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Effects of Thyroid Hormones on Myofibrillar Proteolysis and Activities of Calpain, Proteasome, and Cathepsin in Primary Cultured Chick Muscle Cells

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Summary The effects of thyroxine (T_4) and triiodothyronine (T_3) on growth, muscle protein degradation, and proteases activities in cultured chick muscle cells were studied. The cells were treated with a physiological level of T_4 (60 ng/mL) or T_3 (12 ng/mL) for 6 d. Calpain, cathepsins, and proteasome activities and N^{τ}-methylhistidine release were measured as indexes of myofibrillar protein breakdown. Creatine kinase activity was also measured as an index of myotube formation. Calpain activity was increased by T_4 and T_3. Cathepsin D and proteasome activities and N^{τ}-methylhistidine release were increased by T_3, but not by T_4. Neither were cathepsin B and B+L activities affected by T_3 or T_4. Creatine kinase activity was increased by T_4 and T_3. The results suggest that myotube formation is accelerated by T_4 and T_3, whereas myofibrillar protein degradation is accelerated by T_3, but not by T_4.

Key Words thyroid hormones, calpain, cathepsin, proteasome, cultured chick muscle cells

It is well known that thyroid hormones (thyroxine, T_4; triiodothyronine, T_3) produce a catabolic effect on skeletal muscle. However, the modes of action are largely unknown.

It has been reported that the physiological level of T_3 stimulates degradation of long-lived proteins in cultured hepatocytes (*1*) and in cultured cardiac myocytes (*2*). It has also been shown that skeletal muscle protein degradation is accelerated by T_3 with N^{τ}-methylhistidine as an index (*3*). The intracellular proteolytic process is composed of lysosomal and nonlysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, cysteine proteases in the cytosol, are thought to be the main agents of nonlysosomal Ca^{2+}-dependent proteolysis that exists within the myofibril and which is capable of

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carrying out the initial step in myofibrillar proteolysis (*4*, *5*). Proteasome, multicatalytic proteinase complexes in the cytosol, is also thought to be responsible for nonlysosomal ATP-dependent proteolysis (*6*). Although proteasome has been implicated in the regulation of myofibrillar protein degradation (*7*, *8*), its substrates and the endocrine control of its activity have not been fully studied in muscles. Proteasome may degrade muscle proteins released because of calpain action. Cathepsins, the main agents of lysosomal degradation, have been well established to contribute to muscle protein breakdown (*9*). Lysosomal proteases may degrade sarcoplasmic proteins and released myofibrillar proteins (*10*), but the precise roles of all these protein degradation systems in skeletal muscle are yet to be determined.

We have previously observed that skeletal muscle protein synthesis is accelerated by T_4 and that plasma T_3 concentration is not changed in chicken (unpublished data). Thus the present study was conducted to compare the effects of T_4 and T_3 on the growth, myofibrillar proteolysis, and protease (calpain, proteasome and cathepsins) activities of primary chick muscle cell culture. Creatine kinase activity and N^{τ}-methylhistidine release were also measured as indexes of myotube formation and myofibrillar protein breakdown, respectively. The present study has clearly shown the differential effects of T_4 and T_3 on the growth, protein breakdown, and protease activities in cultured skeletal muscle cells.

MATERIALS AND METHODS

Cell culture. Myoblasts were isolated from thigh muscle of 13-d-old chick embryos (*11*). Briefly, the muscle tissue obtained from the embryo was digested with dispase. The cell suspension was transferred to a 35 mm uncoated culture dish to allow fibroblast attachment. The cell numbers were counted and plated onto gelatin-coated 6-well plates at a density of 2.5×10^5 cells/well. Myoblasts were cultured in M-199 containing 15% calf serum and 2.5% chicken embryo extract. The cells were grown at 37°C in a 5% CO_2-enriched atmosphere of humidified air. After 24 h, the medium was replaced by media containing a physiological level of thyroxine sodium salt (60 ng/mL) or 3,3′,5-triiodothyronine sodium salt (12 ng/mL). Thyroid hormones were dissolved in ethanol and added in the medium. The final ethanol concentration in the culture medium was 0.1%. The media were replaced every other day for a 6-d incubation period. To measure proteasome activity, the cultured cells were separately prepared.

