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Oxidative metabolism of Polytron versus Nagarse mitochondria in hearts of genetically diabetic mice

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We have shown previously that heart mitochondria obtained by the Nagarse method from genetically diabetic mice (C57BL/KsJ db/db) exhibit a defect in oxidizing NAD^+-linked substrates (Kuo, T.H., Moore, K.H., Giacomelli, F. and Wiener, J. (1983) Diabetes 32, 781–787). In this study, the oxidative phosphorylation characteristics of cardiac mitochondria isolated by the Polytron method were compared with that of Nagarse mitochondria. Evidence is presented here that in the diabetic heart both Nagarse and Polytron mitochondria have defective pyruvate oxidation, whereas only the former exhibit impaired fatty acid oxidation. Assay of two rate-limiting β-oxidation enzymes, namely β-hydroxyacyl-CoA dehydrogenase and β-ketothiolase, indicates no alteration in specific activities from diabetic mice vs. controls. The data suggest that two populations of mitochondria are present in myocardium and that the defective oxidative metabolism in the cardiac mitochondria of db/db mice may be linked to deficiencies in total NAD + NADH content.

Introduction

The cardiomyopathy of the genetically diabetic mouse (C57BL/KsJ db/db) is characterized by abnormal accumulation of lipid droplets and progressive degeneration of mitochondria in the cardiac muscle cells [1]. Analysis of the tissue levels of β-hydroxy fatty acids shows a 2–3-fold increase in diabetic hearts versus control hearts as early as 7 weeks of age [2]. Oxidative phosphorylation studies using isolated cardiac mitochondria also indicate impairment in β-oxidation and pyruvate oxidation in diabetic hearts. It was suggested that the defective oxidative metabolism may be due to a deficiency in mitochondrial NAD and NADH content in diabetic hearts or alternatively might be related to defects in the enzymes of the β-oxidation pathway [2]. In this regard, it has been suggested that fatty-acid oxidation in heart may be controlled via the regulation of β-hydroxyacyl-CoA dehydrogenase and β-ketothiolase activities [3]. The present study was therefore designed to determine whether the decreased fatty-acid oxidation in genetically diabetic hearts was due to altered activities of these two rate-limiting enzymes of the β-oxidation pathway.

The existence of two populations of mitochondria (subsarcolemmal and interfibrillar) in cardiac muscle has been reported [4]. Subsarcolemmal mitochondria are preferentially obtained by the Polytron method whereas the interfibrillar mitochondria are only released after Nagarse treatment. Since our earlier study on the defective oxidative metabolism in db/db mice was carried out using mitochondria obtained by the Nagarse method [2], an additional aspect of this study was to compare the properties of Polytron vs. Nagarse prepared mitochondria in control and diabetic mice. Evidence is presented here that in the diabetic heart both Nagarse and Polytron

mitochondria have defective pyruvate oxidation, whereas only the former exhibit impaired fatty-acid oxidation.

Materials and Methods

Animals. Genetically diabetic mice (C57BL/KsJ db/db) and lean littermate controls were obtained from Jackson laboratories, Bar Harbor, ME. Mice were studied between 8 and 14 weeks of age, the period that corresponds to the 'active stage' of cardiomyopathy in db/db mice [2].

Preparation of mitochondria. Polytron mitochondria were isolated using the procedure of Sordahl [5]. Hearts from four mice were pooled, rinsed to remove blood, and weighed. The hearts were cut into halves, placed in 12 volumes of KEA medium (0.18 M KCl/10 mM EDTA/0.5% bovine serum albumin, pH 7.2–7.4) and homogenized with a Polytron (Brinkmann Instruments) for 3–4 s with a rheostat set at 2. One quick pass through the homogenate with a motor-driven Teflon pestle was then made to ensure complete cellular disruption. The homogenate was centrifuged at $1200 \times g$ for 10 min and the supernatant strained through several layers of cheese cloth and centrifuged at $10000 \times g$ for 10 min. The resulting mitochondrial pellet was resuspended in KEA medium and centrifuged at $10000 \times g$ for 10 min. This 'washing' procedure was repeated once. The final mitochondria were resuspended in KEA medium to yield approx. 20 mg protein per ml. Protein concentration was then determined by the biuret method [6]. Heart mitochondria prepared in KEA medium by this method have better oxidative phosphorylation values compared to those isolated in the sucrose-Tris-EDTA medium by the conventional method [5].

