The role of creatine and fatty acids in the regulation of mitochondrial respiration

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In this study, the role of creatine and fatty acid oxidation in the regulation of mitochondrial respiration *in situ* was determined using the saponin-permeabilized rat cardiac fibers.

The data showed that the permeability of the outer mitochondrial membrane (OMM) to ADP was much higher (app. K_m lower) at 20 °C during the palmitoyl–L- carnitine oxidation than during the pyruvate+malate oxidation. However, the stimulating effect of creatine on fiber respiration in the presence of a low (60 μM) concentration of the exogenous ADP with these substrates was similar (it increased about 1.4-fold). In case of the palmitoyl-L-carnitine oxidation, it did not depend on the way of delivery of ADP to mitochondria – from the surrounding medium or from the other organelles of the cell. Creatine also induced a 2.4-fold decrease in app. K_m during octanoyl-DL-carnitine oxidation. It is obvious that the functional coupling between ADP/ATP translocase and creatine kinase is preserved in the mitochondria, despite a significant increase in OMM permeability to ADP induced by fatty acid oxidation.

Key words: saponin-permeabilized heart muscle fibers, outer mitochondrial membrane permeability, fatty acid oxidation, oxidative phosphorylation, effect of creatine

INTRODUCTION

Several authors [1–3] have noted that the outer membrane of mitochondria (OMM), in contrast to the isolated heart mitochondria, possesses a low permeability to ADP *in situ* (high apparent K_m of oxidative phosphorylation for ADP (app. K_m^{ADP})) in saponin- permeabilized cardiac fibers. Activation by creatine of the mitochondrial creatine kinase (mi-CK) reaction in the intermembrane space coupled to adenine nucleotide translocase (ANT) and oxidative phosphorylation overcomes the diffusion difficulties of ADP by amplifying the stimulatory effect of exogenous ADP on respiration. This allows to maximally activate the processes of oxidative phosphorylation in the mitochondria, despite a limited access of ADP (at micromolar concentration) through the OMM.

However, when CoA- or carnitine-esters of palmitate and octanoate were substituted for pyruvate + malate or succinate, used as mitochondrial respiratory substrates in the above-mentioned studies [4], the OMM permeability to ADP dramatically (up to 10-fold) increased (app. K_m^{ADP} decreased).

Thus, the aim of the present work was to study the functional coupling of mitochondrial creatine kinase and ANT under the conditions of the elevated permeability of the OMM to ADP, *i.e.* during fatty acid oxidation in the heart mitochondria located in the saponin-permeabilized rat heart muscle fibers.

MATERIALS AND METHODS

Wistar male rats weighing 250–300 g were used for experiments. Hearts were excised and rinsed in ice-cold 0.9% KCl solution. Bundles of the heart muscle fibers, approximately 0.2–0.3 mm in diameter, were prepared [5] and transferred to cooled solution A containing 20 mM of imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 7.1 mM $MgCl_2$, 50 mM 2-[N-Morpholino]ethanesulfonic acid (MES), 5 mM ATP, 15 mM phosphocreatine, 2.6 mM CaK_2EGTA and 7.4 mM K_2EGTA (free Ca^{2+} concentration 0.1 μM) (pH 7.0 adjusted with KOH at 2 °C), supplemented with 50 μg/ml saponin (from Gypsophila; sapogenin content 17+ %; Sigma) and incuba-

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ted for 30 min. Then the bundles were washed for 10 min in solution B containing 20 mM of imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.6 mM $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4, 3.0 mM CaK_2EGTA and 7.1 mM K_2EGTA (pH 7.1 adjusted with KOH at 37 °C).

Oxygen consumption rates of saponin-permeabilized cardiac fibers were recorded at 20 °C by means of the Clark-type electrode system in solution B supplemented with 2 mg/ml of bovine serum albumin (Fraction V; A4503, Sigma). The solubility of oxygen was taken to be 463 ngatoms/ml. Respiration rates were expressed as ngatoms O/min/mg of dry weight fibers (dry weight = wet weight before respiration measurement/4.85). The ADP regenerative system, consisting of 1.2 IU/ml lyophilized yeast hexokinase (Type V; EC 2.7.1.1; Sigma) and 24 mM glucose (Sigma), was added into the oxygraph chamber before addition of heart muscle fibers. In case of stimulation of respiration by exogenous ATP, the ADP-regenerative system was not used. Skinned fibres were added at approx. 2–4 mg wet weight/ml.

The coupling of mi-CK and ANT was estimated using two approaches. (1) The apparent K_m for ADP (app. K_m^{ADP}) and V_{max} values were assayed from [ADP] *versus* VO_2 rate relationships in the presence or absence of 20 mM creatine; the results were compared with the corresponding kinetic parameters without creatine; (2) 20 mM of creatine was added into oxygraphic medium after recording the respiration in the presence of submaximal concentration of ADP (60 μM). The stimulation of respiration by creatine, *i.e.* the creatine effect, was expressed as the ratio $V_{O2}^{Cr}/V_{O2}^{60ADP}$, where V_{O2}^{Cr} and V_{O2}^{60ADP} denote the respiration rates with and without creatine, respectively. App. K_m^{ADP} was estimated from the least-squares fit to the Michaelis–Menten equation by GraphPad Prism demo v3.0.

