Oxidation of myosin heavy chain and reduction in force production in hyperthyroid rat soleus

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Despite the well-accepted view that loss of muscle strength and exercise intolerance are prominent clinical features of hyperthyroid patients, the mechanisms underlying the depressed capacity of contractile function in skeletal muscle remain incompletely understood. Although protein degradation due to accelerated proteolysis has been found in skeletal muscle of patients with hyperthyroidism (2), it seems unlikely that the protein loss contributes only to the muscle weakness in hyperthyroid skeletal muscles. Based mainly on clinical examinations, muscle strength has been shown to decrease by 40–100%, whereas muscle mass only reduced by ~20% in hyperthyroidism (29, 46), suggesting that the observed reduction in force may result not only from muscle atrophy but also from the failures in the excitation-contraction coupling (EC) process.

In contrast to clinical observations, the effects of thyroid hormone on isometric properties in the animal model are controversial. A hyperthyroid state has been reported to consistently decrease the isometric twitch force (12, 13, 26, 44) and to either decrease (44) or maintain the maximum tetanic force at control levels (12, 13, 26). This discordance seems likely to be related to the dosage of thyroid hormone administered to the animals. For example, Merican and coworkers (26, 44) have studied the effects of two doses of thyroxine treatment on the isometric properties of cat soleus muscles: 25 μg/kg every third day and 250 μg/kg three times per week for 10 wk. The significant decrease in maximum tetanic force was observed only in higher dose (44). Consistent with these experiments, we previously confirmed that the higher dosage (300 μg·kg⁻¹·day⁻¹) of 3,5,3'-triiodo-L-thyronine (T₃) treatment induced the significant depression in maximum tetanic force in rat soleus muscles (43).

Protein oxidation could play an important role in an etiology of contractile dysfunctions in hyperthyroidism because oxidative stress, as mediated by vigorous contractile activity, can modify the structure and function of proteins associated with EC coupling (28, 36) and because in hyperthyroid muscles a hypermetabolic state that occurs with accelerated oxidative metabolism in the mitochondria brings about augmented production of reactive oxygen species (ROS) (3, 4, 11, 40). Sarcoplasmic reticulum (SR) regulates intracellular free Ca²⁺ concentration and plays a central role in the EC coupling process. Recent observations have implicated the impaired SR function as a major contributor to a depression in force induced by exercise to exhaustion (19). There is substantial evidence to indicate that protein oxidation may account at least in part for the decay in SR function (24, 25). However, the possibility seems remote that dysfunction of the SR may be a major factor for the hyperthyroid-induced decline in contractile function because in a previous study from our laboratory (42) it was shown in rat soleus muscles that hyperthyroidism resulted in elevations in SR Ca²⁺ release and uptake rates.

The molecular basis for contractile ability of muscle fiber is the conversion of chemical energy to mechanical work by the cycling attachment and detachment of myosin motor protein with the actin filament. Although the functional behavior of myofibrillar proteins is deleteriously affected by intracellular accumulation of ROS, their susceptibilities to oxidative damage vary among different proteins (17). On the basis of its primary structure, myosin heavy chain (MHC), an asymmetric molecule with distinct head and tail domains, has been argued to be highly susceptible to oxidation (17, 45). MHC comprises sulfhydryl (SH)-containing cysteine residues critical for contractile function (23). Single-fiber study has revealed...
that ROS donor-induced oxidation of SH groups residing in the myosin head decreases isometric force production, maximal shortening velocity, and ATPase activity (31). Because SH groups are not involved in the catalytic activity of myosin ATPase, it is suggested that oxidation may result in a structure perturbation in myosin, leading to impaired myosin function.

Taking these findings into account, one plausible hypothesis arises that elevated ROS production may contribute to the hyperthyroid-induced force reduction by modulating the structural state of MHC. No published study presently exists, however, that examines the redox state of MHC in hyperthyroid skeletal muscle. In this study, two hypotheses were tested in hyperthyroid rat soleus: 1) that a depression in myofibrillar protein function would be responsible mainly for the hyperthyroid-induced force reduction; and 2) that oxidation of MHC would be elicited by hyperthyroidism. To test these hypotheses, forces induced by 4-chloro-m- cresol (4-CmC), a specific activator of Ca\(^{2+}\) release channel (CRC) of the SR, were compared with those induced by depolarization. Moreover, myofibrillar extracts were analyzed for protein oxidation by the assessment of 2,4-dinitrophenylhydrazine (DNPH)-reactive carbonyl groups.

