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Experimental Hyperthyroidism in Rats Increases the Expression of the Ubiquitin Ligases Atrogin-1 and MuRF1 and Stimulates Multiple Proteolytic Pathways in Skeletal Muscle

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ABSTRACT

Muscle wasting is commonly seen in patients with hyperthyroidism and is mainly caused by stimulated muscle proteolysis. Loss of muscle mass in several catabolic conditions is associated with increased expression of the muscle-specific ubiquitin ligases atrogin-1 and MuRF1 but it is not known if atrogin-1 and MuRF1 are upregulated in hyperthyroidism. In addition, it is not known if thyroid hormone increases the activity of proteolytic mechanisms other than the ubiquitin–proteasome pathway. We tested the hypotheses that experimental hyperthyroidism in rats, induced by daily intraperitoneal injections of 100 μg/100 g body weight of triiodothyronine (T3), upregulates the expression of atrogin-1 and MuRF1 in skeletal muscle and stimulates lysosomal, including cathepsin L, calpain-, and caspase-3-dependent protein breakdown in addition to proteasome-dependent protein breakdown. Treatment of rats with T3 for 3 days resulted in an approximately twofold increase in atrogin-1 and MuRF1 mRNA levels. The same treatment increased proteasome-, cathepsin L-, and calpain-dependent proteolytic rates by approximately 40% but did not influence caspase-3-dependent proteolysis. The expression of atrogin-1 and MuRF1 remained elevated during a more prolonged period (7 days) of T3 treatment. The results provide support for a role of the ubiquitin–proteasome pathway in muscle wasting during hyperthyroidism and suggest that other proteolytic pathways as well may be activated in the hyperthyroid state. J. Cell. Biochem. 108: 963–973, 2009.

KEY WORDS: MUSCLE ATROPHY; MUSCLE WASTING; PROTEIN BREAKDOWN; PROTEIN DEGRADATION; THYROID HORMONE; UBIQUITIN; PROTEASOME; THYROTOXICOSIS

Hyperthyroidism is frequently accompanied by muscle wasting and weakness [Hasselgren et al., 1984; Adlerberth et al., 1987; Klein and Ojamaa, 2000; Riis et al., 2005; Brennan et al., 2006]. Previous studies suggest that loss of muscle mass in the hyperthyroid state is mainly caused by increased protein degradation, in particular degradation of the myofibrillar proteins actin and myosin [Goldberg, 1980; Carter et al., 1981; Yates et al., 1981; Rodier et al., 1984; Angeras and Hasselgren, 1985; Adlerberth et al., 1987], although reduced protein synthesis may also contribute to loss of muscle mass in hyperthyroid patients [Flaim et al., 1978; Morrison et al., 1988].

Muscle protein breakdown in various catabolic conditions reflects activation of multiple proteolytic mechanisms, including lysosomal, calpain-, caspase-3-, and ubiquitin–proteasome-dependent protein degradation [Hasselgren et al., 2002; Du et al., 2004]. Among these mechanisms, increased ubiquitin–proteasome-dependent proteolysis is particularly important for the development of muscle atrophy, and increased expression of various components of the ubiquitin–proteasome pathway has been documented in a number of catabolic conditions [for reviews, see Glass, 2003; Hasselgren et al., 2005]. The expression of the muscle-specific ubiquitin ligases atrogin-1 and MuRF1 has been found to be substantially upregulated in atrophying muscle, and there is evidence that the expression and activity of these ubiquitin ligases may be rate limiting for ubiquitin–proteasome-dependent proteolysis in catabolic muscle [Bodine et al., 2001; Gomes et al., 2001]. In fact, elevated mRNA levels for atrogin-1 and MuRF1 are sometimes used as "molecular markers" of muscle wasting, although there is

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not an absolute correlation between stimulated protein breakdown rates and the expression of atrogin-1 and MuRF1 [Fareed et al., 2006; Jin and Li, 2007].

Previous observations suggest that hyperthyroidism stimulates proteasome-dependent protein breakdown in skeletal muscle [Tawa et al., 1997]. It is not known, however, if the expression of atrogin-1 and MuRF1 is increased by thyroid hormone. In addition, the influence of hyperthyroidism on proteolytic mechanisms, other than the ubiquitin–proteasome system, is not known. In the present study, we tested the hypotheses that experimental hyperthyroidism in rats upregulates the expression of atrogin-1 and MuRF1 in skeletal muscle and stimulates lysosomal, calpain- and caspase-3-dependent protein breakdown in addition to proteasome-dependent proteolysis.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Experimental hyperthyroidism was induced in male Sprague–Dawley rats by daily intraperitoneal injections of 100 μg 3,5,3′-triiodothyronine (T3; Sigma, St. Louis, MO) per 100 g body weight during three consecutive days (except in one experiment in which the treatment was extended to 7 days) as described previously [Angeras and Hasselgren, 1985]. Control rats were injected with corresponding volumes (0.2 ml/100 g body weight) of solvent (5 mM NaOH). In a separate experiment, the effects of a lower dose of T3 (10 μg/100 g body weight) were tested. All injections were performed between 9 and 10 AM. The rats had free access to water and food (F6 Rodent Diet #7964, Harland Teklad, Madison, WI) throughout the experiments. Body weight and food intake were measured daily. Twenty-four hours after the final T3 injection, rats were anesthetized with pentobarbital (40 mg/kg) and the extensor digitorum longus (EDL) muscles were gently dissected with intact tendons. Muscles were either weighed and incubated for measurement of protein breakdown rates (see below) or immediately frozen in liquid nitrogen and stored at −80°C for subsequent measurement of mRNA levels, protein levels and caspase-3 activity. In some experiments, muscles were harvested for measurement of mRNA levels 3 and 7 days after the final T3 injection. Blood samples were obtained by heart puncture and serum was stored at −80°C for measurement of T3 and thyroid stimulating hormone (TSH) levels.

The rats were treated and cared for in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Animal Care and Utilization Committee at the Beth Israel Deaconess Medical Center.

MUSCLE INCUBATIONS

Protein degradation was measured in intact EDL muscles incubated at resting length for 2 h under physiological conditions in a shaking water bath at 37°C as described in detail previously [Hasselgren et al., 1990; Fareed et al., 2006; Poylin et al., 2008]. Protein breakdown rates were determined as release of free tyrosine into the incubation medium measured according to Waalkes and Udenfriend [1957]. Because tissue levels of free tyrosine remained stable during incubation and protein synthesis was blocked by the presence of cycloheximide (0.5 mM) in the incubation medium, and because tyrosine is not synthesized or degraded in muscle tissue [Goldberg et al., 1980], release of tyrosine into the incubation medium reflected net protein degradation in the incubated muscles.

