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Article

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Effect of triiodothyronine on mitochondrial energy coupling in human skeletal muscle

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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.

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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 (P_i) and the γ phosphate resonance of ATP (γ ATP); 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 μ g orally every 12 hours). Whole-body oxygen consumption was measured using a DeltaTrac II indirect

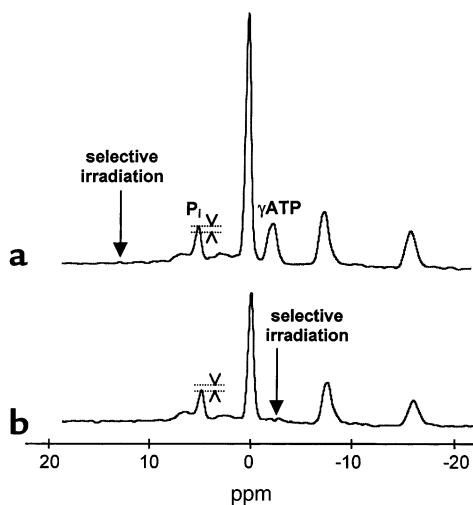


Figure 1
Principle of ATP synthesis rate measurement by ^{31}P NMR spectroscopy. (a) Control spectrum in one volunteer: the saturation pulse is applied symmetrically to the γATP Larmor frequency. (b) Spectrum with γATP saturation acquired in the same volunteer ($NS = 128$). Subtraction of spectrum (b)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

calorimeter (SensorMedics Corp., Yorba Linda, California, USA) before and after T_3 treatment. All measurements were performed at rest following a 12-hour overnight fast at thermoneutrality ($22\text{--}23^\circ\text{C}$).

Informed written consent was obtained from all subjects after the aims and potential risks of the study were explained to them. The protocol was approved by the Yale University School of Medicine Human Investigation Committee.

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.

^{31}P NMR measurement of ATP flux. ^{31}P spectroscopy was performed at 36.31 MHz, using a flat concentric probe made of a 9-cm (diameter) inner coil (for ^{31}P) 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 P_i and ATP. The steady-state P_i magnetization M_z was measured in the presence of selective irradiation of the γATP , and compared with the equilibrium P_i magnetization M_0 in a control spectrum (without irradiation of γATP), as shown in Figure 1.

The fractional reduction of P_i magnetization upon γATP 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: $k_1 = [(M_0 - M_z)/M_0](1/TI^*)$, where TI^* is the spin-lattice relaxation time for the phosphorus nucleus of P_i when ATP is saturated.

TI^* was then measured using a modified version of the inversion-recovery experiment ($180^\circ\text{-}\tau\text{-}90^\circ\text{-acq-ID}$) in the presence of steady-state saturation of γATP during the total interpulse delay (ID) of 4 seconds and during the variable delay (τ). TI^* 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 P_i concentration. The P_i concentration was measured from the control spectrum, assuming a constant ATP concentration of 5.5 mmol/kg in muscle (19).

Total acquisition time for ^{31}P spectra was about 120 minutes. After completion of ^{31}P 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.

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

^{13}C NMR spectra were acquired using either an image-selected in vivo spectroscopy (ISIS) sequence ($TR = 2.3$ sec-