**METHODS**

**Animal care and treatment.** Nine-week-old male Wistar rats at the beginning of the experiment were randomly assigned to an euthyroid (n = 9) and a hyperthyroid (n = 9) group. Animals assigned to the hyperthyroid group received daily intraperitoneal injections of T\(_3\) (300 \(\mu\)g·kg\(^{-1}\)·day\(^{-1}\)) for 21 days. Animals were given food and water ad libitum and housed in an environmentally controlled room (temperature, 22–25°C) with a 12-h light-dark cycle. At the end of a given period, rats were sedated with pentobarbital sodium (50 mg/kg) and the soleus muscles were excised from both legs. The experimental protocol was approved by Animal Care Committee of Hiroshima University.

**Measurement of isometric contractile force.** The muscles were dissected into small bundles weighing ~30 mg. Isometric contractions of the bundles were recorded in a chamber, which had a volume of 200 ml and was filled with continuously stirred, temperature-controlled standard solution (30°C) of the following composition (in mM): 115 NaCl, 5 KHCO\(_3\), 1 MgCl\(_2\), 20 NaHCO\(_3\), 2 CaCl\(_2\), 5 N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 11 glucose, 0.3 glutamic acid, and 0.38 glutamine. The solution was continuously bubbled with 95% O\(_2\)-5% CO\(_2\), which gives a bath pH of 7.4. The bundles were connected to an isometric force transducer, and muscle length was adjusted to optimize twitch force. The stimulation pulses were applied via two platinum plate electrodes placed on each side of the muscle. After 10 min of incubation, we measured isometric forces evoked by direct stimulation at 1 (evokes twitch contraction), 10, 20, 40, 75, 100, and 150 Hz using supramaximal voltage, 1-ms pulses, and trains of 350 ms. These contractions were produced at 1-min intervals. Peak force in each contraction was measured and normalized to cross-sectional area, where cross-sectional area was computed as muscle wet weight divided by the product of muscle length and density (1.96 g/ml).

**Measurement of K\(^{+}\)-, and 4-CmC-induced contracture.** The bundles were connected to an isometric force transducer in a temperature-controlled chamber (30°C) containing a standard solution and set at optimal length. After 10 min of incubation, K\(^{+}\) contracture was evoked by a standard solution in which the NaCl was replaced with 100 mM K\(_2\)SO\(_4\). The bundles were rested for 5 min in a standard solution and then placed in a standard solution containing 10 mM 4-CmC to elicit 4-CmC contracture. This concentration was chosen because previous study indicated that the concentration of 4-CmC >7.5 mM caused maximal contracture force (20). The data were recorded throughout the period of experiments, and peak tensions in both contractures were measured. Specific tension was calculated as described above.

**Na\(^{+}-K\(^{+}\)-ATPase activity.** Na\(^{+}-K\(^{+}\)-ATPase activity was spectrophotometrically measured in muscle homogenates by using the K\(^{+}\)-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) assay, as described by Fraser and McKenna (16). The muscle pieces of ~20 mg were homogenized in 9 volumes of a solution consisting of 5 mM N-2-hydroxyethylpipеразине-N\(^{\prime}\)-2-ethanesulfonic acid, 0.3 M sucrose, 0.2 mM phenylmethylsulfonylfluoride, and 0.2% (wt/vol) NaN\(_3\) (pH 7.4). The resulting homogenates were freeze-thawed four times and then diluted with nine volumes of the homogenate buffer. The assay buffers contained (in mM) 5 MgCl\(_2\), 1.25 EDTA, 1.25 EGTA, 5 NaN\(_3\), and 100 Tris (pH 7.4). After the addition of a 30-μl aliquot of the homogenate, the assay mixture was incubated for 3 min. The reaction was started by adding 3-O-MFP to give a final concentration of 200 μM. Finally, the K\(^{+}\)-stimulated activity of Na\(^{+}-K\(^{+}\)-ATPase was determined by an increase in activity after the addition of 10 mM KCl. Changes in fluorescence were quantified by fluorospectrophotometry (excitation wavelength = 475 nm; emission wavelength = 515 nm). The 3-O-MFPase activity was based on the difference in slope before and after the addition of KCl.

