Effect of T₃-induced hyperthyroidism on mitochondrial and cytoplasmic protein synthesis rates in oxidative and glycolytic tissues in rats

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Short KR, Nygren J, Nair KS. Effect of T₃-induced hyperthyroidism on mitochondrial and cytoplasmic protein synthesis rates in oxidative and glycolytic tissues in rats. Am J Physiol Endocrinol Metab 292: E642–E647, 2007. First published October 17, 2006; doi:10.1152/ajpendo.00397.2006.—Hyperthyroidism increases metabolic rate, mitochondrial ATP production, and protein synthesis, but it remains to be determined whether all tissues and synthesis of specific protein pools are equally affected by hyperthyroidism. Previous studies showed that mitochondrial function was less responsive to elevated triiodothyronine (T₃) levels in the low-oxidative plantaris muscle compared with other tissues in rats. We tested the hypothesis that in T₃-treated animals mitochondrial protein synthesis would increase in oxidative but not glycolytic tissues. Male rats received either T₃ (200 μg/day, n = 10) or saline (controls, n = 9) by subcutaneous pump for 14 days, and then in vivo protein synthesis rates were measured using [¹⁵N]phenylalanine in liver, heart, plantaris, and red gastrocnemius (Red Gast). Mitochondrial protein synthesis rate in T₃-treated rats was higher than in controls by 62% in Red Gast and plantaris and 89 and 115% in liver and heart, respectively (P < 0.01). Cytoplasmic protein synthesis rates in the T₃ group were 107–176% higher than control values (P < 0.01). There was also indirect evidence that protein breakdown was increased in all tissues of the T₃-treated rats. Phosphorylation of selected regulators of protein synthesis in plantaris and Red Gast (mTOR, p70 S6 kinase, 4E-BP1), however, were not significantly affected by T₃. We conclude that T₃ infusion stimulates a general increase in mitochondrial and cytoplasmic protein synthesis rate among tissues and that this does not appear to explain the tissue-specific responses in mitochondrial oxidative capacity.

METHODS

Animals and treatment. The study protocol and procedures were approved by the Institutional Animal Care and Use Committee and followed the guidelines of the National Research Council’s Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 325–350 g were randomly allocated to thyroid hormone-treated (n = 10 animals) or control groups (n = 9). T₃ (Spectrum Quality Products, Gardena, CA) was administered for 14 days via a subcutaneous osmotic pump (Alzet; Alza Scientific Products, Palo Alto, CA) implanted above the shoulders. The pump delivered 200 μg/day of T₃, which was dissolved in saline vehicle containing 20 mM NaOH, 50 mM Na₂CO₃, and 300 mM NaCl.

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and 5% (wt/vol) bovine serum albumin. Control animals were implanted with vehicle-only pumps for 14 days. Animals were housed individually in plastic boxes with standard bedding in a controlled environment (12:12-h light-dark cycle, 20–22°C, 50–60% relative humidity). Food consumption and body mass were recorded on alternate days throughout the treatment period. Standard laboratory rat chow (Lab Diet 5001; PMI International, Brentwood, MO) and water were provided ad libitum ≤3 h before the animals were killed. Experimental conditions and the effects of treatment on mitochondrial function and physical activity in these animals have been reported (17, 30).

Tissue collection. On the morning of day 14 of pump implantation, food was removed from the cages 3 h before tracer infusion. Rats were transferred to a smaller holding cage with light restraint to facilitate access to the tail. Each rat received an intravenous injection of [15N]phenylalanine (99 atom percent excess, 15 mg/kg; Cambridge Isotope Laboratories, Cambridge, MA) for measurement of protein synthesis rate. Pentobarbital anesthesia (Nembutal, 50 mg/kg ip) was given 10 min after tracer injection. After 20 min of tracer incorporation, tissues of interest, including individual hindlimb skeletal muscles (soleus, plantaris, and gastrocnemius [deep red portion subdivided]), liver, and heart, were rapidly and sequentially removed. After blotting of blood and removal of visible fat or connective tissue, samples were quickly frozen in isopentane chilled in liquid nitrogen and stored at –80°C until analysis. Blood was collected via cardiac puncture, separated into serum or plasma, and also frozen until further analysis.

