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Beneficial Effect of Oxygen-Enriched Medium for Isolation of Mitochondoria

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Abstract

We compared oxidative phosphorylation activities and ultrastructure of mitochondria isolated with a normal isolating medium and with an oxygen-enriched medium. RCI, $Q0_3$, $Q0_4$ and OPR values of mitochondria isolated with the oxygen-enriched solution were 14 - 34 % high as compared with those isolated with the normal medium, when succinate was employed as a substrate. Electron microscopic study showed that the ratio of small size of mitochondria (< 1 μm) was 87 % in the total numbers of the fraction isolated with the oxygen-enriched medium, whereas 77 % in that isolated with the normal medium. The results suggest a possible role of oxygen-enriched isolating medium in reserving mitochondrial function and ultrastructural integrity, presumably due to protection of mitochondrial aging or disruption during isolation procedure.

Introduction

It is well admitted that oxygen supply to mitochondria is a crucial factor for energy production in the myocardium (1-5), and exposure of myocardial cells to oxygen-deprived state may result in an induction of mitochondrial functional and morphological changes (6-8). It is also demonstrated that the reversibility of such impaired mitochondria under experimental oxygen-deprived state is conceived to depend upon the interval and the severity induced. For example, Nishi et al. (7) have histologically demonstrated that mitochondrial structure of sino-atrial nodal cells of rabbit heart, once impaired upon hypoxia for shorter than 20 min, was almost completely restored upon reoxygenation of the tissue for a period of 20 min, but reoxygenation of the tissue pretreated with the hypoxic conditions for 60 min, was found not to restore the mitochondrial ultrastructure to the normal level, indicating a significance of oxygen supply for a reservation of mitochondrial histological integrity. Generally, isolation procedure of heart mitochondria takes 1.5 to 2 hrs and leaves mitochondria under the absence of oxygen (9, 10). This may arise a possibility to induce biochemical and morphological changes in mitochondria during the isolation procedure, although the hypoxic conditions mentioned above were

severer than those for the isolation procedure. The present experiment is undertaken to elucidate whether or not, the presence of oxygen in the isolation medium is beneficial for biochemical function and histological structure of isolated mitochondria. For this purpose, two isolation media, an oxygen-enriched and an ordinary solution, were employed, and the mitochondrial oxidative phosphorylation activity and the electron microscopic examination on mitochondria isolated either with these media were performed.

Methods

Heart mitochondria was isolated according to the method described elsewhere (9). Albino rabbits of either sex, weighing 1.8 - 2.0kg, were anesthetized with 30 mg/kg pentobarbital, and the heart was quickly removed and put into a cold 0.25 M sucrose - 1 mM EDTA, pH 7.4. Heart ventricles, free from fat and connective tissues, were minced using scissors and razor, and homogenized with 4 volumes of 0.18 M KCl - 10 mM EDTA - 0.5% albumin, pH 7.4 (KEA solution), for 4 sec with an Ultraturax, then centrifuged for 20 min at 1,000g. The supernatant was filtered through 4 layers of gauze and centrifuged at 10,000 g for 20 min. The pellet thus obtained was suspended in the KEA solution and centrifuged at 8,000 g. Suspension and centrifugation were repeated again. The final pellet was suspended in a small volume of KEA solution or 0.15 M KCl - 20 mM Tris-HCl, pH 7.4 and employed for the assays of mitochondrial oxidative phosphorylation activity or calcium accumulating and ATPase activities, respectively. For the electron microscopic study, the isolation was carried out using the equal amount of the tissue, isolation medium and final suspension medium for the comparison of the different experimentalgroups. The isolation method employed in the present experiment was essentially similar to that of Sordahl et al. (10).

For the isolation procedure using an oxygen-enriched solution, both EDTA - sucrose solution and KEA solution in the absence of tissue proteins were prebubbled with oxygen for 10 min. Furthermore, after suspension of heart homogenate and crude mitochondrial fractions with an oxygen-enriched solution, the suspended fraction at each step during the isolation procedure was transferred into a centrifugation tube and gently bubbled with oxygen for 10 sec, then shielded with a well fitted cap to avoid the loss of oxygen from the suspension solution in the tube. The pO_2 values of the oxygen-enriched solution and suspension treated as mentioned above were 678 - 726 mmHg, whereas those of the ordinary KEA solution (without any bubbling of oxygen) (control solution), 197 - 242 mmHg when measured by Corning Blood Gas analyser 165.

