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Modulation of transglutaminase expression in rat skeletal muscle by induction of atrophy and endurance training

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The persistence of muscle fiber number regardless of size reduction in muscle atrophy has not yet been fully explained. For the mechanism inherent in skeletal muscle tissues for preventing cellular death, the protective function of muscle tissue through transglutaminases has been tested, since the enzyme is responsible for structural stabilization and participates in signal transduction. In the present experiment, hindlimb suspension for two weeks caused a marked muscle atrophy in Wistar female rats. Comparison of muscle weight and histological analysis showed that suspension-induced atrophy in the hindlimb was more prominent in the soleus muscle, comprised mainly of type I fiber than that in the plantaris muscle of type II fibers. The immunohistochemical analysis with antitransglutaminase C antibody (anti TGase C Ab) showed that some atrophic bundles of soleus muscle were positively reacted with the antibody. The anti-TGase C Ab-reactive substances were observed to disappear significantly after endurance exercise, indicating their characteristic atrophy-dependency. The enzymatic analysis of transglutaminase showed the increase in activity in the atrophic soleus muscle tissue, compared with that in the normal or exercise-trained muscle tissues. From these results, the expression of TGase in the atropic muscle is suggested to be the possible marker for muscle atrophy and its expression is probably related with the protective mechanism of the muscle tissue to prevent further cellular damage in the atrophic process.

Key Words : *Muscle atrophy, Transglutaminase, Exercise.*

INTRODUCTION

Half of the body mass is comprised of skeletal muscle, changing readily in mass and function in response to physical activity. For example, the skeletal muscle can be led to atrophy simply by disuse, denervation, tenotomy, or by some hormonal factors(Thomason et al., 1987). The mechanism involved in the process of muscle atrophy is not yet understood clearly. However, the persistence of muscle fiber number despite reduction in muscle size by atrophy indicates the presence of some protective mechanism inherent in muscle tissues

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(Cardenas et al., 1977). Therefore, it would be interesting to identify the muscle defensive mechanism against cellular death. In that aspect, it would be adequate to monitor the changes in transglutaminase expression and activities in response to muscle atrophy and recovery, since transglutaminase is one of the enzymes which build physical barrier and structural stabilization.

Transglutaminases are a group of enzymes which cross-link the protein molecules through covalent isopeptide bonds between a glutamine residue of one protein and a lysine residue of the other protein or another glutamine residue either directly or through a bridge formation with any of the polyamines such as putrescine, spermidine and spermine(Folk, 1980). Therefore, the enzyme has been well studied in the tissues requiring a characteristic tight structure formation such as skin and blood clot. In addition, it was recently suggested that several tissues of varying physiological states might have increased amounts of transglutaminase(Birckbichler et al., 1977 ; Cohen et al., 1979 ; Folk 1980 ; Kim et al., 1990). Though it is not thoroughly understood, the enzymatic crosslinking reaction by transglutaminases may be related to either limiting cellular activities or preventing cellular damage. Moreover, the recent observation that TGase is identified to be the alpha subunit of Gh protein(Ghα) of the signal transduction indicates the active participation of the enzyme in cellular regulation(Baek et al., 1993 ; Das et al., 1993 ; Nakaoka et al., 1994).

In the present experiment, we tried to compare the expression of transglutaminase by biochemical and immunohistochemical analysis between the muscle tissues atrophied by hindlimb suspension and those recovered by endurance exercise.

MATERIALS AND METHODS

Reagents

1,4,C^{14}-putrescine dihydrochloride (118 mCi/mmol) was purchased from Amersham (Arlington Heights, Illinois), and phenylmethysulfonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), dithiothreitol(DTT) and trichloroacetic acid(TCA) were from Sigma Co. (St. Louis, Mo). Other biochemical reagents of analytical grade were obtained from local commercial sources. Antitransglutaminase C antibody was kindly donated from Dr.S.I. Chung of the National Institute of Dental Research, N.I.H. USA.

