Sensitivity of cytochrome c test in intactness assessment of the outer mitochondrial membrane: dependence on the respiratory substrate and metabolic state of the mitochondria

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The effect of exogenous cytochrome c on the respiration rate of the rat ventricular and human heart atrial muscle mitochondria was assessed *in situ*, using the saponin- and saponin+collagenase-permeabilized fibers. It was much more pronounced in State 4 than in State 3 with all the respiratory substrates (pyruvate+malate, succinate, palmitoyl-CoA+carnitine and octanoyl-L-carnitine), and different with different substrates. Total 1 h ischemia *in vitro* a induced a significant increase in the degree of cytochrome c-induced stimulation of respiration of mitochondria in both metabolic states, which was much more intense with succinate than with pyruvate+malate. In conclusion, it should be noted that the highest sensitivity of the cytochrome c test used for evaluation of intactness of the outer mitochondrial membrane was determined in State 4 with succinate as a substrate.

Key words: saponin-permeabilized heart muscle fibers, outer mitochondrial membrane permeability, cytochrome c test, oxidative phosphorylation

Abbreviations: OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

INTRODUCTION

The OMM is a barrier for macromolecules (proteins, enzymes, etc.) and smaller molecules (adenine nucleotides, creatine phosphate, creatine, etc.). Therefore it is relevant in the compartmentation of various metabolites and enzymes as well as in the regulation of oxidative phosphorylation and energy transport in the cell. These processes may be disturbed by various factors (mitochondria isolation, ischemia, hypoosmotic conditions, fatty acids, etc.) leading to disruption of OMM and loss of cytochrome c from the mitochondria. Thus, the evaluation of OMM intactness is relevant for assessment of the quality of mitochondrial preparations and for interpretation of experimental results. For this purpose, the cytochrome c test is used most often. It is based on the oxygraphic measurement of the degree of stimulation of mitochondrial respiration, as a rule in State 3, by the exogenous cytochrome c [1, 2]. However, the role of the particular effector or component of the oxidative phosphorylation system in the regulation of respiration appeared to be crucially dependent on the metabolic state of mitochondria and on the respiratory substrate [3]. Thus, the aim of this work was to assess the influence of these two factors on the sensitivity of cytochrome c test in evaluation of the intactness of OMM.

MATERIALS AND METHODS

Hearts of Wistar male rats weighing between 250 and 300 g were used for the experiments. Total ischemia was induced *in vitro* by autolysis (37 °C, 60 min). The human heart atrial tissue (about 20 mg biopsy specimens) was obtained from the patients with ischemic heart disease. The bundles of the heart muscle fibers,

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approximately 0.2–0.3 mm in diameter, were prepared for investigation according to a method described earlier [4].

The oxygen uptake rates were recorded at 37 °C by means of a Clark-type electrode system [4]. The respiration rates were expressed as ngatoms O/min/mg of fiber dry weight (dry weight = wet weight before respiration measurement/4.85). The results are presented as means ± S.E.M. Statistical analysis was performed using Student's t test, and $p < 0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

As can be seen in Fig. 1 (A and B), the stimulating effect of the exogenous cytochrome c on the respiration of the control rat heart (ventricles) mitochondria in State 3 (+ADP), measured *in situ* using saponin- and saponin+collagenase-permeabilized cardiac fibers, was higher in the case when succinate but not pyruvate+malate was used as a respiratory substrate. The difference was particularly evident in the case of ischemia (1h), which is known to produce the injury to OMM [1, 5].

The injury to OMM in the fibers prepared from the ischemic rat myocardium was detected with both respiratory substrates – pyruvate+malate and succinate (Fig. 1). It is noteworthy that the degree of cytochrome c-induced stimulation of respiration of the ischemic fibers in State 3, *i.e.* the degree of injury to OMM, was much higher in the case of succinate used as a substrate (Fig. 1, A and B). This is also true for State 4 respiration of the mitochondria (Fig. 1, C). In the later experiments, cytochrome c was added to the incubation medium after atractyloside – an inhibitor of adenine nucleotide translocase – which decreases the maximum State 3 respiration rate of the mitochondria to the level characteristic of State 4. It should be noted that the higher cytochrome c effect on succinate oxidation cannot be ascribed to succinate itself, *i.e.* to its deteriorating action on the integrity of OMM, because almost a 4-fold increase in succinate concentration (up to 37 mM) did not increase the cytochrome c effect on State 3 respiration rate of the rat heart fibers (1.17 ± 0.01, compared to 1.13 ± ± 0.03 at 10 mM succinate, n = 3). This is also true for State 4 respiration of the fibers.

