INCREASED FAT DEGRADATION IN THE DENERVATED MUSCLE OF FROG

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Summary: The total lipids triglycerides, free fatty acids, glycerol and acetoacetate levels in the gastrocnemius muscle of frog denervated for 1 month were compared. A significant atrophy without any change in total DNA content per whole muscle was noted on denervation. No significant change in the mitochondrial protein content in the atrophied muscle was noted. Relative to total muscle mass, the total fat, triglyceride, free fatty acids and glycerols decreased on denervation. This suggests the increased degradation of fat during denervation. Concomittantly lypolytic and esterase activities in the atrophied muscle increased. Palmitate and pyruvate oxidations in the mitochondria of atrophied muscle and also the enzymes of β-oxidative scheme increased. ^{14}C-acetate incorporation rates revealed that there is a decrease in fatty acid synthesis in the atrophied muscle. It was suggested that the increased fatty acid oxidation and decreased fatty acid synthesis in the atrophic process may not be the result of a simple functional demand; but may involve more factors in terms of neuro-muscular functions.

Key words: denervation-atrophy, muscle mitochondrial β-oxidation, pyruvate and palmitate oxidations, increased fat degradation, ^{14}C-acetate incorporation

INTRODUCTION

Denervation of frog gastrocnemius muscle disturbs the fat metabolism (28-31), in contrast to vertebrate dystrophic muscles (21). Dystrophy leads to fat accumulation (21) but atrophy alters the levels of fat metabolic intermediates (29) in vertebrate skeletal muscle. It is also reported (28) that there is augmentation of the neutral fat hydrolysis during the atrophic process and consequently, the glycerol content of the muscle increases. The concentration of free fatty acids in the atropic muscles is significantly lower than in controls (28). In order to examine whether such decrease in free fatty acids in the denervated muscle could be related to an enhanced rate of fatty acid oxidation during the atrophic process, the present study was initiated.

MATERIALS AND METHODS

Denervation in the common south Indian frog *Rana hexadactyla* was done as described by Krishnamoorthy and Das (23) by surgical deprivation of about 1 cm. length of sciatic nerve root. Only one leg was denervated while the contralateral innervated (non-operated) leg served as control. The gastrocnemius muscle was chosen for the experiments.

Atrophy: The control and denervated gastrocnemii were excised and weighed. The denervated muscle lost about 24% of its initial mass 1 month after denervation. This loss is referred as atrophy.

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Water content: The percentage of water content was calculated from the dry (110°C) and wet weights of the muscle.

DNA estimations: The muscle was weighed and homogenized in 5 *ml* ice-cold distilled water and treated with 5% PCA (perchloric acid) in order to precipitate the macromolecules. DNA was separated from the lipids by washing the precipitate with alcohol, chloroform and ether but not from protein and RNA. A fluorometric method based on the reaction of deoxyribose with diaminobenzoic acid in a strong acidic medium (10% PCA) was used for determinations of DNA (22). Measurements were made with SPECOL colorimeter equipped with fluorimeter attachment. Yeast DNA was used as standard.

Protein estimations: Proteins in the homogenates as well as mitochondrial suspensions was estimated by micro-biuret method (20). A Beckman DU_2 spectrophotometer was employed for the protein estimations. Bovine serum albumin was used as the standard protein. Determinations in crude homogenates was carried out with biuret-method (24).

Separation of the mitochondria: The muscles were rapidly removed, weighed and suspended in ice-cold medium consisting of 0.1M KCl, 0.04M Tris, pH 7.4, and 0.004M $MgCl_2$ (11, 34). The minced muscle was homogenized in medium containing 0.01M EDTA, and the mitochondria were separated at 14,000 g in an International refrigerated PR-Model centrifuge at 0°(11, 34).

The pyruvate and palmitate oxidation by the mitochondria were studied according to Umbreit *et al.* (33) in a Warburg apparatus (Precision Instruments, Chicago, Ill., U.S.A.). The incubation mixture taken into the flask was much similar to that used by Lin *et al.* (26).

Total lipid and lipid components: Total lipid in the muscle was extracted with 1:2 chloroform:methanol mixture and estimated by the method of Bligh and Dyer (3).

