



Effect of Thyroid Hormone on the Gene Expression of Myostatin in Rat Skeletal Muscle*

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ABSTRACT : Modification of thyroid hormone levels has a profound effect on skeletal muscle differentiation, predominantly through direct regulation involving thyroid hormone receptors. Nevertheless, little is known about the regulation of myostatin gene expression in skeletal muscle due to altered concentrations of thyroid hormone. Thus, the goal of our study was to find out whether altered thyroid states could change the gene expression of myostatin, the most powerful inhibitor of skeletal muscle development. A hyperthyroid state was induced in rats by daily injections of L-thyroxine 20 mg/100 g body weight for 14 days, while a hypothyroid state was induced in another group of rats by administering methimazole (0.04%) in drinking water for 14 days. After a period of 14 days of L-thyroxine treatment we observed a significant increase of myostatin expression both in mRNA and protein level. However, decreased expression of myostatin mRNA and protein were observed in hypothyroid rats. Furthermore, our studies demonstrated that the upregulation of myostatin gene expression might be responsible for the loss of body weight induced by altered thyroid hormone levels. We concluded that myostatin played a role in a metabolic process in muscle that was regulated by thyroid hormone. (**Key Words :** Thyroid Hormone, Skeletal Muscle, Hyperthyroidism, Hypothyroidism, Myostatin, Expression)

INTRODUCTION

Myostatin (MSTN), previously known as growth differentiation factor-8, is a member of the transforming growth factor superfamily, which is predominantly expressed in skeletal muscle throughout life, from the early stages of embryogenesis to late adulthood (McPherron and Lee, 1997). The expression pattern of myostatin suggests that it might play an important role in regulating muscle development or function (McPherron and Lee, 1997). Mice completely lacking myostatin were shown to have dramatic and widespread increases in skeletal muscle mass. Conversely, overexpression of myostatin suppressed cell proliferation and differentiation in rat myoblasts (Eun-Jung et al., 2006). Furthermore, increased myostatin expression has been found in certain physiological conditions in which

the common symptom is muscle atrophy (Gonzalez-Cadavid et al., 1998; Carlson et al., 1999; Lalani et al., 2000; Urban F et al., 2000; Wehling et al., 2000). Thus, myostatin functions as an inhibitor of skeletal muscle growth and development (McPherron and Lee, 1997).

The maintenance of muscle mass is determined by a fine balance between protein synthesis and breakdown, a dynamic homeostatic state modulated by numerous anabolic and catabolic factors at different levels (Jian-hai et al., 2008). The precise processes by which these factors affect skeletal muscle growth or influence myostatin gene expression remain unclear.

However, investigations in the 1950s demonstrated that thyroid hormone (TH) plays a very significant role in skeletal muscle formation (Scow, 1951). Rats, thyroidectomized at birth, had skeletal muscle deficiency characterized by musculature that was thin, pale and flabby. In addition, the subnormal muscle mass was associated with a decrease in the amount of myosin and 'water soluble' protein fractions. Later studies concluded that alterations in circulating thyroid hormone had a significant role in affecting muscle phenotype and contractile properties, even in the presence of the dominant influence on the muscle of the pattern of motor neurone activity (Nicol and Bruce, 1981; Nwokyee et al., 1982). These results indicated that TH

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alters muscle properties, and is correlated with the clinical abnormalities of muscle function associated with thyrotoxicosis or myxoedema. Hyperthyroidism exacerbates a number of human myopathies, including myasthenia gravis and myotonic dystrophy (Nicol and Bruce, 1981). Hypothyroidism changed the muscle phenotype of mdx-dystrophin-deficient mice to resemble that of Duchenne dystrophy; furthermore, hypothyroidism but not hyperthyroidism in mdx mice decreased post-injury regeneration of muscle tissue (McIntosh et al., 1994; Anderson et al., 1994).

