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Mitochondrial respiratory parameters in cardiac tissue: a novel method of assessment by using saponin-skinned fibers

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Respiratory parameters of cardiac mitochondria were determined in the bundles of cardiac fibers skinned by using saponin that specifically removed sarcolemma, but left intracellular structures intact. In the assay medium which simulated the ion composition of cardiac cytoplasm maximal value of state 3 oxygen consumption per mol cytochromes aa_3 was close to that value for isolated mitochondria. Ischemia and isopreterenol treatment were found to affect respiratory parameters of mitochondria in saponin-skinned fibers, among them creatine-stimulated respiration decreased most significantly, (3–4)-times under these conditions. The method described can be easily applied for determination of the mitochondrial respiratory parameters in small (5–10 mg) biopsy samples from human heart.

Introduction

Assessment of the functional parameters of mitochondria is of high importance for evaluation of cardiac energy metabolism. Conventional methods of such assessments include as necessary steps isolation of mitochondria by homogenization of tissue and sedimentation of mitochondria by centrifugation. However, the properties of mitochondria are affected at these steps, as evidenced by appearance of 'light' mitochondrial fraction, and this influence may be very significant when the mitochondria are being isolated from tissue already damaged by a pathological process, for example ischemia [1,2]. Moreover, the fraction of mitochondria which can be isolated in more or less intact state does not exceed 10–15% of total mitochondrial content of the tissue [3,4]. All that makes it difficult to relate the parameters of isolated mitochondrial preparation to the characteristics of mitochondria in situ. To overcome these difficulties, digitonin-treated isolated cardiomyocytes were recently used [5]. However, that method is time-consuming and expensive. In the present work we describe a more simple procedure for determination of mitochondrial functions in small amounts of tissue without any steps of isolation. This method is based on the use of saponin which perforates the sarcolemma due to a high amount of cholesterol in this membrane [6]. The conditions of saponin treatment and the composition of the incubation medium used in this work allowed us to obtain skinned cardiac fibers with morphologically and functionally intact mitochondria. These fibers can be easily used for determination of respiratory parameters of mitochondria in small amounts of biopsy material.

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Materials and Methods

Preparations. White Wistar line male rats were narcotized with ethyl ether, hearts were excised and placed into a cooled Krebs–Henseleit solution. Bundles of fibers, 0.3–0.4 mm in diameter and 5–7 mm in length, were isolated from endocardial surface of left ventricle and transferred into relaxing solution with EGTA (solution A, see below). 7–8 bundles with total weight of 4–6 mg were incubated for 20 min in 1 ml of solution A, containing 50 μg/ml of saponin. After that bundles were washed for 10 min in solution B without high energy phosphates (see below) to remove saponin. All procedures were carried out at +4° C with intensive stirring of solutions.

The extent of the removal of sarcolemma was estimated by determining the activity of lactate dehydrogenase in preparations according to Ref. 7. The content of cytochromes aa_3 was determined spectrophotometrically at 605–630 nm, $E = 24\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ [8]. Protein concentration was determined by a modified Lowry method [9]. For electron microscopic observation bundles were fixed by 3% glutaraldehyde solution in 0.1 M phosphate buffer (pH 8.0) and processed further as described in Ref. 10.

Determinations. The respiratory rates were determined in an oxygraph medium containing 7–8 bundles in 3 ml of solution B by Clarck electrode, Yellow Spring Instruments (OH, U.S.A.) at 22° C with continuous stirring. The solubility of oxygen was taken to be 460 ng atoms/ml.

The bundles of the fibers had been kept in the solution A and skinned in the solution with saponin just before the oxygraphic measurements were carried out. The preparations were stable in the ice-cold solution A at least for a period of 2–3 h.

Solutions. All solutions used contained 10 mM EGTA-Ca EGTA (0.1 μM free Ca^{2+}), 3 mM free Mg^{2+}, 20 mM taurine, 0.5 mM dithiothreitol, 20 mM imidazole (pH 7.0). Ionic strength was adjusted to 0.16 M by addition of potassium 4-morpholineethanesulfonate. Free Ca^{2+} and Mg^{2+} concentrations were calculated by using equations described by Fabiato and Fabiato [11] and known values of dissociation constants [12]. Relaxing solution A, in which skinning by saponin was performed, contained also 5 mM ATP and 15 mM phosphocreatine. Soluiton B contained instead high-energy phosphates: 5 mM glutamate, 2 mM malate, 3 mM phosphate and 5–10 mg/ml fatty-acids-free bovine serum albumin. Solutions A and B were composed on the basis of available information of ionic composition of muscle cell cytoplasm [13].