The values in Table and figures are expressed 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. Where missing, error bars were smaller than the symbol size (Fig. 2).

Chemicals used in this study were: KCl – from Roth, pyruvate – from Merck, KH_2PO_4 and malate – from Serva, all other reagents – from Sigma. Palmitoyl-L-carnitine was dissolved in 40% ethanol solution. The final ethanol concentration in the fiber respiration measurements did not exceed 1% and did not affect respiratory parameters.

RESULTS AND DISCUSSION

Data presented in Table show that exogenous creatine (20 mM) stimulates fiber respiration with pyruvate + malate (1.37 ± 0.05) and palmitoyl-L-carnitine (1.42 ± 0.04) with a similar efficacy. Apparent K_m^{ADP} values for respiration with these substrates, estimated using two different ADP concentrations (60 μM and 1.2 mM), were significantly different: 275 ± 165 and 106 ± 48 μM, respectively (n = 6). It means that the OMM permeability to ADP is much higher in the case of palmitoyl-L-carnitine oxidation.

Table. **Respiration parameters for two different respiratory substrates: the effect of creatine**
Temperature of the measurements was 20 °C. The data of six unpaired experiments are presented.

	Final concentration	Pyruvate + malate (6 mM + 6 mM)	Palmitoyl-L-carnitine + malate (9 μM + 0.24 mM)
Vo		8.7 ± 0.8	7.1 ± 0.6
Vadp	60 μM	16.9 ± 1.1	15.2 ± 1.3
Vcreat	20 mM	22.7 ± 1.6	21.4 ± 2.0
V_{ADP}	1.2 mM	61.1 ± 6.1	45.7 ± 0.9
Effect of creatine[]		**1.72 ± 0.12**	**1.77 ± 0.09**
Vcreat/Vadp		1.37 ± 0.05	1.42 ± 0.04
V_{ADP}/Vo		6.41 ± 0.23	5.53 ± 0.61
$V_{ADP + C}/V_{ADP}$		1.01 ± 0.03	1.08 ± 0.04

V_o, basal respiration rate; Vcreat, respiration rate in the presence of 60 μM ADP and 20 mM creatine; V_{ADP+C}, respiration rate in the presence of 1.2 mM ADP and 30 μM cytochrome c. []The calculation of the effect of creatine is described in Materials and Methods.

In more accurate separate measurements of app. K_m^{ADP} with palmitoyl-L-carnitine as a respiratory substrate, using multiple ADP concentrations (as shown in Fig. 2), a lower value of this parameter was obtained (49 ± 8, n = 5). It should be noted that the exogenous cytochrome c has no significant effect on the respiration rate in State 3 with either of the substrates, demonstrating the intactness of OMM.

In the further experiments, the fiber respiration supported by palmitoyl-L-carnitine was stimulated by exogenous ATP (Fig. 1). In these conditions, endogenous ADP is produced from exogenous ATP by ATPases in the myofibrils and in the sarcoplasmic reticulum and is delivered directly to the mitochondria [6]. The effect of creatine in this case ap-

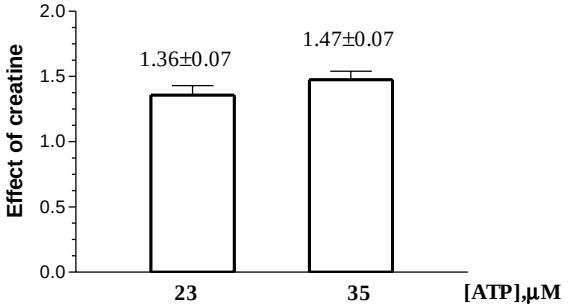

Fig. 1. Effect of creatine in the presence of different concentrations of exogenous ATP. The substrate: palmitoyl-L-carnitine 9 μM + malate 0.24 mM. The data of five separate unpaired experiments are presented. The average respiration parameters (in ngatO/min/mg dry weight) were as follows: V_O = 5.8; V_{atp} = 7.6–8.1; Vcreat[20 mM] = 10.8; VATP[1 mM] = 18.2

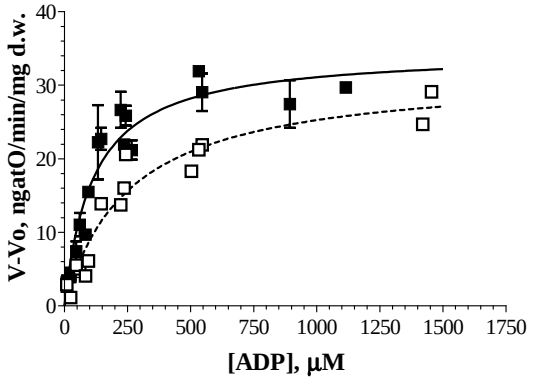

Fig. 2. Dependence of respiration rates of rat cardiac fibers on the external ADP concentration: effect of creatine. The substrate: octanoyl-DL-carnitine 0.36 mM + malate 0.24 mM. The data of five separate paired experiments are presented. V_{MAX} values (in ngatO/min/mg dry weight) were found to be similar: 29.9 ± 1.8 (medium without creatine) and 31.1 ± 1.6 (medium with creatine)