Nagarse mitochondria were isolated by the method of Mela and Seitz [7] as we described previously [2], except that 0.5% bovine serum albumin was included in the isolation medium.

Oxidative phosphorylation. Using a Clark oxygen electrode, oxygen consumption was monitored in 1.5 ml of medium at 30°C. The medium contained 0.25 M sucrose/10 mM Tris-HCl/10 mM potassium phosphate (pH 7.4) for assay of Polytron mitochondria, or 0.225 M mannitol/0.07 M sucrose/1 mM EDTA/10 mM potassium phosphate (pH 7.5) for assay of Nagarse mitochondria. The concentration of different substrates were as follows: 5 mM pyruvate + 2.5 mM malate, 50 μM palmitylcarnitine + 2.5 mM malate; or 5 mM succinate + 2.5 μM rotenone. Approx. 1 mg mitochondrial protein was used in each assay. State-3 (ADP-stimulated) respiration was initiated by addition of ADP (273 μM) and was followed by state-4 (ADP-limiting) respiration. Respiratory control ratios and ADP/O ratios were calculated as described previously by Chance and Williams [8].

Enzyme assays. Assays for β-oxidation enzymes were performed at 25°C according to Fong and Schulz [3]. Briefly, β-hydroxy acyl-CoA dehydrogenase activity was determined spectrophotometrically at 340 nm by the oxidation of NADH. The assay mixture (1 ml) contained 0.05 M KP_i (pH 7), 0.06% Triton X-100, 0.12 mM NADH, 2.7 μM bovine serum albumin, and approx. 2–10 μg of mitochondrial protein. The reaction was started by the addition of acetoacetyl-CoA to a final concentration of 30 μM. β-Ketothiolase was determined by following spectrophotometrically the disappearance of the Mg^{2+}-enolate complex at 303 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 8), 25 mM $MgCl_2$, 30 mM KCl, 0.06% Triton X-100, 7 μM bovine serum albumin and 30 μM acetoacetyl-CoA. The reaction was started by the addition of CoASH to a final concentration of 0.13 mM. Molar extinction coefficient of 18000 was used to calculate the rate determined with acetoacetyl-CoA.

Electron Microscopy. Mitochondria pellets were fixed in 2.5% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C and processed as previously described [2].

Statistical analysis. Data are expressed as mean ± S.D. The Student's *t*-test for unpaired samples was used and significance defined as $P < 0.05$.

Results

Comparison of Polytron mitochondria vs. Nagarse mitochondria

It was previously shown that the cardiomyopathy in genetically diabetic mice begins to appear at approx. 5 weeks of age [1]. Thus the animals (control and diabetic) chosen for this study were

between 8 and 14 weeks of age, corresponding to the 'active state' of cardiomyopathy. The yield of mitochondrial protein per g of heart by the polytron method was 9.9 ± 2.1 mg for control animals ($n = 14$) and 13.5 ± 3.1 mg for diabetic mice ($n = 16$). In contrast, the yield of mitochondrial protein per g of heart of the Nagarse method was 24.4 ± 4.2 mg for controls ($n = 18$) and 31.2 ± 6.0 mg for diabetic animals ($n = 17$). Thus it appears that the diabetic hearts consistently give higher mitochondrial yield than controls.

* Since the method for isolating Polytron mitochondria was similar to that used by Palmer [4] to obtain the subsarcolemmal mitochondria, these can be considered as an enriched subsarcolemmal mitochondrial population. However, the Nagarse mitochondria prepared in this study may represent a mixture of both subsarcolemmal and interfibrillar subpopulations. Due to limitations imposed by the small amounts of cardiac tissue, attempts were not made to use the two-step isolation technique by Palmer [4] for obtaining the 'clean' interfibrillar mitochondria. In the assumption that Polytron mitochondria represent mostly the subsarcolemmal population whereas Nagarse mitochondria represent both subsarcolemmal and interfibrillar populations, a comparison of protein yield of the two gives the estimation that Nagarse mitochondria contain roughly 40% subsarcolemmal mitochondria and 60% interfibrillar populations.

