Effect of triiodothyronine on mitochondrial energy coupling in human skeletal muscle

Vincent Lebon,1 Sylvie Dufour,1 Kitt Falk Petersen,2 Jianming Ren,3 Beat M. Jucker,2 Lori A. Slezak,4 Gary W. Cline,2 Douglas L. Rothman,5 and Gerald I. Shulman1, 2, 6

1Howard Hughes Medical Institute, and
2Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, USA
3Department of Metabolic Research, Bristol-Myers Squibb Co., Princeton, New Jersey, USA
4Department of Surgery,
5Department of Diagnostic Radiology, and
6Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA

Address correspondence to: Gerald I. Shulman, Howard Hughes Medical Institute, Yale University School of Medicine, BCMM 254C, 295 Congress Avenue, New Haven, Connecticut 06510, USA.
Phone: (203) 785-5447; Fax: (203) 737-4059; E-mail: gerald.shulman@yale.edu.

Vincent Lebon, Sylvie Dufour, and Kitt Falk Petersen contributed equally to this work.

Received for publication November 13, 2000, and accepted in revised form July 24, 2001.

The mechanism underlying the regulation of basal metabolic rate by thyroid hormone remains unclear. Although it has been suggested that thyroid hormone might uncouple substrate oxidation from ATP synthesis, there are no data from studies on humans to support this hypothesis. To examine this possibility, we used a novel combined 13C/31P nuclear magnetic resonance (NMR) approach to assess mitochondrial energy coupling in skeletal muscle of seven healthy adults before and after three days of triiodothyronine (T3) treatment. Rates of ATP synthesis and tricarboxylic acid (TCA) cycle fluxes were measured by 31P and 13C NMR spectroscopy, respectively, and mitochondrial energy coupling was assessed as the ratio. Muscle TCA cycle flux increased by approximately 70% following T3 treatment. In contrast, the rate of ATP synthesis remained unchanged. Given the disproportionate increase in TCA cycle flux compared with ATP synthesis, these data suggest that T3 promotes increased thermogenesis in part by promoting mitochondrial energy uncoupling in skeletal muscle.


Introduction
The regulation of basal metabolic rate by thyroid hormone has been known since the 18th century (1), but the mechanism by which it occurs remains controversial. Thyroid hormone has been shown to increase energy expenditure by activating Na+/K+-ATPase, stimulating futile cycling as well as promoting mitochondrial biogenesis (2–6). However, in vitro observations have raised the possibility that thyroid hormone also promotes mitochondrial uncoupling of substrate oxidation from ATP synthesis (7, 8). The absence of an identified mechanism that accounts for uncoupling and the inability to assess the degree of mitochondrial coupling in vivo have long hampered investigation into the regulation of this process. Recently, a skeletal muscle–specific uncoupling protein (UCP-3) has been cloned in humans (9). This protein is thought to create a pathway that allows exothermic movement of protons through the inner mitochondrial membrane. A positive correlation between the concentration of thyroid hormone in plasma and UCP-3 mRNA expression in skeletal muscle has been demonstrated in mice, rats, and humans (10–18), raising the possibility that UCP-3 might be involved in regulating energy expenditure and body weight in humans by promoting mitochondrial energy uncoupling.

In order to examine whether thyroid hormone is capable of inducing mitochondrial uncoupling in human skeletal muscle, we developed a combined 13C/31P nuclear magnetic resonance (NMR) approach to assess mitochondrial energy coupling in vivo. The rate of tricarboxylic acid (TCA) cycle flux was assessed by monitoring the rate of appearance of 13C label into the C4 and C2 carbon atoms of muscle glutamate during an intravenous infusion of [2-13C] acetate (12). The rate of ATP synthesis was measured using 31P NMR spectroscopy by direct observation of 31P magnetization transfer between inorganic phosphate (Pi) and the γ phosphate resonance of ATP (γATP); mitochondrial energy coupling was determined by the ratio of ATP synthesis to TCA cycle oxidation. To assess the effect of thyroid hormone on mitochondrial energy coupling, these measurements were performed in healthy volunteers before and after three days of treatment with triiodothyronine (T3) to induce a state of mild hyperthyroidism.