Mitochondrial oxidative phosphorylation activity was measured at 25 °C with Kyusui Oxygraph in a medium containing about 1.0 mg mitochondrial protein/ml, 0.25 M sucrose, 10 mM K_2HPO_4, 10 mM Tris-HCl, pH 7.4 and either 10 mM glutamate or 10 mM succinate as a substrate. State 3 respiration ($Q0_3$) of mitochondria was initiated by adding 200 nmoles ADP, whereas state 4 respiration ($Q0_4$) refers to the conditions when the whole ADP in the medium has been phosphorylated. The respiratory control index (RCI) was calculated as the ratio of oxygen consumption during state 3 and 4. ADP/

O ratio was estimated as nmoles ATP formed/natom oxygen consumed. The phosphorylation rate(OPR) was calculated by multiplying the values for ADP/O and state 3 respiration.

Mitochondrial calcium binding activity was measured at 25 °C in a medium containing 100 mM KCl, 20 mM Tris-HCl, 10 mM $MgCl_2$, 4mM ATP and 0.1mM $^{45}CaCl_2$, pH 6.8, according to the Millipore filtration technique described previously (9). Mitochondrial calcium uptake activity was assayed at 37°C in a medium containing 100 mM KCl, 20 mM Tris-HCl, 10 mM $MgCl_2$, 4 mM ATP, 0.1 mM $^{45}CaCl_2$, 4 mM KH_2PO_4 and 5 mM Tris-succinate pH 6.8. Mitochondrial ATPase activity was measured at 37°C in a medium containing 100 mM KCl, 20 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 4 mM Tris-ATP, pH 6.8. The details for measurement of mitochondrial activity were described previously (9).

For electron microscopic examination, isolated mitochondria were fixed with cold 2% glutalaldehyde in Millonig's phosphate buffer at pH 7.4 for 3 hr, rinsed with the phosphate buffer, and postfixed in 1% osmium tetraoxide in the same buffer for 2 hr. The preparation was dehydrated during a 12-hour period in a series of increasing concentrations of ethanol, and then embedded in Epon. The section was cut with a LKB ultratome, stained with uranyl acetate and lead citrate, and examined in a Hitachi UH 12 A electron microscope.

Protein concentrations were determined by the method of Lowry et al. (11). Statistical significance was estimated using Student's t-test ($p < 0.05$).

Results

Heart mitochondria were isolated using either an oxygen-enriched KEA solution and a control KEA solution, and their oxidative phosphorylation activities were measured. When succinate was employed as a substrate, respiratory control index (RCI) of mitochondria isolated with the oxygen-enriched solution showed about 14% high value in comparison with that of mitochondria isolated with the normal solution. Furthermore, oxidative phosphorylation activities of mitochondria isolated with the oxygen-enriched medium, such as rate of oxygen consumption, state 3 respiration (QO_3) and oxidative phosphorylation rate (OPR), also revealed about 30- 35% higher values than those isolated with the control KEA solution. However, employment of the oxygen-enriched solution for isolation medium did not elicit any changes in ADP/O of mitochondria and rate of oxygen consumption in state 4. In contrast, when glutamate was employed as a substrate, there were no appreciable changes in the oxidative phosphorylation activity between the mitochondria isolated with the oxygen-enriched solution and with the control solution.

We also measured calcium binding, calcium uptake and ATPase activities of isolated mitochondria, which may be considered to represent one of the marker activities for an integrity of mitochondrial membrane. The control values (isolated with the control KEA solution) of mitochondrial calcium binding, calcium uptake and ATPase activities were 54.3 ± 4.2 nmoles Ca^{2+}/mg protein /5 min, 142.3 ± 10.2 nmoles Ca^{2+}/mg protein/5 min

Table 1. Oxidative phosphorylation activities of mitochondria isolated with the oxygen-enriched solution (oxygen) and the control solution (control).

	RCI	ADP/O	QO_3	QO_4	OPR
Succinate					
control	2.94 ± 0.09	1.83 ± 0.06	160 ± 16	76 ± 6	249 ± 32
oxygen	3.35 ± 0.15*	1.83 ± 0.06	223 ± 18*	98 ± 6*	324 ± 34*
Glutamate					
control	11.4 ± 1.4	2.75 ± 0.05	137 ± 18	14 ± 1	383 ± 54
oxygen	11.9 ± 0.3	2.85 ± 0.03	158 ± 8	15 ± 1	451 ± 24

RCI : Respiratory control index (ratio of oxygen consumed in the presence of ADP to that after phosphorylation of ADP).