Blood analysis. Blood samples were used for determination of circulating thyroid hormone levels. Total thyroxine (T4) and T3 were separated into serum or plasma, and also frozen until further analysis. Blood was collected via cardiac puncture, quickly frozen in isopentane chilled in liquid nitrogen and stored at –80°C until analysis. Blood was collected via cardiac puncture, separated into serum or plasma, and also frozen until further analysis.

Muscle protein synthesis. Due to the small size of the soleus muscle and its use for other assays, there was not sufficient tissue for mitochondrial protein separation for protein synthesis measurements. Therefore, we used the red portion of the gastrocnemius as a representative skeletal muscle with high oxidative capacity (10). A portion of each tissue was used for measurement of activity of citrate synthase and cytochrome c oxidase (36). Mitochondrial and cytoplasmic protein fractions were separated from red gastrocnemius and plantaris muscles and heart and liver samples (150 mg each) from individual animals, as previously described (28). Another 25-μg piece of each tissue was to isolate the tissue-free fluid according to established methods (18). Protein samples were hydrolyzed for 24 h in 0.05 mol/l HCl at 110°C in the presence of cation exchange resin (AG 50W-X8; Bio-Rad Laboratories, Hercules, CA). Amino acids from the protein hydrolysates and tissue fluid fractions were then purified over individual columns of the same resin. Samples were derivatized using N-methyl-N-(t-butyldimethylsilyl)-triﬂuoroacetamide in acetonitrile. Isotopic enrichment in the protein-bound amino acids was measured using gas chromatography–mass spectrometry (Finnigan-MAT, Bremen, Germany) and expressed as atom percent excess (APE) after adjustment to a six-point standard curve and subtraction of background enrichment (12). Enrichment of the free amino acids in tissue fluid was measured using gas chromatography-mass spectrometry and expressed as molar percent excess (MPE) after adjustment to an eight-point standard curve and subtraction of background enrichment (12).

Fractional protein synthesis was calculated using the equation FSR (%/h) = (Epro × 100)/ETRF × time, where Epro and ETRF are the isotopic enrichments in muscle protein and in tissue fluid, respectively, and time is the number of hours of tracer incorporation.

Western blotting. The effect of T3 on selected intracellular signaling proteins involved in protein synthesis regulation was performed by Western blotting. These analyses were performed only in the plantaris and red gastrocnemius muscles, as there was insufficient tissue available for the liver and heart from both groups of animals. Frozen samples were homogenized on ice in cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing (in mmol/l) 20 Tris-HCl (pH 7.5), 150 NaCl, 1 Na3EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 Na2VO4, 1 leupeptin, and 1% Triton X-100. After centrifugation at 10,000 g for 10 min, the supernatant was collected and protein concentration measured (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA). Samples were mixed with 4× SDS sample loading buffer (Invitrogen, Carlsbad, CA), and equal amounts of proteins were separated on NuPage Bis-Tris gels by electrophoresis (Invitrogen). Proteins were transferred with an XCell II blot module (Invitrogen) to PVDF membranes. Following blocking in 5% nonfat milk (Bio-Rad), membranes were incubated overnight at 4°C with primary antibodies (Cell Signaling) directed against the total or phosphorylated (Ser2448) form of mammalian target of rapamycin (mTOR), the total or phosphorylated (Thr389) form of p70 S6 kinase-1, and the total or phosphorylated (Thr37/46) form of 4E-BP1. The blots were then incubated with horse-radish peroxidase-conjugated secondary antibodies and the ECL-Plus detection system (Amersham Biosciences, Piscataway, NJ). Images were captured on Biomax XR film (Kodak Scientific, New Haven, CT) and analyzed using Kodak Molecular Imaging software. Values were expressed as the relative signal for the phosphorylated/total forms of each protein and adjusted so that the average value for the control group was equal to 1.

Statistics. Differences between treatment groups for most variables were analyzed with Student’s t-tests. To assess whether T3 treatment had a differential impact among tissues, analysis of variance and Tukey’s post hoc tests were employed. In all cases, P values of <0.05 were considered statistically significant.