For estimations of triglycerides, the phospholipid in the total lipid fraction was first removed by employing the Zeolite method of Handel and Zilversmit (18). The saponification of the triglyceride and the determination of the glycerol were then made by the method of Carlson and Wadstrom (5). The muscle was homogenized in 10 *ml* of 1:2 chloroform: methanol mixture with 10 g anhydrous sodium sulfate and the supernatant was collected by centrifugation. The total free fatty acids in this were estimated by the method of Duncombe (8). The muscle was weighed and homogenized in 5% TCA (trichloroacetic acid) and centrifuged. The glycerol content of the supernatant was estimated colorimetrically by the method of Lambert and Neish, as modified by Korn (9). The acetoacetate in the above supernatant was estimated by the method of Lehninger (25).

The lipase activity was assayed in the aqueous extracts of the muscle according to Mahadevan *et al.* (27) using olive oil as substrate. The liberated fatty acids in the assay were determined by the method of Duncombe (8).

The esterase activity in muscle extracts was estimated titrimetrically using, methyl salicylate as substrate according to Cherry and Crandall (6).

Mitochondrial β-oxidative enzymes: The acyl-dehydrogenase activity of the mitochondrial suspensions estimated according to the method of Bainert (1) using thioethylamine as substrate.

Acetoacetate-thiokinase activity was measured according to the method described by Berg (2).

Crotonase activity was assayed according to Stern (32). Glycerol dehydrogenase activity was estimated according to Burton (4).

^{14}C-Acetate incorporation into muscle fats: The acetate-1-^{14}C was injected (50 μCi in 1 *ml*) into the dorsal lymphatic duct of the frog. After 3 hours the animal was sacrificed and the lipids and fatty acids were extracted according to the methods described in earlier paragraphs. The extracted lipid was evaporated to dryness under vacuum and was dissolved in hyamine hydroxide solution diluted to 5 *ml* with methanol. One *ml* of this was further diluted with 10 *ml* scintillation solution (200 *g* naphthalene, 10 *g* POP, 0.25 *g* POPOP in a liter of dioxane - BDH) for radioactivity measurement in glass vials. A Packard Tricarb scintillation counter Model 2002 was employed for counting the radioactivity. The specific activity of radioactive incorporation was expressed as counts min^{-1} mg^{-1} lipid.

Reagents and chemicals: The lipid solvents used in this study were purchased from BDH Glaxo Laboratories (India) Ltd. The substrates for enzyme studies were obtained from Sigma Chemical Co., St. Louis, Missouri and BDH Glaxo Laboratories (India) Ltd.

Statistical analysis: The statistical analyses of the data was done by employing the methods described by Croxton (7).

RESULTS

A significant atrophy (i.e. loss of muscle mass) is noticed in the denervated muscle (Table I). When the DNA content is expressed per whole muscle mass, no significant change in the former is noticed after denervation. Similarly the mitochondrial protein content does not exhibit a significant change on denervation, when expressed per whole muscle mass. When expressed to unit wet weight, the protein content does not vary significantly but when considered to whole muscle mass it decreases after denervation. All these results (Table I) demonstrate that the muscle mass is lost due to denervation which involves the reduction in the fibre volume but not the number of muscle fibres.

When expressed per whole muscle mass, the denervated muscle undergoes significant reduction in the levels of total lipids, triglycerides, glycerols and free fatty acids (Table II). Acetoacetate level per whole muscle mass also exhibits a significant decrease (Table II).

TABLE I: Changes in the composition of gastrocnemius muscle of frog denervated for 1 month.

	No. of observations	Control muscle	Denervated muscle	Incidence of change on denervation
1. Atrophy (% initial mass)	16	—	24±1.5	—
2. Water content (% wet weight)	6	72±1.9	71±2.2	t = 0.8273 P > 0.001 no change.
3. DNA (μg/whole muscle)	16	175±6	176±5	t = 0.5143 p > 0.001 no change.
4. Total protein (*mg*/*g* wet weight)	8	282±28	236±31	t = 3.129 p < 0.001 decrease.
5. Mitochondrial protein (*mg*/whole muscle)	6	2.67±0.24	2.86±0.21	t = 1.463 p > 0.001 no change

Values are mean±S.D.

TABLE II: The levels of total fat, triglyceride, free fatty acids, glycerol and acetoacetate in the gastrocnemii of frog denervated for 1 month.

mg or μg per whole muscle mass	*No. of observations*	*Control muscle*	*Denervated muscle*	*Incidence of change on denervation*
1. Total lipid (*mg*)	16	115±16	35±4	t = 19.4 p < 0.001 decrease
2. Triglycerides (*mg*)	12	171±38	32±5	t = 14.5 p < 0.001 decrease
3. Free fatty acids (μg)	14	117±14	23±8	t = 21.56 p < 0.001 decrease
4. Glycerol (μg)	24	273±45	208±9	t = 6.97 p < 0.001 decrease
Acetoacetate (*μmol*)	18	529±119	640±18	t = 10.3 p < 0.001 decrease

Values are mean ± S.D.

