FATTY ACID METABOLISM IN DENERVATED MUSCLE OF FROG IN VITRO

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Summary

Observations on fatty acid metabolism in the denervated muscle of frog contrast both in respect to fat accumulation and fatty acid metabolism with the data obtained for dystrophic muscle elsewhere. Dystrophy leads to fat accumulation in the skeletal muscle. Denervation-atrophy in the gastrocnemius muscle of frog for one month resulted in the depletion of fat composition and fatty acid synthesis. Unlike dystrophic muscle the denervated muscle does not undergo reduction in mitochondrial protein, and shows an increase in palmitate and pyruvate oxidation. Although these changes are diametrically opposite, both the disorders viz. denervation-atrophy and dystrophy, are due to changes in mitochondrial activity.

Although denervation-atrophy and dystrophic phenomena have more common features with reference to histochemical changes (1, 2, 3) the mechanisms underlined in them seem to be totally different with reference to lipid metabolism (4). Dystrophy leads to fat accumulation in the skeletal muscle (4). The accumulation of fat in the dystrophic muscle has been postulated due to an impaired capacity of mitochondria to oxidize the fat (5). A study of fatty acid synthesis revealed a highly significant increase in the incorporation of acetate-2-¹⁴C into the fatty acids of dystrophic muscle as compared with controls (5). Fatty acid oxidation decreased in the dystrophic

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muscle (5). Denervation atrophy in frogs seems to be different (6). Denervation is known to deplete the muscle fat composition (7, 8) in frogs. No conclusive and clear reports on the changes in fat metabolism due to denervation are available even though the muscle fats contribute towards the energy metabolism (9). In view of the above findings the fatty acid synthesis and the mitochondrial oxidation of pyruvate and palmitate have been investigated in the denervated gastrocnemius muscle of frog.

Material and Methods

Denervation in the common South Indian Frog Rana hexadactyla was done as described by Krishnamoorthy and Das (10) by surgical deprivation of about 1 cm length of sciatic nerve root in the leg of the frog. Only one leg was denervated while the contralateral innervated (non-operated) leg served as control. The denervation was carried out for 30 days. The gastrocnemius muscle was chosen for the biochemical analysis. The muscles were rapidly removed, weighed and suspended in ice-cold medium consisting of 0.1 M KCl, 0.04 M tris, pH 7.4 and 0.004 M $MgCl_2$ (11, 12).

The homogenization medium contained 0.01 M EDTA in addition to this composition. EDTA was used to chelate the adhering Ca^{2+} ions to the mitochondria. The muscle was cut into small pieces and homogenized. The homogenization was first performed with a loose fitting glass homogenizer and then moderately tight apparatus. The resulting homogenate was centrifuged according to Winnick and Winnick (12) using an International refrigerated PR-Model-6 centrifuge to sediment the

mitochondria. The pellet was suspended in 0.2 M K_2PO_4 buffer, pH 7, for enzyme studies. The protein in the mitochondrial suspension was determined by the microbiuret method (13). The SDH-cytochrome-c-oxidase activity in the mitochondrial suspension was measured manometrically according to the method of Schneider and Potter as described by Umbreit *et al.* (14). The activity was measured at 25°C using cytochrome-c purchased from Biochemicals Unit, V.P. Chest Institute, New Delhi.

P:O ratios: The measurement of P:O ratios in isolated mitochondrial suspension was done in Warburg flasks at 25°C. For this experiment the mitochondrial pellets were suspended in 0.1 M KCl-tris buffer, pH 7.4. The reaction system was much similar to that of Maley and Lardy (24) containing hexokinase (purchased from Sigma Chemicals, St. Louis, Missouri) and glucose. In the reaction system the glucose and hexokinase are added from the side arm after 10 minutes to temperature equilibration and 'zero'-time flasks are immediately taken off, chilled and deproteinized with 0.2 ml of 10% trichloroacetic acid. The inorganic phosphate in the contents of control and sample flasks was determined by isobutanol extraction of phosphomolybdate (25).

Mitochondrial respiration rates were studied according to Umbreit *et al.* (14). The incubation mixture taken into the flask was much similar to that used by Lin *et al.* (5). Fatty acid synthesis in the muscle was studied using the method of Allman and Gibson (15). The amount of acetate-2- ^{14}C (obtained from Atomic Energy, Government of India) incorporation into the combined non-saponified and saponified fatty acids fraction was determined by the method of Fritz and Hsu (16) in a Packard

Tricarb Scintillation Counter Model-2002. The specific activity of isotope incorporation was examined as counts $\text{min}^{-1}\text{mg}^{-1}$ lipid. All chemicals used in the analysis were obtained from the Sigma Chemical Company. The statistical analysis of the data were done according to Croxton (17).

Results

One month denervation produced $28 \pm 7.5\%$ atrophy as judged by the loss of bulk or initial muscle mass.

The mitochondrial yield expressed per whole muscle did not change due to one month denervation (Table I).

The mitochondrial yield is known to vary in accordance with the isolation procedure (26). The validity of the present observation was further confirmed by measuring the specific activity of mitochondrial marker enzyme viz. SDH-cytochrome-c-oxidase which was not changed on denervation (Table I).