In order to assess the contribution of different proteolytic pathways to protein degradation in muscles from control and T3-treated rats, muscles were incubated in the absence or presence of different inhibitors and any difference in protein degradation observed in the absence or presence of the inhibitor was interpreted as reflecting the contribution of that specific proteolytic pathway. In order to assess proteasome-dependent proteolysis, muscles were incubated in the absence or presence of 10 μM of the proteasome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) (Sigma–Aldrich, St. Louis, MO). The use of this inhibitor in the present study was based on a previous report in which 10 μM MG132 was used to assess proteasome-dependent protein breakdown in muscles from control and T3-treated rats [Tawa et al., 1997]. MG132 is a rapidly acting and reversible inhibitor of the proteasome described in previous reports [Polombella et al., 1994; Lee and Goldberg, 1996]. Although it is a selective and potent inhibitor of the proteasome, it also has some inhibitory effects on other proteases, including calpains and cathepsins. In order to assess overall lysosomal protein degradation, muscles were incubated in the absence or presence of 100 μM leupeptin (Sigma–Aldrich). Leupeptin has been used in previous reports to assess lysosomal protein degradation in incubated rat muscles and inhibits lysosomal activity by inhibiting serine, thiol, and cysteine proteases [Lowell et al., 1986; Tiao et al., 1994]. Among different lysosomal proteases, the contribution of cathepsin L, a lysosomal cysteine protease, to total protein degradation was assessed by incubating muscles in the absence of presence of 80 μM Cathepsin L Inhibitor IV (EMD Chemicals, Gibbstown, NJ). Cathepsin L Inhibitor IV was used in previous experiments examining the role of cathepsin L in muscle protein degradation [Fareed et al., 2006] and selectively blocks cathepsin L cysteine protease activity [Yasuma et al., 1998]. Calpain-dependent protein degradation was calculated by incubating muscles in the absence of presence of 100 μM calpeptin (EMD Chemicals). This drug blocks calpain activity by inhibiting calpain cysteine protease activity and is commonly used as a specific calpain inhibitor in incubated muscles [Ebisui et al., 1994; Goll et al., 2003]. Caspase-3-dependent proteolysis was assessed by incubating muscles in the absence or presence of 10 μM Ac-DEVD-CHO. Ac-DEVD-CHO is a selective caspase-3 inhibitor and was used in previous experiments in our [Wei et al., 2005] and other laboratories [Du et al., 2004] to assess caspase-3-dependent muscle protein degradation.

MEASUREMENT OF T3 AND TSH

Serum levels of T3 and TSH were measured by radioimmunoassay (Millipore Laboratories, St. Charles, MO).

DETERMINATION OF CASPASE-3 ACTIVITY

Caspase-3 activity was measured as described in detail recently [Du et al., 2004; Wei et al., 2005] using the caspase-3 specific fluorogenic substrate Ac-DEVD-AMC (Calbiochem-Novabiochem,

San Diego, CA) and the caspase-3 inhibitor Ac-DEVD-CHO (Calbiochem-Novabiochem) for validation of the assay.

MEASUREMENT OF MRNA LEVELS

For determination of mRNA levels, RNA was extracted and real-time PCR was performed by Taqman as described in detail recently [Wei et al., 2005; Fareed et al., 2006]. The sequences for the forward, reverse, and double-labeled oligonucleotides for atrogin-1 were, respectively: 5′-CTT TCA ACA GAC TGG ACT TCT CGA-3′, 5′-CAG CTC CAA CAG CCT TAC TAC GT-3′, and 5′-TGC CAT CCT GGA TTC CAG AAG ATT CAA C-3′. The corresponding sequences for MuRF1 were 5′-GGA CTC CTG CCG AGT GAC C-3′, 5′-GCG TCA AAC TTG TGG CTC AG-3′, and 5′-AGG AAA ACA GCC ACC AGG TGA AGG AGG-3′. The corresponding sequences for ubiquitin were 5′-GGC AAG CAG CTG GAA GAT G-3′, 5′-CAG GTG CAA GGT GGA CTC CT-3′, and 5′-CCG CAC CCT GTC AGA CTA CAA CAT CCA-3′. The corresponding sequences for proteasome subunit C3 were 5′-GCC TGG GTC ATG CCA CTC T-3′, 5′-CCA CCA CCT GAC CAA CCT TT-3′, and 5′-TGG TAC CGA TGT CGA TGC AGC TAA CCT C-3′. The corresponding sequences for caspase-3 were 5′-CAC TGG AAT GTC AGC TCG CA-3′, 5′-TCA GGG CCA TGA ATG TCT CTC-3′, and 5′-TGC TGG TTG TGG CCC TGC CAG T -3′. The corresponding sequences for μ-calpain were 5′-GGG TCA GCC TGT GCA CTT G-3′, 5′-GCT CTG ATT GTG CCC GAG A-3′, and 5′-AGC GTG ACT TCT TCC TGG CCA ATG C-3′. The corresponding sequences for m-calpain were 5′-GCC AGG GAG CGG TCA GAT-3′, 5′-GGG CAG CTT GAA GCG GTT-3′, and 5′-CCT TCA TCA ACC TCC GGG AGG TCC T-3′. Cathepsin L mRNA levels were determined using the ABI TaqMan Gene Expression Assay (Assay ID: Rn00565793_m1). Amplification of 18S rRNA was performed in the same reaction tubes as an internal standard with an alternatively labeled probe (VIC-labeled probe) to distinguish its product from that derived from the specific genes studied here. The mRNA concentrations were normalized to the 18S mRNA levels and expressed as arbitrary units (AU). Measurements were performed in duplicate for each standard and rat muscle sample.