Creatine kinase activity. After the medium was collected, the cell monolayer was washed three times with ice-cold PBS, and the cells were scraped with a rubber policeman using 2 mL Tris buffer (20 mM, pH 6.8). The cells were then homogenized by a microhomogenizer and centrifuged at 2,500 rpm for 10 min at 4°C. The supernatants were rapidly frozen and stored at −80°C until analysis. Creatine kinase activity was analyzed by the method of Rosalki (*12*), as follows. ATP formed by action of the enzyme on ADP and creatine phosphate is linked to the reduction of nicotinamide-adenine dinucleotide phosphate with glucose, hexokinase, and

glucose-6-phosphate dehydrogenase. The increase in optical density at 340 nm, which depends on NADP reduction, was monitored by spectrophotometry, and it provided a measure of creatine kinase activity. One enzyme unit is the amount of creatine kinase that catalyzes the formation of 1 μmol of ATP/min at 30°C, pH 6.8.

Measurement of protease activities. The cells were washed three times with the homogenization solution (20 mM Tris-HCl, pH 7.4, containing 0.25 μM sucrose, 2 mM EDTA, and 2 mM EGTA). It was then homogenized with 1 mL of the homogenization solution containing 0.2% Triton X-100 and lysed by sonication. The homogenate was centrifuged at 18,000 × *g* for 15 min. The supernatant was dialyzed against the same amount of glycerol and stored at −80°C until analysis. The activities of cathepsins B and B+L were measured by the method of Barrett and Kirschke (*13*) by using fluorogenic peptides. Cathepsin B activity was assayed with 10 mM Z-Arg-Arg-MCA as a substrate at pH 6.0. Cathepsin B+L activity was assayed by the same method as cathepsin B with Z-Phe-Arg-MCA as a substrate. Since this synthetic substrate is hydrolyzed by cathepsin L and cathepsin B, the activity is shown as cathepsin B+L activity. The activity of cathepsin D was measured by the method of Barrett and Kirschke (*14*) with 2% hemoglobin as a substrate. Calpain activities were assayed with fluorescein isothiocyanate-labeled casein as a substrate, and the fluorescence of a 2.5% trichroloacetic acid supernatant was measured (*15*).

Proteasome activity was analyzed by the method of Tanaka et al (*16*) by using crude enzyme prepared by differential centrifugation of tissue homogenates. In brief, cells were washed three times with the homogenization buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, and 0.25 M sucrose), then homogenized in a microhomogenizer. The resulting suspension was centrifuged at 10,000 × *g* for 20 min. The supernatant was collected and centrifuged at 105,000 × *g* for 1 h. It was centrifuged again for 5 h at 105,000 × *g* and the resulting protein pellet was dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, and 20% (v/v) glycerol and stored at −80°C. The proteasome activity was determined with succinyl-Leu-Leu-Val-Tyr-MCA as a substrate (*16*).

Proteins were measured by Lowry's method by using bovine serum albumin as a standard (*17*).

N^{τ}-methylhistidine analysis. N^{τ}-methylhistidine was analyzed by the method of Hayashi et al (*18*) with a modification. The media were hydrolyzed with 6 N HCl at 110°C for 20 h. After the hydrolysate was cooled and passed through filter paper, the hydrochloric acid was removed by evaporation. The residue was dissolved in 5 mL of 0.2 M pyridine; 4.5 mL of this was applied to a cation-exchange resin column (7 × 60 mm, Dowex 50 × 8, 200 to 400 mesh, pyridine form). After eluting most of the acidic and neutral amino acids with 20 mL of 0.2 M pyridine, N^{τ}-methylhistidine was eluted with 20 mL of 1 M pyridine and collected. The eluent was then dried, and the residue was dissolved in 1 mL of mobile phase (15 mM sodium octane sulfonate in 20 mM KH_2PO_4). Fifty milliliters of this was used for