RESULTS

Serum total T3 concentration was higher (P < 0.01) in the T3-treated group (755 ± 57 ng/dl) than the control group (57 ± 1 ng/dl). Serum total T4 was 1.8 ± 0.1 μg/dl in control rats but was suppressed below the detectable level of the assay (<20 ng/dl) in the T3-treated group. T3-treated rats had higher food intake but gained less weight than control rats, attributable to an increase in energy expenditure (17, 30).

In red gastrocnemius muscle, the activity of citrate synthase and cytochrome c oxidase was higher in T3-treated rats compared with controls. For citrate synthase the increase was 45% (T3, 55.6 ± 5.2 μmol·min⁻¹·g tissue⁻¹; Control, 38.2 ± 1.8, P < 0.021) whereas for cytochrome c oxidase activity the increase was 82% (T3, 57.1 ± 4.6 μmol·min⁻¹·g tissue⁻¹; Control, 31.4 ± 1.4, P < 0.01). Thus, the response in mitochondrial oxidative capacity of the red gastrocnemius is similar to what was previously shown for the heart, liver, and soleus muscles of these animals, whereas there was no change in the activity of these enzymes in plantaris (30).

Tissue fluid [15N]phenylalanine enrichment was reduced 37–54% (P < 0.01) in all tissues of the hyperthyroid rats compared with controls (Table 1). Protein-bound enrichments in mitochondrial and cytoplasmic proteins were not statistically different between treatment groups in any of the four tissues (Table 1). The calculated fractional synthesis rates of mitochondrial and cytoplasmic proteins were much higher (P < 0.01, effect sizes = 2.1–3.3) in T3-treated rats than in controls in each of the four tissues (Fig. 1). Compared with the control group, synthesis rates were higher in the T3 animals by an average of 89, 115, 62, and 62% for mitochondrial proteins from liver, heart, red gastrocnemius, and plantaris, respectively. For cytoplasmic proteins, the corresponding increase in the T3 group vs. controls was 148, 141, 95, and 87% in liver,
The effect of thyroxine (T₃) treatment on protein synthesis rates of mitochondrial and cytoplasmic proteins in various tissues of hyperthyroid rats was studied. The synthesis rate of mitochondrial proteins was higher (P < 0.05) than cytoplasmic proteins in liver, heart, and red gastrocnemius, and plantaris, respectively. The synthesis rate of mitochondrial proteins was increased in multiple tissues of hyperthyroid rats. The synthesis rate of cytoplasmic proteins was also increased in hyperthyroid rats, but there were no significant differences among tissues for the effect of T₃ on increasing mitochondrial protein synthesis rate. For cytoplasmic protein synthesis rate, the only difference among tissues was a greater mitochondrial protein synthesis rate. For cytoplasmic protein synthesis rate, the only difference among tissues was a greater mitochondrial protein synthesis rate.

To determine whether the effect of T₃ treatment varied among tissues and proteins, the individual fractional synthesis rate data were first transformed to z-scores, distributed around the mean of the control group within each tissue and protein type. Subsequent analyses revealed that there were no significant differences among tissues for the effect of T₃ on increasing mitochondrial protein synthesis rate. For cytoplasmic protein synthesis rate, the only difference among tissues was a greater T₃ effect in the liver vs. the plantaris muscle. Additionally, within the liver, the cytoplasmic protein synthesis rate was increased by T₃ to a greater magnitude than the mitochondrial protein synthesis rate (P = 0.034). No further treatment differences were detected among tissues or proteins.

Western blotting for phosphorylation of selected proteins in plantaris and red gastrocnemius is shown in Fig. 2. There were no statistically significant differences between groups in the signals for the total or phosphorylated forms of each protein, nor for the relative phosphorylation when normalized for total form of the protein. There was, however, a trend for mTOR phosphorylation to be reduced (P = 0.063) in the red gastrocnemius of rats infused with T₃.