Methods
Protocol. NMR studies were performed on seven healthy volunteers (three men and four women, aged 25 ± 2) before (basal) and after three days of T3 treatment (100 µg orally every 12 hours). Whole-body oxygen consumption was measured using a DeltaTrac II indirect
NMR spectroscopy. NMR studies were performed on a 2.1 T whole-body (1-m bore) magnet connected to a modified AVANCE spectrometer (Bruker, Billerica, Massachusetts, USA). Subjects remained supine in the magnet with the main body of the right calf muscles lying on top of a home-built radio-frequency NMR probe.

31P NMR measurement of ATP flux. 31P spectroscopy was performed at 36.31 MHz, using a flat concentric probe made of a 9-cm (diameter) inner coil (for 31P) and a 13-cm outer coil tuned to proton frequency for scout imaging and shimming. Unidirectional rates of ATP synthesis were measured using the saturation transfer experiment applied to the exchange between Pi and ATP. The steady-state Pi magnetization $M_z$ was measured in the presence of selective irradiation of the $\gamma$ATP, and compared with the equilibrium Pi magnetization $M_0$ in a control spectrum (without irradiation of $\gamma$ATP), as shown in Figure 1.

The fractional reduction of Pi upon $\gamma$ATP saturation ($M_0 - M_z$/$M_0$) was measured from our spectra and was used in the calculation of the pseudo-first-order rate constant according to the equation of Forsen and Hoffman: $k_1 = ([M_0 - M_z]/M_0)(1/T1^*)$, where $T1^*$ is the spin-lattice relaxation time for the phosphorus nucleus of Pi, when ATP is saturated.

$T1^*$ was then measured using a modified version of the inversion-recovery experiment (180°-τ-90°-acq-ID) in the presence of steady-state saturation of $\gamma$ATP during the total interpulse delay (ID) of 4 seconds and during the variable delay (τ). $T1^*$ was evaluated using a nonlinear least-squares fitting method. Unidirectional flux of ATP synthesis was then calculated by multiplying the constant $k_1$ by the Pi concentration. The Pi concentration was measured from the control spectrum, assuming a constant ATP concentration of 5.5 mmol/kg in muscle (19).

Total acquisition time for 31P spectra was about 120 minutes. After completion of 31P measurements, the volunteer was removed from the magnet for a 10-minute break. The NMR radio-frequency probe was changed, and the volunteer was repositioned for measurement of the TCA cycle rate.

13C NMR measurement of TCA cycle flux. For 13C spectroscopy, the probe consisted of one 13C circular coil and two 1H quadrature coils for 1H acquisitions and decoupling. After tuning, shimming with the FASTERMAP procedure (20), and acquiring scout images, 13C NMR spectra were acquired for 20 minutes before and during a 120-minute [2-13C]acetate infusion (350 mmol/l sodium salt, 99% 13C enriched; Isotec Inc., Miamisburg, Ohio, USA) at an infusion rate of 2.9 mg/kg/min.

13C NMR spectra were acquired using either an image-selected in vivo spectroscopy (ISIS) sequence (TR ~ 2.3 sec-

Figure 1
Principle of ATP synthesis rate measurement by 31P NMR spectroscopy. (a) Control spectrum in one volunteer: the saturation pulse is applied symmetrically to the $\gamma$ATP Larmor frequency. (b) Spectrum with $\gamma$ATP saturation acquired in the same volunteer (NS = 128). Subtraction of spectrum (b) from (a) gives the fraction of P, involved in the synthesis of ATP, as shown on the P, peak. ppm, parts per million.

Figure 2
13C NMR data. (a) 13C spectra acquired in a volunteer before and at the end of the [2-13C]acetate infusion. (b) Time course of glutamate C4 and glutamate C2 peak areas during the same experiment. Glutamate C4 appears soon after the beginning of the [2-13C]acetate infusion. The increase in glutamate C2 is delayed in comparison to that of glutamate C4, reflecting the extra turn of the TCA cycle required to randomize the 13C-labeled carbon into this position.
Muscle 

Typical model fit of the 13C NMR data of glutamate C4 in skeletal muscle (R^2 = 0.93).

