rate of transition from *2AP* to *2R* metabolic state and from *3* to *4R* (V_2 and V_3, respectively) were studied. The results were processed by paired Wilcoxon—Mann—Whitney's test.

RESULTS

The results provide a new interpretation of changes in PNR in MC during transition from one metabolic state to another. Due to a better preservation of the MC structure [4], we demonstrated stability of PNR and

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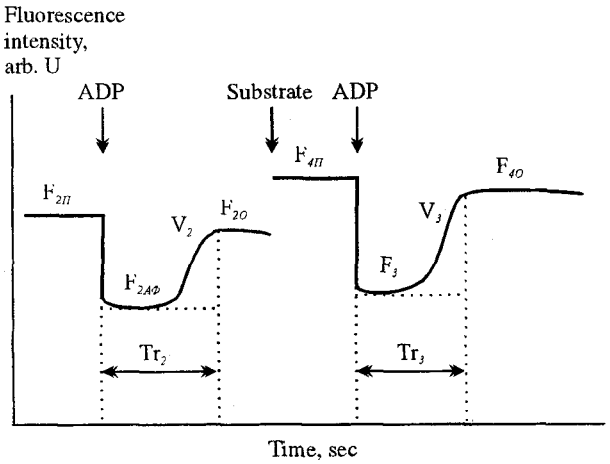

Fig. 1. Scheme and parameters of fluorimetric recording of the level of pyridine nucleotide reduction in mitochondria in different metabolic states.

high lability of its kinetic characteristics (Table 1), reflecting the ratio between the entry of reduction equivalents to NADH dehydrogenase and their oxidation by the respiratory chain.

The time of phosphorylation increased by 20-28% and the rate of *2AP* to *2R* transition decreased by 25-30% in animals exposed to hypoxia. These changes reflect the development of compensatory inhibition of SDH by oxaloacetate generated during hypoxia-induced hyperactive oxidation of succinate [3]. In the control, succinic acid and NAD-dependent substrates added to MC in the *2R* state increased the PNR by 19% ($F_{2R}\rightarrow F_{4S}$), while the rate of PN transition from the oxidized to reduced state after addition of ADP increased by 110 and 150%, respectively ($V_2<V_3$), depending on the substrate. Our data indicate that besides a traditional evaluation of the contribution of metabolic pathways (oxidation of NAD-dependent substrates or succinic acid) to net production of reduction equivalents to PN, the contribution of these reactions to the maintenance of $F_3\rightarrow F_4$ transition rate should be evaluated, which is essential for understanding of the regulation of metabolic pathways, particularly for tissues with intense metabolism. Succinic acid in a concentration of 0.5 mM did not affect the time of reduction of oxidized PN, while NAD-dependent substrates decreased it by 35%. Addition of succinic acid to MC in a metabolic state *2S* from animals exposed to hypoxia promoted a 21% increase in PNR ($F_{2R}\rightarrow F_{4S}$), a decrease in Tr_3, and reduced V_3 by 16 and 35%, respectively, in comparison with the control. During oxidation of the malate+glutamate mixture, PNR increased by 18% ($F_{2R}\rightarrow F_{4S}$) and V_3 increased by 35%, while Tr_3 remained unchanged. This seems to confirm the SDH inhibition in hypoxia and points to an essential contribution of endogenous succinic acid to PN reduction during oxidation of NAD-dependent substrates through reverse electron transfer [6].

From the viewpoint of contribution of reactions of rapid metabolic cluster of MC to maintenance of the energy homeostasis in the brain [1,2] our findings suggest that acceleration of transition from metabolic state *3* to *4R* and shortening of the period of reduction of oxidized PN during utilization of NAD-dependent substrates are due to oxidation of the endogenous succinic acid, specifically, that formed in the glutamate transamination reactions. In the control group, SDH inhibitor malonate modified the rate of PN transition from oxidized into reduced state, while aminotransferase inhibitor aminooxyacetic acid prolonged the time of reduction of oxidized PN 3-fold, decreasing the rate of transition from metabolic state *3* to *4R* 10-fold. In animals exposed to hypoxia, malonate added to MC oxidizing NAD-dependent substrates decreased

TABLE 1. Effect of Hypoxia on Parameters of Rat Brain MC (n=5)

Parameters	Control				Hypoxia			
	1	2	3	4	1	2	3	4
F_{2S}	0.489	0.502	0.489	0.491	0.512	0.501	0.492	0.496
F_{2AP}	0.400	0.402	0.392	0.393	0.425	0.411	0.399	0.399
F_{2R}	0.445	0.449	0.447	0.449	0.465	0.454	0.451	0.445
Tr_2	38.6	39	40.8	42.4	49.5*	46.5*	46.3*	47.3*
F_{4S}	0.529	0.534	0.509	0.51	0.562	0.540	0.518	0.536
F_3	0.424	0.447	0.428	0.413	0.443	0.434	0.432	0.429
F_{4R}	0.507	0.514	0.495	0.441	0.542	0.518	0.505	0.447
Tr_3	38.2	26	26.6	75.0	43.5	26.3	27.0	78.5
V_2	3.16	3.26	3.91	3.73	2.34*	2.32*	2.20*	2.45*
V_3	6.58	8.12	3.72	0.87	4.17*	5.8*	3.41*	0.61

Note. *p<0.05 *vs.* the control; 1) succinic acid; 2) malate+glutamate; 3) malonate+malate+glutamate; 4) aminooxyacetic acid+malate+glutamate.

V_3 by 70% without affecting Tr_3, and leveled the differences between MC in the control and experimental groups. The use of another inhibitor, aminooxyacetic acid, prolonged the time of reduction of oxidized PN 3-fold and decreased the rate of transition from metabolic state *3* to *4R* 10-fold; aminooxyacetic acid completely abolished the differences between the control and experimental MC. Termination of oxidation of endogenous succinic acid by SDH inhibition or by limiting substrate production in the transamination reactions modifies the energy status of MC.

Our data demonstrate that PNR strongly depends on the rate of NAD-dependent oxidation and on the state of endogenous succinic acid production and oxidation pathway, since succinic acid activates SDH by stimulating transfer of reduction equivalents along the respiratory chain, including the reverse electron transfer to PN.

The most important finding is high sensitivity of the kinetic characteristics of the MC respiratory system to the state of succinate-dependent energy production. Each individual MC works in a pulsed mode [1], and the dynamic characteristics of the predominant metabolic pathways determine the pattern of adaptive reaction of the energy production system. From this viewpoint, our previous data on the role of SDH in adaptation and dysadaptation to extreme factors [2,3] are essential for understanding the regulatory shifts in MC in different metabolic states.

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