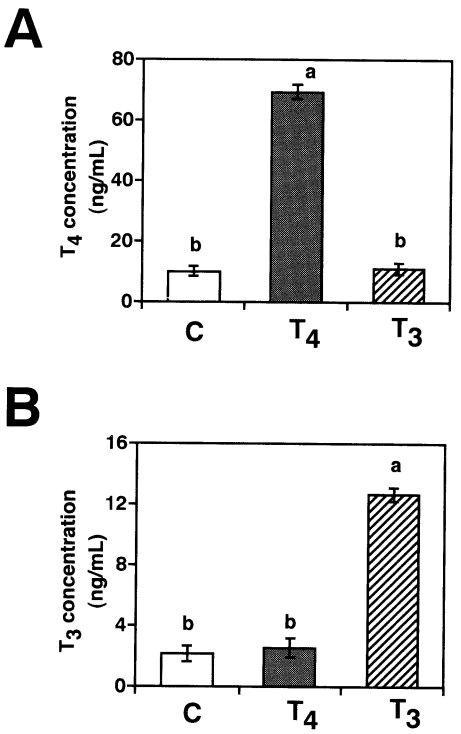

Fig. 1. The effects of T_4 and T_3 on creatine kinase activity (A) and N^{τ}-methylhistidine release (B) in cultured muscle cells. Primary chick muscle cells were treated with T_4 (60 ng/mL in the medium) or T_3 (12 ng/mL in the medium) for 6 d. Values not sharing a common superscript are significantly different ($p<0.05$). The values given are means $\pm$ SD. C, control; T_4, thyroxine; T_3, triiodothyronine.

HPLC analysis. The HPLC system incorporated the reverse-phase separation with ion pairing, using Shim-pack octadecyl-silica gel (ODS) column (6.0 × 150 mm) and the postcolumn fluorescence derivatization with orthophthalaldehyde.

Statistical analysis. Data were analyzed by analysis of variance, and means were further tested by Duncan's multiple-range test. A p value <0.05 was considered statistically significant. Each result is expressed as the mean $\pm$ standard deviation of the values obtained from six replicates.

RESULTS AND DISCUSSION

In the present experiment, the effects of T_4 and T_3 on growth, myofibrillar proteolysis, and protease activities in primary cultured muscle cells were investigated. The results of creatine kinase activity and N^{τ}-methylhistidine release are shown in Fig. 1. Creatine kinase activity (Fig. 1A) was significantly ($p<0.05$) increased by T_4 and T_3, showing that both hormones induced myotube formation. This is

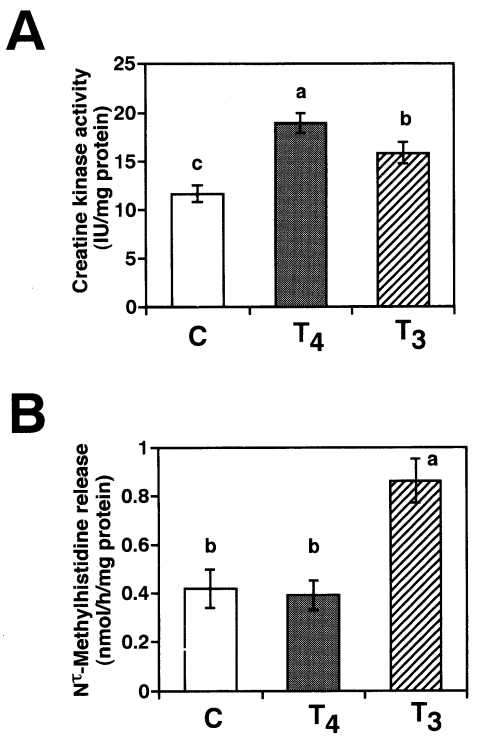

Fig. 2. The concentrations of T_4 (A) and T_3 (B) in the media of cultured muscle cells. Primary chick muscle cells were treated with T_4 (60 ng/mL in the medium) or T_3 (12 ng/mL in the medium) for 6 d. Concentrations of T_4 and T_3 were measured in the medium on the final day of incubation. Values not sharing a common superscript are significantly different ($p < 0.05$). Values given are means ± SD. C, control; T_4, thyroxine; T_3, triiodothyronine.

consistent with the results of Marchal et al (*19*), who reported that a physiological amount of T_3 reduces the proliferation of myoblasts and stimulates myotube formation.

It is well known that T_4 is converted to the biologically potent T_3 in many tissues by 5′-deiodinase. However, the activity of 5′-deiodinase in skeletal muscle is much lower than those of liver and kidney (*20*), and little or no monodeiodination of T_4 occurs in skeletal muscle (*21*). The concentrations of T_4 and T_3 in the media measured on the final day of incubation are shown in Fig. 2. T_3 concentration (A) was not increased by T_4 supplementation, indicating that little deiodination of T_4 occurred. Therefore it is unlikely that T_4 increased creatine kinase activity after conversion to T_3. It is very interesting that T_4 was effective in increasing creatine kinase activity.

