Depletion of NADH determines the inhibition of the respiratory chain in heart mitochondria by calcium overload

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We investigated the reasons for a higher sensitivity of the respiratory subsystem to calcium overload in heart mitochondria oxidizing various NAD-dependent substrates than in mitochondria respiring with succinate. To this end, the activity of Complex I in freeze-fractured heart mitochondria at various concentrations of NADH and Ca^{2+} ions was determined. The results showed that increase in Ca^{2+} concentration in the medium from 1 μM to 10 μM or 30 μM did not affect the activity of Complex I either at a saturating concentration (100 μM) or at subsaturating concentrations (20 μM and 5 μM) of substrate. However, we found that the content of NADH in mitochondria oxidizing pyruvate + malate was substantially reduced and the level of NAD^+ was increased under exposure to Ca^{2+} overload. Therefore we conclude that although Ca^{2+} does not directly inhibit Complex I, it may effectively diminish the activity of Complex I by causing conversion of NADH to NAD^+ in mitochondria.

Key words: heart mitochondria, Complex I, NADH, calcium

INTRODUCTION

Mitochondria possess active Ca^{2+} transport systems that enable them to respond to changes in cytosolic calcium concentration and to play the dominant role among several mechanisms for clearing large loads of cytosolic Ca^{2+} under various pathologic conditions (*e.g.*, ischemia, intoxication) [1]. However, excessive sequestration of Ca^{2+} in mitochondrial matrix leads to impairment of oxidative phosphorylation. Numerous studies concentrated on the mitochondrial permeability transition induced by high Ca^{2+} concentration (reviewed in [2, 3]), although increase in calcium concentration above the physiological level (1 μM) may affect mitochondrial function by mechanisms other than the opening of the PTP. In our previous study we investigated the effect of increased the external Ca^{2+} concentrations (1–30 μM) on different parts of the mitochondrial oxidative phosphorylation system, using modular kinetic analysis (MKA) [4, 5]. The results showed that under our experimental conditions Ca^{2+} accumulation in mitochondria did not lead to PTP opening, but respiration and phosphorylation were severely impaired. MKA revealed that overall inhibition was caused by multiple effects on the mitochondrial oxidative phosphorylation system, resulting in the inhibition of both respiratory and phosphorylation subsystems and partial uncoupling of heart mitochondria. It was evident from our results that the respiratory subsystem is inhibited by calcium overload to a much higher degree in mitochondria oxidizing various NAD-dependent substrates (2-oxoglutarate, palmitoylcarnitine, glutamate + malate, pyruvate + malate) than in mitochondria respiring with succinate [5].

In this study, we investigated the reasons for a different sensitivity of the respiratory subsystem to Ca^{2+} in these two cases.

MATERIALS AND METHODS

Mitochondria were isolated from the hearts of male Wistar rats as described earlier [6]. Activity of Complex I was measured spectrophotometrically [7] by following the kinetics of NADH oxidation at 340 nm in fractured mitochondria (by a rapid freezing–thawing of mitochondria, repeated four times) in the medium containing 110 mM KCl, 30 mM Tris-HCl, 50 mM creatine, 1 mM EGTA, 10 mM NaCl, 5 mM NTA,

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5 mM KH_2PO_4, antimycin A (1 μg/ml), 0.1 mM NADH, fractured mitochondria (0.02 mg/ml of mitochondrial protein) and/or 0.875 mM $CaCl_2$ (1 μM free Ca^{2+}) and 5.17 mM $MgCl_2$ (1 mM free Mg^{2+}) or 1.3 mM $CaCl_2$ (10 μM free Ca^{2+}) and 4.95 mM $MgCl_2$ (1 mM free Mg^{2+}) or 1.5 mM $CaCl_2$ (30 μM free Ca^{2+}) and 4.8 mM $MgCl_2$ (1 mM free Mg^{2+}), pH 7.2, 37 °C. The reaction was started by adding 40 μM of coenzyme Q_1. Enzymatic activity was calculated for the NADH extinction coefficient 6.81 $mM^{-1}\,cm^{-1}$.

