T₃ stimulates resting metabolism and UCP-2 and UCP-3 mRNA but not nonphosphorylating mitochondrial respiration in mice

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J ekabsons, Mika B., Francine M. Gregoire, Nancy A. Schonfeld-Warden, Craig H. Warden, and B. A. Horwitz. T₃ stimulates resting metabolism and UCP-2 and UCP-3 mRNA but not nonphosphorylating mitochondrial respiration in mice. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E380–E389, 1999.—The molecular basis for variations in resting metabolic rate (RMR) within a species is unknown. One possibility is that variations in RMR occur because of variations in uncoupling protein 2 (UCP-2) and uncoupling protein 3 (UCP-3) expression, resulting in mitochondrial proton leak differences. We tested the hypothesis that UCP-2 and -3 mRNAs positively correlate with RMR and proton leak. We treated thyroidectomized and sham-operated mice with triiodothyronine (T₃) or vehicle and measured RMR, liver, and skeletal muscle mitochondrial nonphosphorylating respiration and UCP-2 and -3 mRNAs. T₃ stimulated RMR and liver UCP-2 and gastrocnemius UCP-2 and -3 expression. Mitochondrial respiration was not affected by T₃ and did not correlate with UCP-2 and -3 mRNAs. Gastrocnemius UCP-2 and -3 expression did correlate with RMR. We conclude 1) T₃ did not influence intrinsic mitochondrial properties such as membrane structure and composition, and 2) variations in UCP-2 and -3 expression may partly explain variations in RMR. One possible explanation for these data is that T₃ stimulates the leak in vivo but not in vitro because a posttranslational regulator of UCP-2 and -3 is not retained in the mitochondrial fraction.

proton leak; thyroid hormone; mitochondria; uncoupling protein

EIGHTY-FIVE TO NINETY PERCENT of whole body oxygen consumption is attributable to the mitochondria (54). In liver and skeletal muscle, proton leak is an important contributor to mitochondrial respiration (55). Brand et al. (3) have reported that 26% of isolated rat hepatocyte and 50% of perfused rat hindlimb skeletal muscle mitochondrial respiration occur to support proton leak in these tissues. Moreover, liver and skeletal muscle mitochondrial proton leak has been estimated to account for 20% of rat resting metabolic rate (RMR; Refs. 53, 54). Thus the well-documented effects of thyroid hormone on RMR may involve changes in proton leak. In support of this, Hafner et al. (21) and Harper and Brand (22, 24) have observed higher rates of proton leak in isolated mitochondria as well as in mitochondria within isolated hepatocytes from hyperthyroid compared with euthyroid rats; they have also reported lower hepatocyte mitochondrial proton leak rates in hypothyroid compared with euthyroid rats.

Thyroid hormone status can affect the total surface area of the inner mitochondrial membrane as well as its phospholipid composition (5, 12, 28, 31, 32, 56). Because phospholipid bilayers are inherently leaky to protons (7–9, 18), a change in inner membrane surface area is one mechanism by which triiodothyronine (T₃) may influence proton leak. Phospholipid composition may also be important because the degree of mitochondrial proton leak correlates with the number and type of unsaturated fatty acids found within mitochondria (4, 52). However, liposomes of differing fatty acid compositions (prepared from mitochondria differing in composition) do not exhibit differences in proton leak (6). Although this indicates that compositional differences do not affect the inherent leakiness of lipid bilayers, this does not exclude the possibility that such differences influence proton leak in intact mitochondria via interactions with membrane structure or integral membrane proteins (6, 7). Thus the inner mitochondrial membrane phospholipid bilayer may be one target of thyroid hormone-induced changes in proton leak.

A third possible mechanism by which thyroid hormone may affect proton leak is by inducing expression and synthesis of one or more proteins that facilitate proton transport across the inner mitochondrial membrane. This effect may involve uncoupling protein 2 (UCP-2) and/or uncoupling protein 3 (UCP-3) because these proteins, when expressed in yeast or myoblast cells, reduce mitochondrial membrane potentials (1, 17, 19, 20, 41) and are upregulated at the mRNA level by relatively high concentrations of T₃ (20, 38, 39, 42). However, there is no direct evidence that UCP-2 or UCP-3 facilitates proton leak in mammalian tissues. Furthermore, integrating studies of proton leak with those of UCP expression in an effort to define the role of these proteins in the thermogenic action of thyroid hormone is difficult because 1) no study to date has examined the effect of thyroid hormone on whole body oxygen consumption, mitochondrial nonphosphorylating respiration (an index of proton leak), and UCP-2 and UCP-3 expression in the same animal; 2) studies that have addressed the effects of thyroid hormone on proton leak have primarily been confined to liver, whereas those that have addressed the effects of thy-
roid hormone on UCP-2 and UCP-3 expression have focused on heart, skeletal muscle, and adipose tissue; and 3) the T₃ concentrations used in these studies were variable and of different durations. Thus the goal of our study was to measure whole body and mitochondrial oxygen consumption as well as UCP-2 and UCP-3 expression from the same animal treated with a relatively low concentration of T₃ to approximate physiological levels (49). In this way, we could test the hypothesis that UCP-2 and UCP-3 expression positively correlates with RMR and with mitochondrial proton leak. For this, we treated hypothyroid and euthyroid mice for 6 days with 2.5 µg T₃ per 100 g body mass or vehicle and subsequently measured whole animal RMR, liver and skeletal muscle mitochondrial oxygen consumption, and UCP-2 and UCP-3 expression in these tissues as well as in heart.

