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ON THE EXISTENCE OF TWO POPULATIONS OF MITOCHONDRIA IN A SINGLE ORGAN. RESPIRATION, CALCIUM TRANSPORT AND ENZYME ACTIVITIES

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SUMMARY: Mitochondria isolated from rat heart and kidney cortex by Polytron treatment of the tissues exhibit lower state 3 rates of respiration than mitochondria isolated by Nagarse method. Addition of cytochrome c to Polytron mitochondria isolated from heart, but not from kidney, increases oxygen uptake to values approaching those of Nagarse-treated preparations. Similar results were observed for Ca^{2+} uptake. Kidney Polytron mitochondria exhibited lower mitochondrial, but higher non-mitochondrial enzyme activities compared to kidney Nagarse mitochondria. Enzyme activities were the same in Polytron and Nagarse mitochondria from heart. The differences between Polytron and Nagarse mitochondria appear to be mainly due to lower cytochrome c content of Polytron mitochondria from heart and higher contamination of Polytron mitochondria from kidney.

INTRODUCTION: The existence of different populations of mitochondria in cardiac muscle in normal and disease states has been proposed by ourselves and others (1-6) Multiple populations subserving different functions in other organs such as kidney have also been suggested (7). It is not inconceivable that subpopulations of mitochondria may be differently affected in certain pathological conditions and by various pharmacological interventions. There is a growing interest in studying isolated mitochondria representative of possible in vivo subpopulations.

In a recent report, Palmer et al (4) described the isolation of biochemically distinct "subsarcolemmal" and "interfibrillar" mitochondria from rat cardiac muscle by Polytron and Nagarse treatment methods, respectively. However, some of the biochemical differences observed by them between these mitochondria prepared by these two methods may be due to the isolation and assay procedures and may not represent physiological differences. The purpose of this study is to examine mitochondria from contractile and non-contractile organs isolated by procedures which putatively release different populations of the organelle.

MATERIALS & METHODS: Isolation of Mitochondria: Female Sprague-Dawley rats (200-300g) were used. Mitochondria were isolated from heart and kidney by Polytron treatment according to Sordahl and Schwartz (8) and by Nagarse treatment according to Palmer et al (4). The isolation medium contained 180mM KCl, 10mM EGTA[1], 0.5% BSA (Fraction V) and 10mM HEPES buffer, pH 7.4 (KEAH). The atria and the right

Abbreviations: EGTA: ethylene glycol bis (β-amino-ethyl) N,N'-tetra-acetic acid; BSA: bovine serum albumin; HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid.

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ventricular free wall were removed from hearts. Only the cortical portion of kidney was used. Homogenate after Polytron treatment was centrifuged at 400xg for 10 minutes. The resulting pellet was used to isolate Nagarse mitochondria according to Palmer et al (4). The supernatant solution was divided into two halves. One half was treated with Nagarse (5mg Nagarse/g tissue the homogenate represents). The supernatant solutions were then centrifuged at 5000xg for 10 minutes. The pellets were washed twice before finally suspended in a small volume of KEAH containing 0.1mM EGTA. Kidney mitochondria were isolated similarly except that the tissue was either treated with a Polytron very briefly and slower speed, or homogenized in a Potter-Elvejhem homogenizer (5 passes). Mitochondria isolated from untreated and from Nagarse-treated first low speed supernatant from heart were designated as HP and HPN, respectively; similarly from kidney as KP and KPN, respectively. Mitochondria isolated from the first low speed pellet from heart and kidney homogenate were designated as HN and KN, respectively.

Measurement of Respiration and Phosphorylation: Oxygen uptake was measured with a Clark oxygen electrode and a Gilson polarograph at 30°C in 1.5ml of medium. The medium contained 0.25M sucrose, 2.66mM Pi and 10mM HEPES, pH 6.8. Each assay was carried out with 1mg protein and 6.6mM glutamate + 6.6mM malate. State 3 rate was initiated with 403 nmoles ADP. State 3, state 4 rates, respiratory control (RC) and ADP/O ratios were calculated as described previously (9,10).

Calcium Uptake: Calcium uptake was carried out at room temperature by the murexide method (11,12) in the same medium used for the measurement of respiration with 5mM succinate as substrate. 1.2mg mitochondrial protein and 66μM $CaCl_2$ was used in a final volume of 3ml. A decrease in absorbance at 472-508nm was recorded in an Aminco dual beam spectrophotometer model DW2-a.

