Effects of the Thyroid Hormone on Differentiation, Growth, and Proteolysis in Cultured Muscle Cells during Serum Deprivation

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Summary The actions of the thyroid hormone (T₃) are modified by other hormones. Therefore, in the normal cell culture system, with serum as a medium ingredient, it is difficult to eliminate the influences of other hormones derived from the serum. In the present study, two experiments were conducted to clarify the effects of T₃ on differentiation, growth and proteolysis (experiment 1), and concerted effects of T₃ and insulin (experiment 2) in a serum-free culture with the use of muscle cells originated from chick embryo. Protein content and creatine kinase (CK) activity were examined as indices of growth and differentiation of the muscle cells, respectively, and N⁎-methylhistidine (MeHis) release was examined as an index of myofibrillar proteolysis. It was observed that T₃ suppressed both the muscle differentiation and proteolysis in the serum-free medium (experiment 1), though in our previous experiment they were enhanced by T₃ in the serum-supplemented normal medium. On the other hand, T₃ increased myofibrillar proteolysis and had no effect on muscle differentiation when insulin was included in the serum-free medium (experiment 2). These results clearly show that the effects of thyroid hormones on muscle differentiation and proteolysis are apparently different when serum is deprived from the medium, and these differential effects of thyroid hormone could be partially explained by an interaction with insulin, one of the growth factors in the serum.

Key Words thyroid hormones, insulin, protein metabolism, serum-free culture

A continuous turnover of protein (synthesis and breakdown) maintains the functional integrity and quality of skeletal muscle. Hormones are important regulators of this remodeling process, but the effect of the individual hormones on muscle and their mechanisms of action remain to be clearly defined. It is well known that thyroid hormones play important roles in basal metabolic rate and protein synthesis and breakdown to maintain homeostasis. The physiological level of thyroxine (T₄) or triiodothyronine (T₃) induces the growth of hypothyroid rats because they stimulate muscle protein synthesis more than they promote breakdown (1). Although thyroid hormone is essential during muscle growth, an excess and a deficiency both cause muscle wasting. The growth promoting level of thyroid hormone stimulates the rate of protein synthesis and, to a lesser extent, proteolysis, whereas the higher catabolic level causes a further stimulation of protein degradation (2, 3).

Recent advances in the understanding of the mechanism of hormone action at the cellular level have been obtained through the employment of primary muscle cell culture, which retains many muscle functions. We have previously reported the effects of thyroid hormone on proteolysis in primary cultured muscle cells in a serum-supplemented normal media (4). However, in the serum-supplemented media, a detailed analysis of the mechanism of hormone action underlying proteolysis may be difficult because hormonal content varies widely among serum lots. Therefore the cultivation of muscle cells in a serum-free medium is needed to clarify factors affecting proteolysis.

The present experiment was undertaken to examine the effects of T₄ and T₃ on growth, differentiation, and proteolysis in the muscle cells cultured in a serum-free medium. Furthermore, in the present study the concerted effects of T₃ and insulin, a growth factor in the serum, was clarified because it was shown in the preceding experiment that the effects of thyroid hormones on muscle cells were modified by serum.

It is well documented that thyroid hormones potentiate the effect of insulin-like growth factors (IGFs), and these growth factors induce cell proliferation. Milne et al. reported that T₃ treatment upregulates IGF-I protein secretion by vertebral marrow stromal cell cultures (5), and Forhead et al. reported that thyroidectomy suppressed muscle IGF-I gene expression in the skeletal muscle of fetal sheep (6). Therefore in the present study, the concerted effects of T₃ and insulin on muscle growth, differentiation, and proteolysis were also investigated.

MATERIALS AND METHODS

Cell culture. Myotubes were isolated from the thigh muscle of 13-day-old chick embryos (4, 7). The muscle
The cell suspension was then passed through a net and centrifuged 1,000 rpm for 3 min. The supernatant was aspirated, and the cell pellet was dispersed into 3 mL of basal growth medium (Medium 199, M-199; LIFE TECHNOLOGIES, containing 15% calf serum and 2.5% chicken embryo extract). The cell suspension was transferred to a 35 mm uncoated culture dish to allow fibroblast attachment. After 40 min, the unattached cells were recovered and transferred to another uncoated dish. This protocol was repeated three times, and the cell number was counted and plated onto gelatin-coated 6-well plates at a density of 2.5×10^5 cells/well. The cells were grown at 37°C in a 5% CO₂-enriched atmosphere of humidified air. After 4 days, the basal growth medium was changed to the differentiation medium (M-199 containing 2% horse serum), which stimulates cell fusion. Cytosine arabinoside (Ara-C, 4 μM) was also added to the differentiation medium to eliminate proliferating myoblasts and fibroblasts. The media were replaced every other day for the incubation period. On day 6 the medium was replaced with a serum-free medium (M-199 containing 1% BSA). Thyroxine sodium salt (T₄, Nacalai Tesque, Japan, 60 ng/mL) and 3,3′,5-triiodothyronine sodium salt (T₃, Nacalai Tesque, 12 ng/mL) were added to the medium after being dissolved in ethanol. Insulin (from human recombinant, Sigma Co., USA, 10 nM) was added to the medium after being dissolved in 0.01 M HCl. The final concentrations of ethanol and HCl in the culture medium were 0.1% and 0.1 nM, respectively. After a 24-hour treatment with T₄ or T₃ or insulin, the cells were harvested. The medium was also collected and stored at −20°C until being analyzed for MeHis.