WESTERN BLOTTING

Atrogin-1, MuRF1, and caspase-3 protein levels were determined by Western blotting of extracted muscle proteins as described in detail previously [Wei et al., 2005; Poylin et al., 2008]. In short, muscle protein extracts were subjected to SDS-PAGE using 10% gels, followed by transfer to PVDF membranes. The membranes were blocked with 5% non-fat milk in TTBS buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% Tween-20, pH 7.4) and incubated with the following primary antibodies and the appropriate secondary antibodies: a rabbit polyclonal anti-mouse atrogin-1 antibody (1:2,000, a gift from Dr Stuart H. Lecker, Harvard Medical School, Beth Israel Deaconess Medical Center, MA); a rabbit polyclonal anti-rat MuRF1 antibody (1:1,000, ECM Biosciences, Versailles, KY); a rabbit polyclonal anti-human MuRF1 antibody (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA); a rabbit polyclonal anti-rat caspase-3 antibody (1:1,000, Cell Signaling Technology, Danver, MA). A mouse monoclonal anti-rat α-tubulin antibody (1:2,000, Sigma–Aldrich) was used for loading control. Immunoreactive protein bands were detected by using the Western Lightning kit for enhanced chemiluminescence detection (Perkin-Elmer Life Sciences) and analyzed using the public domain Image J program (http://rsb.info.nih.gov/ijl). The bands were quantified by densitometry and normalized to the appropriate loading controls.

STATISTICAL ANALYSIS

Results are reported as means ± SEM. Statistical analysis was performed using Student's *t*-test or ANOVA followed by Holm Sidak's or Dunn's post-hoc analysis as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

When rats were treated with 100 μg/100 g body weight of T3 for 3 days, serum concentrations of T3 were increased to a level consistent with severe hyperthyroidism (Table I). As expected, the T3 treatment also resulted in suppressed TSH levels. The initial body weight was almost identical in control and T3-treated rats whereas after 3 days of T3 treatment, body weight was significantly lower in hyperthyroid than in control rats, despite unchanged food intake (Table I). After T3 treatment for 3 days, muscle protein breakdown rates were increased by approximately 45% and muscle weight and protein content were reduced by approximately 15% (Fig. 1). Taken together, the results in Table I and Figure 1 suggest that the experimental protocol used in the current study resulted in changes in hormone levels, body weight and muscle mass similar to changes seen in patients with severe hyperthyroidism [Yates et al., 1981; Hasselgren et al., 1984; Rodier et al., 1984; Adlerberth et al., 1987; Klein and Ojamaa, 2000; Riis et al., 2005; Brennan et al., 2006] and that were reported previously in T3-treated rats [Carter et al., 1981; Angeras and Hasselgren, 1985; Tawa et al., 1997].

One of the main purposes of the present study was to determine the influence of experimental hyperthyroidism on the expression of atrogin-1 and MuRF1 in skeletal muscle. As can be seen in Figure 2A,B, muscle atrogin-1 and MuRF1 mRNA levels were increased approximately twofold 1 day after the final T3 injection. The effects of T3 treatment on atrogin-1 and MuRF1 expression were reversible; no differences between control and T3-treated rats in atrogin-1 mRNA levels were noticeable 3 and 7 days after cessation of the T3 treatment and MuRF1 mRNA levels were normalized 7 days after the T3 treatment was stopped.

The increase in atrogin-1 mRNA levels in T3-treated rats was accompanied by increased atrogin-1 protein levels (Fig. 3). Despite

TABLE I. Serum Hormone Levels, Body Weight, and Food Intake in Rats Treated for 3 days With 100 μg/100 g Body Weight of T3 or Corresponding Volumes of Solvent

	Control (n = 8)	T3 (n = 8)
Serum T3 (μg/ml)	1.0 ± 0.2	17.4 ± 0.7*
Serum TSH (μg/ml)	1.33 ± 0.26	N.D.
Initial body weight (g)	84 ± 2	83 ± 1
Final body weight (g)	108 ± 2	99 ± 1*
Food intake (g/100 g bw/day)	10.2 ± 0.17	9.9 ± 0.03

Results are means ± SEM. N.D., not detectable.
*$P < 0.05$ versus control.

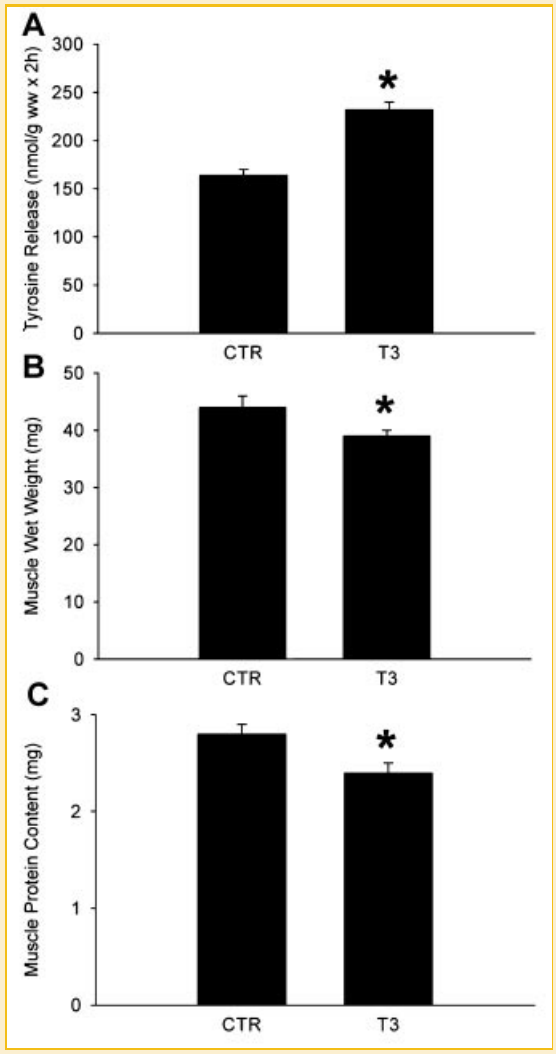

Fig. 1. (A) Muscle protein breakdown rates, (B) muscle weight, and (C) muscle protein content in control and T3-treated rats. Rats were treated for 3 days with 100 μg/100 g body weight of T3 or corresponding volumes of solvent. Twenty-four hours after the last T3 injection, EDL muscles were incubated for measurement of protein degradation rates (assessed as release of tyrosine into the incubation medium as described in Materials and Methods Section). Other muscles were weighed, and total muscle protein content was determined. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus control.