P/O ratios with pyruvate and malate as substrates increased on denervation. The results on palmitate and pyruvate oxidations (Table I) by muscle mitochondria of control and denervated muscles in the same frog showed a significant increase. A Kreb's cycle intermediate is required as shown in Table I. An increase in both palmitate and pyruvate oxidations in the muscle mitochondria of denervated frogs were observed using either malate or succinate.

A study of fatty acid synthesis revealed a highly significant decrease in the incorporation of acetate-2- ^{14}C into the fatty acids of denervated muscle as compared with the contralateral control (Table II). The results indicate that fatty acid synthesis is decreased in denervated muscle.

TABLE I

The oxidation of Pyruvate and Palmitate by mitochondria from the control and denervated gastrocnemius muscle of Frog (60 to 65 g) denervated for one month.

Substrate added	No. of experiments	Control muscles mean $\pm$ S.D.	Denervated muscles mean $\pm$ S.D.	Incidence of change on denervation
1. Mitochondria (mg. protein/whole muscle)	6	2.67 $\pm$ 0.21	2.86 $\pm$ 0.24	t = 1.460 p > 0.01 no change.
2. P/O ratio (Substrate: pyruvate + malate)	4	1.90 $\pm$ 0.26	2.70 $\pm$ 0.11	t = 5.665 p < 0.001 increase.
3. SDH-cytochrome-c-oxidase activity (μ l O ₂ /mg/hr)	4	2182 $\pm$ 152	2268 $\pm$ 182	t = 0.7256 p > 0.001 no change.
4. Pyruvate oxidation (μ l O ₂ /mg/hr)				
a) Malate	4	2120 $\pm$ 282	3648 $\pm$ 231	t = 8.336 p < 0.001 increase.
b) Succinate	4	1946 $\pm$ 216	3426 $\pm$ 161	t = 12.06 p < 0.001 increase.
5. Palmitate oxidation (μ l O ₂ /mg/hr)				
a) Malate	4	865 $\pm$ 76	1120 $\pm$ 46	t = 5.666 p < 0.001 increase
b) Succinate	4	598 $\pm$ 116	1349 $\pm$ 120	t = 8.303 p < 0.001 increase.

TABLE II

The in vitro synthesis of Fatty Acid from Acetate-2- ^{14}C by the homogenates of denervated and contralateral control gastrocnemius muscle of Frog (60 to 65 g) denervated for one month.

Number of experiments	Acetate-2- ^{14}C incorporation (n moles/mg protein $\pm$ S.E.)		Pair comparison: Incidence of change on denervation
	Denervated muscle	Control muscle	
5	3.81 $\pm$ 0.11	6.78 $\pm$ 0.19	t = 13.54 p < 0.001 decrease.

Discussion

Recent biochemical studies of mitochondria in dystrophic muscle have revealed metabolic defects that are typically a major factor in the disease process. Ernster and Luft (19) and Van Wijngaarden et al. (20) described recently a type of skeletal muscle disease, similar to dystrophy in which the mitochondria showed a loosely coupled state of oxidative phosphorylation. Coleman et al. (21) have also described a type of myopathy with mitochondrial enzyme hyperactivity. They found an abnormal accumulation of fat in dystrophy and postulated that it is due to an impaired capacity of mitochondria to oxidize fat. An abnormal increase of lipid during progressive dystrophy is well known and it is probable that mitochondria have an important role in its accumulation. Lin et al. (5) examined the fatty acid oxidation in muscle homogenate and also of pyruvic and fatty acid oxidation in muscle mitochondria of dystrophic mice to determine whether a defect exists. The evidence obtained from them indicates that two types of defect

may contribute to an accumulation of fat in the skeletal muscle of dystrophic mice. Both the impaired capacity of mitochondria to oxidize pyruvic and fatty acid, and an increased synthesis of fatty acid exist in the dystrophic muscle. Further, they have pointed out that there is a marked reduction of mitochondrial protein in dystrophic muscle. Because of the reduced oxidative activity per unit weight of mitochondrial protein, they argued that it may be probable that some mitochondria are functioning poorly. They have also pointed out that the increase in fatty acid synthesis is probably secondary to the disorder in mitochondria and indicates either an enhanced production of fatty acid synthetase or modification in the activity of this enzyme.

The results on the mitochondrial oxidations (Table I) of the present study are very interesting in view of the results obtained by Lin et al. (5). No reduction in mitochondrial protein per whole muscle was noticed in the present study. The P/O ratio is increased (Table I). Pyruvate and palmitate oxidations by the mitochondria are significantly increased. These results corroborate the data on increased fat hydrolysis and increased fatty acid degradation (8). It can be envisaged from these results that the denervation also involves the mitochondrial activity changes but the nature of change is totally different from that in dystrophic mice (5).

Unlike dystrophic muscle (22), the denervated muscle does not undergo reduction in mitochondrial number, but the latter are relatively more active in phosphorylating mechanism and oxidizes fat and pyruvate more than synthesizing of fat.

It should be emphasized in view of these findings that

the denervation and dystrophy are different disorders as far as the fat metabolism is concerned. Both the disorders exhibit changes in mitochondrial activity but the changes are diametrically opposite. The activity towards fatty acid oxidation determines the atrophic nature of the muscle.

The determining factor is likely to be found in the trophic influence of the nerve either through a primary response like change in the permeability properties or secondary response like change in the mitochondrial activity, since denervation caused change in the permeability of the membrane (23) and in the mitochondrial activity.

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