**DISCUSSION**

The main new finding in the present study is that both mitochondrial and cytoplasmic protein fractional synthesis rates are increased in multiple tissues of hyperthyroid rats. In contrast to the effect of excess T₃ to selectively enhance mitochondrial ATP production rate in tissues with high-oxidative capacity but not in the low-oxidative plantaris muscle, the present study showed a similar increase of mitochondrial protein synthesis in all tissues. Tracer enrichment of tissue fluid was reduced in all tissues of T₃-treated animals compared with controls, providing indirect evidence that the rate of tissue protein breakdown is also increased in hyperthyroid rats. There was also no apparent effect of T₃ treatment on the phosphorylation of mTOR, p70 S6 kinase, or 4E-BP1 in skeletal muscles, suggesting that other steps in the regulation of protein synthesis must be activated by T₃ action.

Previous animal studies showed that experimental hyperthyroidism results in increased mitochondrial biogenesis, most frequently in heart, liver, and oxidative skeletal muscle, but skeletal muscles with lower oxidative capacity appear to be less responsive to hyperthyroidism (2, 7, 37). It is intriguing, then, that, despite increased mitochondrial content and oxidative capacity, greater muscle fatigability was reported in the soleus but not in the predominantly glycolytic extensor digitorum longus muscle of hyperthyroid animals (11). The T₃-treated rats used in the present study had higher mitochondrial ATP production, cytochrome c oxidase activity, and cytochrome c oxidase mRNA abundance in red skeletal muscle (soleus and/or red gastrocnemius) but not in the lower oxidative plantaris (present study and Ref. 30). Collectively, these findings demonstrate tissue-specific regulation of thyroid hormone action on oxidative capacity and muscle contractile properties. This could be due to differences in thyroid hormone binding affinity among tissues (14). We therefore hypothesized that regulation of protein synthesis rate by T₃ treatment would also vary among tissues on the basis of their oxidative capacity, but we found that mitochondrial and cytoplasmic proteins were both synthesized in vivo at significantly increased rates in each tissue of T₃-treated animals.

**Table 1. Enrichment of [¹⁵N]Phe in muscle proteins and tissue fluid**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mitochondrial Protein</th>
<th>Cytoplasmic Protein</th>
</tr>
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<tbody>
<tr>
<td>Liver</td>
<td>0.172 ± 0.008</td>
<td>0.017 ± 0.003</td>
</tr>
<tr>
<td>Heart</td>
<td>0.067 ± 0.005</td>
<td>0.015 ± 0.003</td>
</tr>
<tr>
<td>Red gastroc.</td>
<td>0.147 ± 0.006</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>Plantaris</td>
<td>0.152 ± 0.007</td>
<td>0.016 ± 0.004</td>
</tr>
</tbody>
</table>

Values are means ± SE. T₃, triiodothyronine. Enrichment values for tissue fluid are given as molar percent excess and for protein as atom percent excess.

*Different from Control, P < 0.05.

Fig. 1. Fractional synthesis rates (FSR) of mitochondrial and cytoplasmic proteins in liver, heart red gastrocnemius muscle (Red Gast), and plantaris (Plant) muscle. T₃, triiodothyronine. Protein FSR was calculated from the incorporation of [¹⁵N]phenylalanine. *T₃ greater than Control, P < 0.01.
of the tissues from T3-treated rats. Thus the rate of mitochondrial protein synthesis does not appear to be the main determinant for altered mitochondrial ATP production rate in response to hyperthyroidism.

The exact mechanisms responsible for the relatively similar increase in protein synthesis among tissues are not yet established. As synthesis rates of both protein fractions were increased in all tissues, it is possible that there is a general stimulation of the protein synthesis machinery. We tested whether three key regulatory elements of muscle protein synthesis, mTOR, p70 S6 kinase, and 4E-BP1, were activated by T3 treatment (by measuring phosphorylation status), since this has not, to our knowledge, been previously reported. Phosphorylation of these molecules increases in response to anabolic stimuli, such as meals (13) or insulin-like growth factor I treatment (36), and decreases in response to catabolic conditions such as sepsis (15). We did not find differences between groups in phosphorylation of mTOR, p70 S6 kinase, and 4E-BP1 in skeletal muscles between groups. However, Cao et al. (6) recently showed that 12-h exposure of human fibroblasts to T3 resulted in enhanced phosphorylation of mTOR and p70 S6 kinase. Whether these contrasting results are due to differences in the length of T3 exposure, tissue type, or in vitro vs. in vivo approaches is not known.