![Figure 3](image)

Results

Three days of T3 treatment induced a mild hyperthyroid state in these subjects, as reflected by a significant increase in plasma T3 concentration that was associated with suppression of the mean concentration of thyroid stimulating hormone (TSH) in plasma, a 30% increase in heart rate, a 17% increase in the mean rate of whole-body oxygen consumption (Table 1), and a 15% increase in whole-body energy expenditure (basal: 1819 ± 129 kcal/24 h vs. 2089 ± 141 kcal/24 h after T3 treatment; P < 0.02) as assessed by indirect calorimetry.

The rate of ATP synthesis as assessed by 31P NMR was 5.2 ± 0.6 μmol/g/min in the basal state and it did not change significantly after T3 treatment (4.6 ± 0.8 μmol/g/min; P = 0.16 vs. basal), as shown in Figure 4.

In contrast, the mean rate of the TCA cycle as assessed by 13C NMR from the rate of 13C incorporation into glutamate C4 and C2 increased by 70% after T3 treatment (P = 0.01 vs. basal). The TCA cycle flux was 0.064 ± 0.008 μmol/g/min in the basal state, and increased to 0.109 ± 0.013 μmol/g/min after T3 administration (Figure 4).

An estimate of mitochondrial energy coupling was obtained by expressing the ratio of ATP synthesis to TCA cycle flux calculated. The muscle tissue was quickly blotted on filter paper and immediately frozen in liquid nitrogen. The time from obtaining the tissue specimen until freezing was less than 30 seconds. Glutamate concentrations were measured using a 2300 STAT Plus analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA).

Statistical analysis. All data are expressed as mean ± SE. Comparisons of values measured before and after T3 treatment were made using the paired Student’s t test.

Table 1

<table>
<thead>
<tr>
<th>Effect of T3 treatment on clinical and metabolic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
</tr>
<tr>
<td>(kg)</td>
</tr>
<tr>
<td>Basal</td>
</tr>
<tr>
<td>Post-T3</td>
</tr>
<tr>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Body weight; heart rate; plasma thyroxine; thyroid binding capacity; estimated free thyroxine, T3, and TSH concentrations; and whole-body oxygen consumption measured by indirect calorimetry in the subjects before and after T3 treatment.
TCA cycle flux, normalized to the basal state and this was found to decrease by 45% after T₃ treatment ($P = 0.001$ vs. basal) (Figure 4).

**Discussion**

While it is well established that thyroid hormone regulates metabolic rate in humans, the mechanism by which it does so remains unknown. Although T₃ has been shown to increase energy needs by activating ion pumping across membranes (2), substrate cycling (3, 4), and protein synthesis (5), regulation of basal metabolic rate by uncoupling substrate oxidation from ATP synthesis has been postulated (6) but never before demonstrated in humans.

In order to address this question, we used a novel $^{13}$C/$^{31}$P NMR approach to measure T₃-induced changes in mitochondrial energy coupling in human skeletal muscle. We found that three days of T₃ treatment increased skeletal muscle TCA cycle flux by 70% that was associated with no significant change in the rate of ATP synthesis. Since there was a disproportionate increase in TCA cycle flux compared with ATP synthesis, these results suggest that T₃ promotes increased thermogenesis in part by promoting mitochondrial energy uncoupling in skeletal muscle.

The TCA cycle is coupled to muscle oxygen consumption via a stoichiometric relationship with substrate utilization (24). Therefore it is likely that the 70% T₃-induced increase in TCA cycle flux reflects a similar increase in muscle oxygen consumption. In contrast, whole-body oxygen consumption increased by only 17% following T₃ treatment. The difference in magnitude between the T₃-induced increase in muscle versus whole-body oxygen consumption can probably be ascribed to the limited contribution of resting skeletal muscle to whole-body oxygen consumption (25), and demonstrates the important advantage of this tissue-specific NMR method over whole-body indirect calorimetry. Furthermore, if one extrapolates the observed T₃-induced increase in muscle energy metabolism to the whole body, it can be estimated to account for an increase of approximately 7% in whole-body oxygen consumption. The remainder (~10%) can most likely be explained by the contribution of other tissues such as liver, kidney, and heart to whole-body oxygen consumption (26).