To study the effects of ischemia on the functional properties of mitochondria the excised hearts were incubated in closed moist chamber at 37° C for 15 and 30 min and fiber bundles were isolated from these totally ischemic hearts.

To study the effect of isoproterenol, 80 mg drug per kg body weight were injected subcutaneously into rats 3 h before experiment.

Results and Discussion

Fig. 1 shows the electron micrograph of a cardiac fiber skinned by using a saponin procedure described in the previous section. It is clear from this figure that sarcolemma is completely removed; however, the ultrastructure of the intracellular membraneous systems, such as mitochondria and sarcoplasmic reticulum, stays intact; that is in agreement with several earlier data [14,15]. This selective effect of saponin could be explained by its affinity to cholesterol [16] which content is high in sarcolemma and low in mitochondria or in reticulum [6]. After 20 min of saponin treatment only 1% of lactate dehydrogenase activity initially present in cardiac fibers was found in skinned preparations. That shows complete removal of cytoplasmic components of the cells and accessibility of exogeneous substrates to all intracellular systems due to total desintegration of sarcolemma. Cytochromes aa_3 content of skinned preparations was found to be 20.4 ± 1.8 nmol per g wet weight (mean value and standard deviation for six preparations) and did not differ from the value of this parameter for intact heart muscle (22.5 ± 2.0 nmoles per g of wet tissue). Thus, mitochondrial content of fibers stayed constant during saponin treatment.

Fig. 2 shows the oxygraph traces of recordings of the respiratory parameters of mitochondria in saponin-treated cardiac fibers. Fig. 2A shows that in the absence of substrates of mitochondrial

Fig. 1. Electron micrograph of saponin-skinned fiber from rat heart (16000×).

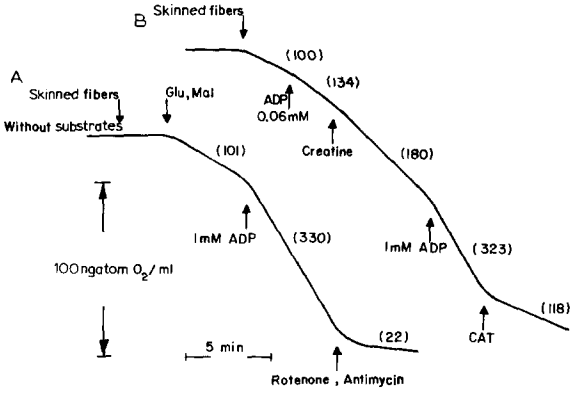

Fig. 2. Oxygraph traces of respiratory activities of mitochondria in skinned fibers. The reaction medium (volume, 3 ml) is described in 'Materials and Methods'. (A) The substrates of mitochondrial oxidation were initially absent; (B) the reaction medium initially contained glutamate (5 mM) and malate (2 ml) as substrates; creatine was added to 20 mM; rotenone to 20 μM; antimycin A to 3 μg/ml and to carboxyatractyloside to 35 μM. Numbers in parentheses indicate the rate of oxygen consumption in ngatoms/min per nmol cytochrome aa_3, 22° C.

oxidation there was no oxygen uptake by fibers. If skinned fibers were transferred into medium containing malate and glutamate, the respiration rate was relatively high even in the absence of externally added ADP. This respiration was stimulated further by addition of 60 μM ADP (Fig. 2B). The respiration rate in the presence of 60 μM ADP was submaximal. Maximal rate of respiration was observed at 1 mM ADP (Fig. 2A and B). The State 3 respiration was not followed by transition into State 4 as it is the case if isolated mitochondria are used. This is due to the presence of endogenous ATPases which continuously regenerate ADP. Fibers' respiration was effectively inhibited by respiratory chain inhibitors (rotenone, antimycin A) and by that of adenine nucleotide translocase - carboxyatractyloside (Fig. 2). Submaximal activation of respiration at 60 μM ADP was most probably due to diffusion difficulties for ADP and its binding to the cellular structures, since this concentration significantly exceeds the apparent K_m for ADP for adenine nucleotide translocase (about 5–10 μM [17]). The respiration rate in the presence of 60 μM ADP was enhanced further by addition of 20 mM creatine (Fig. 2B) which activates mitochondrial creatine kinase and in this way increases the local concentration of ADP near the inner mitochondrial membrane [18].