Atrophy and exercise model

The adult female Wistar rats of approximately 200 g in weight were obtained from the Seoul National University animal breeding house. The animals were accommodated in our animal laboratory for two weeks prior to the experiment. During the whole period, the rats were maintained in the controlled circadian rhythm by alternating light and darkness for 12 hours and were provided with food and drinking *ad libitum*.

The rats were divided into two major groups of control(Group B) and hypokinetic groups(Group C). The hypokinetic group was subjected to a hindlimb suspension for two weeks. The hindlimb was suspended with a tail-suspension device so it could not reach the cage floor. After two weeks, each group was divided again into two sub-groups such as sedentary group(Group D & F) and exercise group-(Group E & G).

The sedentary group remained in cages throughout the recovery, while the exercise group were forced to swim for four weeks with a weekly increase of exercise intensity from 5 minutes every day in the 1st week to 7 minutes in the 4th week. To prevent the advantage of tail effect on floating we attached lead weights equivalent to 5% of body weight to the tail during swimming. The grouping of the experimental animals is summarized in Fig. 1.

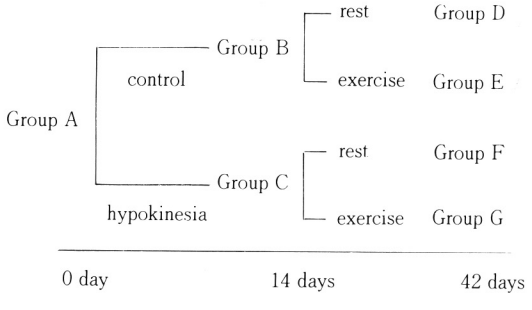

Fig. 1. Summary of experimental groups.

Determination of weight changes

The animals were sacrificed in anesthesia with

pentothal (4 mg/100 g body weight, intraperitoneally) and the soleus and plantaris muscle samples were obtained. The total body weights and the wet weights of soleus and plantaris were determined.

Preparation of the samples

The muscle samples were divided into two parts ; one part was stored frozen in a liquid nitrogen tank for biochemical analysis and the other part was fixed in Carnoy's solution for immunohistochemical analysis. For the biochemical analysis, the muscle was homogenized in Tris-acetate buffer (0.1 M, pH 7.6) including 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine after pulverization in liquid nitrogen. The homogenate was subjected to high speed centrifugation for 1 hour at 30,000 x g, after which the supernatant was used as the cytosol fraction and the precipitate as the particulate fraction for transglutaminase activity determination.

Analysis of transglutaminase

The activity of transglutaminase was monitored by determination of radio-labelled putresine incorporation into dimethylated casein. For the routine assay, the 50 μl sample was incubated in 0.5 ml assay mixture including C^{14}-putresine 0.5 μCi, 1% dimethylated casein, 50 mM Tris acetate buffer (pH 7.5), 1 mM EDTA, 10 mM $CaCl_2$, 1 mM DTT, 0.15 M NaCl, 0.5% lubrol. After 30 minutes of incubation, the sample was acidified with 10% TCA solution. The precipitated casein containing the incorporated radio-labelled putrescine was recovered on Whatman GF/A filters, which were subjected to radioactivity monitoring in a β-liquid scintillation spectrophotometer after rinsing twice with ice cold 5% TCA solution.

Immunohistochemical analysis

The muscle tissues fixed in Carnoy's solution and embedded in paraffin was subjected to immunohistochemical analysis with successive treatment of primary antibody to transglutaminase C, biotinylated secondary antibody and avidin-biotin-peroxidase complex, followed by visualization with diaminobenzidine peroxidation.

RESULTS

Effect of atrophy and exercise on soleus and plantaris muscle mass

The disuse of the hindlimb by suspension caused a significant decrease in muscle weight of both the soleus and plantaris(Table 1). The decrease in muscle size in the soleus (50%) was more marked than that in the plantaris (33%) between group B and group C. The atrophy-induced muscle mass reduction was completely recovered to the control level by 4 weeks of endurance swimming exercise(Group G), whereas the sedentary group showed incom-

Table 1. Effect of hypokinesia and exercise on muscle mass and relative muscle weight of soleus and plantaris muscles