ADP/O : ADP/O ratio (nmoles ADP/natom oxygen consumed).

QO_3 : Oxygen uptake at state 3 (natoms oxygen/mg protein/min).

QO_4 : Oxygen uptake at state 4 (natoms oxygen/mg protein/min).

OPR : Oxidative phosphorylation rate (nmoles ATP produced during state 3/mg protein/min).

Each value represents a mean ± S. E. M. of 8 experiments.

* Significantly different from the control value ($p<0.05$).

and 345 ± 21 nmoles Pi/mg protein/min (n=5), respectively. Mitochondria isolated with the oxygen-enriched solution showed the similar values to those of the control; that is, calcium binding, calcium uptake and ATPase activities of the mitochondria isolated with the oxygen-enriched solution were 50.0 ± 5.0 nmoles Ca^{2+}/mg protein/5 min, 145 ± 12 nmoles Ca^{2+}/mg protein/5 min and 326 ± 15 nmoles Pi/mg protein/min, respectively (n=5).

Electron microscopic examination of mitochondria isolated with the oxygen-enriched solution and with the control solution was carried out (Table 2, and Figure 1A and 1B). Assessment of size (long axis) of isolated mitochondria was performed using, at least, six different samples derived from six rabbit hearts. Four electron microscopic pictures were taken from one sample, and their mean value was expressed as one experimental datum. Numbers in total mitochondria, whose sizes are bigger than 0.25 μm, were 136 - 167 per 320 μm^2 (control group : 153 ± 4, oxygen-enriched group : 157 ± 5 per 320 μm^2, n=6), indicating no significant difference in numbers of mitochondria between the two groups. With respect to sizes in mitochondria, 1.5 - 0.5 μm of mitochondria were mostly seen in the fraction which was essentially similar to the results of Anand et al. (12), and their shapes were rounder than those of mitochondria of the intact myocardial cells (13). The difference may be due to a direct contact of isolated mitochondria with several solutions (because of a lack of the cell membrane) for dehydration and fixation of specimen during the preparation procedure. In the fraction of the oxygen-enriched group, there was much population in small sizes of mitochondria in comparison with that in the control

Table 2. Electron microscopic examination on sizes of mitochondria, A) isolated with the oxygen-enriched solution and the control solution, and B) isolated after the treatment with or without oxygen-bubbling.

	% of total numbers	
	1 μm	0.25 – 1.0 μm
A : control	22.7 ± 1.0	77.3 ± 1.0
oxygen-enriched	13.1 ± 1.0*	86.9 ± 1.0*
B : control	20.2 ± 1.6	79.8 ± 1.6
oxygen-enriched	22.1 ± 1.5	77.9 ± 1.5

Numbers of total mitochondria(> 0.25 μm) were 136 to 167 / 320 μm².
Each value represents a mean ± S. E. M. of 6 experiments.
* Significantly different from the control value ($p < 0.05$).