Though skinned fibers are known to have not only mitochondrial creatine kinase but also isoenzyme bound to myofibrils and probably to sarcoplasmic reticulum (cytosolic enzyme is removed, as shown by absence of LDH, a marker of soluble proteins), it is very likely that that is the mitochondrial creatine kinase which plays the major role in the stimulation of respiration. The effect of creatine on respiration rate was studied at low level of ATP (ADP). K_m(ATP) for myofibrillar creatine kinase is around 0.9 mM [19] and at that ATP concentration myofibrillar creatine kinase reaction is not significantly activated. On the other hand, coupling of the mitochondrial creatine kinase reaction to oxidative phosphorylation decreases the K_m(ATP) value to 0.014 mM [20], and this coupled reaction is therefore significantly activated at the low adenine nucleotide level.

Moreover, it has been recently shown that the myofibrillar enzyme is very stable and does not

alter its properties in skinned fibers even after 90 min of total ischemia [21]. Nevertheless, in the present work we have shown that shorter period of ischemia results in very significant depression of the stimulation of the oxygen consumption in the presence of creatine (see below). Taking together with the data by Bittl et al. [22] about the progressive loss of mitochondrial creatine kinase during ischemia, these facts support the statement that the degree of the acceleration of respiration after the creatine addition reflects the function of mitochondrial creatine kinase.

Statistically analyzed data of nine similar experiments are shown in Table I. The maximal values of the respiration rates of fibers achieved in the presence of 1 mM ADP are 323 ng atoms/min per nmole of cytochrome aa_3 and this value is approaching the State 3 respiration rate of isolated mitochondria (375 ngatoms/min per nmole of cytochrome aa_3) under the same conditions. Thus, the maximal functional properties of mitochondria can be directly assessed in saponin-treated fibers. The salt composition of oxygraph medium described was found to be optimal for respiratory activity of mitochondria, and behaviour of isolated heart mitochondria in this physiological salt solution was described in detail in our previous publication [23]. This medium closely simulates the composition of cardiac cells cytoplasm and its use may be important if one wishes to describe mitochondrial parameters in situ. The ratio of maximal rate to the basal respiration rate can be taken to be an analog of respiratory control index and is around three for the fibers. For heart mitochondria in the presence of Mg^{2+} (activation of ATPases) this is a reasonably good value. Of interest is also the relative extent of stimulation of respiration by creatine, $(V_{Cr} - V_{ADP})/V_{ADP}$, which characterizes the control of respiration by the mitochondrial creatine kinase reaction under given experimental conditions. Finally, the percentage of inhibition of respiration by carboxyatractiloside shows the intactness of the inner mitochondrial membrane [17].

Fig. 3 shows the changes in these three parameters in fibers isolated from hearts after 15 and 30 min of ischemia and from hearts of animals to which high dozes of isoproterenol were injected. In both these cases the functional parameters of mitochondria were affected, and most significantly the creatine-stimulated respiration, which

TABLE I

RESPIRATION OF SKINNED FIBERS (NINE EXPERIMENTS) FROM RAT HEART MUSCLE

Additions	Respiration rates measured in ngatoms O_2 per min	
	per mg protein	per nmol cytochrome aa_3
Basal respiration rate (V_0)	15.5 ± 1.3	101 ± 12
In the presence of:		
0.06 mM ADP (V_{adp})	20.4 ± 1.6	134 ± 15
20 mM creatine (V_{Cr})	27.1 ± 2.6	179 ± 23
1.00 mM ADP (V_{ADP})	49.0 ± 4.0	324 ± 38
35 μM carboxyatractiloside (V_{CAT})	18.0 ± 1.5	118 ± 14
Percentage of inhibition of the respiratory rate by carboxyatractiloside	69 ± 2	
Ratio of maximal (1.00 mM) ADP-stimulated respiration rate to basal respiration rate (V_{ADP}/V_0)	3.2 ± 0.2	
Respiration rate increase after the addition of 20 mM creatine at low (0.06 mM) ADP concentration ($V_{Cr} - V_{adp})/V_{adp}$ (an index of the creatine kinase system state)	0.34 ± 0.04	