Mitochondrial respiration was measured as described in [5]. The aliquots (1 ml) of medium with mitochondria (2 mg/ml) respiring with pyruvate + malate in state 2 or state 3 were taken from the incubation chamber and placed to the tubes containing either 100 μl 25% $HClO_4$ or 250 μl of saturated KOH in alkohol (99%), for extraction of NAD^+ and NADH, respectively [8]. The amount of NAD^+ and NADH in neutralized extracts was determined spectrofotometrically [8] by monitoring light absorbance changes at 340 nm.

RESULTS AND DISCUSSION

Different response of the respiratory subsystem to Ca^{2+} overload in mitochondria oxidizing succinate and NAD-dependent substrates (*e.g.*, pyruvate + malate) might be explained by interaction of Ca^{2+} with Complex I, which is involved in the electron transport from NAD-dependent substrates, but not from succinate. However, our preliminary results showed that the rate of NADH oxidation in disrupted heart mitochondria did not depend on Ca^{2+} concentration, at least at saturating concentrations (2 mM) of NADH [5]. Therefore we determined the rate of NADH oxidation in freeze-fractured heart mitochondria in the presence of different concentrations of NADH and Ca^{2+}. Under our experimental conditions, the determined K_m value of Complex I was 18 ± 1 μM NADH, and V_{max} was 803 ± 72 nmol NADH/min per mg of mitochondrial protein at 1 μM Ca^{2+}. The kinetic constants did not change upon increase of Ca^{2+} concentration in the medium to 10 or 30 μM (n = 3, data not shown). The results showed (Figure) that Ca^{2+} did not affect the activity of Complex I either at saturating (100 μM) or at subsaturating (20 and 5 μM) concentrations of the substrate. The obtained data confirm that Complex I is not affected by Ca^{2+}. This finding implies that the sensitivity of oxidation of NAD-dependent substrates to Ca^{2+} cannot be explained by a direct inhibition of Complex I by calcium ions.

Another possible reason for the diminution in the activity of Complex I is the decrease of NADH concentration in mitochondria overloaded by calcium. Recently, it has been suggested [9] that opening of mitochondrial permeability transition pore causes depletion of both mitochondrial and cytosolic NAD^+ by the process catalysed by NAD^+ glycohydrolase, the enzyme localized in the outer mitochondrial membrane [10]. Although the permeability transition pore does not operate under the experimental conditions used in this work [5], we determined the amount of NADH and NAD^+ extracted from mitochondria respiring with pyruvate + + malate in the medium containing 1 and 30 μM Ca^{2+}. The results indicated (Table) that in the medium with 1 μM Ca^{2+} the amount of NAD^+ and NADH in mitochondria respiring in state 2 and state 3 did not differ. Under the exposure of mitochondria to Ca^{2+} overload, the amount of NADH in mitochondria substantially reduced, whereas the quantity of NAD^+ increased, so that the total amount of NAD^+ + NADH remained the same (Table). The evidence that we measured the amount of pyridine nucleotides inside mitochondria comes from the experiments on the measurement of oxidation of external NADH [5]. The rate of 2 mM NADH oxidation by intact mitochondria in State 3 was 39 ± 5 and 37 ± 1 nmol O/min per mg, whereas the stretching of the mitochondrial membrane, by freezing and thawing, increased the rate of NADH oxidation up to 567 ± 60 and 598 ± ± 49 nmol O/min per mg at 1 μM and 30 μM Ca^{2+}, respectively (n = 3). Therefore we conclude that the inner membrane in intact mitochondria was not permeable to NADH (and NAD^+) in these experiments.