Examination by electron microscopy indicate that Polytron and Nagarse mitochondria are homogeneous and devoid of any major contamination (Fig. 1). The parameters of oxidative phosphorylation for Polytron and Nagarse mitochondria (Table I) using pyruvate plus malate, palmitylcarnitine plus malate, and succinate as substrates show that both preparations from the control hearts are well coupled. In addition, the Nagarse mitochondria oxidize all three substrates faster than Polytron mitochondria, in agreement with earlier findings of Palmer et al. [4]. Comparison of the diabetic mice with controls (Table I) also indicates that Nagarse mitochondria from the diabetic hearts have a decreased capacity to utilize NAD^+-linked substrates. Both the state-3 oxidation rates for pyruvate plus malate and palmitylcarnitine plus malate are depressed by 56 and 26%,

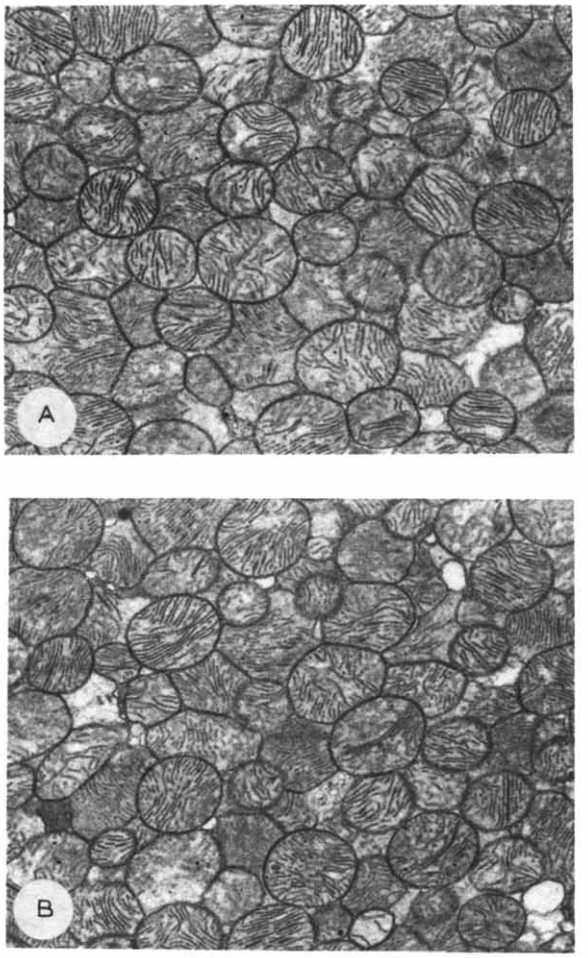

Fig. 1. Electron micrographs of Polytron mitochondria obtained from 10-week-old control (A) and diabetic (B) mice. The majority of mitochondria appear intact and free of other cytoplasmic components. No differences are discernible between control (A) and diabetic (B) mitochondria. Magnification, ×11 000.

respectively, while that for succinate is not significantly different from controls. These data confirm our earlier report [2] using Nagarse mitochondria prepared in the absence of bovine serum albumin, where the decreases in state-3 rates were 30% for pyruvate plus malate, and 24% for palmitylcarnitine plus malate. Thus the results indicate that oxidative phosphorylation characteristics of mouse heart mitochondria prepared by Nagarse with or without bovine serum albumin are similar.

The findings obtained with Polytron mitochon-

TABLE I

OXIDATIVE PHOSPHORYLATION IN POLYTRON AND NAGARSE PREPARED MITOCHONDRIA

The Polytron and Nagarse mitochondria were prepared as described in the text. Rates of oxygen consumption are expressed as natom O/min/mg of mitochondrial protein (mean ± S.D.) from 8–10 mitochondrial preparations.

