Myofibrillar protein oxidation and contractile dysfunction in hyperthyroid rat diaphragm

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Myofibrillar protein oxidation and contractile dysfunction in hyperthyroid rat diaphragm. J Appl Physiol 102: 1850–1855, 2007. First published February 15, 2007; doi:10.1152/japplphysiol.01177.2006.—The purpose of the present study was to test the hypothesis that administration of thyroid hormone [3,5,3'-triiodo-l-thyronine (T3)] could result in oxidation of myofibrillar proteins and, in turn, induce alterations in respiratory muscle function. Daily injection of T3 for 21 days depressed isometric forces of diaphragm fiber bundles across a range of stimulus frequencies (1, 10, 20, 40, 75, and 100 Hz) (P < 0.05). These reductions in force production were accompanied by a remarkable increment (104%; P < 0.05) in carbonyl groups of myosin heavy chain. In additional experiments, we have also tested the efficacy of carvedilol, a nonselective β1-β2-blocker that possesses antioxidative properties. Treatment with carvedilol dramatically improved isometric tetanic force production at stimulus frequencies from 40 to 100 Hz (P < 0.05). Carvedilol also prevented T3-induced contractile protein oxidation (P < 0.05). These data suggest that the oxidative modification of myofibrillar proteins may account, at least in part, for an impairment of diaphragm in hyperthyroidism.

reactive oxygen species; hyperthyroidism; specific force reduction; myosin heavy chain

BREATHLESSNESS is a common complaint in patients with hyperthyroidism, and a decrease in maximum respiratory muscle strength has been reported (17, 20). Although little is known about the effects of thyroid hormone on the contractility of the respiratory muscle, reductions in the in vivo transdiaphragmatic pressure evoked by tetanic stimulation have been found in experimental hyperthyroidism (21), suggesting a loss of contractile regulation in the diaphragm. However, the mechanisms responsible for the hyperthyroid-induced diaphragmatic dysfunction are less well understood.

The cellular redox balance may have an important influence on contractile function (27). Many studies have shown that hypermetabolic state in hyperthyroidism is associated with tissue oxidative injury (5, 16, 31, 32). Lipid peroxidation in slow oxidative muscles has been shown to increase in hyperthyroid rats (4, 36). Consistent with these experiments, we previously demonstrated in the soleus muscle that 3,5,3'-triiodo-l-thyronine (T3) treatment induced both oxidation of myofibrillar proteins and reductions in specific force generation (35). However, to our knowledge, no information was given in previous studies as to whether T3 treatment increases the diaphragmatic oxidative stress and induces the impairment of contractile properties.

A number of studies have suggested that increased reactive oxygen species (ROS) production may be responsible for a component of the respiratory muscle dysfunction in some pathophysiological conditions (6, 24, 28, 30, 37, 38). In support of this contention, an exogenous ROS donor has been shown to decrease force generation in diaphragm fiber bundles (18, 29) and skinned fibers (9, 29). In addition, the role of ROS as a contributor to respiratory muscle dysfunction is emphasized by previous observations by Shindoh et al. (28) and Supinski and Callahan (30), who found that administration of antioxidants is capable of improving the force production of diaphragm in inflammatory disease processes.

As a role of ROS in muscle contractility is widely investigated, there is increasing evidence suggesting that oxidative modification of contractile and regulatory proteins is responsible for a depression in skeletal muscle force production (10, 12, 37). For instance, it is a well-known fact that alkylaition of the sulfhydryl groups of myosin has significant functional effects (11). Andrade et al. (1), using intact single muscle fibers, have shown that prolonged application of hydrogen peroxide resulted in decreased force production that was accompanied by a fall in myofibrillar Ca2+ sensitivity. Moreover, they also demonstrated that functions of the sarcoplasmic reticulum are less susceptible to ROS than those of myofibrillar proteins. These findings are in agreement with the observation that, in diaphragm skinned fiber, the maximum Ca2+-activated force is decreased by the addition of an exogenous ROS donor (9, 13).

Taking these findings into account, one plausible hypothesis arises that administration of T3 may result in oxidative modifications in myofibrillar proteins and, in turn, may induce alterations in respiratory muscle function. The purpose of this study was to investigate this issue by 1) comparing protein oxidations in diaphragm muscle samples taken from control animals and T3-treated animals; and 2) examining the effect of administration of carvedilol, an antioxidant, on diaphragm force generation in animals with hyperthyroidism.