Groups (Number of rats)	A (10)	B (10)	C (10)	D (10)	E (8)	F (9)	G (8)
Wet weight of soleus(mg)	96.6±14.3	105.7±6.6	53.0±5.5*	131.6±12.9	113.9±7.6**	93.7±20.8	116.5±13.2
Ratio of weight (soleus/body, mg/g)	0.49±0.03	0.52±0.03	0.30±0.04*	0.55±0.05	0.51±0.04**	0.41±0.07**	0.50±0.05
Wet weight of plantaris(mg)	185.8±24.4	213.9±12.0	144.0±26.3*	241.8±21.9	242.2±15.2	226.8±23.6	242.7±11.0
Ratio of weight (plantaris/body, mg/g)	0.95±0.07	1.05±0.07	0.79±0.10	1.01±0.10	1.08±0.07	0.99±0.09	1.04±0.06

All the data are means±S.D.
*Significantly different from group B($p<0.001$)
**Significantly different from group D($p<0.05$)
The numbering of the groups is explained in Fig. 1.

plete recovery(Group F). The ratio of muscle weight to total body weight also showed a significant decrease in both the soleus and plantaris by hindlimb suspension(Group B & C). However, the soleus was more significantly affected than the plantaris. The exercise also improved the ratios to the control level(Group G).

Effect of atrophy and exercise on transglutaminase expression in the skeletal muscles

The prolonged hindlimb suspension caused a significant increase in transglutaminase activities in the soleus and plantaris muscles of the rats(Table 2). The atrophy-induced soleus muscle showed increased activities in the particulate fraction, while no significant change was found in the plantaris. However, the supernatant enzymic activities of atrophic muscle fibers were increased in both the soleus and plantaris muscles(Table 2). As illustrated in Fig. 2, the atrophy-induced soleus muscle showed a strong positive reaction to anti-TGase antibody, while the sedentary group showed a marked decrease in the reaction and the exercise group was completely eliminated of anti-TGase Ab positive fibers. In contrast, the plantaris muscle was very weakly reactive to anti-TGase antibody, regard-

Table 2. Comparison of transglutaminase activities in atrophic muscle tissues

Class	Soleus		Plantaris	
Source	Normal	Atrophy	Normal	Atrophy
Supernatant(%)	3828(100)	4814(126)	2111(100)	3423(162)
Particulate fraction(%)	6950(100)	13321(192)	5317(100)	5735(108)