The experiments with the saponin+collagenase-treated human cardiac atrial fibers respiring on succinate (Fig. 2) demonstrated that, as with the rat cardiac ventricular fibers (compare with Fig. 1, A, C), after addition of cytochrome c to the incubation medium in State 4, the respiration rate increased much more significantly (about 1.4-fold) than in State 3 (when no increase was observed).

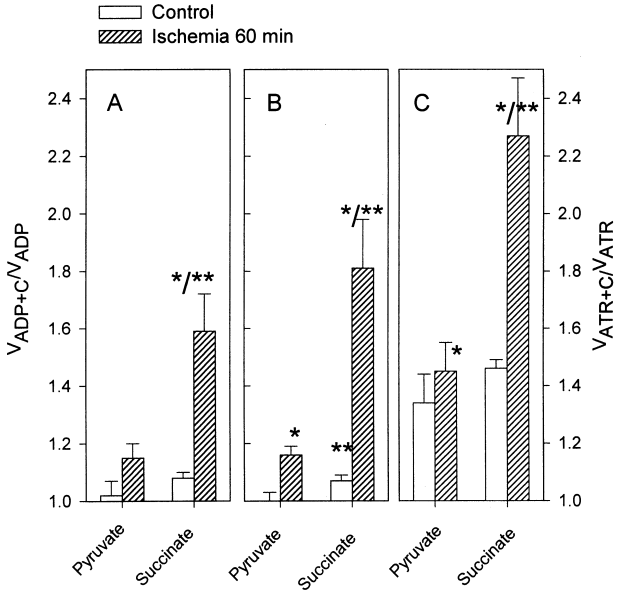

Fig. 1. The effect of cytochrome c on State 3 (A and B) and State 4 (C) respiration rate of saponin- (A, C) and saponin+collagenase-permeabilized (B) rat cardiac fibers – the influence of ischemia
The standard incubation medium [4] was supplemented with pyruvate + malate (6 mM+6 mM, n = 5), or succinate + rotenone (10 mM + 5 μM, n = 5). Additions: 1 mM ADP, 30 μM cytochrome c, 0.12 mM atractyloside. V_{ADP+C} / V_{ADP} – the effect of cytochrome c on State 3 respiration rate; V_{ATR+C} / V_{ATR} – the effect of cytochrome c on State 4 respiration rate. *P < 0.05 *vs.* control, **P < < 0.05 *vs.* pyruvate+malate as substrates

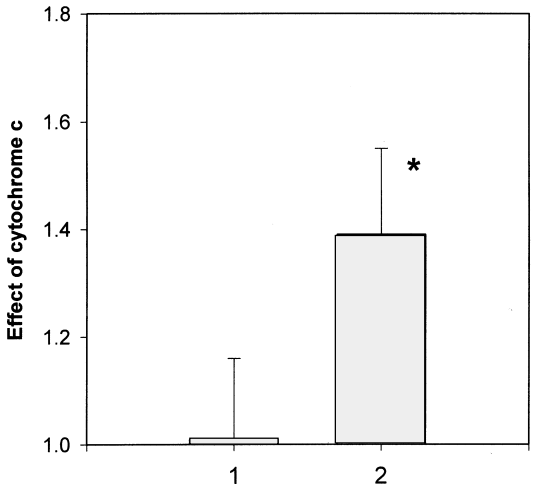

Fig. 2. The effect of cytochrome c on State 3 and State 4 respiration rate of human cardiac atrial fibers permeabilized with saponin plus collagenase
The standard incubation medium [4] was supplemented with succinate + rotenone (10 mM + 5 μM, n = 4). Additions: 1 mM ADP, 0.12 mM atractyloside, 30 μM cytochrome c. 1 – the effect of cytochrome c on State 3 respiration rate; V_{ADP+C} / V_{ADP}; 2 – the effect of cytochrome c on atractyloside-insensitive respiration rate, V_{ATR+C} / V_{ATR}. *P < 0.05 vs V_{ADP+C} / V_{ADP}

Differences in cytochrome c effect on respiration with different substrates and in different metabolic states were confirmed also with palmitoyl-CoA (+L-carnitine) and octanoyl-L-carnitine (Fig. 3). Their oxidation in the saponin-treated rat cardiac fibers was investigated in parallel with pyruvate+malate and succinate oxidation (for control purposes), and cytochrome c was applied in State 3 and in State 4.