Substrate	Mice	Polytron mitochondria				Nagarse mitochondria			
		State 3 [a]	State 4	RCI	ADP/O	State 3	State 4	RCI	ADP/O
Pyruvate + malate	control	113 ± 22	23 ± 6	5.1 ± 1.0	2.9 ± 0.3	154 ± 34	43 ± 11	3.6 ± 0.4	2.7 ± 0.5
	diabetic	64 ± 15 [b]	15 ± 4 [b]	4.4 ± 1.5	2.9 ± 0.3	68 ± 15 [b]	29 ± 8	2.4 ± 0.2 [b]	2.7 ± 0.5
Palmityl carnitine + malate	control	164 ± 35	43 ± 13	3.9 ± 0.5	2.3 ± 0.2	318 ± 48	74 ± 12	4.4 ± 0.6	2.5 ± 0.2
	diabetic	157 ± 34	36 ± 10	4.4 ± 0.6	2.3 ± 0.3	236 ± 41 [b]	65 ± 17	3.4 ± 0.5 [b]	2.5 ± 0.3
Succinate	control	177 ± 36	55 ± 17	3.6 ± 1.8	1.7 ± 0.2	344 ± 25	141 ± 22	2.4 ± 0.2	1.6 ± 0.3
	diabetic	157 ± 27	55 ± 16	3.1 ± 1.0	1.8 ± 0.3	309 ± 70	139 ± 36	2.3 ± 0.2	1.4 ± 0.2

[a] State 3 represents oxidation in the presence of 273 μM ADP and state 4 that in the absence of ADP.
[b] Indicates significant difference from control values ($p < 0.05$).

dria of the diabetic mice were unexpected. Table I shows that while the diabetic mitochondria remain defective with respect to pyruvate oxidation (state-3 rate is depressed 43% relative to controls, $p < 0.005$), they are functionally normal with regard to palmitylcarnitine oxidation (compare state-3 rate at 157 ± 34 for diabetic mitochondria vs. 164 ± 35 natom O/min/mg for controls, $p < 0.2$).

The effect of Nagarse treatment on Polytron mitochondria

Preliminary experiments from this laboratory indicated that mouse cardiac mitochondria prepared by either Polytron or Nagarse methods contain proteolytic activities capable of hydrolyzing [^{3}H]acetylcasein at pH 8.0. It should be noted, however, that the amount of proteolytic activity at pH 8.0 in Nagarse mitochondria is 10-fold higher than that in Polytron mitochondria (Kuo, T.H. et al., unpublished data). It was found that this excess amount of proteolytic activity was attributable to the residual nagarse contamination that persists even after three washings of the mitochondria preparation. It has also been shown that nagarse may cause the loss of long-chain fatty-acid activating enzyme(s) from the isolated mitochondria [9].

TABLE II

OXIDATIVE PHOSPHORYLATION IN POLYTRON MITOCHONDRIA TREATED WITH NAGARSE

The Polytron mitochondria were first isolated and then nagarse (3 mg/g heart) added to the mitochondrial suspension during the washing step. The mitochondrial suspension was then centrifuged and washed twice as described in Materials and Methods. Rates of oxygen consumption are expressed as natom O/min/mg (mean ± S.D.) from 10 mitochondrial preparations.

Substrate	Mice	State 3 [a]	State 4	RCI	ADP/O
Pyruvate + malate	control	104 ± 25	21 ± 3	5.2 ± 1.5	3.1 ± 0.5
	diabetic	68 ± 10 [b]	15 ± 4 [b]	4.6 ± 1.0	3.1 ± 0.6
Palmityl carnitine + malate	control	174 ± 23	39 ± 5	4.6 ± 1.0	2.5 ± 0.4
	diabetic	160 ± 21	30 ± 5	5.4 ± 1.1	2.4 ± 0.3
Succinate	control	140 ± 24	50 ± 13	2.9 ± 0.7	2.2 ± 0.3
	diabetic	132 ± 32	49 ± 12	2.8 ± 0.6	2.3 ± 0.3

[a] State 3 represents oxidation in the presence of 273 μM ADP and state 4 that in the absence of ADP.
[b] Indicates significant difference from control values ($p < 0.05$).