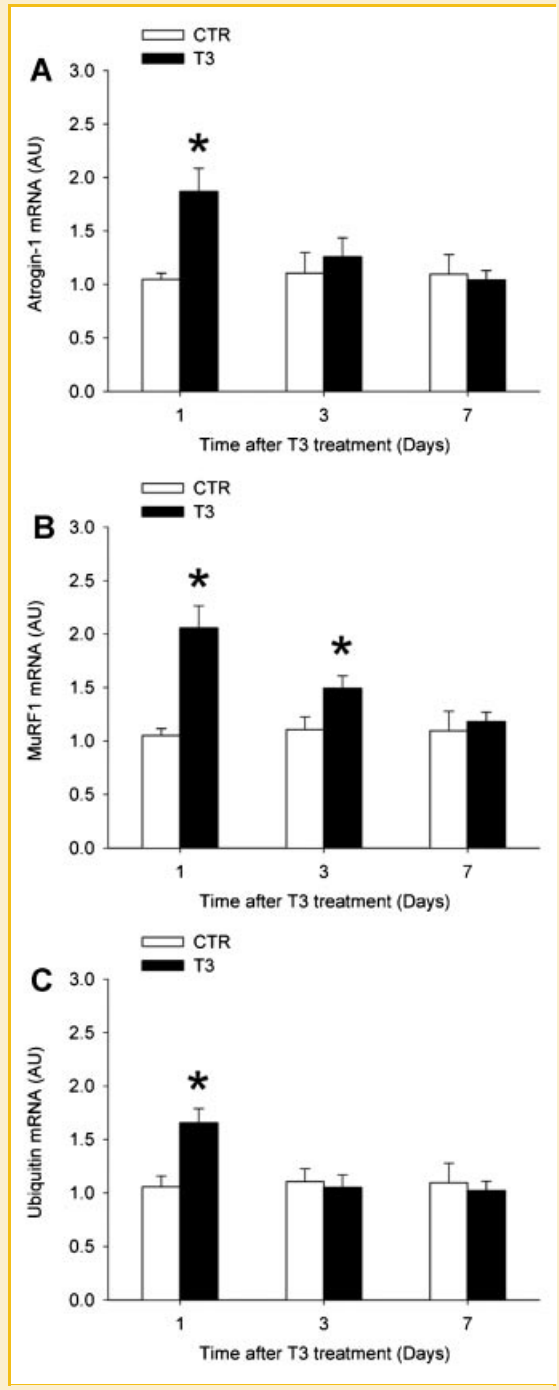

Fig. 2. mRNA levels for (A) atrogin-1, (B) MuRF1, and (C) ubiquitin in EDL muscles at various time points (1, 3, and 7 days) after treatment of rats for 3 days with T3 (100 μg/100 g body weight) or corresponding volumes of solvent. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus control.

multiple attempts using different commercially available anti-MuRF1 antibodies, we were not successful in generating interpretable Western blots for MuRF1. It is therefore unclear at this point whether the increased MuRF1 mRNA levels in muscle from T3-treated rats were accompanied by increased MuRF1 protein levels.

In addition to increased expression of the ubiquitin ligases atrogin-1 and MuRF1, muscle wasting in various catabolic conditions is also characterized by increased expression of ubiquitin [Tiao et al., 1994]. In the present study, treatment of rats with T3 for 3 days resulted in an approximately 1.6-fold increase in ubiquitin mRNA levels (Fig. 2C), further supporting the notion that the ubiquitin–proteasome system is activated in skeletal muscle during hyperthyroidism. Similar to the changes in atrogin-1 and MuRF1 expression, the changes in ubiquitin mRNA were reversible and differences between control and T3-treated rats were no longer present 3 and 7 days after the last T3 injection.

In order to test whether the upregulation of atrogin-1, MuRF1, and ubiquitin expression was a sustained response to

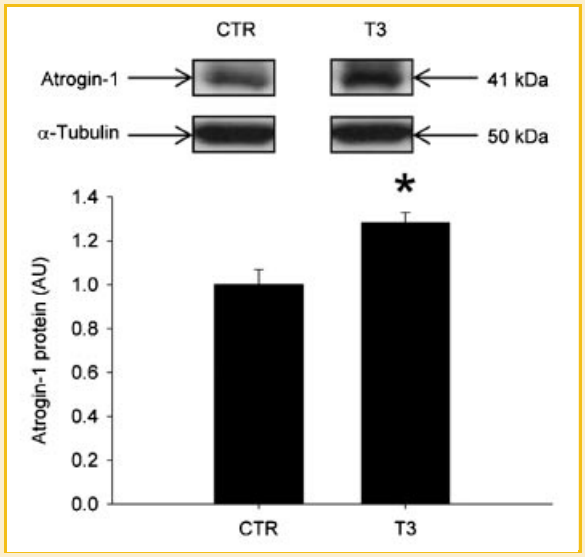

Fig. 3. Atrogin-1 protein levels in EDL muscles after T3 treatment (100 μg/100 g body weight) for 3 days. Muscles were studied 24 h after the last T3 injection. Atrogin-1 levels were determined by Western blotting. Representative blots are shown in the upper panel. Densitometric quantifications are shown in the lower panel with n = 8 in each group. Results are means ± SEM. *$P < 0.05$ versus control (CTR).

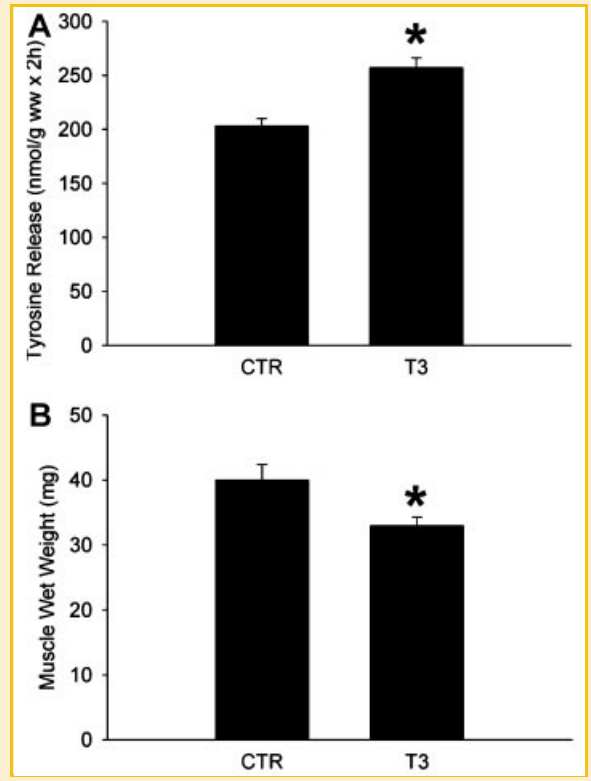

Fig. 4. A: Muscle protein breakdown rates and (B) muscle weight in control and T3-treated rats. Rats were treated for 7 days with 100 μg/100 g body weight of T3 or corresponding volumes of solvent. Muscles were studied 24 h after the last T3 injection. Results are means ± SEM with n = 8 in each group. *$P < 0.05$ versus control.