Uncoupling effects of high doses of thyroid hormone have previously been reported in vitro (6, 7), and more recently T₃-induced proton leaks in the inner mitochondrial membrane have been demonstrated in vitro in rat liver (27) and rat skeletal muscle (28, 29). However, no evidence of a similar uncoupling effect in humans has yet been demonstrated, due to the difficulty in extrapolating in vitro measurements of isolated mitochondrial membrane permeability to the in vivo situation. Another difficulty in extrapolating the in vitro data to in vivo conditions is that the in vitro measurements are usually performed on isolated mitochondria under nonlimiting concentrations of ADP (state 3 respiration), in which proton leaks play a minor role (30). Under in vivo basal conditions, skeletal muscle respiration is likely to be operating under conditions of limited ADP (state 4 respiration), where proton leaks may account for a more important fraction of oxygen consumption (6, 31). The in vivo NMR method used in this study circumvents these limitations by assessing mitochondrial energy coupling in intact skeletal muscle under ambient ADP concentrations.

The mechanism by which thyroid hormone might promote increased mitochondrial uncoupling is unknown. However, a skeletal muscle-specific uncoupling protein (UCP-3) has recently been cloned in humans, and has been shown to be unregulated by thyroid hormone (9–18). This protein is thought to create a pathway that allows exothermic movement of protons through the inner mitochondrial membrane, and it has been speculated that UCP-3 might be involved in regulating energy expenditure and body weight in humans. Whether UCP-3 mediates mitochondrial uncoupling or not remains unclear. It has been reported recently that UCP-3 null mice have a normal response to thyroid hormone (32). On the other hand, transgenic mice with UCP-3 overexpression in skeletal muscle are hyperphagic and lean (33). However, the very high level of UCP-3 overexpression reported in these mice (~60-fold) does not argue in favor of UCP-3 being an efficient mitochondrial uncoupler. Taken together, these data suggest that an increase in UCP-3 expression may not be the only factor responsible for the observed T₃-induced increase in mitochondrial uncoupling.

Given the increasing prevalence of obesity in the industrialized nations (34), there is considerable interest in the regulation of uncoupling protein expression and activity as a means of promoting nonshivering thermogenesis and weight loss. Although thyroid hormone...
promotes UCP-3 expression, the ubiquitous distribution of thyroid hormone receptor and action throughout the body, especially in the heart and central nervous system, limits its use in this regard. In contrast, UCP-3 is expressed almost exclusively in skeletal muscle, and the present data would support UCP-3 as a possible pharmacological target to promote weight loss through selective increases in mitochondrial energy uncoupling in this tissue. Furthermore, conversion of intramyocellular lipid into heat by this mechanism would also be predicted to reverse fat-induced insulin resistance (35).

In summary, we used a novel NMR approach to measure T3-induced changes in mitochondrial energy coupling in human skeletal muscle. We found that three days of T3 treatment induced a disproportionate increase in TCA cycle flux compared with ATP synthesis in skeletal muscle. These data demonstrate that T3 can increase thermogenesis in skeletal muscle by promotion of mitochondrial uncoupling that may be partly mediated by an increase in UCP-3 protein expression. This in vivo NMR approach should be useful for exploring the roles of mitochondrial energy uncoupling processes in regulating energy metabolism and obesity in humans.

Acknowledgments

The authors would like to thank Joan Boyer, Laura Burden, Damien Nuzzo, and the staff of the Yale–New Haven Hospital General Clinical Research Center for expert technical assistance, Terry Nixon for maintenance and upgrades to the spectrometer, Peter Brown for coil construction, Jun Shen for assistance in pulse sequence implementation, Graeme Mason for assistance in modeling, and Gerard Burrow for helpful discussions. This work was supported by grants from the United States Public Health Service (R01 DK-49230, P30 DK-45735, and M01 RR-00125) and a K-23 award from the United States Public Health Service (R01 DK-49230, and M01 RR-00125) and a K-23 award from the National Institute of Diabetes and Digestive and Kidney Diseases. This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (R01 DK-49230, and M01 RR-00125) and a K-23 award from the United States Public Health Service (R01 DK-49230, and M01 RR-00125) and a K-23 award from the National Institute of Diabetes and Digestive and Kidney Diseases.