**Glutathione contents.** The amounts of total, reduced (GSH), and oxidized glutathione (GSSG) were determined by spectrophotometric methods, as described by Baker et al. (5). The tissue samples, weighing ~30 mg, were minced, placed on ice in nine volumes of 5% (wt/vol) 5-sulfosalicylic acid for 30 min, and then centrifuged at 16,000 g for 10 min to remove precipitated materials. For total glutathione (GSH + GSSG), triethanolamine was added to the supernatant to give a final concentration of 6% (vol/vol). For GSSG measurements, 2% (vol/vol; final concentration) 2-vinylpyridine was additionally added. The assay buffers contained 1.52 mM NaH\(_2\)PO\(_4\), 7.6 mM Na\(_2\)HPO\(_4\), 0.485 mM EDTA, 1 U/ml glutathione reductase, and 0.1 mM NADPH (pH 7.5). After the addition of an aliquot of the sample, the assay mixture was incubated for 2 min. The reaction was started by adding 5.5'-dithiobis-(2-nitrobenzoic acid) to give a final concentration of 0.4 mM. The glutathione concentration was determined spectrophotometrically at a wavelength of 412 nm. The GSH content was calculated as the difference between total glutathione and GSSG contents.

**Myofibrillar protein extraction.** Myofibrillar protein concentrations were measured according to Tsika et al. (39). Approximately 60 mg of muscle were homogenized in 10 volumes of a solution containing (in mM) 250 sucrose, 100 KCl, 20 imidazole, and 5 EDTA (pH 6.8). The homogenate was centrifuged at 3,000 g for 10 min, and the supernatant was discarded. The resulting pellet was rehomogenized in 10 volumes of 175 mM KCl containing 0.5% (vol/vol) Triton X-100 (pH 6.8) and processed through two additional washes. After these washes, the pellet was subjected to two more washes in a solution of 150 mM KCl and 20 mM imidazole (pH 7.0). The resulting pellet was then homogenized in the same buffer, and protein concentration was determined according to Bradford (6), using bovine serum albumin as a standard. Just before use, all solutions used were stirred under vacuum to reduce the oxygen tension. Aliquots of myofilibr extracts were used for measures of the carbonyl group content in myofibril and MHC.

**MHC content in myofibrillar proteins.** To separate myofibrillar proteins, SDS-PAGE was performed using a 10–20% (wt/vol) gradient separating gel. Aliquots of myofilibr extracts containing 5 μg of protein were subjected to electrophoresis at 22°C for 5 h, applying a current of 20 μA. The gels were stained with Coomassie blue R in 45% (vol/vol) methanol. On basis of densitometry of total myofibrillar proteins, the relative content occupied by MHC in myofibrillar proteins was estimated.

**Carbonyl content in total myofibrillar proteins.** The carbonyl content in total myofibrillar proteins was determined by spectrophotom-
et method, as described by Levine et al. (21). Briefly, myofibrillar extracts containing 200 μg of protein were incubated for 30 min at room temperature in 10 mM DNPH in 2 N HCl. Derivatization was stopped by the addition of 20% (wt/vol) trichloroacetic acid, and protein was pelleted by centrifugation for 3 min at 11,000 g. The pellets were washed three times with ethanol-ethyl acetate (1:1). The protein was solubilized in 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). Insoluble material was removed by centrifugation, and maximal absorbance of the supernatant was measured at the wavelength of 360 nm.

Carbonyl content in MHC. An aliquot of myofibrillar extracts was added to 12% (wt/vol) SDS and then reacted with 20 mM DNPH for 10 min. The reaction was stopped by the addition of a solution containing 2 M Tris and 30% (vol/vol) glycerol. Aliquots of the DNPH-derivated samples containing 1.25 μg of MHC were applied ontoalkali phosphatase (Sigma). The contents of carbonyl group in MHC were densitometrically evaluated using National Institutes of Health image.