For these reasons, experiments were carried out to exclude the possibility that the depressed palmitylcarnitine oxidation observed in Nagarse mitochondria from hearts of diabetic mice was due to artifacts induced by nagarse treatment. Table II shows that firstly, in control mice, the nagarse treatment does not increase the rates of oxidation of the Polytron mitochondria for substrates tested to those rates found in the Nagarse mitochondria (Tables II and I). Secondly, in diabetic mice, the same defect toward pyruvate but not palmitylcarnitine utilization is observed in the Polytron mitochondria with or without nagarse treatment (Tables II and I).

Activities of β-oxidation enzymes in isolated heart mitochondria

Our previous study [2] suggested that the depressed fatty acid and pyruvate oxidation occuring in Nagarse mitochondria from diabetic hearts can be explained by the deficiencies in total NAD + NADH content. However, other abnormalities such as altered specific activities for the β-oxidation enzymes or pyruvate dehydrogenase can also be contributing factors. For this reason, the assay of the two rate-limiting enzymes in β-oxidation [3] was carried out. Table III indicates that the specific activities of the β-hydroxyacyl-CoA dehydrogenase and β-ketothiolase in mitochondria from diabetic animals prepared by either Polytron or Nagarse methods were not statistically different from the controls. Kinetic parameters for the dehydrogenase were studied also by measuring dehydrogenase activity as a function of NADH concentration (in the range 0.05–0.25 mM) at fixed concentration of acetoacetyl CoA and also as a function of acetoacetyl CoA concentration (in the range of 0.02–0.16 mM) at fixed NADH concentration. Analysis of these parameters showed

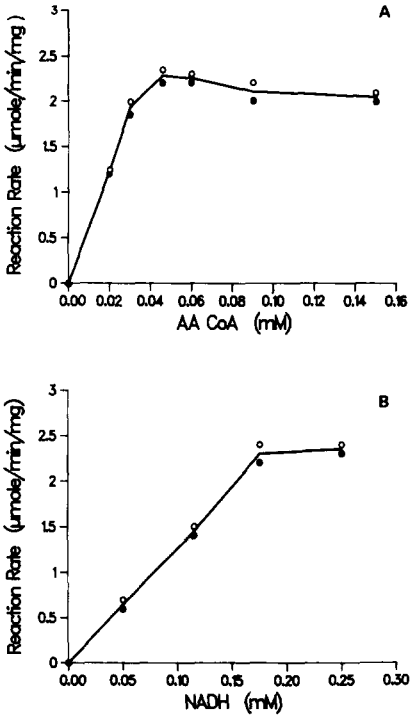

Fig. 2. β-Hydroxyacyl-CoA dehydrogenase rate as a function of acetoacetyl-CoA (A) or NADH concentration (B). The initial rate of reaction were measured at 25°C using 2 μg of mitochondrial protein in 0.8 ml assay medium containing 0.05 M KP_i (pH 7), 0.06% Triton X-100, 2.7 μM bovine serum albumin and variable amounts (as indicated) of AACoA and NADH. The reaction was started by the addition of AACoA (see Materials and Methods) and the initial rates are expressed as μmol NADH oxidized/min/mg protein. ○, diabetic; ●, control.

TABLE III

SPECIFIC ACTIVITIES OF β-OXIDATION ENZYMES IN ISOLATED MITOCHONDRIA

Polytron and Nagarse mitochondria were prepared and β-oxidation enzymes assayed as described in Materials and Methods. Numbers in parentheses indicate the number of mitochondrial preparations.

Assay	Mice	Polytron mitochondria (μmol/min/mg protein)	Nagarse mitochondria (μmol/min/mg protein)
β-hydroxy acyl-CoA dehydrogenase	control	3.03 ± 0.33 (5)	3.05 ± 0.49 (10)
	diabetic	3.19 ± 0.48 (5)	3.39 ± 0.46 (10)
β-Ketothiolase	control	0.17 ± 0.015 (4)	0.41 ± 0.035 (8)
	diabetic	0.18 ± 0.014 (4)	0.39 ± 0.033 (8)

no significant changes in V_{max} and K_m (Fig. 2) between control and diabetic mitochondria. Similarly, the kinetic parameters for the thiolase studied as a function of substrate concentration (data not shown) indicated no alteration in V_{max} and K_m for thiolase between diabetic and control mitochondria. It is also interesting to note that while the dehydrogenase activities are similar for both Polytron and Nagarse prepared mitochondria, the thiolase activities are significantly different; being at least 2-fold higher in Nagarse mitochondria as compared to Polytron mitochondria.