Numbers are means of triple experiments in cpm/mg protein

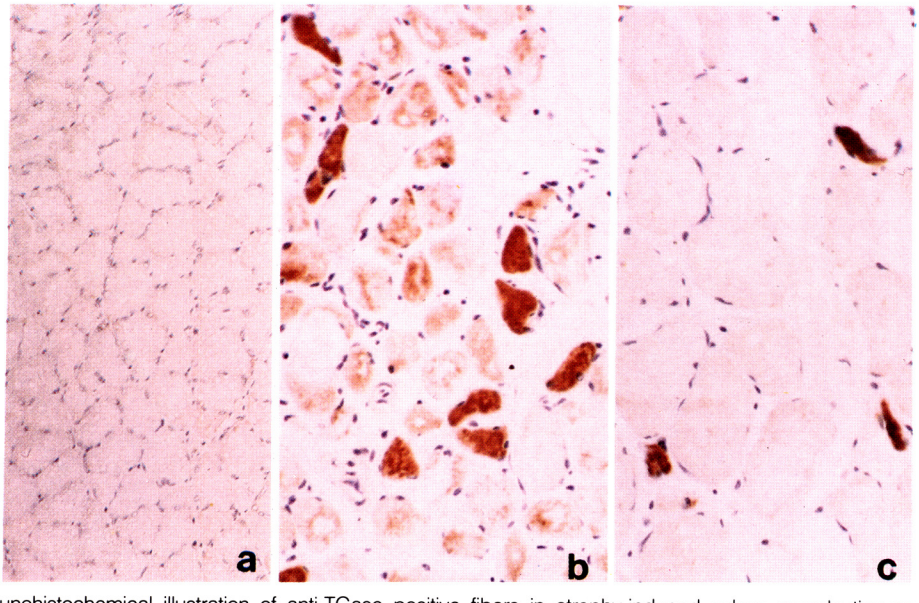

Fig. 2. Immunohistochemical illustration of anti-TGase positive fibers in atrophy-induced soleus muscle tissues of rats.
(a) Exercise group after atrophy induction(Group G) (100×fold)
(b) Atrophy-induced group(Group C)(400×fold)
(c) Sedentary group after atrophy induction(Group F)(400×fold)
The lesions are visualized by immunohistochemical method using anti-TGase Ab and peroxidase staining as explained in Materials and Methods. Darkly stained TGase positive fibers are smaller in bundle size than the adjacent TGase negative fibers.

Table 3. Immunohistochemical distribution of cytosolic transglutaminase in rat soleus muscle tissues

Groups	Normal (Group B)		Atrophy (Group C)		Atrophy-sedentary (Group F)		Atrophy-exercise (Group G)	
Reactivity to TGase Ab of fibers	+	−	+	−	+	−	+	−
Number of fibers(%)	0	100	23	77	4	96	0	100
Volume(%)	0	100	12	88	1	99	0	100
Mean size of single fiber(μm^2)	0	2054	610	1378	284	1806	0	2250

All the numbers are means of triple experiments

less of atrophy or exercise(data not shown).

In the soleus muscle of the hypokinesia group (Group C), 23% of the whole muscle fibers are strongly positive to anti-TGase Ab, reaching 12% in volume which indicated that the size of TGase-positive fiber is significantly smaller than TGase-negative fibers(Table 3). The resting after the atrophy induction decreased the anti-TGase Ab positivity in the soleus muscle significantly. The numbers of TGase-positive fibers were decreased to 4%, and the volume of those fibers to 1%. Moreover, the exercise significantly eliminated anti-TGase Ab positive fibers in the soleus muscle (Table 3). In regard to fiber size, the atrophy caused a reduction in the mean size of each fiber (Table 3). The mean size of fibers in the normal tissues was 2054 μm^2, while that of fibers in the atrophy group was decreased to 1378 μm^2 (67% of normal) in TGase-negative bundles and to 610 μm^2 (29%) in TGase-positive bundles. The strenuous exercise increased the mean size of single muscle bundles to 2250 μm^2. However, the sedentary rest after induction of atrophy caused a decrease of muscle size to 284 μm^2 in TGase-positive fibers, while it caused its increase to 1806 μm^2 in TGase-negative bundles.

DISCUSSION

The prolonged hindlimb suspension caused marked atrophy in the rat skeletal soleus and plantaris muscles, determined by the decrease in muscle mass and ratio of muscle weight to body weight (Table 1). But the effect of atrophy was more prominent in the soleus muscle. In the present study, the weight of the soleus muscle was decreased to 61.2% of the control after two weeks of hindlimb suspension, while that of the plantaris muscle, only to 83.2% (Table 1). The data indicated that the soleus muscle was more vulnerable to atrophy, compatible with atrophy-sensitivity of type I fiber (Newsholme & Leech 1983; Thomason et al., 1987, 1989), since the soleus muscle is enriched with type I muscle fiber whereas the plantaris muscle is enriched with type II fibers.

In contrast to atrophy induction by disuse, the exercise program could restore the atrophic muscle to the normal state readily. As summarized in Table 1, the weight of soleus muscle in sedentary state for 4 weeks was recovered to 88.6% of the normal (but increased to 176.7% of the atrophied muscle), while in the swimming exercise group, it was increased to 110.2% of the normal (but to 219.8% of the hypokinetic muscle). In the case of plantaris muscle, the sedentary state caused the increase of muscle weight to 106% of the normal control (but 157.5% of the hypokinetic plantaris), while the exercise training increased that of the plantaris to 114% of the normal (168.7% of the hypokinetic muscle). These data indicated that the exercise could induce the recovery of hypokinetic muscles significantly in both the soleus and plantaris muscles compared with the sedentary state. Therefore, it is evident that exercise has a positive effect on prevention or treatment of muscle atrophy. The significant reversion of prednisone-induced myopathy, by prolonged isokinetic training, was also observed(Horber et al., 1985). It was illustrated in murine treadmill exercise model that cortisone acetate-induced muscle atrophy could be prevented by endurance exercise(Hickson et al., 1984). Therefore, it may be summarized that exercise can prevent glucocorticoid-induced myopathy as well as hypokinetic atrophy.