Besides any potential direct effects of T3 on protein synthesis, increased physical activity may contribute to the changes in protein synthesis in hindlimb muscles. We previously reported that T3-treated animals were more physically active compared with the euthyroid controls, and much of the increased movement involved rearing on the hindlegs (17). This activity should result in increased contractile recruitment of the ankle flexor muscles (soleus, plantaris, and gastrocnemius). Exercise is a known stimulant of muscle protein synthesis and mitochondrial biogenesis (4, 34) and therefore may contribute to the changes in skeletal muscle protein synthesis. However, this still does not fully explain how T3 administration enhances protein synthesis in liver and heart or why mitochondrial functional changes were selectively increased in soleus and red gastrocnemius muscle but not in plantaris.
We measured fractional synthesis rates of mitochondrial and cytoplasmic proteins, which reflect the average synthesis rates of many proteins within each of these pools. It is possible that synthesis of individual proteins is differentially regulated by thyroid hormone status, although techniques to measure the fractional synthesis rates of multiple individual mitochondrial and cytoplasmic proteins in vivo are not yet available. Changes in mRNA transcript abundance among individual mitochondrial proteins in response to T3 treatment can vary among liver, heart, and skeletal muscles (29, 30); however, it remains to be seen how many of these mRNAs are translated into proteins and whether this alters protein composition among tissues. Development of novel technologies will eventually allow us to examine how hyperthyroidism alters the synthesis rate of individual mitochondrial and cytoplasmic proteins in individual tissues (24). Such advances will allow, for example, for further exploration of which proteins account for the relatively greater effect of T3 treatment on synthesis rate of the liver cytoplasmic fraction.

Besides increasing protein synthesis, T3 treatment may affect protein expression through regulation of protein breakdown. It has been shown in vitro (tyrosine release method) that the global breakdown rate of mixed proteins in diaphragm and hindlimb muscles is increased in young rats infused with T3 for 4–10 days in a process regulated through the proteosome pathway (1, 33). Although muscle protein breakdown rate cannot be directly measured in vivo, the present study provides indirect support that tissue protein breakdown rate was increased in T3-treated rats. All animals received a standard dose of [13C]phenylalanine, and yet the enrichment of tracer in tissue fluid (free amino acid pool) was consistently lower in all tissues of T3-treated animals. This reduction of the isotopic tissue fluid (free amino acid pool) was consistently lower in all tissues of T3-treated animals. This reduction of the isotopic enrichment within the tissue is most likely to occur by dilution from unlabeled amino acids derived from catabolism of existing intracellular proteins. These data strongly suggest that turnover of proteins, both synthesis and breakdown, was significantly increased in all tissues of the T3-treated animals.

It is not yet known whether the present results in rats made hyperthyroid by T3 infusion may be directly translated to humans. Most animal studies showed that the mixed protein synthesis rate in skeletal muscle, liver, and heart is stimulated by excess thyroid hormone (1, 3, 8, 9, 16, 23). In humans, whole body rates of protein synthesis and breakdown are increased in some (20, 27, 32, 35) but not all (19, 21) studies of hyperthyroid patients or short-term experimental hyperthyroidism. From studies using an arterial-venous balance approach it appears that hyperthyroidism increases muscle protein breakdown across the forearm (27) and leg (21) in humans, but there was only a nonsignificant increase in forearm protein synthesis (27). To our knowledge, there are no published reports on the effect of hyperthyroidism on in vivo protein fractional synthesis rates using tissue sampling techniques in humans. As whole body protein synthesis is typically increased in the hyperthyroid state, it is likely that protein synthesis is also increased in one or more tissues in humans, but further studies are required.

In conclusion, we provide evidence of a general enhancement of mitochondrial and cytoplasmic protein synthesis rate in multiple tissues of hyperthyroid rats. Unlike changes in mitochondrial oxidative phosphorylation and mRNA abundance of mitochondrial genes, there was not a tissue-specific regulation of mitochondrial protein synthesis by T3. Therefore, the differences in mitochondrial function among red and white muscle groups from hyperthyroid rats cannot be explained on the basis of alterations in protein synthesis as measured in the present study. There may, however, be selective changes in the synthesis of individual proteins that could explain differences in functional responses among tissues.

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GRANTS

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