METHODS

Experimental design. Nine-week-old male Wistar rats at the beginning of the experiment were randomly assigned to one of four groups: euthyroid, hyperthyroid, euthyroid plus carvedilol, and hyperthyroid plus carvedilol. Hyperthyroidism was elicited by treatment with daily administration of carvedilol, an antioxidant, on diaphragm force generation in animals with hyperthyroidism.
intra peritoneal injections of T₃ (300 μg·kg⁻¹·day⁻¹) for 21 days. In this study, we employed carvedilol as an antioxidant since this agent has been shown to improve force production by preventing the oxidation of myofibrillar proteins in rat soleus muscle undergoing oxidative stress (12). Carvedilol is also a nonselective β₁-β₂-blocker. It is possible, therefore, that the properties of carvedilol as a β-blocker rather than an antioxidant could act on the diaphragm. However, β-blockers without known antioxidative properties have been shown to exert no effect on force production in hyperthyroidism (3, 36).

Carvedilol (2 mg·kg⁻¹·day⁻¹) was given orally for 21 days, dissolved in ethanol and then in drinking water (ethanol final concentration 0.2%). Animals were given food and water ad libitum and housed in an environmentally controlled room (temperature, 22–25°C) with a 12:12-h light-dark cycle. At the end of a given period, rats were sedated with pentobarbital sodium (50 mg/kg). In each animal, two diaphragm bundles from left hemidiaphragm were dissected with the associated ribs and central tendon intact. One bundle was used to measure contractile function and the other to measure protein oxidation. The experimental protocol was approved by the Animal Care Committee of Hiroshima University.

Measurement of isometric contractile properties. Isometric contractions of the diaphragm bundles were recorded in a chamber that was filled with a temperature-controlled standard solution (30°C) of the following composition (in mM): 115 NaCl, 5 KHCO₃, 1 MgCl₂, 20 NaHCO₃, 2 CaCl₂, 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% O₂-5% CO₂, which gives a bath pH of 7.4. Bundles were connected to an isometric force transducer, and 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, and 100 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 was normalized to cross-sectional area, where cross-sectional area was computed as muscle wet weight divided by the product of muscle length and density (1.06 g/ml).

Carbonyl content in total myofibrillar proteins. Small muscle pieces were homogenized in a glass homogenizer in 20 volumes of a solution containing (in mM) 300 KCl, 100 KH₂PO₄, 5 K₂HPO₄, and 1 EDTA (pH 6.5). The solutions were prepared with 1:5,000 dilution of monoclonal anti-dinitrophenyl antibody coupled to alkaline phosphatase (Sigma). The contents of carbonyl group in MHC were densitometrically evaluated using National Institutes of Health Image software.

RESULTS

Body and heart weights. The body weights of T₃-treated animals were significantly lower than those of euthyroid group (Table 1). Similar to what previous studies showed (34, 35), T₃ treatment elicited significant increase in the absolute and normalized (the heart-to-body weight ratio) heart weights, suggesting that this protocol was effective in inducing a hyperthyroid state. In contrast, carvedilol treatment caused no change in body and heart weights from both euthyroid and hyperthyroid animals.

Isometric contractile force. In agreement with a previous study (21), T₃ administration induced contractile dysfunction in the diaphragm. Maximum tetanic force developed by diaphragm fiber bundles treated with T₃ (1,156.4 ± 48.6 g/cm²) was less than that of euthyroid group (1,456.0 ± 49.7 g/cm²) (Fig. 1). T₃ treatment also decreased twitch force in diaphragm fiber bundles treated with T₃ (1,156.4 ± 48.6 g/cm²) was less than that of euthyroid group (1,456.0 ± 49.7 g/cm²) (Fig. 1). T₃ treatment also decreased twitch force in diaphragm fiber bundles treated with T₃ (1,156.4 ± 48.6 g/cm²) was less than that of euthyroid group (1,456.0 ± 49.7 g/cm²) (Fig. 1). T₃ treatment also decreased twitch force in diaphragm fiber bundles treated with T₃ (1,156.4 ± 48.6 g/cm²) was less than that of euthyroid group (1,456.0 ± 49.7 g/cm²) (Fig. 1).