Statistical analyses. A two-way variance analysis was performed to evaluate the influence of T3 treatment. If an overall F value was obtained, a Scheffe’s post hoc analysis was used to isolate the significantly different means. All comparisons were performed at the 95% confidence level. Data were presented as means ± SE.

RESULTS

Body, heart, and muscle weights. Animals subjected to T3 treatment maintained an ~2.8-fold increase in serum-free T3 levels 24 h postinjection compared with controls (result not shown). This elevation was higher than that observed in previous studies (15, 30) using the lower dosage but less than that of hyperthyroid human patients (38).

The body and absolute soleus muscle weights from T3-treated animals were significantly lower than those of euthyroid group (Table 1). T3 treatment, however, caused no change in the normalized (the muscle-to-body weight ratio) soleus muscle weights. In contrast, T3 injection elicited significant increases in the absolute and normalized heart weights, suggesting that this protocol was effective in inducing a hyperthyroid state.

Isometric contractile force. Similar to what previous studies showed (12, 26, 27, 44), twitch force developed by soleus muscles treated with T3 was less (P < 0.01) than that of controls (414.0 ± 38.7 vs. 618.9 ± 19.2 g/cm2; Fig. 1). Hyperthyroidism also led to significant reductions (P < 0.01) in maximum tetanic force (2,257.9 ± 123.0 vs. 3,093.5 ± 44.7 g/cm2). Examined more broadly, T3 treatment depressed force across the entire range of soleus muscle activation (P < 0.01). The force reduction in hyperthyroid group was more prominent at low stimulation frequency at which the tetanic contractions appeared unfused.

K+ and 4-Cmc-induced contracture force. K+-induced contracture force was reduced by 47% (P < 0.01) in hyperthyroid group compared with euthyroid group.

![Fig. 1. Isometric forces in euthyroid (EU) and hyperthyroid (HYPER) rat soleus muscles. HYPER was induced by daily injection of 3,5,3′-triiodo-t-thyronine (T3) for 21 days. Isometric forces were evoked by direct stimulation at 1 (evokes twitch contraction), 10, 20, 40, 75, 100, and 150 Hz. Data are expressed as a percentage of maximum tetanic tension (Po) in control group. Mean ± SE of the 100% value is 3,093.5 ± 44.7 g/cm2. Soleus muscles from HYPER group developed lower forces than EU group at all stimulus frequencies. Values are means ± SE of n = 9 per group. * P < 0.01, compared with paired euthyroid group.](http://jap.physiology.org/)

![Fig. 2. K+- and 4-chloro-m-cresol (4-Cmc)-induced contracture forces recorded in fiber bundles from soleus muscles. Contracture forces were elicited by 200 mM K+ or 10 mM 4-Cmc. Force is expressed as a percentage of EU force. No differences in the magnitude of the T3-induced depressions were observed between K+- and 4-Cmc-induced forces. Values are means ± SE of n = 9 per group. * P < 0.01, compared with EU group.](http://jap.physiology.org/)
thyroid group relative to control groups (Fig. 2). 4-CmC contracture force was also decreased by 39% (P < 0.01) in T3-treated muscles. No differences in the magnitude of the T3-induced depressions were observed between K+- and 4-CmC-induced forces.

**Na+-K+-ATPase activity.** The observed decline in K+-induced contracture force could be explained by the depressed sarcolemmal excitability. To investigate this possibility, Na+-K+-ATPase activity was measured in muscle homogenates. In contrast to our results of force, the activity, as assessed by 3-O-MFPase, was significantly (P < 0.05) increased by 41% in hyperthyroid group (Table 2).

**MHC content in myofibrillar proteins.** The concentration of myofibrillar proteins contained in muscle was not different between hyperthyroid and euthyroid rats (results not shown). T3 treatment resulted in significant reductions (P < 0.05) in the relative concentration of MHC in total myofibrillar proteins (Fig. 3B). MHC content in T3-treated muscles amounted to 86% of the euthyroid level.