Enzyme Assay: Citrate Synthase activity in sonicated mitochondria was assayed at 30°C according to the method of Srere (13). Cytochrome c oxidase activity was assayed at room temperature as described previously (14) using mitochondria which were swelled in 100mM phosphate buffer (pH 7.4) at 30°C for 12 minutes. 5'-nucleotidase activity (15) and glucose-6-phosphatase activity (16) were assayed according to published procedures. Cytochrome c content in isolated mitochondria was determined from the reduced-oxidized spectra obtained in an Aminco dual beam model DW-2a spectrophotometer after reduction with sodium dithionite. A millimolar extinction coefficient of 19 at 550-540nm for cytochrome c and 16.5 at 607-630 for cytochrome a + a_3 were used. Protein concentration in mitochondria was determined according to Lowry et al (17) with BSA as standard.

RESULTS: Mitochondria isolated by Nagarse treatment from heart and kidney exhibited significantly ($P = .001$) higher state 3 rates of respiration compared to mitochondria isolated by the Polytron treatment of heart and kidney when the rates are expressed in terms of mitochondrial protein after isolation (Table I). The addition of 6.6μM cytochrome c to the assay medium increased the state 3 rates of the Polytron mitochondria from heart by 42% ($P = .0013$) and of Nagarse mitochondria by 8% (not significant). The state 3 rate of Polytron mitochondria was increased by cytochrome c to near those of Nagarse mitochondria (the difference was no longer significant). Heart mitochondria "released" by the Polytron treatment were then treated with Nagarse and then sedimented by centrifugation as described under "MATERIALS & METHODS." The state 3 rate of these mitochondria was slightly higher than Polytron mitochondria. Nagarse per se had no effect on state 3 rate of mitochondria isolated by Polytron treatment. Kidney cortical mitochondria isolated similarly showed state 3 rates of respiration significantly higher ($P = .001$) than those of mitochondria prepared by homogenization in a Potter-Elvejhem homo-

genizer. Cytochrome c (up to 150μM) did not stimulate state 3 respiration of mitochondria isolated from kidney by homogenization or Nagarse treatment.

The cytochrome c and a + a_3 content of isolated mitochondria were determined (Table I). The cytochrome c content of Polytron mitochondria from heart was significantly lower compared to that of Nagarse mitochondria (P = 0.0001). Cytochrome a + a_3 content was slightly lower in Polytron mitochondria compared to Nagarse mitochondria, but the difference was not significant (P = 0.30). Significantly lower cytochrome c (P = .006) and a + a_3 (P = 0.0038) content mitochondria prepared by homogenization was observed compared to that of Nagarse mitochondria isolated from kidney cortex.

The state 3 rate of respiration was then calculated on the basis of cytochrome c or a + a_3 content of the mitochondrial preparations (Table I). The advantage of expressing the results in this way is that it eliminates the possible contribution of proteins from contaminating membranes. It also determines the effect of loss of respiration due to loss of cytochrome c which is very easily released in high ionic medium from mitochondria with leaky outer membranes (18,19). The data expressed in this manner revealed no significant differences between state 3 rates of respiration of Polytron and Nagarse mitochondria from heart. Similar results were obtained with kidney cortical mitochondria. Although large variations in Ca^{2+} uptake of isolated mitochondria were observed between experiments, the Ca^{2+} uptake properties were also similarly affected (Table I).

Specific mitochondrial enzymes, cytochrome c oxidase activity tightly bound to the inner membrane and citrate synthase activity localized in the matrix compartment were also studied (Table II). Cytochrome c oxidase activity expressed in terms of mg mitochondrial protein exhibited slightly lower activity in heart Polytron mitochondria compared to Nagarse mitochondria (not significant; P = .0521). Kidney mitochondria isolated by homogenization, on the other hand, showed significantly (P<0.0001) lower cytochrome c oxidase activity. Nagarse treatment of mitochondria released by Polytron treatment of kidney increased cytochrome oxidase activity significantly (P = .001). When the data are expressed in terms of cytochrome a + a_3 content of the mitochondrial preparations, no significant differences in activity of the enzyme was observed (Table II). Citrate synthase activity of heart mitochondria expressed in terms of per mg protein revealed no difference between Polytron and Nagarse (Table II). Kidney mitochondria, on the other hand, isolated by the Nagarse method revealed significantly higher citrate synthase activity (P = .0006). Nagarse treatment of mitochondria previously "released" by Polytron treatment from kidney tissue also showed significantly higher activity compared to that of the Polytron mitochondria. However, the difference in citrate synthase activity in kidney Polytron and Nagarse mitochondria disappear if the data are expressed in terms of nmoles of cytochrome a + a_3 (Table II).