N^{τ}-methylhistidine release (Fig. 1B) was significantly ($p < 0.05$) increased about twofold by T_3, but not by T_4. This result indicates that T_3, but not T_4, induces

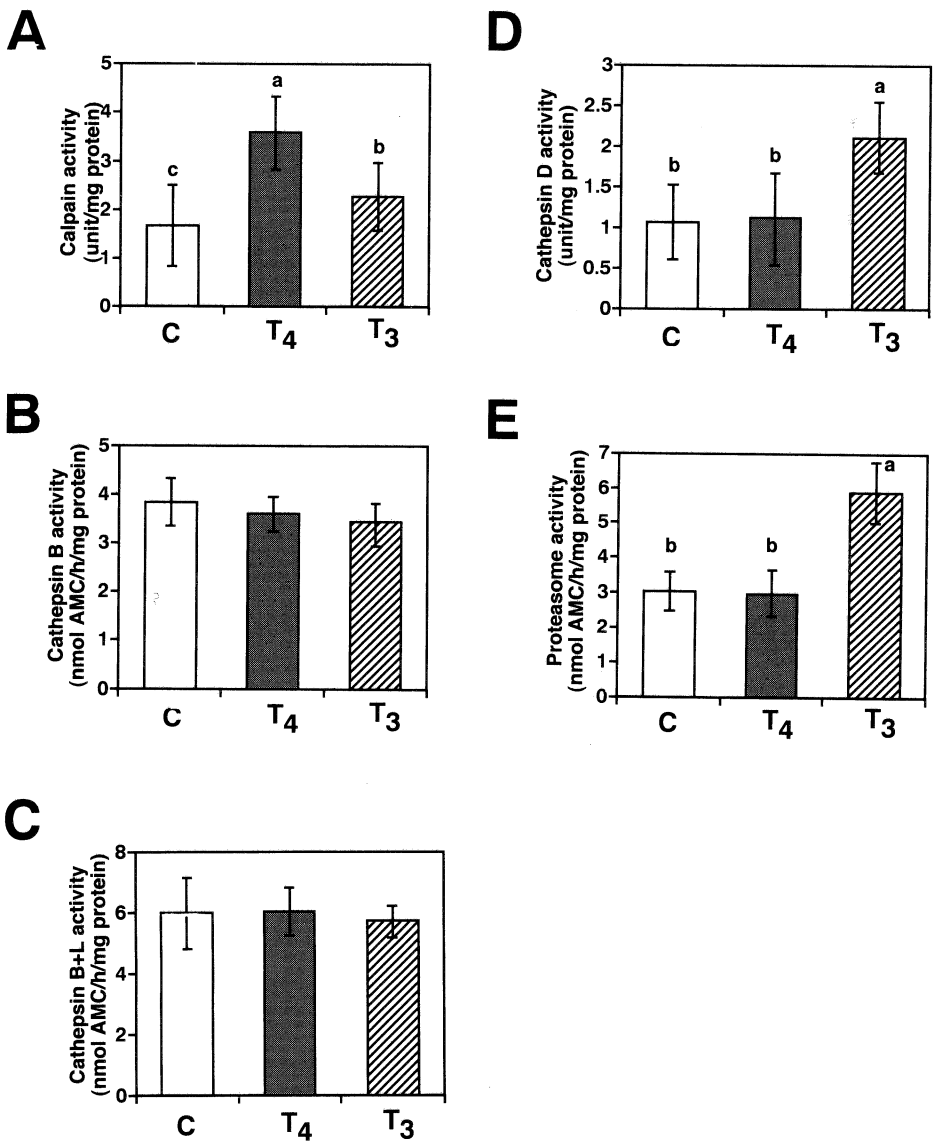

Fig. 3. The effects of T_4 and T_3 on calpain (A), cathepsin B (B), cathepsin B+L (C), cathepsin D (D), and proteasome (E) activities in cultured muscle cells. Primary chick muscle cells were treated with T_4 (60 ng/mL in the medium) or T_3 (12 ng/mL in the medium) for 6 d. Values not sharing a common superscript are significantly different ($p<0.05$). Values given are means $\pm$ SD. C, control; T_4, thyroxine; T_3, triiodothyronine.

myofibrillar proteolysis in cultured chick muscle cells. In cultured cells, T_3 is known to stimulate the degradation of long-lived protein in hepatocytes (*1*) and cardiac myocytes (*2*).