**Carbonyl group and glutathione contents.** T3 administration resulted in an ~1.5-fold increase (P < 0.05) in the carbonyl content in total myofibrillar proteins (2.07 ± 0.19 vs. 2.91 ± 0.26 nmol/mg protein). This alteration was accompanied by marked reductions in both GSH and GSSG contents (Table 2). The reduction in force production was accompanied by a remarkable increment in DNPH-reactive carbonyls in MHC, suggesting that oxidative modification of MHC may be responsible, at least in part, for an inability of cross bridges to generate force in hyperthyroidism.

**Level of EC coupling failure in hyperthyroid soleus muscle.** Our results of the marked depressions in isometric forces, even when tensions were normalized by cross-sectional area, indicate that EC-coupling impairment may be responsible for hyperthyroid-induced contractile dysfunctions. The fact that the magnitude of T3-induced depressions showed no significant differences between peak twitch force and K+ contracture force suggests that a failure in the EC coupling process occurs at or below the level of the voltage sensor, given that K+ contractures are evoked by depolarizing transverse-tubular membranes adjacent to the voltage sensors (10) and that no reductions were observed in Na+-K+-ATPase activity (Table 2).

One of the important results in this study is that, in T3-treated muscles, reductions in 4-CmC-induced force approximately correspond to those in K+-induced force. 4-CmC is a reagent specific for the CRC and activates the CRC directly by putting a carbonyl group and glutathione contents.

Table 2. **Na+-K+-ATPase activity and glutathione content in euthyroid and hyperthyroid rats**

<table>
<thead>
<tr>
<th>Rat</th>
<th>n</th>
<th>Na+-K+-ATPase Activity, nmol/mg protein^-1·h^-1</th>
<th>Total Glutathione, μmol/g wet wt</th>
<th>GSH, μmol/g wet wt</th>
<th>GSSG, μmol/g wet wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>9</td>
<td>43,913.3 ± 2,390.6</td>
<td>6.77 ± 0.34</td>
<td>6.48 ± 0.36</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>9</td>
<td>61,776.5 ± 7,614.5*</td>
<td>2.39 ± 0.17†</td>
<td>2.30 ± 0.17†</td>
<td>0.09 ± 0.02†</td>
</tr>
</tbody>
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Values are means ± SE. GSH, reduced glutathione; GSSG, oxidized glutathione. Significant difference compared with euthyroid group: *P < 0.05; †P < 0.01.

**DISCUSSION**

The present study is the first to indicate that the force deficit in hyperthyroid rat soleus muscle would stem primarily from a failure in the EC coupling process at the level of myofibrillar proteins (see Level of EC coupling failure in hyperthyroid soleus muscle). The reduction in force production was accompanied by a remarkable increment in DNPH-reactive carbonyls in MHC, suggesting that oxidative modification of MHC may be responsible, at least in part, for an inability of cross bridges to generate force in hyperthyroidism.

**Level of EC coupling failure in hyperthyroid soleus muscle.** Our results of the marked depressions in isometric forces, even when tensions were normalized by cross-sectional area, indicate that EC-coupling impairment may be responsible for hyperthyroid-induced contractile dysfunctions. The fact that the magnitude of T3-induced depressions showed no significant differences between peak twitch force and K+ contracture force suggests that a failure in the EC coupling process occurs at or below the level of the voltage sensor, given that K+ contractures are evoked by depolarizing transverse-tubular membranes adjacent to the voltage sensors (10) and that no reductions were observed in Na+-K+-ATPase activity (Table 2).

One of the important results in this study is that, in T3-treated muscles, reductions in 4-CmC-induced force approximately correspond to those in K+-induced force. 4-CmC is a reagent specific for the CRC and activates the CRC directly and maximally. Although it is obvious that decreases in CRC function attenuate the 4-CmC-induced contracture force, our