Table 1. Body and heart weights of rat groups

<table>
<thead>
<tr>
<th></th>
<th>EU</th>
<th>EU-CAR</th>
<th>HY</th>
<th>HY-CAR</th>
</tr>
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<tr>
<td>BW, g</td>
<td>401.5±8.5 (10)</td>
<td>379.0±11.2 (10)</td>
<td>338.5±9.2† (10)</td>
<td>318.9±7.7† (10)</td>
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<td>HW, mg</td>
<td>1,061.5±40.9 (10)</td>
<td>978.5±24.9 (10)</td>
<td>1,605.0±52.6† (10)</td>
<td>1,544.2±54.1† (10)</td>
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<tr>
<td>HW/BW, mg/g</td>
<td>2.64±0.07 (10)</td>
<td>2.59±0.06 (10)</td>
<td>4.75±0.11† (10)</td>
<td>4.84±0.11† (10)</td>
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Values are means ± SE (n of samples); BW, body weight; HW, heart weight; EU, euthyroid; HY, hyperthyroid; CAR, carvedilol. *P < 0.01 compared with EU group; †P < 0.05 compared with EU-CAR group.
carvedilol treatment significantly increased tetanic force at stimulation frequencies from 40 to 100 Hz ($P < 0.05$).

In Table 2, we show the properties of contraction and relaxation in isometric force of diaphragm preparation. The tetanic parameters were obtained from stimulus frequency at 40 Hz where tension was significantly reduced in hyperthyroid animals compared with the other three groups. Twitch contraction time was shorter in hyperthyroid animals than in euthyroid and carvedilol-treated euthyroid animals. In contrast, the rate of contraction and relaxation from twitch and tetanic forces did not show any significant differences among the four groups.

**Carbonyl group content.** $T_3$ administration resulted in an ~2-fold increase ($P < 0.05$) in the carbonyl content in diaphragmatic myofilbrillar proteins compared with the euthyroid group (2.71 ± 0.37 vs. 5.54 ± 0.63 nmol/mg protein) (Fig. 2). Administration of carvedilol had no effect on the carbonyl content in euthyroid animals, whereas carvedilol treatment dramatically reduced the level of myofilbrillar protein oxidation in hyperthyroid animals ($P < 0.05$). Figure 3A shows the representative Western blots illustrating the carbonyl content in MHC. The densitometric evaluation of the blot revealed that treatment with $T_3$ or carvedilol evoked no changes in the carbonyl content in MHC (Fig. 3B).

**MHC content in myofilbrillar proteins.** There were no significant differences in the concentration of myofilbrillar proteins among all groups (results not shown). In contrast, $T_3$ treatment resulted in significant reductions ($P < 0.05$) in the relative concentration of MHC in total myofilbrillar proteins compared with euthyroid animals given carvedilol (Fig. 4).

**DISCUSSION**

This study provides the first evidence that, in hyperthyroid rat diaphragm, the myofilbrillar proteins exhibit increased levels of oxidative stress, and carvedilol, an antioxidant, is capable of preventing both contractile dysfunction and oxidation of myofilbrillar proteins. These data suggest that the oxidative modification of myofilbrillar proteins may account, at least in part, for an impairment of diaphragm in hyperthyroidism.

**$T_3$-induced oxidative stress in diaphragm.** Available data indicate that hyperthyroid tissues exhibit an increased ROS production, as documented by enhanced levels of indicators of lipid and protein oxidation (reviewed in Ref. 32). In both rat (4) and cat (36), $T_3$-induced increases in lipid peroxidation were found in the soleus composed mainly of slow-twitch oxidative fibers but not in the extensor digitorum longus composed mainly of fast-twitch glycolytic fibers. Taken together, it is conceivable that the degree of $T_3$-induced oxidative modifications correlates with properties in oxidative metabolism in muscles, given that these effects seem to be primarily determined through increased mitochondrial production of ROS. Our results demonstrated for the first time that protein oxidation occurs in hyperthyroid diaphragm, which is composed predominantly of glycolytic fibers. The reason for the oxida-
tion of diaphragm remains unknown, but this discrepancy could be explained by the fact that the diaphragm contains relatively large amounts of mitochondria compared with locomotor muscles consisting of similar fiber-type composition (8).