hyperthyroidism, an additional experiment was performed in which rats were treated for 7 days with 100 μg/100 g body weight of T3 or corresponding volumes of solvent. Rats with lower initial body weights were used in this experiment in order to make the final body and muscle weights comparable between the two experiments. This was important because small muscles are needed for the measurement of protein breakdown rates during in vitro incubation [Hasselgren et al., 1990]. As can be seen in Table II, serum levels of T3 and TSH confirmed the hyperthyroid state in rats treated with T3 for 7 days. The final body weight was lower in the hyperthyroid than in the control rats. Protein breakdown rates were increased by approximately 30% and muscle weight was reduced by 17% after T3 treatment for 7 days (Fig. 4). Importantly, atrogin-1, MuRF1 and ubiquitin mRNA levels remained elevated during T3 treatment for 7 days and the upregulation of these genes was somewhat more pronounced than seen after T3 treatment for 3 days (Fig. 5 and compare with Fig. 2). These observations suggest that the ubiquitin–proteasome pathway remains activated during extended periods of untreated hyperthyroidism.

TABLE II. Serum Hormone Levels, Body Weight, and Food Intake in Rats Treated for 7 days With 100 μg/100 g Body Weight of T3 or Corresponding Volumes of Solvent

	Control (n = 8)	T3 (n = 8)
Serum T3 (μg/ml)	2.9 ± 0.5	16.8 ± 2.2*
Serum TSH (μg/ml)	1.36 ± 0.32	N.D.
Initial body weight (g)	57 ± 1	55 ± 1
Final body weight (g)	106 ± 2	95 ± 2*

Results are means ± SEM. N.D., not detectible.
*$P < 0.05$ versus control.

Because treatment of rats with 100 μg/100 g body weight of T3 resulted in a pronounced increase in serum T3 levels consistent with severe hyperthyroidism (see Tables I and II), we next wanted to test the effects of a lower dose of T3. When rats were treated for 3 days with 10 μg/100 g body weight of T3, the increase in serum T3 levels and the suppression of TSH were less pronounced than noticed after treatment with 100 μg/100 g body weight of T3 (Table III). Muscle mRNA levels for atrogin-1, MuRF1, and ubiquitin were not altered after treatment with 10 μg/100 g body weight of T3 (data not shown). Thus, upregulation of atrogin-1, MuRF1, and ubiquitin in skeletal muscle may occur in severe, but not in more moderate, hyperthyroidism.

The second objective of the current report was to determine the influence of experimental hyperthyroidism on different proteolytic mechanisms in skeletal muscle. In a previous study [Tawa et al., 1997], treatment of hypophysectomized rats with subcutaneous injections of T3 (100 μg/100 g) for 4 days resulted in an approximately 40% increase in protein breakdown in incubated diaphragm muscles. In the same study, by using the proteasome inhibitor MG132, the calculated proteasome-dependent protein breakdown was increased by 42% in T3-treated rats. In order to test whether a similar increase in proteasome-dependent proteolysis occurred under the present experimental conditions, muscles from control rats and from rats treated with T3 for 3 days were incubated

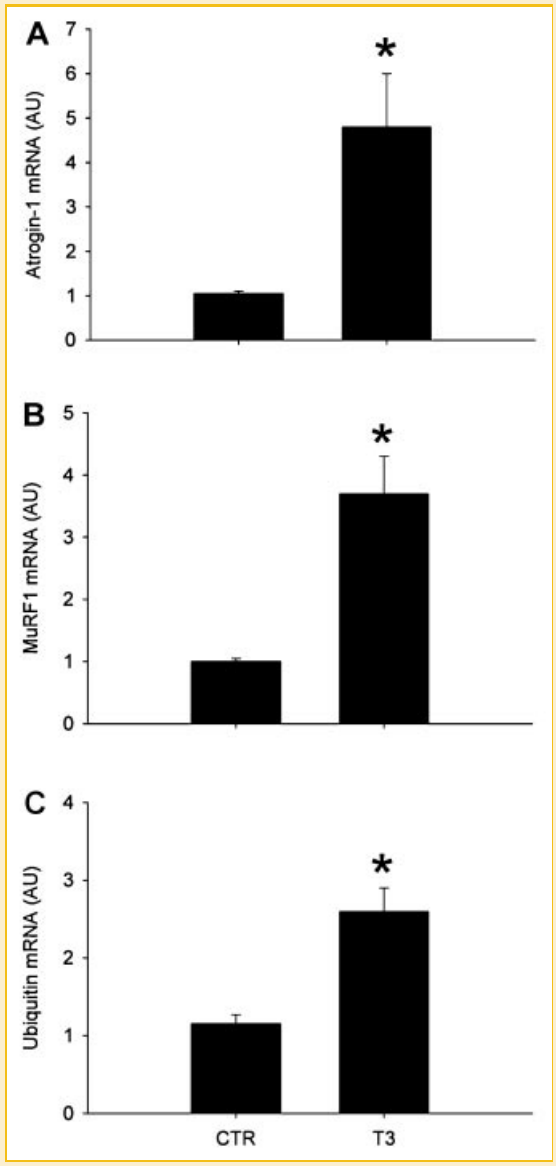

Fig. 5. mRNA levels for (A) atrogin-1, (B) MuRF1, and (C) ubiquitin in EDL muscles from rats treated for 7 days with T3 or corresponding volumes of solvent. Results are means ± SEM with n = 8 in each group. *$P < 0.05$ versus control.

TABLE III. Serum Hormone Levels in Rats Treated for 3 days With 10 μg/100 g Body Weight of T3 or Corresponding Volumes of Solvent

	Control (n = 8)	T3 (n = 8)
Serum T3 (μg/ml)	2.3 ± 0.2	6.8 ± 2.5*
Serum TSH (μg/ml)	0.53 ± 0.001	0.28 ± 0.02*

Results are means ± SEM.
*$P < 0.05$ versus control.