**Analyses of cells.** After the medium was collected, the cell monolayer was washed three times with ice-cold PBS, and the cells were detached with a cell scraper using 1 mL of 0.1 M Tris-HCl buffer (pH 6.8). The cells were then homogenized by a microhomogenizer, and the aliquots were collected for a measurement of protein contents. The remaining homogenate was then centrifuged at 2,500 rpm for 10 min at 4°C. The supernatant was rapidly frozen and stored at −80°C until the analysis of creatine kinase (CK) activity.

CK activity was analyzed by the method of Rosalki (8). ATP formed by the action of the enzyme on ADP and CP is linked to the reduction of nicotinamide-adenine dinucleotide phosphate (NADP) with glucose, hexokinase, and glucose-6-phosphate dehydrogenase. The increase in optical density at 340 nm, which depends on NADP reduction, was followed by spectrophotometry and provided a measure of CK activity. One enzyme unit (IU) is the amount of CK that catalyzes a formation of 1 μM/min at 30°C, pH 6.8.

Protein was determined by the method of Lowry et al. (9) by the use of bovine serum albumin as a standard.

N²-methylhistidine (MeHis) was analyzed by a modification of the method of Hayashi et al. (10). 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, MeHis 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 1-octanesulfonate in 20 mM KH₂PO₄). Fifty microliters of this was used for HPLC analysis. The HPLC system incorporated the reverse-phase separation with ion-pairing, using Shodex C18-5B column (4.6×250 mm) and the postcolumn fluorescence derivatization with orthophthalaldehyde.

**Statistical analysis.** The data were analyzed by an analysis of variance (ANOVA), and means were further tested by Duncan’s multiple-range test. A p value <0.05 was considered statistically significant. Each result is the mean±standard deviation of the values obtained from six replicates.

**RESULTS**

**Experiment 1**

In the present experiment, the effects of T₄ and T₃ on growth and myofibrillar proteolysis in muscle cells cultured in a serum-free medium were investigated. Microscopic observations showed little difference of cell growth between the control and the hormone treatment groups (Fig. 1, A to D). As shown in Fig. 2A, the protein contents of the cells were significantly decreased by serum deprivation, and there was no difference between serum-deprived control and the hormone groups. CK activity was measured as an index of muscle differentiation. However, as shown in Fig. 2B, it was significantly decreased by serum deprivation. In the serum-deprived muscle cells, T₄ further decreased the CK activity, and T₃ had no effects on CK activity. MeHis release was measured as an index of myofibrillar protein breakdown (Fig. 2C). The MeHis release was significantly increased by serum deprivation, showing an enhanced myofibrillar proteolysis. It was very interesting, however, that in the serum-deprived muscle cells, MeHis release was significantly decreased by both T₄ and T₃ (Fig. 2C). This shows that enhanced myofibrillar proteolysis by serum deprivation was suppressed by both T₄ and T₃.

**Experiment 2**

In the present experiment 2, the concerted effect of T₄ and insulin was investigated. As shown in Fig. 3A, there were no effects of T₃ and insulin on protein content. CK activity was significantly increased by insulin (Fig. 3B), and it was interesting that CK activity was decreased by T₃ alone (Fig. 2B, 3B), though T₃ had no effect when insulin was present. MeHis release tended to be decreased by T₃ and significantly decreased by insulin. However, T₃ tended to increase MeHis when insulin was included in the medium, indicating that T₃ in-
Fig. 1. Microscopic images of chick muscle cells. Muscle cells were cultured for four days in a basal growth medium containing 15% calf serum and 2.5% chicken embryo extract and allowed to grow to approximately 80–90% confluency. To induce differentiation, cells were switched to differentiation medium containing 2% horse serum and 4 μM Ara-C and allowed to differentiate for 2 days. The medium was then switched again to a serum containing basal growth medium (A), serum-free medium (B), serum-free media containing 60 ng/mL T₄ (C) or 12 ng/mL T₃ (D) and cultured for 24 h.

creases myofibrillar protein breakdown if insulin is present. These results indicate that T₃ affects growth and myofibrillar proteolysis differently in the cultured muscle cells when insulin is present.