In our immunohistochemical analysis as shown in Fig. 2 and Table 2 and 3, it was observed that the

atrophic fiber bundles of the soleus muscle were strongly positive to anti-TGase C antibody reaction. Instead of the diffuse localization of anti-TGase C Ab-positive substances in the muscle fiber bundles, the limited number of specific fiber bundles in the atrophic muscle tissue showed a strong reaction, the signficance of which is not yet clear. However, with the anti-TGase antibody reactions, the positive bundles were not only markedly decreased by sedentary rest but almost disappeared in the exercise group. Therefore, it can be suggested that the anti-TGase C antibody-reacted substance in the skeletal muscle is atrophy-related, and readily affected by exerise. Moreover, a previous immunohistochemical analysis of human tissues with anti-TGase C antibody showed that the skeletal muscle was negative, while the smooth muscle was positive to the antibody (Thomazy and Fesus, 1989). The positive reaction of the atrophic skeletal muscle to anti-TGase C antibody in the present experiment suggests strongly the physiological significance of the transglutaminase enzyme in the process of muscle atrophy. Furthermore, the biochemical analysis indicated that the atrophic muscles showed higher transglutaminase activities than the control tissues, regardless of whether soleus or plantaris (Table 2). These results indicate that expression of transglutaminase can be used as a biological marker for muscle atrophy.

Since it was suggested that the expression of TGase C might be related with the apoptotic process(Fesus et al., 1984 ; Park et al., 1991), anti-TGase C antibody positive muscle bundles of varying sizes might indicate the various steps of on-going muscle atrophy presumably in association with apoptosis of atrophic muscle. The mean sizes of the fibers were markedly decreased in the atrophy group. TGase-positive muscle bundles were more prominently reduced in size. With the increase of the mean size of muscle fibers either by exercise or sedentary rest, the positivity to anti TGase C antibody was decreased (Table 3). However, the persistence of muscle fiber number in the atrophic muscle and the ready restoration of muscle size by exercise suggest that the mechanism of muscle atrophy is probably not related with the apoptosis (programmed cell death) (Cardenas et al., 1977 ; Nicks et al., 1989). Therefore, the induction of TGase in the atrophic muscle bundle can not be explained in terms of apoptosis. Rather, the biochemical characteristics of TGase as the crosslinking enzyme as well as the signal transduction mediator may indicate the role of TGase as the protective mechanism in muscle atrophy to prevent further cellular damage (Baek et al., 1993 ; Nakaoka et al., 1994).

The probable substrates for TGase in the skeletal muscle are probably myosin and actin, already known to be crosslinked by fibrinoligase (blood clotting factor Ⅷ)(Mui and Ganguly, 1977 ; Cohen et al., 1979). If molecules in skeletal muscle can be irreversibly crosslinked by the newly induced transglutaminase in the atrophic state, the role of the enzyme may be important in the mechanism of atrophy. Recently, fructose 1,6 biphosphate aldolase A, an intracelluar protein, was identified as a substrate of transglutaminase (Lee et al., 1992). Since aldolase A is dominantly expressed in skeletal muscle tissue, the crosslinking of aldolases by the induced transglutaminase may block glycolysis, which may limit the metabolic waste of the muscle in the atrophic process.

In conclusion, it can be suggested that the expression of TGase in the muscle fiber is induced by immobilization atrophy, which is reversibly decreased by endurance exercise. Since the biochemical roles of transglutaminase are related with protein cross-linking and signal transduction, its expression in the atrophic muscle fibers may be responsible for the protective mechanism of the muscle against further damage in the atrophic process rather than muscle apoptosis. And since its expression is dependent on the atrophic status of the muscle, it can be proposed as the marker for muscle atrophy.

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