The activities of proteases are shown in Fig. 3. Calpain activity (Fig. 3A) was significantly ($p<0.05$) increased by T_4 and T_3. This is consistent with the changes in creatine kinase activity. It has been shown that calpain plays an essential role in the fusion of myoblasts and causes a limited degradation of membrane and cytoskeletal muscle proteins during the myogenesis (*22*, *23*). However, the interaction between the fusion and proteolysis has not yet been clarified. Calpain has been implicated in the degradation of myofibrillar elements of muscle (*4*, *5*). Calpain activation may trigger a dissociation of myofibrillar elements. Toyo-oka (*24*) has also reported that T_4 stimulates calpain activity, but not overall protein degradation in skeletal muscle, indicating that other functions are needed to complete proteolysis.

The activities of cathepsin B (Fig. 3B) and cathepsin B+L (Fig. 3C) were affected neither by T_4 nor T_3 in the present experiment. However, cathepsin D (Fig. 3D) activity was significantly ($p<0.05$) increased about twofold by T_3, but not by T_4. This is consistent with the change in N^{τ}-methylhistidine release. Lysosomal cathepsins B, L, and D are endopeptidases that are thought to play major roles in intracellular protein degradation (*25*). Lysosomal proteases may degrade released myofibrillar proteins (*10*).

Thyroid hormone is known to alter protein degradation by altering levels of cathepsins B and D in liver and muscle (*26*, *27*). In the present experiment, however, T_3 stimulated cathepsin D activity, but not cathepsin B activity. We used a specific synthetic substrate for the assay of cathepsin B activity. However, De Martino and Goldberg used hemoglobin as a substrate of cathepsins B and D, and cathepsin B activity was estimated by using pepstatin as an inhibitor of cathepsin D. Cathepsin D activity might not be completely inhibited by pepstatin in their experiments.

Proteasome (Fig. 3E) activity was measured by using separately cultured cells. It was also significantly ($p<0.05$) increased about twofold by T_3, but not by T_4. This is consistent with changes in N^{τ}-methylhistidine release and cathepsin D activity. Recently, Tawa et al (*28*) have observed that the administration of T_3 raises the proteasome activities and also the lysosomal protease activities in rat muscle in vivo.

Our results suggest that accelerated muscle proteolysis by T_3 is associated with the activities of calpain, proteasome, and cathepsin D. The role of calpain in the proteolysis is controversial. Although T_4 stimulates calpain, it might not stimulate myofibrillar proteolysis. It would be necessary to examine the relation between calpain activity and the proteolysis by using complete myotubes, though in these experiments myotube formation was almost completed on day 6 when it was evaluated by fusion index.

Autophagy is a physiologically regulated intracellular process that plays a major role in the turnover of long-lived protein (*29*). It has also been reported that the ubiquitin-proteasome pathway is associated with autophagy (*30*), and once dissociated from myofibrils, the isolated myofibrillar proteins may be readily

ubiquitinated and degraded by proteasome, as reported by Solomon and Goldberg (*8*). The resultant peptide fragments may be delivered to lysosome and further degraded by cathepsins.

Cathepsin activity might not be activated by T_4 because it did not activate proteasome. Proteasome is an ATP-dependent multicatalytic proteolytic system (*6*). The multiple proteolytic pathways are essential to complete proteolysis. Thus we speculate that T_3, but not T_4, stimulates ATP production in mitochondria; thus proteasome is activated by T_3, but not by T_4. Indeed, it has been shown that T_3, but not T_4, regulates diet-induced heat production (*31*). Furthermore, we have observed that muscle oxygen consumption is not accelerated by T_4 with the in vitro system (unpublished data).

Our findings clearly show that the degradation of myofibrillar protein is accelerated by T_3, but not by T_4, because cathepsin D and proteasome are not activated by T_4, and T_4 and T_3 both stimulate muscle cell growth. The multiple proteolytic pathways must be activated to complete skeletal muscle protein breakdown; thus it is thought that T_4 cannot activate the whole proteolytic pathways.

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