**Diaphragmatic force production in hyperthyroidism.** While a locomotor muscle weakness is frequently recognized (25, 26, 39), respiratory muscle function has not hitherto been studied in detail in hyperthyroidism. It has been shown in experimental hyperthyroidism that microscopic examination reveals atrophy of diaphragm muscle fiber, indicating that loss of muscle mass may account for decreased muscle contractility (21). However, our results of the marked depression in isometric forces, even after data were corrected for differences in muscle cross-sectional area, suggest that hyperthyroid-induced diaphragmatic contractile dysfunctions result not only from muscle atrophy but also from the failure in the muscle force-generating capacity.

There have been no previous demonstration, however, of a role of oxidation in diaphragm in an animal model of hyperthyroidism. Our data provide the first evidence that administration of carvedilol is capable of preventing both hyperthyroid-induced protein oxidation and reductions in specific force generation. Because carvedilol is a widely used β-blocker that possesses antioxidative properties (12), it is possible that their antiadrenergic activity could influence the improvement of diaphragm dysfunction in our animal model. However, this possibility seems remote because previous studies have revealed that β-blockers such as metoprolol and propranolol, without known antioxidative properties, have no effect on muscle weakness and wasting in hyperthyroidism (3, 36).

**T3-induced myofibrillar protein oxidation.** There are a number of studies indicating that oxidative modifications of myofibrillar proteins have a large impact on the function of skeletal muscle (1, 2, 22, 23). Myofibrillar proteins appear to exhibit high sensitivity to redox modulation (10, 12, 37). For instance, Zergeroglu et al. (37) studied the effects of mechanical ventilation, which evokes protein oxidation and contractile dysfunction, on entire proteins in diaphragm and found more severe oxidation in myofibrillar proteins than in others. It is still an open question which proteins constituting the myofibrillar complex are primarily influenced by changes in intracellular redox balance. As previously shown by our carbonyl data, MHC, the most abundant protein in myofibrils, is highly susceptible to oxidation in hyperthyroid rat soleus (35). The alterations in carbonyl groups were accompanied by reductions in MHC protein content (35). It has been hypothesized that...
enhanced carbonylation may trigger protein degradation (35), as carbonylation is characterized by an irreversible modification that requires the proteolytic removal followed by the resynthesis of the affected protein (14). Our results of the changes in the protein and carbonyl contents of MHC resemble, at least qualitatively, those occurring in the soleus, although the elevation in the carbonyl content did not reach a significant level. Alternatively, our data imply that diaphragm and limb muscle could differ with regard to their responses to oxidative stress, although the mechanism is unclear.

The depressions in force production observed in the diaphragm could be explained by alterations in myofibrillar Ca\(^{2+}\) sensitivity. Moopanar and Allen (22, 23) found that repetitive contraction decreased force in the absence of tetanic Ca\(^{2+}\) concentration in the myoplasm and suggested that ROS, the production of which is elevated by muscle activity, might elicit oxidative modification of troponins I and C, leading to loss of Ca\(^{2+}\) sensitivity (15). On the basis of these findings, it is speculated that treatment with carvedilol could prevent or attenuate T\(_3\)-induced oxidative damage of regulatory proteins in the myofibril.

In conclusion, we have presented data suggesting that hyperthyroid-induced loss of respiratory muscle strength may be ascribed to oxidative modification in myofibrillar proteins. A possible role of ROS in hyperthyroid rat diaphragm weakness is further supported by the observations showing that exogenous administration of antioxidant prevents both myofibrillar oxidation and reduction in specific force generation. Moreover, the fact that there is no overt loss of myofibrillar proteins in hyperthyroid animals argues that, in early stages of hyperthyroidism, contractile dysfunction in diaphragm does not result from protein degradation but more likely results from the failure in the force-generation capacities. Although the present experiment revealed that MHC is the unlikely target of ROS in diaphragm, additional work is required to determine whether T\(_3\)-induced ROS production could affect other proteins in myofibrils and induce contractile dysfunctions.

REFERENCES