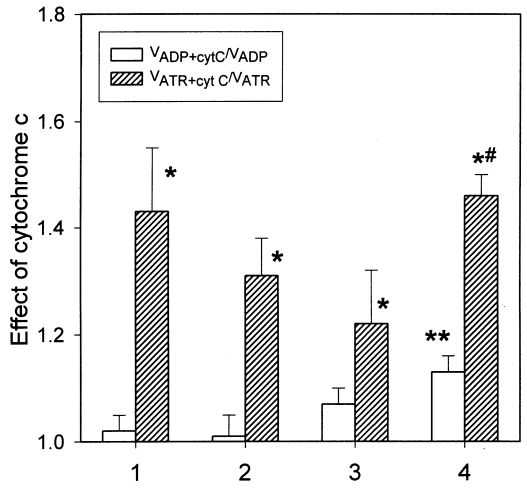

Fig. 3. The effect of cytochrome c on State 3 and State 4 respiration rate of the saponin-permeabilized rat cardiac ventricular fibers
1 – 6 mM pyruvate + 6 mM malate (n = 4); 2 – 12.2 μM palmitoyl-CoA + 2.41 mM L-carnitine + 0.24 mM malate (n = 7); 3 – 0.36 mM octanoyl-L-carnitine + 0.24 mM malate (n = 5); 4 – 10 mM succinate + 5 μM rotenone (n = 4). Cytochrome c concentration was 33 μM, *P < 0.05 *vs*. V_{ADP+C} / V_{ADP}, **P < 0.05 *vs*. pyruvate+malate as substrates. #P < 0.05 *vs*. octanoyl-L-carnitine+malate as substrates

The data presented in Fig. 3 show that cytochrome c, when applied in State 4, estimates the intactness of OMM more sensitively than in State 3. The dependence of cytochrome c test sensitivity on the respiratory substrate is also obvious.

The revealed findings may be explained by the known fact that the role of a particular component of the multienzyme system in the control of the flow through the system depends on the metabolic state and respiratory substrate [3]. On the other hand, estimation of the intactness of OMM by cytochrome c test in the ischemia-damaged mitochondria respiring on NAD-dependent substrates as pyruvate+malate, etc., is complicated (OMM injury underestimated), because severe ischemia damages OMM, leading to the loss of cytochrome c from the mitochondria, and IMM, leading to the loss of NAD(H) as well as the other components of the mitochondrial matrix (enzymes, adenine nucleotides, etc.). These alterations significantly decrease the State 3 respiration rate of mitochondria when the NAD-dependent substrates (pyruvate, malate, glutamate, etc.) are used, whereas succinate oxidation is affected to a lesser degree and, in contrast to pyruvate+malate oxidation, can be nearly completely restored by a mere addition of cytochrome c to the incubation medium.

In conclusion, it is possible to affirm that in most cases the intactness of OMM is most sensitively estimated by cytochrome c test when the mitochondria oxidize succinate in State 4. Probably, in these conditions, the role of cytochrome c in the control of mitochondrial respiration is expressed best.

ACKNOWLEDGEMENTS

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MITOCHONDRIJŲ IŠORINĖS MEMBRANOS INTAKTIŠKUMO ĮVERTINIMAS CITOCHROMO C TESTU: PRIKLAUSOMYBĖ NUO MITOCHONDRIJŲ METABOLINĖS BŪSENOS IR SUBSTRATŲ

S a n t r a u k a

Tyrėme egzogeninio citochromo c poveikį mitochondrijų kvėpavimui žiurkės širdies (skilvelių) ir žmogaus prieširdžių raumens skaidulose, permeabilizuotose saponinu bei saponinu ir kolagenaze. Nustatėme, kad esant įvairiems kvėpavimo substratams (piruvatui ir malatui, sukcinatui, palmitoil-KoA+karnitinui ir oktanoil-L-karnitinui), 4-oje metabolinėje būsenoje citochromas c mitochondijų kvėpavimo greitį stimuliuoja labiau nei 3-ioje metabolinėje būsenoje. Citochromo c efektas po vienos valandos totalinės išemijos stebimas abiejose metabolinėse būsenose, tačiau efektas didesnis oksiduojant sukcinatą negu piruvatą ir malatą. Gauti rezultatai rodo, kad išorinės mitochondrijų membranos intaktiškumui įvertinti geriausia naudoti citochromo c testą 4-oje metabolinėje būsenoje, oksiduojant sukcinatą.