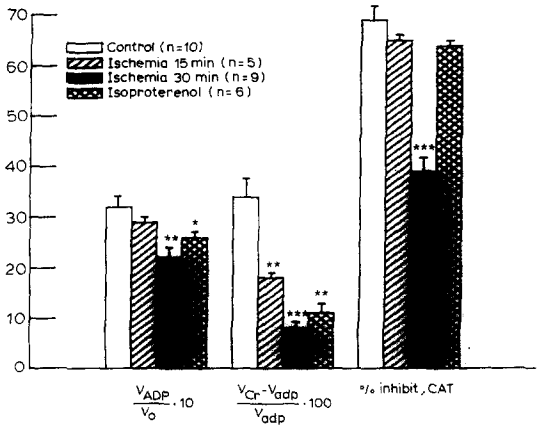

Fig. 3. Effects of ischemia (37 ° C) and isoproterenol (80 mg/kg) on the respiratory parameters of the mitochondria in cardiac skinned fibers. V_{ADP}/V_0 is the maximal ADP-stimulated to basal respiration rate; $(V_{Cr} - V_{adp})/V_{adp}$ the respiration increase rate after addition of creatine (20 mM) at low ADP concentration (an index of creatine kinase reaction); % inhibit, CAT is the percentage of inhibition of the respiration rate by carboxyatractiloside.

decreased 4 times after 30 min of ischemia, and 3 times after injection of isoproterenol. The similarity of the actions of ischemia and isoproterenol administration is not unexpected as it is well known, since Rona et al. [24] demonstrated that catecholamines can produce the ischemia-like lesions in myocardium. Excessive β-stimulation leads to mitochondrial swelling, alterations in mitochondrial function and enzyme activities and creatine kinase release from the heart [25–27]. It is likely that the catecholamine stress results in dissociation of creatine kinase from the inner mitochondrial membrane.

Ischemia decreased also the sensitivity of mitochondria to carboxyatractiloside that shows the rupture of significant part of mitochondrial membrane. The results of Fig. 3 indicate that the mitochondrial creatine kinase is the most sensitive part of phosphocreatine shuttle and is easily damaged during ischemia, in accordance with the data by Bittl et al. [22].

In conclusion, an assessment of respiration rates in mitochondria in saponin-treated fibers allows to detect quickly and precisely the changes in mitochondrial properties in different pathological states of heart muscle. One of the values of this method is that it requires not more than 10–20 mg of tissue to determine the mitochondrial functional properties and therefore, it can be used for an analysis of biopsy samples taken from human hearts. An example of this analysis is given in Fig. 4, where an oxygraphic trace of respiratory activity of mitochondria in skinned fibers obtained from a patient is shown. This patient had the dilated form of cardiomyopathy, and the biopsy sample weighing 5 mg was taken with bioptome from the endomyocardium of left ventricle during an angiographic examination. As it can be seen, this quantity of material is enough for the determination of the functional state of heart mitochondria.

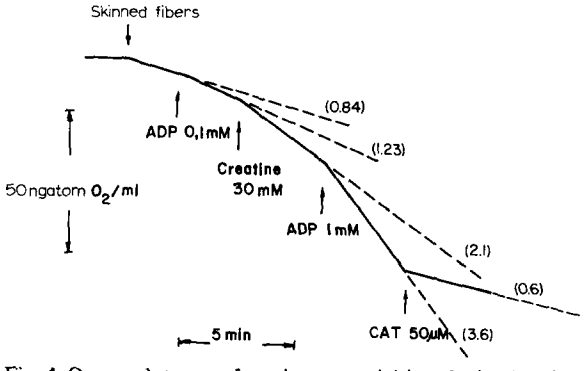

Fig. 4. Oxygraph traces of respiratory activities of mitochondria in skinned fibers obtained from human heart by bioptome during an angiographic cardiac examination. Biopsy samples weighting 5 mg was treated with saponin as described above and measurements were carried out in 2 ml of reaction medium. The numbers indicate the respiration rates in ngatoms O_2 per min per mg wet weight. CAT, carboxyatractiloside.

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