Both thyroid hormone and myostatin have an important influence on the skeletal muscle. So, what is the relationship between them? Furthermore, because the myostatin promoter region was found to contain putative thyroid hormone response elements (Ma et al., 2001), we considered the possibility that TH might regulate myostatin gene expression. Therefore, to answer the above questions, we investigated the effects of thyroid hormone on myostatin transcriptional activity by using real-time PCR. Subsequently, we examined the effects of thyroid hormone on endogenous myostatin protein expression in the hyperthyroid (HYPER) state and hypothyroid (HYPO) state.

MATERIALS AND METHODS

Animal preparation

The Expert Committee of Tianjin Institute of Animal Science and Veterinary approved the investigation and the animals were treated in agreement with the guidelines of the Care and Use of Animals from Tianjin Academy of Agricultural Science (Tianjin, PRC).

Male adult Wistar rats (weight of 250-260 g, from the Experimental Animal Center, Xiehe University, Beijing, China) were kept in a room with controlled lighting (12 h light, 12 h darkness, lights on at 0800 h, off at 2000 h) and temperature (22°C). 36 adult male rats were divided into 3 groups (12 in each group) with even initial body weight distribution in each group. Before the pharmacological treatment, all the rats in three groups were anesthetized and 2 ml of blood was taken from individual rat for evaluation of initial T4 concentration. Then the first group was regarded as the control group. The second group (hypo-group) was administered 0.04% methimazole (Sigma, USA) in drinking water for consecutive 14 days (Isman et al., 2003). The last group (hyper-group) was treated with daily injections of L-thyroxine (T4, Sigma, USA) 20 mg/100 g body weight for 14 days (Bruno et al., 2005). All rats had unlimited access to standard laboratory rodent diet (LabDiet, USA) and clean water. Feed intake was recorded daily. Animals of each group were weighed and killed by decapitation 18 h after the last day of treatment. Blood samples were then collected for hormonal evaluation

(Ortiga-Carvalho et al., 1997). Muscle tissues from the legs were dissected out as quickly as possible and frozen in liquid nitrogen for real-time PCR and western blotting.

Serum T4 quantification

The concentrations of total T4 in serum were measured by a specific and highly sensitive RIA kit (Diagnostic Products Corporation, USA), using a standard curve made with rat serum free of hormones.

Skeletal muscle transcript levels

Total RNA was isolated from frozen muscle tissues using TRIZOL extraction (Gibco BRL, USA). RNA concentration was measured by absorption at 260 nm and the purity of the RNA was guaranteed based on a 260 nm/280 nm absorption ratio of ~2.0. A total of 2 mg of RNA per reaction was reverse transcribed using M-MLV reverse transcriptase (Promega, USA) into cDNA using Oligo dT (Promega, USA) as the primer according to the manufacturer's protocol. Primers for real-time PCR were designed as (Sense primer) 5'-AAAGAGGGGCTGTGTAATGCG-3' and (Anti-sense primer) 5'-TCCGTGGTAGCGTGATAATCG-3' for amplification, based on the gene sequence of myostatin in GenBank (AF019624).

Quantification of transcript levels was performed using the 7900HT Sequence Detection System (Applied Biosystems, USA) on cDNA. A sample of 1 ml of 1:10 diluted cDNA was analyzed using SYBR Green PCR master mix (Applied Biosystems, USA). All samples were analyzed in triplicate along with the gel purified PCR product standard. The PCR reaction vessel (25 ml) contained: 12.5 ml 2x PCR master mix (1x), 0.2 µM sense and anti-sense primers. Two-step amplification was performed as follows: An initial hold at 95°C for 10 min was followed by 40 cycles of denaturing at 95°C for 20 s followed by annealing/extension at 61°C for 40 s. The success of each reaction was deduced based on observing a single reaction product (245 bp) on an agarose gel and a single peak on the DNA melting temperature curve determined at the end of the reaction.