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**Fig. 3.** Electrophoresis of myofibrillar proteins (A) and myosin heavy chain (MHC) content in total myofibrillar proteins in EU and HYPER rat soleus muscles (B). Myofibrillar proteins were separated by using polyacrylamide gradient (10–20%) gel electrophoresis and evaluated densitometrically. MHC content is expressed as a percentage of total myofibrillar proteins. Values are means ± SE of n = 9 per group. M, molecular marker; αTM and βTM, α- and β-subunits of tropomyosin, respectively. *P < 0.05, compared with EU group.
Susceptibility of MHC to ROS-induced damage. Skeletal muscle proteins have differential susceptibilities to ROS-mediated oxidation (17, 18). The susceptibility of lipids to ROS-mediated peroxidation has been described previously (36). The transverse tubule, SR, plasma membrane, and mitochondria comprise the relatively large amounts of lipid. Of these, the inner membrane of mitochondria is reported to be the most susceptible region (18). Numerous studies have identified cysteine as the likely target of ROS other than lipids (36). Although the reason is unknown, MHC containing cysteine residues critical for contractile function has been shown to exhibit a high susceptibility to ROS-induced modification (23). For example, Zergeroglu et al. (45) studied the effects of mechanical ventilation, which evokes oxidative modification, on entire proteins in diaphragm and found more severe oxidation in MHC than in others.

Reductions in MHC content. It has widely been accepted that introduction of carbonyl groups into amino acid residues of protein (carbonylation) can be a hallmark for oxidative modification (37). The results obtained in this study clearly point out that oxidation of MHC and the reduced content of MHC are brought about by hyperthyroidism. The precise mechanism for the decay in protein is unclear. However, it is conceivable that protein degradation due to the carbonyl formation may be responsible, at least partly, for the decreased MHC content, given that carbonylation is characterized by an irreversible modification that requires the proteolytic removal followed by the resynthesis of the affected protein (9).

Whereas the maximum velocity of shortening is highly correlated with the rate of cross-bridge dissociation, isometric peak tension is purported to be determined primarily by the number of cross bridges and the force per cross bridge (14). The decreased MHC content makes it likely that the observed reduction in 4-CmC-induced force might be ascribed to the decreased number of attached cross bridges. It is unlikely, however, that this is the only mechanism because a 14% depression in the MHC content found in T3-treated muscle is too small to explain a 39% attenuation in 4-CmC-induced force (Figs. 2 and 3).

Acute effects of MHC oxidation on contractile function.

There is evidence to suggest that oxidation would induce not only quantitative but also qualitative alterations in MHC proteins, leading to a decrease in force generation. For instance, acute exposure to millimolar levels of H2O2 has been shown to reduce the maximal Ca2+-activated force of both fast- and slow-twitch fibers (32). It is suggested that the deleterious influence of ROS on myosin function may be mediated through critical SH groups located on MHC, since loss of free SH groups in myosin decreases the population of myosin heads in the strong-binding structural state (23). This suggestion is reinforced by a very recent study (8) indicating, in the soleus muscle from heart failure rat, the organs that undergo oxidative stress, that administration of antioxidant is capable of preventing carbonylation in myofibrillar proteins and improving force production. On the basis of these previous findings and the results in this study, we propose that T3-mediated depressions in force production may be attributable to enhanced MHC oxidation, which not only triggers protein degradation but also results in dysfunction of cross-bridge kinetics.

Perspective. This study advances our understanding of the mechanism by which the elevated level of thyroid hormone...
depresses contractile function in skeletal muscle. Our results implicate that the hyperthyroidism-induced loss of muscle strength and exercise intolerance may be ascribed to oxidative modification of MHC. In accordance with the results in rats, enhanced myofibrillar protein degradation also appears to occur in hyperthyroid human patients (1). Clinical examination has shown that, even after the circulating levels of thyroid hormone are normalized with medical treatment, impairment of muscle function occasionally lasts for prolonged periods (29). From the present results, it would be expected that the protection of organs against oxidation-induced damage may prevent or inhibit decreases in tension developed by human skeletal muscles. Studies using excised muscles in vitro or intact muscle in situ have suggested that treatment with antioxidants is capable of offsetting the effects of ROS (35). However, it was difficult to demonstrate this positive influence in humans. Supplementation of antioxidant nutrients such as vitamin C, vitamin E, and β-carotene has been evaluated as ineffective (7). In further study, it would appear productive to examine antioxidant interventions available for use in humans.

REFERENCES