TABLE IV. Protein Breakdown Rates (nmol tyrosine/mg wet weight × 2 h) in Incubated EDL Muscles From Control and T3-Treated Rats

Inhibitor	Control (n = 8)	T3 (n = 8)	T3-induced change (%)
No inhibitor	177 ± 5	213 ± 6*	
MG132	101 ± 5	108 ± 5	
Difference	76 ± 3	105 ± 6*	+38
No inhibitor	137 ± 4	195 ± 6*	
Leupeptin	107 ± 3	132 ± 6*	
Difference	30 ± 3	63 ± 8*	+110
No inhibitor	201 ± 10	268 ± 15*	
Cathepsin L inhibitor IV	99 ± 8	129 ± 9*	
Difference	102 ± 7	138 ± 20*	+36
No inhibitor	186 ± 8	227 ± 11*	
Calpeptin	111 ± 7	122 ± 6	
Difference	75 ± 6	106 ± 10*	+41

Muscles were harvested after 3 days of treatment with T3 or corresponding volumes of solvent and incubated in the absence or presence of inhibitors as indicated in the table.
Results are means ± SEM. The different inhibitors were added to the incubation medium at the following concentrations: MG132, 10 μM; leupeptin, 100 μM; cathepsin L inhibitor IV, 80 μM; calpeptin, 100 μM.
*$P < 0.05$ versus corresponding control value.

in the absence or presence of 10 μM MG132. Using this experimental approach, the estimated proteasome-dependent proteolysis was increased by 38% in muscles from T3-treated rats (Table IV).

We next assessed the influence of experimental hyperthyroidism on the role of other proteolytic pathways by using additional inhibitors. In order to determine the effect of T3 treatment for 3 days on lysosomal protein degradation, muscles were incubated in the absence or presence of the lysosomal inhibitor leupeptin [Lowell et al., 1986]. Calculated from results in that experiment, lysosomal protein degradation was doubled in muscles from hyperthyroid rats (Table IV). Among lysosomal enzymes that may be involved in muscle wasting, previous studies suggest that cathepsin L is particularly important [Deval et al., 2001]. In order to test whether cathepsin L-dependent proteolysis was increased in hyperthyroid rats, muscles were incubated in the absence or presence of the cathepsin L inhibitor IV [Komamura et al., 2003]. Results from that experiment showed that cathepsin L-dependent proteolysis was increased by 36% in muscles from T3-treated rats (Table IV). When calpeptin was used to block calpain-dependent proteolysis [Ebisui et al., 1994], results suggested that T3-treatment resulted in a 41% increase in calpain-dependent muscle protein breakdown (Table IV).

Because none of the inhibitors used in the experiments shown in Table IV is completely specific, results based on their use need to be interpreted with caution. In order to further assess the influence of T3 treatment on the proteolytic pathways examined in Table IV, we next determined the gene expression for the different pathways. The mRNA levels for the proteasome subunit C3, cathepsin L, μ-calpain, and m-calpain were higher in muscles from T3-treated than control rats (although the difference in C3 mRNA levels between control and T3-treated rats did not reach statistical significance; Fig. 6).

An additional mechanism that may be involved in muscle wasting is caspase-3 dependent proteolysis [Du et al., 2004]. Although evidence has been reported that this mechanism is activated during various catabolic conditions, including uremia and diabetes [Du et al., 2004], the involvement of caspase-3 does not

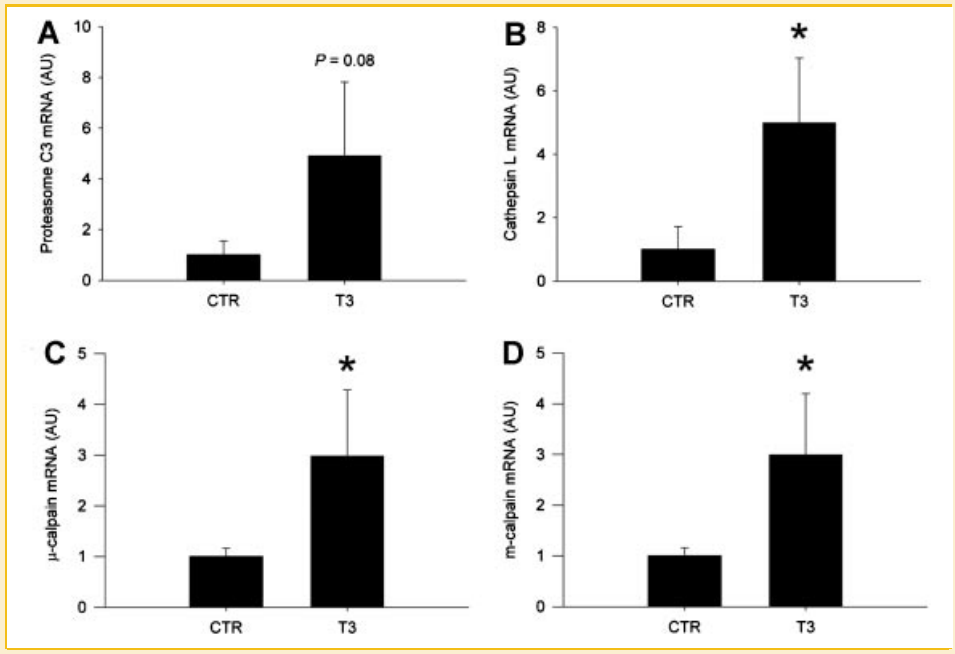

Fig. 6. mRNA levels for (A) the proetasome subunit C3, (B) cathepsin L, (C) μ-calpain, and (D) m-calpain in EDL muscles from rats treated for 3 days with T3 (100 μg/100 g body weight) or corresponding volumes of solvent. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus control.

seem to be universal for all muscle wasting conditions. For example, we found recently that caspase-3 may not be involved in sepsis-induced muscle wasting [Wei et al., 2005]. Here, we tested the potential role of caspase-3 in rats with experimental hyperthyroidism by determining caspase-3 mRNA and protein levels, enzyme activity, and caspase-3-dependent protein breakdown. Caspase-3 mRNA levels were increased in muscle from T3-treated rats (Fig. 7A). In contrast, caspase-3 protein levels and activity were not influenced by T3 treatment (Fig. 7B,C). In order to test whether caspase-3-dependent protein degradation was stimulated by T3 treatment, muscles were incubated in the absence or presence of 10 μM Ac-DEVD-CHO. Ac-DEVD-CHO did not reduce protein degradation rates in muscles from control or T3-treated rats (Fig. 7D), suggesting that caspase-3-dependent proteolysis did not contribute to total protein degradation under the present experimental conditions. These results are similar to those observed in recent experiments in which we found that Ac-DEVD-CHO did not influence protein breakdown in muscles from control or septic rats [Wei et al., 2005] but differ from the report by Du et al. [2004] in which the caspase-3 inhibitor reduced protein degradation in muscles from diabetic rats. Of note, the concentration of Ac-DEVD-CHO used in the experiment depicted in Figure 7 was the same as used by Du et al. [2004].