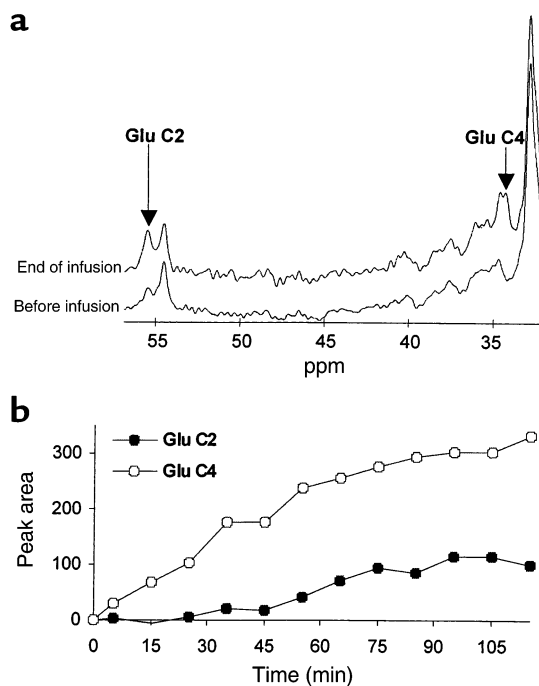


Figure 2
 ^{13}C NMR data. (a) ^{13}C spectra acquired in a volunteer before and at the end of the $[2\text{-}^{13}\text{C}]\text{acetate}$ infusion. (b) Time course of glutamate C4 and glutamate C2 peak areas during the same experiment. Glutamate C4 appears soon after the beginning of the $[2\text{-}^{13}\text{C}]\text{acetate}$ 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 ^{13}C -labeled carbon into this position.

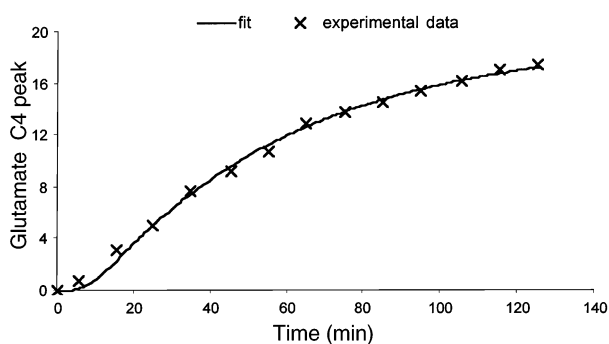


Figure 3
Typical model fit of the ^{13}C NMR data of glutamate C4 in skeletal muscle ($R^2 = 0.93$).

onds), or a nonlocalized sequence (TR = 1.4 seconds) with suppression of the 34.4 parts per million (ppm) lipid signal after inversion by an adiabatic pulse. Temporal resolution was 10 minutes. After Fourier transformation and baseline subtraction, glutamate C2 peaks were integrated between 55.3 and 55.8 ppm, and glutamate C4 peaks were integrated between 33.6 and 35.0 ppm (Figure 2).

Fractional enrichments and plasma acetate were measured from blood samples collected at 10-minute intervals and analyzed on a Hewlett-Packard 5890 gas chromatograph (HP-1 capillary column, 12 m \times 0.2 mm \times 0.33 mm film thickness) interfaced to a Hewlett-Packard 5971A mass selective detector operating in the electron impact ionization mode (12).

TCA cycle flux was assessed by monitoring the incorporation of ^{13}C into glutamate from $[2-^{13}\text{C}]$ acetate and modeling the data using Cwave software (21, 22). An intracellular concentration of glutamate in skeletal muscle of 2.41 ± 0.28 mmol/l (as measured in the muscle biopsies described below) was used in the model. The model describes the increase in glutamate C4 labeling resulting from $[2-^{13}\text{C}]$ acetate conversion into $[2-^{13}\text{C}]$ acetyl CoA, which in turn condenses with oxaloacetate to produce $[4-^{13}\text{C}]$ citrate; this in turn labels α -ketoglutarate C4, which then equilibrates with glutamate C4. Following the second turn of the cycle, C2 and C3 of glutamate become labeled. Nonlinear least squares fit of glutamate C4 and C2 yielded an estimate of TCA cycle flux. A typical fit of the C4 glutamate data is shown in Figure 3. It is important to note that the TCA cycle flux calculated from our mathematical model is independent of both the amount of acetate incorpo-

rated into the TCA cycle (flux through acetyl CoA synthetase) and the substrate source (glucose, fatty acids, or acetate). This approach is similar to the methodology developed and validated by Chatham et al. in the perfused heart, which found a 1:1 relationship between ^{13}C NMR-determined rates of oxygen consumption and measured rates of oxygen consumption (23).