Western blotting

The frozen muscles were homogenized in lysis buffer (10 mM Tris-HCl; 1 µg/ml Aprotinin; 1 mM EDTA; 100 mM NaCl; 100 µg/ml PMSF). The homogenate was centrifuged at 13,000×g for 20 min, and the protein content of the supernatant was measured using the Bradford assay. Cytosolic protein samples (40 µg per lane) were treated with Laemmli sample buffer (Laemmli, 1970), heated in a boiling water bath for 4 min and electrophoretically separated using 15% SDS-PAGE gel. Electro transfer of proteins from the gel to the permeabilized polyvinylidenedifluoride (PVDF) membrane was performed

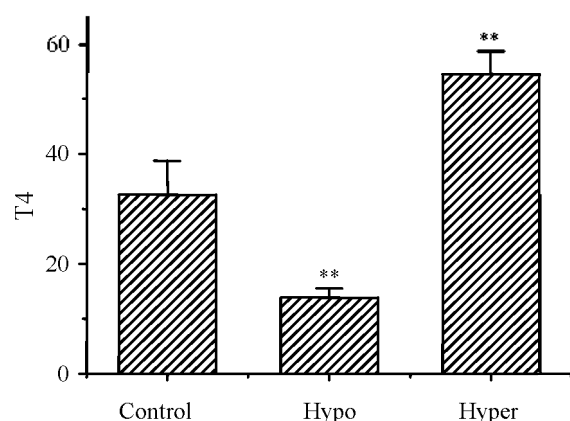


Figure 1. Levels of serum T4 (ng/ml) in different TH states after pharmacological treatment. Concentrations of serum T4 were measured using RIA. Data represents the mean±SEM, * p<0.05; ** p<0.01 vs. control group (n = 12 per group).

for 90 min at 300 mA and the uniformity of loading was checked with Ponceau S on completion of transfer. Nonspecific protein binding to the membrane was reduced by incubating the filter 2 h at room temperature in blocking buffer (TBS with 0.1% Tween-20; 5% w/v non-fat milk powder). The membrane was incubated with primary antibody, goat anti-GDF-8/myostatin IgG (1:500, Santa Cruz, CA, USA), diluted in blocking buffer overnight at 4°C. The following morning the membrane was rinsed in wash buffer (TBS with 0.1% Tween-20) three times for 5 min each time. The membrane was then incubated for 1 h at ambient temperature with gentle agitation in blocking buffer containing the appropriate secondary antibody, HRP-linked mouse anti-goat IgG (1:2,000, Santa Cruz, CA, USA) and anti-biotin antibody (1:1,000, CellSignaling, USA) to detect biotinylated standards. The membrane was then cleaned three times for 5 min in wash buffer. Membranes were exposed to ECL chemiluminescent detection HRP reagents (Santa Cruz, USA) mixed in the proportion 1:1 in 10 ml for 1 min. Membranes were partially dried, wrapped in saran and exposed to X-ray film. To adjust the protein sample loading, the membranes were stripped in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris·HCl, pH 6.7) at 50°C for 30 min and then reprobbed with a rat monoclonal antibody for GAPDH. The concentrations of myostatin and GAPDH proteins were quantified using densitometry scanning by a ChemiDoc XRS System (Bio-Rad, USA). Concentrations of myostatin protein were corrected by the optical density (OD) of GAPDH protein. Each sample was analyzed in triplicate.

Statistical analysis

Mean values between different groups were compared by one-way analysis of variance (ANOVA). Post hoc multiple comparisons between each group and control

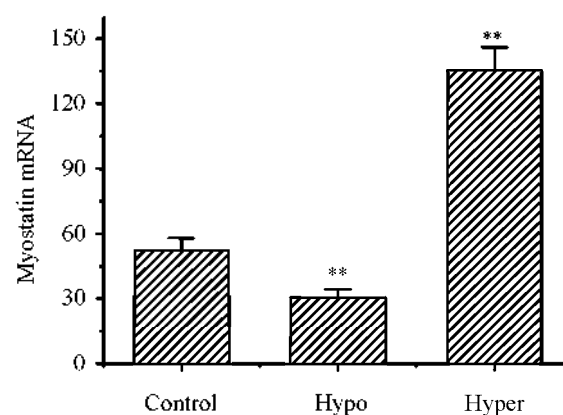


Figure 2. MSTN mRNA expression (fg/μg RNA) in rat skeletal muscle in different TH states after pharmacological treatment. MSTN mRNA was quantified using real-time PCR. Data represents the mean±SEM. * p<0.05; ** p<0.01 vs. control group (n = 12 per group).

groups were made using the least squares difference (LSD) test. The statistical significance level was set to 0.05. All analysis was performed with the SPSS program (v.12.0, Abacus Concepts, Berkeley, CA, USA). Data are presented in the text as mean±SEM unless otherwise stated.