DISCUSSION

In the present study, treatment of rats with T3 resulted in upregulated expression in skeletal muscle of the ubiquitin ligases atrogin-1 and MuRF1. In addition, lysosomal, calpain- and proteasome-dependent protein degradation was increased in T3-treated rats, supporting the concept that multiple proteolytic pathways are activated in skeletal muscle during hyperthyroidism. The present observations are important because they provide novel information about mechanisms involved in the regulation of muscle proteolysis during hyperthyroidism, a condition commonly associated with loss of muscle mass [Yates et al., 1981; Hasselgren et al., 1984; Rodier et al., 1984; Adlerberth et al., 1987; Klein and Ojamaa, 2000; Riis et al., 2005; Brennan et al., 2006].

Evidence of increased muscle protein breakdown was reported in several previous studies in patients with hyperthyroidism [Yates et al., 1981; Hasselgren et al., 1984; Rodier et al., 1984; Adlerberth et al., 1987; Riis et al., 2005; Brennan et al., 2006] and in experimental animals treated with thyroid hormone [Carter et al., 1981; Angeras and Hasselgren, 1985; Tawa et al., 1997]. In previous studies, an experimental protocol similar to the protocol used here resulted in increased protein degradation in multiple muscles, including the diaphragm, epitrochlearis, EDL and soleus muscles [Carter et al., 1981; Angeras and Hasselgren, 1985; Tawa et al., 1997]. Thus, the catabolic response to hyperthyroidism probably reflects a generalized effect in skeletal muscle (although different types of skeletal muscle may respond with varying degrees of protein loss), underscoring the clinical significance of muscle wasting in hyperthyroidism.

It is not known from the present study whether the increased protein degradation and expression of atrogin-1 and MuRF1 in T3-treated rats reflected a direct or indirect effect of T3 in muscle. The effects of thyroid hormones are mainly mediated by activation of gene transcription by thyroid hormone-occupied thyroid hormone receptors [Wu and Koenig, 2000; Zhang and Lazar, 2000]. Because, in previous studies, high affinity T3 receptors were present in cultured L6 muscle cells [Koenig and Smith, 1985] and total and

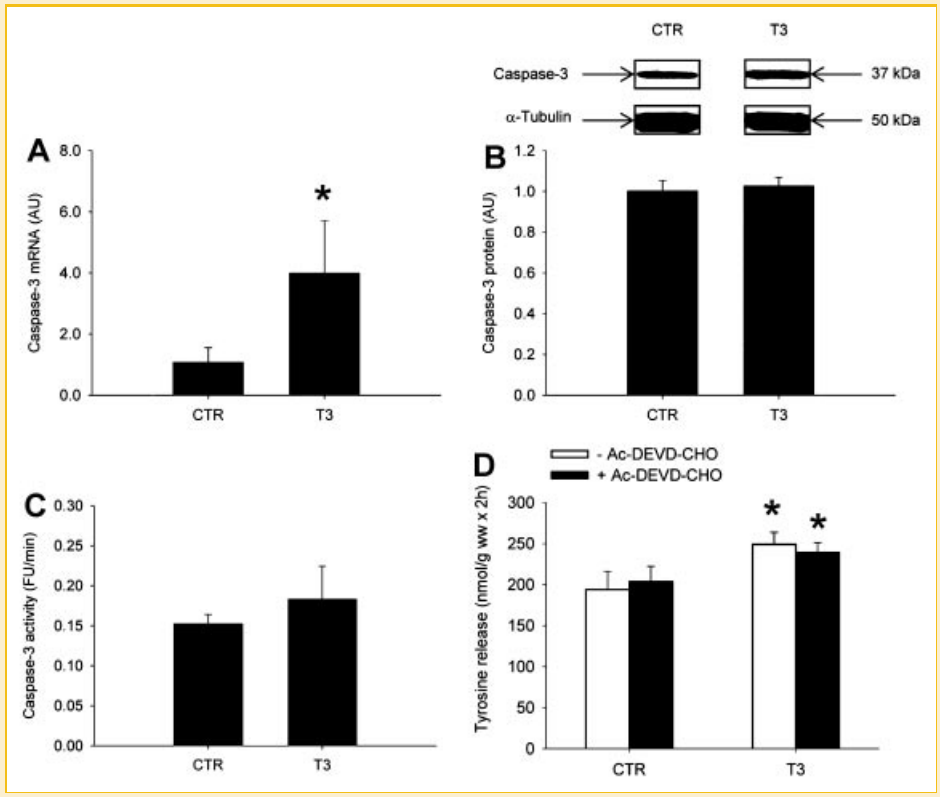

Fig. 7. A: Caspase-3 mRNA levels, (B) protein levels, and (C) activity in EDL muscles from rats treated for 3 days with 100 μg/100 g body weight of T3 or corresponding volumes of solvent. (D) Protein degradation rates in incubated EDL muscles from rats treated for 3 days with 100 μg/100 g body weight of T3 or corresponding volumes of solvent. Muscles were incubated in the absence or presence of the caspase-3 inhibitor Ac-DEVD-CHO (10 μM) and protein breakdown rates were determined as net release of free tyrosine as described in Materials and Methods Section. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus corresponding control group. AU, arbitrary unit; FU, fluorogenic unit.

myofibrillar protein degradation (measured as release of 3-methylhistidine) was increased in T3-treated primary cultures of chick muscle cells [Nakashima et al., 1998; Doi et al., 2003], it is possible that the effects of T3 treatment noticed in the current study, at least in part, reflected a direct effect of the hormone. It will be important in future studies to determine if transcription of genes in the ubiquitin–proteasome pathway is directly responsive to T3. It should be noted that indirect mechanisms may also be involved in muscle wasting during hyperthyroidism, including increased levels of IGF binding proteins (reducing the bioavailability of IGF-I) [Jenkins et al., 2000], insulin resistance Shen and Davidson, 1985; Ikeda et al., 1990], altered levels of adipocytokines [Iglesias et al., 2003], and reduced levels of cortisol binding globulin (increasing glucocorticoid bioavailability) [Mishra et al., 2007].