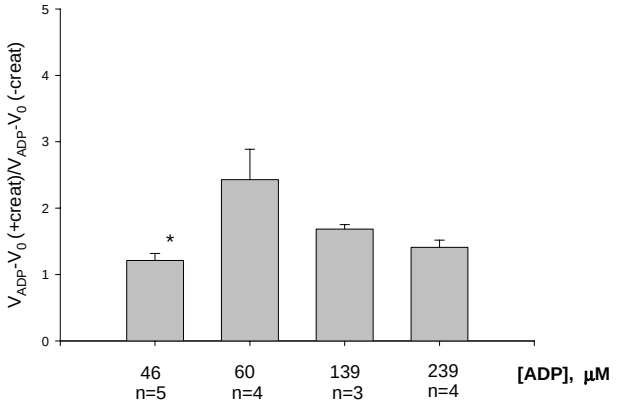

Fig. 3. Effect of creatine in the presence of different concentrations of exogenous ADP. The substrate: octanoyl-DL-carnitine 0.36 mM + malate 0.24 mM. *P < 0.05 compared to 60 μM ADP

peared to be very close to that observed in the above experiments with exogenous ADP. Moreover, it was almost identical in the presence of different concentrations of ATP, 23 μM and 37 μM: 1.36 ± 0.07 and 1.47 ± 0.07, respectively.

Besides, we investigated also the dependence of the effect of creatine on the mitochondrial respiration upon the concentration of exogenous ADP (Figs. 2 and 3). We found that the maximum effect of creatine (2.43 ± 0.46) in these experiments (with octanoyl-DL-carnitine as the substrate) was shown in the presence of 60 μM ADP (Fig. 3). The maximum effect of creatine (1.88 ± 0.14) on the pyruvate + malate (6 + 6 mM) oxidation was achieved at a 50–70 μM concentration of ADP (n = 4). These two observations conform to each other.

The app. K_m^{ADP} values, estimated at 20 °C, when octanoyl-DL-carnitine was used as a respiratory substrate (Fig. 2), were significantly different in two different media: 235 ± 16 μM ADP (medium without creatine) and 98 ± 11 μM ADP (medium with creatine). The finding that creatine significantly decreases app. K_m^{ADP} in the mitochondria oxidizing octanoyl-DL-carnitine is in a good agreement with the data of other investigators [8], when pyruvate + malate was used as a respiratory substrate. The creatine-induced decrease in app. K_m^{ADP} reflects maintenance of functional coupling in the intermembrane space between ANT and mi-CK in the mitochondria respiring on fatty acids. The same conclusion can be drawn also from the above-described creatine effects assessed only at a 60 μM concentration of ADP.

Our unpublished observation showed that elevation of the temperature from 20 °C to 37 °C was accompanied by about a twofold decrease in the apparent K_m for ADP with octanoyl-DL-carnitine. These results are in good accordance with our earlier data obtained with saponin-permeabilized cardiac fibers respiring on succinate [7].

Thus, the data presented in this paper for the first time demonstrate that the functional coupling between ANT and mi-CK is preserved in the mitochondria, despite a significant increase in the OMM permeability to ADP induced by fatty acid oxidation. The latter phenomenon is observed not only at 37 °C [4], but also at a lower temperature (20 °C).

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KREATINO IR RIEBALŲ RŪGŠČIŲ OKSIDACIJOS POVEIKIS MITOCHONDRIJŲ *IN SITU* KVĖPAVIMO REGULIAVIMUI

S a n t r a u k a

Šiame darbe tirta kreatino ir riebalų rūgščių oksidacijos poveikis mitochondrijų *in situ* kvėpavimo reguliavimui (saponinu permeabilizuotose žiurkės širdies raumens skaidulose). Iš duomenų matyti, kad išorinės mitochondrijų membranos pralaidumas ADP 20 °C temperatūroje yra daug didesnis (mažesnė tariamoji K_m^{ADP}) oksiduojantis palmitoil-L-karnitinui negu piruvatui + malatui. Tačiau esant mažai ADP koncentracijai (60 μM) abiem atvejais gautas panašus skaidulų kvėpavimą stimuliuojantis kreatino efektas. Šis parametras, mitochondrijoms oksiduojant palmitoil-L-karnitiną, nepriklauso nuo ADP patekimo į mitochondrijas būdo (iš terpės ar iš kitų ląstelės organelių). Pažymėtina, kad, oksiduojantis oktanoil-DL-karnitinui, kreatinas sumažino tariamąją K_m^{ADP} reikšmę 2,4 karto. Akivaizdu, kad mitochondrijose išsaugoma funkcinė sąveika tarp ADP/ATP nešiklio ir kreatinkinazės, nepaisant ženkliai padidėjusio išorinės mitochondrijų membranos pralaidumo ADPui, indukuoto oksiduojantis riebalų rūgštims.