RESULTS

The effectiveness of T4 treatment was determined by the concentrations of total serum T4 and body weight (BW). Before the treatment, the initial T4 concentration was 36.51±4.36 ng/ml for the control group, 37.29±4.87 ng/ml for the hypo-group and 35.86±4.41 ng/ml for the hyper-group, there were no significant differences between them (p>0.05). After the treatment, the total serum T4 concentration in the hyperthyroid rats was shown to increase approximately 2-fold higher (Figure 1) than that detected in the control group, while the value for the hypothyroid rats was at least 2-fold lower than that of the control group (Figure 1). The BW of rats in the hyperthyroid group decreased, but the BW of rats in the hypothyroid group did not change significantly (Figure 4). Feed intake was significantly increased in the hyper-group (28.31±2.74 g/d, p<0.01) and decreased in the hypo-group (23.95±2.18 g/d, p<0.05) compared with that of control group (25.17±2.32 g/d). These results confirmed the induction of hypothyroidism and hyperthyroidism in our studies.

To explore the effect of different thyroid states on myostatin mRNA expression in skeletal muscle, we detected the mRNA level using two step quantitative real-time PCR. The results showed a significant elevation of expression of myostatin mRNA in hyperthyroid rats after pharmacological induction (Figure 2). This trend correlated

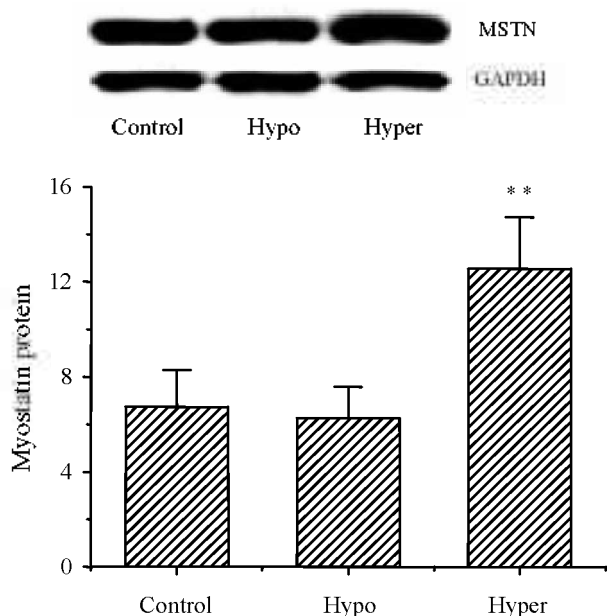


Figure 3. MSTN protein expression in rat skeletal muscle in different TH states after pharmacological treatment. Top: MSTN protein expression in skeletal muscle, analyzed by western blotting. Blots from representative experiments are shown. Bottom: MSTN protein was quantified by densitometry. Data represents the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs. control group. Sample loadings were adjusted by the OD of GAPDH ($n = 12$ per group).

with the total level of T4 in serum ($r = 0.883$, $p < 0.01$). Meanwhile, we found a significant decrease of myostatin mRNA in hypothyroid rats (Figure 2). Moreover, like the situation in hyperthyroid rats, the change of myostatin mRNA also correlated with the total T4 in serum ($r = 0.791$, $p < 0.01$). We also found the expression of myostatin mRNA was significantly negatively correlated with the body weight ($r = -0.757$, $p < 0.01$) in the hyperthyroid rats. The protein levels followed the same profile as myostatin mRNA expression, except that the expression of myostatin protein in hypothyroidism decreased, but not significantly compared with that of euthyroidism. We observed a significant increase of myostatin protein content in hyperthyroid rats after stopping pharmacological treatment (Figure 3).