**DISCUSSION**

It is well documented that the rate of proteolysis is increased and the growth is suppressed when cells are cultured in the medium deprived of serum. Serum contains many factors, including hormones, and the factors affect the regulation of muscle cell growth. In the present experiments, protein content and CK activity were decreased and MeHis release was increased by serum-free culture, showing that serum deprivation produces a catabolic response in muscle cell protein metabolism. These results are consistent with the reports of Berger and Dice (11) and Conejo and Lorenzo (12), indicating that when cultured cells are deprived of serum, cell growth is arrested and protein content declines. In skeletal muscle, amino acids, together with hormones, are key regulators of protein metabolism (13, 14). However, because medium-199 contains enough amino acids to meet the cell demand, the contribution of the serum to medium amino acids concentrations was only minor in the present study.

The present study also showed that the effects of thyroid hormone on protein metabolism are modified by the serum. The effects of T₄ and T₃ were summarized in Table 1. The results of the serum (+) group were from our previous report (4), which has shown that the degradation of myofibrillar protein is accelerated by T₃, but not by T₄ in the serum-supplemented normal cell culture. However, the present study clearly shows that thyroid hormone has different effects on protein metabolism in the muscle cells cultured without serum.

It is well known that T₄ is converted to T₃ in many tissues by 5′-deiodinase (15–17). However, the activity of 5′-deiodinase in muscle is much lower than those of the liver and kidney (18), and little or no monodeiodination of T₄ or T₃ occurs in skeletal muscle (4, 19). Thus it is a very important observation that T₄ was also effective to reduce the proteolysis of the muscle cells in the serum-free culture because T₄ is currently assumed to be a prohormone of the biologically potent T₃.

The effect of serum could be explained by a concerted effect of thyroid hormone and insulin. It has been well documented that insulin and insulin-like growth factors (IGFs) play important roles in muscle cell growth and that a withdrawal of these factors enhances intracellular proteolysis in a variety of cell culture (20, 21). It has also been clearly shown in the present study that myofibrillar proteolysis is enhanced by serum depriva-
Fig. 2. Effects of T₄ and T₃ on protein contents, creatine kinase (CK) activity and N⁵-methylhistidine (MeHis) release in serum-free culture. Muscle cells were cultured as described in the legend of Fig. 1. Values given are means±SD (n=6). Values not sharing a common superscript are significantly different (p<0.05).

Fig. 3. Effects of T₃ and insulin on protein contents, creatine kinase (CK) activity, and N⁵-methylhistidine (MeHis) release in serum-free culture. Muscle cells were cultured as described in the legend of Fig. 1. Values given are means±SD (n=6). Values not sharing a common superscript are significantly different (p<0.05).

In a serum-free culture, thyroid hormones may induce the synthesis of antiproteolytic factors such as IGFs and result in reducing the enhanced proteolysis. Forhead et al. have reported that thyroidectomy suppressed the growth hormone receptor (GHR) and IGF-I gene expression in the skeletal muscle of fetal sheep (6), and Ramos et al. have reported a direct effect of thyroid hormone on the hepatic secretion of IGFs both in vivo and in vitro (22). In the present study, T₄ decreased MeHis release as well as insulin in serum-free medium, but T₃ was not effective when insulin was added to the medium. In the absence of insulin, T₃ reduced myofibrillar proteolysis, whereas in the presence of insulin, there was a trend for enhancing proteolysis by T₃. These results suggest that when muscle cells are cultured with serum, thyroid hormone may interact with other fac-
Table 1. Effects of T4 and T3 on protein content, creatine kinase (CK) activity, and N-methylhistidine (MeHis) release in muscle cell culture with or without serum.

|                        | Serum(+) | Serum(-) |
|------------------------|----------|----------|
|                        | T4       | T3       | T4       | T3       |
| Growth (protein content) | ↑        | ↑        | →        | →        |
| Differentiation (CK activity) | ↑        | ↑        | →        | ↓        |
| Protein degradation (MeHis release) | →        | ↑        | ↓        | ↓        |

The results of serum(+) are cited from Nakashima et al. (4). The results of serum(-) are from the present study. T4, thyroxine; T3, triiodothyronine.

Tors such as glucocorticoids and result in enhancing muscle proteolysis. This hypothesis is supported by the results of Hayashi et al., demonstrating that thyroid hormone enhances the catabolic action of glucocorticoids (3).

Further investigation is in progress in our laboratory to understand the precise role of thyroid hormone in muscle proteolysis.

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