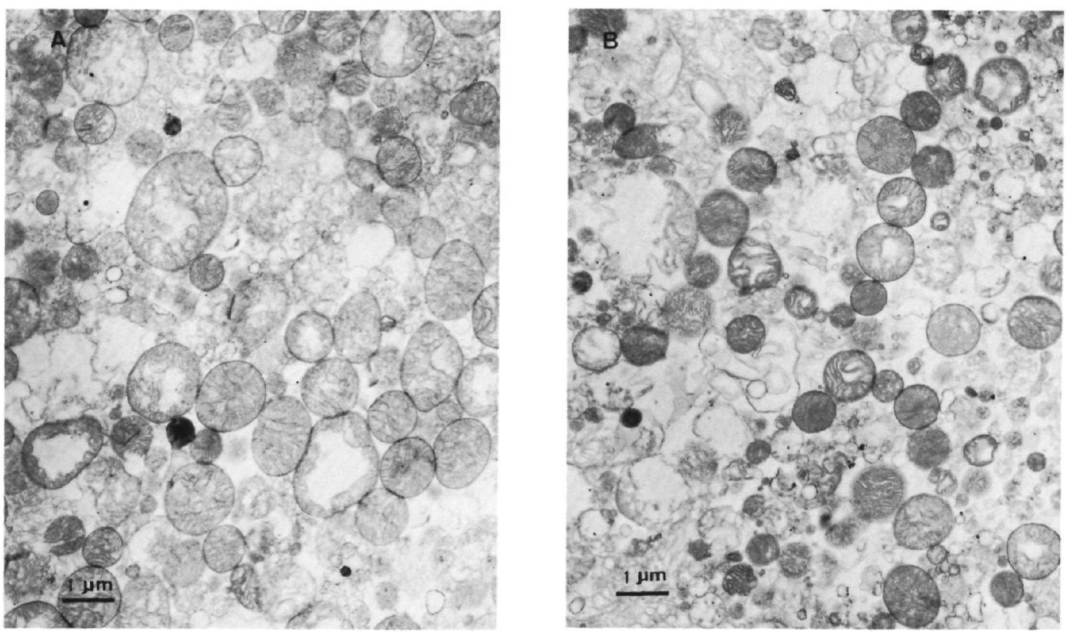

Fig 1. Ultrastructural appearance of heart mitochondria isolated with the control medium (A) and with the oxygen-enriched medium (B). Calibration = 1 μm.

mitochondrial fraction, as was seen in Figure 1A and 1B. Table 2 shows ratios of population in sizes of mitochondria between the two groups. It should also be mentioned that about 12 - 15% of large sizes of mitochondria (> 1 μm) were found to be broken or vacuolated in their morphological structure.

In order to ascertain whether or not, oxygen bubbling into a solution containing crude pellets of mitochondrial fraction, which was obtained upon the first centrifugation at 10,000 g for 20 min, is beneficial, measurements of oxidative phosphorylation activity of mitochondria isolated with and without treatment of gentle oxygen-bubbling for 5 min and following by subsequent washing and centrifugation, was performed. RCI, ADP/O, $Q0_3$, $Q0_4$ and OPR of mitochondria with treatment of oxygen-bubbling were 91 ± 10, 101± 2, 90 ± 3, 122 ± 20 and 98 ± 7 % (n=4) of each control value, respectively. Furthermore, in electron microscopic study on mitochondria thus isolated, there was no appreciable difference in both total numbers and numbers in large sizes of mitochondria (> 1 μm) between the fraction isolated upon treatment of oxygen bubbling and the control fraction (Table 2). By contrast, treatment of isolated mitochondria with oxygen-bubbling for 10 min resulted in a reduction of the oxidative phosphorylation activity (21 - 36 %).

Discussion

The present experiment has shown that oxidative phosphorylation activities, such as RCI, $Q0_3$ and OPR of mitochondria isolated with the oxygen-enriched solution were higher than those isolated with the control solution when succinate was employed as a substrate, whereas there was no appreciable difference in the oxidative phosphorylation activities between mitochondria isolated with the oxygen-enriched solution and those with the control solution, when glutamate was employed as a substrate. Different sensitivities of mitochondrial oxidative phosphorylation activity due to difference in substrate employed were also seen in the mitochondria derived from the ischemic heart (14). Calcium binding and uptake as well as ATPase activities of mitochondria isolated with the oxygen-enriched solution were not different from those with the control solution. The results imply that slight, but significant difference in mitochondrial activity was elicited by the treatment of mitochondrial fraction with the oxygen-enriched solution during isolation procedure. Furthermore, electron microscopic study showed significant difference in sizes of mitochondria isolated between the two solutions. These results suggest two possibilities as a role of oxygen-enriched solution during isolation procedure. At first, an usage of oxygen-enriched solution during isolation of mitochondria might protect the intact mitochondria against their aging or disruption which may occur during isolation procedure. Secondly, oxygen-enriched solution or oxygen-bubbling into the isolation medium even for a short period (about 30 sec) might disrupt old, swollen mitochondria contaminated in the fraction, and eliminate ruptured mitochondria through the subsequent procedure. To

examine the latter possibility, crude mitochondrial fraction was further bubbled with oxygen for 5 min, and the oxidative phosphorylation activities of mitochondria thus isolated were measured. It was found that there were neither appreciable changes in the biochemical activity and the ultrastructure of mitochondria receiving oxygen-bubbling treatment. We rather observed that the treatment of isolated mitochondria with oxygen-bubbling for 10 min aggravated the mitochondrial phosphorylation activity, indicating that a direct contact of mitochondria with excess oxygen seems to be harmful for their function.

It is considered that mitochondrial function is subjected to be influenced by several enzymes located in or on the subcellular organelles in the cell. For example, lysosomal enzymes, which are generally believed to function in a degradation of cellular constituents such as glycoproteins and phospholipids and, accordingly, to deteriolate enzyme activities of the subcellular membranes (15). Beneficial effects of the usage of oxygen-enriched solution may be probably due to a protection of intact mitochondria against their aging or damage during isolation procedure.

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