Muscle biopsies. Muscle tissue (50–100 mg) was obtained from the vastus lateralis muscles of three additional subjects by a punch biopsy procedure using a 5-mm Bergstrom biopsy needle (Life Medical Equipment, Miami, Florida, USA); this was done before and after T_3 treatment in order to determine muscle glutamate concentration for the TCA cycle flux calculations. 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 \pm SE. Comparisons of values measured before and after T_3 treatment were made using the paired Student's *t* test.

Results

Three days of T_3 treatment induced a mild hyperthyroid state in these subjects, as reflected by a significant increase in plasma T_3 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 T_3 treatment; $P < 0.02$) as assessed by indirect calorimetry.

The rate of ATP synthesis as assessed by ^{31}P NMR was 5.2 ± 0.6 $\mu\text{mol/g/min}$ in the basal state and it did not change significantly after T_3 treatment (4.6 ± 0.8 $\mu\text{mol/g/min}$; $P = 0.16$ vs. basal), as shown in Figure 4.

In contrast, the mean rate of the TCA cycle as assessed by ^{13}C NMR from the rate of ^{13}C incorporation into glutamate C4 and C2 increased by 70% after T_3 treatment ($P = 0.01$ vs. basal). The TCA cycle flux was 0.064 ± 0.008 $\mu\text{mol/g/min}$ in the basal state, and increased to 0.109 ± 0.013 $\mu\text{mol/g/min}$ after T_3 administration (Figure 4).

An estimate of mitochondrial energy coupling was obtained by expressing the ratio of ATP synthesis to

Table 1
Effect of T_3 treatment on clinical and metabolic parameters

	Body weight (kg)	Heart rate (beats/min)	Thyroxine T_4 ($\mu\text{g/dl}$)	Thyroid binding capacity ($\mu\text{g/dl}$)	Estimated free thyroxine (ng/dl)	T_3 (ng/dl)	TSH ($\mu\text{U/ml}$)	Whole-body oxygen consumption (ml/min)
Basal	67 ± 4	63 ± 2	7.1 ± 0.4	19.1 ± 1.1	1.77 ± 0.09	93 ± 5	1.66 ± 0.35	257 ± 11
Post- T_3	66 ± 4	82 ± 5	3.6 ± 0.4	17.8 ± 1.0	1.65 ± 0.10	552 ± 30	0.04 ± 0.01	295 ± 12
	$P < 0.05$	$P < 0.001$	$P < 0.0005$	$P < 0.005$	$P = 0.11$	$P < 0.0001$	$P < 0.01$	$P = 0.04$

Body weight; heart rate; plasma thyroxine; thyroid binding capacity; estimated free thyroxine, T_3 , and TSH concentrations; and whole-body oxygen consumption measured by indirect calorimetry in the subjects before and after T_3 treatment.

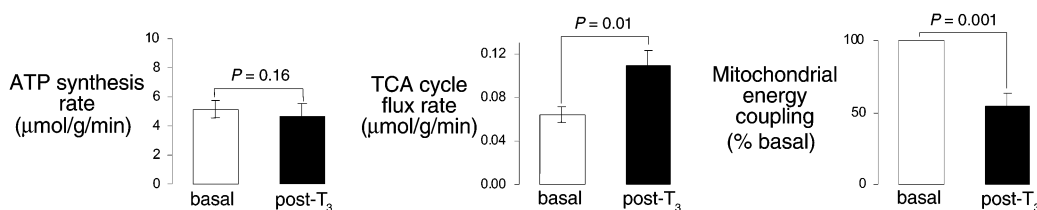


Figure 4

Muscle TCA cycle and ATP synthesis fluxes measured in seven volunteers before (basal) and after T₃ treatment (post-T₃). Thyroid hormone induces a significant increase in muscle TCA cycle flux, whereas the rate of ATP synthesis does not show any significant change. The resulting ATP/TCA ratio (normalized to the basal state) reflects a decrease in mitochondrial energy coupling following 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 ¹³C/³¹P 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.

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