TABLE I

STATE 3 RATES OF RESPIRATION AND CYTOCHROME CONTENTS OF MITOCHONDRIA ISOLATED BY POLYTRON AND NAGARSE TREATMENTS FROM HEART AND KIDNEY

TISSUE & TREATMENT	CYT c IN ASSAY	STATE 3 nAtoms /min/mg Protein	CYTOCHROME CONTENT nmoles/g Protein		STATE 3 nAtoms/min		Ca^{2+}-UPTAKE nmole/min	
			Cyt c	Cyt a + a3	per nmole cyt c	per nmole cyt a + a3	per mg Protein	per nmoles cyt a + a3
HP	-	271 ± 28 (8)	4572 ± 219 (5)	5283 ± 496 (5)	561 ± 20 (5)	506 ± 86 (5)	165 ± 48 (4)	11.5 ± 5.5 (3)
	+	386 ± 12 (9)			788 ± 38 (5)	755 ± 89 (5)	190 ± 45 (4)	12.1 ± 5.2 (3)
HN	-	401 ± 15 (8)	6668 ± 209 (6)	6005 ± 435 (6)	562 ± 27 (6)	580 ± 40 (6)	219 ± 25 (4)	5.5 ± 0.8 (4)
	+	434 ± 20 (8)			588 ± 31 (6)	663 ± 42 (6)	215 ± 31 (4)	5.5 ± 1.0 (4)
HPN	-	345 ± 23 (6)	5469 ± 317 (6)	6899 ± 383 (6)	579 ± 16 (6)	467 ± 32 (6)	174 ± 59 (4)	5.5 ± 2.2 (4)
	+	410 ± 26 (6)			715 ± 13 (6)	570 ± 38 (6)	208 ± 46 (4)	6.5 ± 1.9 (4)
KP*	-	149 ± 4 (9)	3382 ± 200 (6)	3323 ± 194 (6)	456 ± 20 (6)	481 ± 37 (6)	223 ± 7 (5)	11.0 ± 1.0 (4)
	+	162 ± 7 (9)			520 ± 43 (6)	530 ± 46 (6)	242 ± 19 (5)	12.0 ± 1.7 (4)
KN	-	285 ± 19 (9)	6806 ± 325 (6)	5132 ± 443 (6)	393 ± 11 (6)	544 ± 46 (6)	381 ± 35 (5)	13.4 ± 1.9 (4)
	+	283 ± 15 (9)			424 ± 14 (6)	578 ± 48 (6)	293 ± 33 (5)	9.8 ± 2.0 (4)
KPN	-	221 ± 20 (6)	4459 ± 204 (6)	4337 ± 249 (6)	459 ± 16 (6)	475 ± 13 (6)	297 ± 67 (5)	11.0 ± 3.2 (4)
	+	242 ± 21 (6)			503 ± 27 (6)	518 ± 24 (6)	303 ± 57 (5)	10.6 ± 2.6 (4)

*Kidney mitochondria were isolated by homogenization with a Potter-Elvejhem homogenizer. No difference in State 3 rate was observed when mitochondria were isolated by brief Polytron treatment or homogenization. The treatments and isolation procedures were as described under "Materials and Methods." 1 μM cytochrome c was used for Ca^{2+} uptake since higher concentrations inhibit the uptake.