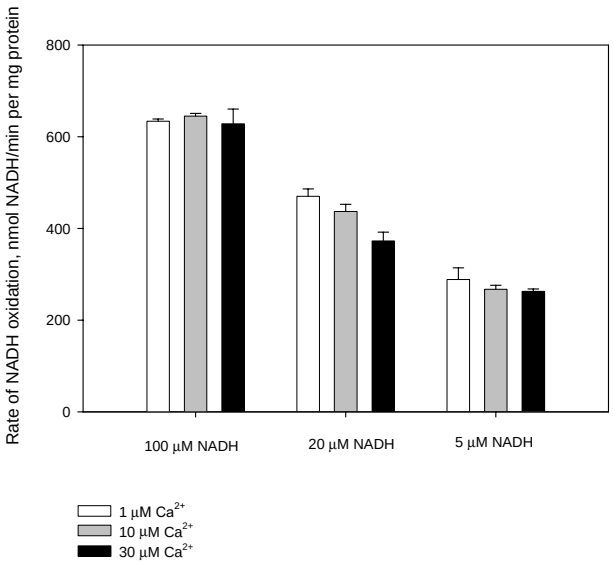

Figure. The rate of NADH oxidation at various NADH and Ca^{2+} concentrations in the medium

Thus, Ca^{2+} overload is responsible for the substantial decrease in the concentration of the NADH – the substrate of the respiratory chain. It

Table. **NAD^+ and NADH content (nmol/mg of protein) in mitochondria oxidizing pyruvate (1 mM) + malate (1 mM) in state 2 (V_2) and in state 3 (V_3) at different concentrations of Ca^{2+} in the medium**

1 μM Ca^{2+}			30 μM Ca^{2+}		
NAD^+	NADH	NAD^++ NADH	NAD^+	NADH	NAD^++ NADH
V_2					
2.2 ± 0.3	2.1 ± 0.4	4.3 ± 0.6	2.8 ± 0.4	1.6 ± 0.4	4.4 ± 0.5
V_3					
2.5 ± 0.5	2.3 ± 0.4	4.8 ± 0.9	3.0 ± 0.6	0.7 ± 0.1*	3.7 ± 0.7

* – statistically signifficant difference (n = 4).

is clear that inhibition of Complex I by calcium should lead to the opposite change – accumulation of NADH. Therefore we conclude that although Ca^{2+} does not directly inhibit Complex I, it may effectively diminish the activity of Complex I by causing the conversion of NADH to NAD^+ in mitochondria. The inhibition of oxidation of NAD-substrates by calcium overload may be explained by this reason as well.

The processes responsible for the conversion of NADH to NAD^+ in mitochondria exposed to calcium overload remain to be determined. Meanwhile, it is clear that the effect observed in our study is disparate from the depletion of NAD^+ involving permeability transition and NAD^+ glycohydrolase [9]. Further investigations are directed to distinguish whether NADH production (substrate transporters or dehydrogenases) is inhibited or NADH oxidation by other processes than respiration (*e.g.*, involvement of NADH/$NADP^+$ transhydrogenase [11] is possible) is stimulated by calcium.

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KALCIO JONŲ PERKROVOS POVEIKIS KVĖPAVIMO GRANDINĖS INHIBAVIMUI, MAŽINANT NADH KIEKĮ ŠIRDIES MITOCHONDRIJOSE

S a n t r a u k a

Šio darbo tikslas buvo nustatyti priežastis, kurios lemia didesnį kvėpavimo grandinės jautrumą kalcio jonų perkrovai širdies mitochondrijose, oksiduojančiose NAD-priklausomus substratus, lyginant su mitochondrijomis, oksiduojančiomis sukcinatą. Įvertinome I komplekso aktyvumą suardytose mitochondrijose, kai terpę sudarė įvairios NADH bei Ca^{2+} jonų (1, 10 ir 30 μM) koncentracijos. Nustatėme, kad Ca^{2+} jonai nekeitė I komplekso aktyvumo tiek esant prisotinančiai (100 μM), tiek neprisotinančiai (20 μM ir 5 μM) substrato koncentracijai terpėje. Mitochondrijose, oksiduojančiose piruvatą + malatą, dėl kalcio jonų perkrovos ženkliai sumažėjo NADH kiekis, tuo tarpu NAD^+ kiekis išaugo. Atlikti tyrimai parodė, kad Ca^{2+} jonai tiesiogiai neveikia I komplekso, tačiau lemia mažesnį NADH oksidacijos greitį, sukeldami mitochondrijose NADH perėjimą į NAD^+ formą.