The lipase and esterase activities of the muscle show significant increase on denervation (Table III).

TABLE III: Changes in the lipolytic activity in the gastrocnemius muscle of frog on denervation (60 to 65 *g* frogs denervated for 30 days are used for assay).

Enzymes	*Number of observations*	*micro Eq acids/mg protein/hr*		
		Control muscle	*Denervated muscle*	*Incidence of change on denervation*
1. Lipase	11	326±102	716±86	t = 30.68 p < 0.001 increase
2. Esterase	8	116±27	226±34	t = 7.101 p < 0.001 increase

Values are mean ± S.D.

The mitochondria of the denervated muscle show greater pyruvate and palmitate oxidations (Table IV) than those of contralateral control muscle. The mitochondrial enzymes of the β-oxidative scheme in the denervated muscle show higher specific activities than those of

TABLE IV: Pyruvate/palmitate oxidations by the mitochondria from the gastrocnemius muscle of frog.

Oxidation with malate substrate	*µl O_2/mitochondria of whole muscle/hr*	
	Control	*Denervated*
1. Pyruvate (4)	5660±558	10433±1106
2. Palmitate (4)	2309±257	3193±314

Number in parenthesis indicate number of observations.
Values are mean ± S.D.

control (Table V). The mitochondrial glycerol dehydrogenase activity decreased in the denervated muscle (Table V).

The incorporation of the ^{14}C-acetate into the lipids of denervated muscle is considerably lesser than that of contralateral control (Table VI).

TABLE V: Changes in the activities of mitochondrial enzymes of fat metabolism in the gastrocnemius muscle of frog (60 to 65 *g*) denervated for 30 days.

Enzyme activity	*Number of observations*	*Control muscle*	*Denervated muscle*	*Incidence of change on denervation*
1. Acyl dehydrogenase units	6	162±29	279±38	$t = 2.803$ $p < 0.001$ increase
2. Acetate thiokinase units	6	58±14	79±8	$t = 1.456$ $p < 0.001$ increase
3. Crotonase units	6	32±28	186±32	$t = 4.051$ $p < 0.001$ increase
4. Glycerol dehydrogenase units	8	56±14	20±7	$t = 4.051$ $p < 0.001$ decrease

Valnes are mean ± S.D.

TABLE VI: Acetate-1-^{14}C incorporation (when isotope is injected into the dorsal lymphatic duct of frog) into the muscle lipids of frog (60 to 65 *g*) denervated for 30 days.

	No. of observations	*Control muscle*	*Denervated muscle*	*Incidence of change on denervation*
1. Counts/min/mg lipid	4	172±18	32±7	$t = 10.26$ $p < 0.001$ decrease
2. Counts/min/total lipid of the muscle	4	19952±2080	1120±245	$t = 17.9$ $p < 0.001$ decrease

12 hours after injection the muscle lipids were extracted.
Values are mean ± S.D.

DISCUSSION

The steady level of DNA content per whole muscle mass indicates that the denervated muscle is not undergoing changes in the muscle fibre number but undergoes reduction in the muscle mass (28). As is clear from Tables I and II, this reduction is mainly due to the degradation of protein and fat but not due to dehydration. It is known that denervation of muscle (a) alters the membrane permeability properties, (b) imbalances the neurotroph c phenomena, (c) brings forth disuse and (d) impairs the metabolism of muscle (12, 15). The permeability changes and the neurotrophic phenomena in their turn may lead ultimately to disuse and simulatenously influence the metabolic changes in the muscle as described earlier. Thus the denervation-atrophy is a complex phenomena affecting several muscle specific functions.

The present results demonstrate that the denervation involves mitochondiral activity changes but the nature of changes is totally different from that in dystrophic mice (26).

A preferential increase in muscle mitochondrial oxygen uptake and aerobic work capacity occur as adaptive responses to altered functional demands (10,19). The present results (Table I) show that the mitochondrial protein content does not change due to denervation whereas, the p/o ratios (3), mitochondrial oxidative enzymes (Table V) and greater palmitate and pyruvate oxidations (Table IV) increase on denervation. The changes reported in the present investigation thus suggest that they are resulted as adaptive responses due to disuse phenomenon during denervation. It is suggested (12, 13, 15-17) that the increased oxidations in the denervated muscle may not be a result of simple functional demand. More factors in terms of neuromuscular functions may be involved.

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