TABLE II

ENZYME ACTIVITIES OF ISOLATED MITOCHONDRIA FROM HEART AND KIDNEY

TISSUE & TREATMENT	CYTOCHROME c OXIDASE 1st ORDER RATE CONSTANT		CITRATE SYNTHASE		5'-Nucleotidase	Glucose-6-Pase
	U/mg Protein	U/nmole cyt a + a_3	U/mg Protein	U/nmole cyt a + a_3	µmoles Pi/hr/mgP	µmoles Pi/hr/mgP
HP	5.79 ± 0.22 (7)	17.2 ± 1.6 (3)	2.55 ± 0.46 (3)	4.38 ± 0.46 (3)	2.27 ± 0.55 (5)	0.578 ± 0.323 (4)
HN	8.04 ± 1.02 (7)	16.2 ± 0.6 (4)	2.78 ± 0.18 (3)	4.60 ± 0.34 (3)	2.02 ± 0.36 (5)	0.636 ± 0.302 (4)
HPN	8.49 ± 0.42 (7)	16.3 ± 1.3 (4)	2.91 ± 0.40 (3)	3.93 ± 0.57 (3)	2.63 ± 0.38 (5)	0.537 ± 0.177 (4)
KP*	3.07 ± 0.21 (7)	12.8 ± 2.1 (4)	0.59 ± 0.04 (3)	1.93 ± 0.19 (3)	12.39 ± 1.27 (6)	16.23 ± 0.56 (4)
KN	6.48 ± 0.47 (7)	16.9 ± 1.4 (4)	0.93 ± 0.07 (3)	1.79 ± 0.17 (3)	6.36 ± 1.56 (6)	6.95 ± 2.40 (4)
KPN	4.69 ± 0.31 (7)	13.4 ± 1.5 (4)	0.77 ± 0.09 (3)	1.72 ± 0.10 (3)	8.61 ± 1.32 (6)	4.38 ± 0.55 (4)

*Kidney mitochondria were isolated by homogenization with a Potter-Elvejhem homogenizer as described under "Materials and Methods." One Unit (U) of enzyme activity equals 1 µmole of substrate utilized or product formed per minute.

5'-Nucleotidase activity and glucose-6-phosphatase activity were similar in Nagarse mitochondria from heart (Table II). In contrast, these enzyme activities were higher in mitochondria isolated by homogenization from kidney suggesting that the possible presence of extramitochondrial material in higher quantity in these preparations compared to mitochondria prepared by Nagarse treatment (Table II). Nagarse did not inhibit activities of these enzymes.

DISCUSSION: This study was prompted by a recent report by Palmer et al (4) describing the isolation of "subsarcolemmal" and "interfibrillar" mitochondria from cardiac muscle. In the present study, experiments were designed to yield these two different populations of mitochondria and to characterize the nature of the biochemical differences between them. A non-contractile tissue, kidney cortex, was included to gain further information on possible preparative artifacts. The cortex was selected because it contains a relatively homogenous cell type.

Our results indicate that differences in state 3 respiration of mitochondria isolated from heart by Polytron and Nagarse treatment were due mainly to a decreased cytochrome c content of the Polytron-treated mitochondria. The decreased cytochrome c content of these organelles may have been due to a loss of cytochrome c during their isolation in high ionic media. This is possible since added cytochrome c in the assay medium stimulated state 3 respiration of Polytron mitochondria but not that of Nagarse mitochondria. This observation suggests that either Polytron treatment makes the mitochondrial outer membrane permeable to cytochrome c or that these mitochondrial outer membranes were permeable to cytochrome c in situ. Normally, mitochondrial outer membranes are not permeable to cytochrome c (20).

Kidney cortical mitochondria isolated by homogenization in a glass vessel with teflon pestle or by very brief Polytron treatment and by Nagarse treatment also show similar differences in state 3 rates of respiration. These differences were probably due to higher levels of contamination of mitochondria isolated by teflon pestle homogenization or Polytron treatment compared to mitochondria isolated by Nagarse treatment. It appears that the kidney mitochondria isolated by homogenization or by very brief Polytron treatment are not permeable to added cytochrome c. Since Nagarse per se had no effect on state 3 rate or enzyme activities, it appears that Nagarse effectively removed contamination probably by digestion.

This study demonstrates a novel aspect, that of expressing mitochondrial function based upon an intrinsic mitochondrial component whereby the problem of contamination and loss of loosely-bound cytochrome c during isolation is effectively eliminated. Moreover, the results presented here do raise doubt as to the validity of separating "subsarcolemmal" and "interfibrillar" mitochondria

by Polytron and Nagarse treatment of tissue as described by Palmer et al (4). Although it could be argued that Polytron mitochondria were isolated in low ionic medium and that there should have been no loss of cytochrome c during isolation, it should also be noted that high ionic medium was used by those coworkers for measurement of respiration (4). Our preliminary data on Polytron mitochondria isolated in low ionic medium indicate no loss of cytochrome c but release of cytochrome was observed when these mitochondria were later suspended in high ionic medium.

It is possible and indeed intriguing that different populations of mitochondria exist within a single cell type. However, current procedures are not entirely adequate for completely solving this problem.

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