DISCUSSION

The purpose of this study was to measure the effect of different TH levels on the expression of myostatin in skeletal muscle. It is well known that thyroid hormone is an important hormone functioning in many tissues, including skeletal muscle. Early studies showed that thyroid hormone plays a very significant role in skeletal muscle formation (Scow, 1951; 1953). The substantial effects of TH on muscle fiber composition and MHC gene expression in

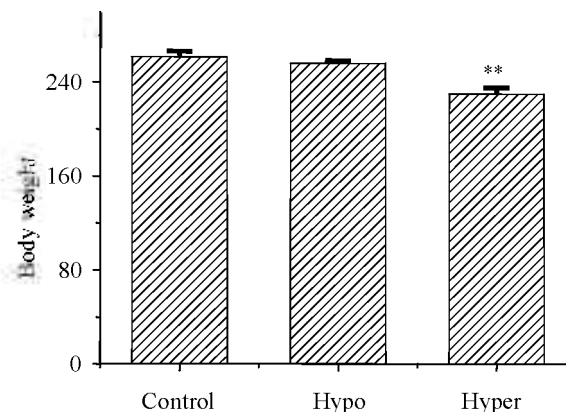


Figure 4. Body weights (g) of rats in different thyroid TH states after pharmacological treatment. Data represents the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs. control group ($n = 12$ per group).

skeletal muscles have also been established (Izumo et al., 1986). T3 could promote the terminal differentiation of the myogenic cell line, C2.7, increase MyoD gene transcription and cause the precocious expression of the MyoD gene and contractile protein mRNAs (Camac et al., 1992). As myostatin is an important negative regulator of skeletal muscle, TH must have some effect on the expression of myostatin. Some researchers have used pharmacological induction of a hyperthyroid state in an attempt to enhance the impact of bed rest on skeletal muscle properties (Lovejoy et al., 1999; Zachwieja et al., 1999), observing an increase in levels of myostatin circulation (Zachwieja et al., 1999; Lalani et al., 2000), and the putative TH response element has been identified in human myostatin (Ma et al., 2001), but much is still unknown about the exact relationship between the thyroid hormone level and myostatin expression.

This study investigated, for the first time, the role of different TH levels on the myostatin mRNA and protein expression in rat skeletal muscle. Studies have shown that many of the known cellular effects of TH result from its interaction with nuclear receptors (THRs) bound to DNA elements of particular genes, a mechanism by which TH either promotes or inhibits gene transcription (Shupnik et al., 1985; Samuels et al., 1989; Glass and Holloway, 1990). The putative THR response element has been identified in the human myostatin gene (Ma et al., 2001). This suggested the expression of myostatin might be regulated by thyroid hormone. This was confirmed by the results of our study. We found increased expression of myostatin mRNA in the hyper-group and decreased expression of myostatin in the hypo-group compared with the control group, which suggested TH could induce the upregulation of myostatin in the hyper-group compared with the control group. This result accorded with the increased levels of circulating myostatin observed in other studies (Zachwieja et al., 1999; Lalani et al., 2000). But in these studies, it was unclear if

such an observation represented the response to bed rest or was primarily a function of pharmacological intervention. However, the physical activity of the animals in our experiment was not restricted, so the possibility of the animal's activity level influencing gene expression was excluded. Thus, we thought the exogenous TH could upregulate the mRNA expression of myostatin in skeletal muscle. However, myostatin could also be regulated by many other factors. For example, recombinant human follistatin could change the myostatin expression pattern (Kocamis et al., 2004). Moreover, in addition to the THR response element, the myostatin promoter contains other elements important for muscle growth. The presence of myocyte enhancer factor-2 and nuclear factor- κ B, among others (Ma et al., 2001), indicated that myostatin was probably regulated not only by TH but also by other factors. The role of these factors in acute and chronic regulation of myostatin gene expression during different TH states remains to be elucidated.