Discussion

Genetically diabetic (db/db) mice are characterized by defective oxidative metabolism in vitro, as demonstrated by impairment of fatty-acid oxidation [2]. This is in contrast to chemically induced diabetes where decreased pyruvate but increased fatty-acid oxidation have been observed [10]. The present study was carried out to further characterize this defective fatty acid oxidation in the cardiac mitochondria of db/db mice. It has been shown that this decrease in state-3 oxidation for palmitylcarnitine plus malate is age dependent and its occurrence coincides with the accumulation of β-hydroxy fatty acids in diabetic hearts [2]. In order to protect the mitochondria from the possible harmful effects of this excess fatty acid, we have included bovine serum albumin in the medium for the preparation of Nagarse mitochondria. The results obtained with Nagarse mitochondria prepared either in the presence (Table I) or absence [2] of bovine serum albumin are similar.

Table I shows that Polytron mitochondria from diabetic mice differ from the Nagarse mitochondria in that the former exhibit a defect only in pyruvate oxidation but not in palmitylcarnitine oxidation. A similar observation of differential response in disease by the subsarcolemmal and interfibrillar mitochondria has been reported in cardiomyopathic hamsters [11]. Treatment of Polytron mitochondria from diabetic hearts with Nagarse did not cause a decrease in palmitylcarnitine oxidation (Table II). This, together with the time-course study [2] showing that depressed palmitylcarnitine oxidation is only observed in Nagarse mitochondria of the diabetic mice after but not prior to 7 weeks of age, suggest that the defect is not an artifact of Nagarse treatment. Since the use of different assay media has been reported to be the basis for differential activities of subsarcolemmal and interfibrillar mitochondria [12], we have examined the oxidative phosphorylation parameters of Polytron and Nagarse mitochondria using the same isolation and assay media. The results once again demonstrated the occurrence of the same defect in pyruvate but not in palmitylcarnitine oxidation in the Polytron mitochondria from diabetic hearts. Thus the difference in the oxidative defect in Polytron vs. Nagarse mitochondria from the diabetic mice suggest that Polytron and Nagarse mitochondria represent two different subpopulations of heart mitochondria.

The studies on the specific activities of the two β-oxidation enzymes (Table III) indicate that the decreased oxidation of fatty acid in Nagarse mitochondria of diabetic hearts is not due to depressed enzymatic activities of β-oxidation. This is in contrast to the case of depressed pyruvate oxidation in diabetic mitochondria where evidence indicates that there is decreased activity in pyruvate dehydrogenase from the heart of db/db mouse (Kuo, T.H. et al., unpublished data). It has long been known that activity of the mammalian pyruvate dehydrogenase is regulated by a phosphorylation-dephosphorylation cycle [13,14]. In rat heart, increased conversion of the enzyme into the phosphorylated (inactive) form occurs in alloxan-diabetes [14]. We have also obtained evidence that the decreased pyruvate oxidation in the db/db mouse heart mitochondria may be controlled via a similar mechanism (Kuo, T.H. et al., unpublished data). While the defective pyruvate oxidation of diabetic mouse-heart mitochondria may be explained by the inactivation of pyruvate dehydrogenase through phosphorylation, there is no evidence that fatty-acid oxidation enzymes are regulated by the phosphorylation-dephosphorylation mechanism. On the other hand, it has been suggested that fatty-acid oxidation enzymes are regulated by substrate availability or product inhibition [15–17]. Thus the activity of the β-hydroxyacyl-CoA dehydrogenase may be controlled by the availability of cofactors such as NAD as determined by the NAD^+/NADH ratio [15]. Since no alteration in the enzyme activities of β-oxida-

tion has been detected from the Nagarse mitochondria of the db/db mice, it follows that the depressed palmitylcarnitine utilization and the accumulation of β-hydroxy fatty acids are not due to abnormalities of the enzyme but may be rather linked to the deficiencies in total NAD + NADH content in the heart mitochondria of db/db mice, as reported previously [2].

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