In a previous report, proteasome-dependent protein degradation was increased in skeletal muscle during experimental hyperthyroidism in rats [Tawa et al., 1997]. In other experiments, proteasome activity was increased in T3-treated cultured muscle cells [Doi et al., 2003]. The present study expanded previous observations by demonstrating that the expression of atrogin-1 and MuRF1 was upregulated during similar experimental conditions, providing further support for a role of the ubiquitin–proteasome pathway in muscle wasting during hyperthyroidsim.

It should be noted that although the present finding of increased expression of atrogin-1 and MuRF1 is consistent with the concept that muscle proteins are ubiquitinated at a higher rate during hyperthyroidism for subsequent degradation by the 26S proteasome, it is possible that increased expression of the ubiquitin ligases also reflected increased ubiquitination of proteins targeted for degradation by other mechanisms, for example autophagy-regulated lysosomal proteolysis [Lenk et al., 1992; Kraft and Peter, 2008; Sou et al., 2008].

Activation of calpain-dependent muscle proteolysis, as observed in the present study, was reported previously in other catabolic conditions as well, including sepsis [Bhattacharyya et al., 1991; Wei et al., 2005; Smith et al., 2008], cancer [Costelli et al., 2001], and muscular dystrophies [Tidball and Spencer, 2000]. Because the proteasome does not degrade intact myofibrils [Koomharaie, 1992; Solomon and Goldberg, 1996], it is possible that calpain activation plays an important role in muscle wasting by cleaving cytoskeletal proteins, resulting in disruption of the sarcomere and release of myofilaments that are subsequently ubiquitinated and degraded by the proteasome [Huang and Forsberg, 1998; Williams et al., 1999; Hasselgren and Fischer, 2001]. Calcium is the most important (albeit not the only) regulator of calpain activity [Goll et al., 2003]. Interestingly, there is evidence that thyroid hormone stimulates calcium uptake in skeletal muscle [Koenig and

Smith, 1985; Hudecova et al., 2004] suggesting a potential mechanism of increased calpain-dependent muscle proteolysis in hyperthyroidism.

Increased activities of the lysosomal enzymes cathepsin B and D were reported previously in T3-treated rats [DeMartino and Goldberg, 1978] but the contribution of lysosomal protein breakdown to T3-induced muscle proteolysis has not been reported. The present result of activated lysosomal protein degradation in skeletal muscle of hyperthyroid rats suggests that the increased cathepsin B and D activities reported previously [DeMartino and Goldberg, 1978] may contribute to muscle wasting during hyperthyroidism. Results in the present study suggest that other lysosomal enzymes as well, in particular cathepsin L, regulate muscle protein breakdown after treatment with T3. Recent studies provided evidence that cathepsin L-regulated protein breakdown is involved in muscle wasting caused by sepsis, cancer, and glucocorticoid treatment [Deval et al., 2001] but the influence of hyperthyroidism on cathepsin L-dependent muscle proteolysis has not been reported previously.

Evidence for activation of caspase-3 and caspase-3-dependent proteolysis in skeletal muscle was reported recently in rats with muscle wasting caused by kidney failure and diabetes mellitus [Du et al., 2004]. Apparently conflicting results were reported from our laboratory in sepsis-induced muscle wasting [Wei et al., 2005] suggesting that caspase-3 may not be universally involved in all muscle wasting conditions but may be disease-specific. The results reported here suggest that muscle wasting during hyperthyroidism is not associated with increased caspase-3 activity or caspase-3-dependent proteolysis, at least not during experimental hyperthyroidism in rats. An interesting observation was that of increased caspase-3 mRNA levels but unchanged caspase-3 protein levels in skeletal muscle of T3-treated rats. Although the mechanism behind that finding is not known at present, the result may reflect decreased translational efficiency or increased degradation of caspase-3 during hyperthyroidism.

Although thyroid hormone-regulated uptake of calcium may be a mechanism of calpain [Goll et al., 2003] and proteasome [Menconi et al., 2004] activation, other cellular mechanisms may also be involved in muscle wasting during hyperthyroidism. Importantly, energy balance in skeletal muscle is influenced by T3 [Doi et al., 2003], and this may contribute to activation of the energy-dependent ubiquitin–proteasome system. Interestingly, in previous studies, T3 treatment resulted in increased muscle ATP production and levels [Doi et al., 2003] despite increased expression of uncoupling proteins [Nagase et al., 1999].

Upregulated expression of genes and gene products that regulate protein breakdown may be an additional, and perhaps the most important, mechanism of thyroid hormone-induced muscle wasting. In addition to increased expression of atrogin-1, MuRF1, and ubiquitin, muscle wasting in various catabolic conditions is associated with altered transcription of several other genes, so called atrogenes [Lecker et al., 2004]. In recent experiments in healthy human subjects treated with T3 for 14 days, microarray analysis of muscle RNA provided evidence of altered expression of multiple genes involved in the regulation of protein degradation, including genes in the ubiquitin–proteasome pathway [Clement et al., 2002]. Although the influence of T3 on atrogin-1 and MuRF1 was not reported in that study, a 1.66-fold increase in mRNA levels for a protein "similar to mouse ubiquitin-protein ligase E3-α" was observed after T3 treatment. Results in the present study suggest that the gene expression of several additional proteolytic pathways, including cathepsin L, μ-, and m-calpain, may be upregulated in the hyperthyroid state.

It should be noted that although the present study provided novel information regarding the expression of atrogin-1 and MuRF1 and the involvement of different proteolytic mechanisms in T3-induced muscle wasting, a number of potential limitations of the study need to be taken into account when interpreting the results. First, the experiments were conducted in rats with experimental hyperthyroidism, and it remains to be determined whether hyperthyroidism in patients is associated with changes in gene expression and activity in different proteolytic pathways similar to those observed here. Second, rats were treated with T3 for a relatively short period of time (3 or 7 days); patients with hyperthyroidism typically have symptoms for longer periods of time before the diagnosis is made and therapy is initiated. Third, the assessment of the role of different proteolytic pathways in part relied on the use of various inhibitors and the potential non-specific effects of the inhibitors need to be considered. Finally, it remains to be determined if the increased expression of atrogin-1 and MuRF1 in muscle from hyperthyroid rats is accompanied by stimulated ubiquitin ligase activity and increased rates of protein ubiquitination.

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