The loss of body weight was increased in the hypergroup compared with that of the control group. This could be attributed to the upregulation of myostatin in hyperthyroidism. Myostatin is the most powerful inhibitor of muscle growth. Normal function of myostatin in adult muscle keeps satellite cells in a quiescent state (Thomas et al., 2000; Joulia et al., 2003; McCroskery et al., 2003), and myostatin level does not fluctuate much in normal adult muscle (Kambadur et al., 1997). Muscle-wasting-related disorders are correlated with upregulation of myostatin. Patients with chronic diseases such as cancer and AIDS often develop cachexia, and the myostatin level in serum also increase. For example, elevated levels of circulating myostatin have been reported in HIV-infected patients with muscle wasting (Gonzalez-Cadavid et al., 1998). Overexpression of myostatin in mice could induce a wasting syndrome that has many of the hallmarks of human cachexia, including fat and muscle loss in the setting of normal caloric intake (Zimmers et al., 2002). Taking the biological function of myostatin into account, this cachexia probably results from the increased myostatin level. Unfortunately, the precise molecular mechanism is still unclear. In our study, we noted the loss of body weight in hyperthyroid animals resembled the clinical symptoms of hyperthyroidism (Nicol and Bruce, 1981; Anderson et al., 1994; McIntosh et al., 1994). Furthermore, the loss of body weight was concomitant with the upregulation of myostatin expression, which was similar to the symptoms of cachexia. This result indicated that the loss of body weight, observed in hyperthyroidism, might at least partly be induced by the increased expression of myostatin. The upregulated myostatin blocked proliferation and differentiation of myoblasts (Thomas et al., 2000; Joulia et al., 2003), inhibited the protein synthesis in cells (Taylor et al., 2001),

and even induced the wasting syndrome.

We observed the expression of myostatin decreased in hypothyroid rats compared with euthyroid rats. However, body weights of hypothyroid rats did not change significantly compared with those of euthyroid rats. Obviously, the downregulation of myostatin in muscle would not answer for the constant body weight in hypothyroid state. Although it was generally thought that hypothyroidism can lead to the increase of body weight (Weaver et al., 2008), some studies had shown opposite results (Kokkinos et al., 2007; Weng et al., 2007). We did not observe the same result either, which suggested the complexity of the body weight in hypothyroidism. We noticed that the duration of hypothyroidism in these studies changed, from newly established (diagnosed) hypothyroidism to long-lasting hypothyroidism (Rebello et al., 1993; Kokkinos et al., 2007; Weng et al., 2007; Fazylov et al., 2008). Thus, we guessed the difference of duration of hypothyroidism might account for the difference of body weight. But unfortunately, our study focused on only the gene expression of myostatin in different thyroid hormone states, and our results were lacked of a long-time observation. As a result, we could not determine this hypothesis from our data. However, these problems could be solved if we considered the duration of hypothyroidism in future studies.

In addition, TH levels might play an important role in inducing the loss of body weight, but they are not the only factor involved in weight loss. Exogenous TH could be expected to impact all thyroid-sensitive tissues, including, but not limited to, skeletal muscle. As an example of a potential unintended action, T3 treatment could induce a dramatic decrease in TSH levels (Zachwieja et al., 1999). It has become evident that TSH has direct biological activity separate from its actions as part of the thyroid axis (Bell et al., 2000; Sellitti et al., 2000; Monici et al., 2002). For example, it had been suggested that TSH might exert anti-apoptotic activity in human skeletal muscle (Monici et al., 2002). Taken together, these data suggested that metabolic processes induced by TH levels might result from a complicated mechanism, in which myostatin plays an important role.

In summary, we demonstrated that myostatin expression could be upregulated in hyperthyroidism and downregulated in hypothyroidism. Furthermore, our studies demonstrated that TH might induce loss of body weight through the upregulation of myostatin gene expression. Previous reports have shown that recombinant myostatin inhibits muscle cell replication and protein synthesis. Therefore, it is possible that an increase in myostatin expression resulting from hyperthyroidism may cause a decrease in protein synthesis that in turn might lead to a loss of body weight. This hypothesis needed to be tested *in vitro*.

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