MATERIALS AND METHODS

Animals. Thyroidectomized-parathyroidectomized (T₃) and sham-operated male C57BL/6 mice were obtained from Taconic (Germantown, NY). Surgeries were performed when the mice were 1–2 mo old. The mice were delivered to us within 3 days after the surgery. They were housed four per cage for 5–8 wk at 22°C on a 14–10 light-dark cycle (lights on 0600) with free access to food (Purina 5001 chow) and water. Because the parathyroid gland was also removed, T₃ mice received 2% calcium lactate in their drinking water. At the beginning of the experiment, mice were housed two per cage according to their surgical category. One mouse per cage received T₃ injections at 2.5 µg/100 g body mass (in 100–140 µl), whereas the other received vehicle (5 mM NaOH; 100–140 µl). Injections were given intraperitoneally once a day for 6 consecutive days. Mice were weighed daily and allowed free access to chow and water during the treatment. Pooled serum thyroid-stimulating hormone levels averaged 964 ng/ml in T₃ + vehicle mice (range 1673–255), 208 ng/ml in T₃ + T₃ mice (range 218 to 198), 223 ng/ml in sham + vehicle mice (range 232–213), and 196 ng/ml in sham + T₃ mice (range 199–193). Mouse thyroid-stimulating hormone RIA was developed and performed by Dr. A. F. Parlow, Scientific Immunology, National Hormone and Pituitary Program, Harbor-University of California Los Angeles Medical Center (Torrance, CA).

RMR measurements. Immediately after the sixth and final injection of T₃ or vehicle (given between 1130 and 1200), mice were transported to the laboratory and kept overnight in a quiet room having the same light cycle and environmental temperature as previously described. Between 0630 and 0700 the day after the final injection, mice were placed into individual 220-ml metabolic chambers after measurement of their colonic temperatures and body masses. The chambers were positioned in a water bath to maintain chamber temperatures at 28 ± 1°C, which is near the 30–32°C thermoneutral zone for mice. The rate of desiccated air delivered to each chamber was held constant at 300 ml/min with mass flow controller calibrated to a single gas flow gauge. The air exiting each chamber was passed through dessicant (Drierite) and CO₂ absorbent (Baralyme) before the fractional oxygen content was measured with an Ametek oxygen analyzer. Over the course of the measurements, drift in the oxygen analyzer was checked periodically by sampling air exiting an empty chamber. Any drift in the oxygen analyzer over the time period of measurement was assumed to be linear. Measurements were documented with a strip chart recorder. Oxygen consumption was calculated as described by Hill (27).

On any given trial day, two mice were measured. They were acclimated to the chambers for ~4 h before oxygen consumption measurements were started, which were made over the next 4–5 h. RMR was assessed from oxygen consumption measurements during periods in which the mice were inactive. At the end of the measurements, mice were killed by decapitation and colonic temperatures were immediately measured. Liver, hindlimb skeletal muscle, and heart were rapidly removed for mitochondrial isolation and/or RNA analysis. One liver lobe was saved for RNA analysis, and the rest of it was immediately used for isolation of mitochondria. The gastrocnemius and soleus muscles from one leg were removed and saved for RNA analysis, and the rest of the hindlimb skeletal muscle on both legs was immediately used for isolation of mitochondria.

RMR changes with body mass, and this change is described by a power function (RMR = aMᵇ, where a is the mass coefficient, M is body mass, and b is the mass exponent). Although there has been debate as to the value of b, Heusner has provided both theoretical and empirical evidence that b = 2/3 (25, 26). That is, when b = 2/3, the variation in RMR that can be explained by the variation in body mass is removed, and any difference remaining in the RMR values can be attributed to intrinsic differences in the animals being studied. Because the body masses of the sham-operated mice in this study were significantly greater than those of the T₃ mice, we wanted to express RMR on a mass-independent basis so that we could ask the question: would RMR differ between the groups if all the mice had the same weight? We found that 25% of the variation in RMR of all mice could be accounted for by variations in body mass, as evidenced by regression analysis (data not shown). Differences in RMR manifested after dividing by body mass to the power 2/3 suggested that thyroid status affected one or more intrinsic properties of the mass itself. Changes in body composition and in the metabolic activity of the mass (including proton leak) are two primary factors dictating the intrinsic nature of the mass.

Mitochondrial isolations and measurement of nonphosphorylating respiration. Liver and hindlimb skeletal muscle (excluding the gastrocnemius and soleus muscles on one leg, which were used for RNA isolation) were homogenized in (in mM) 250 sucrose, 30 HEPES, 10 EDTA, 1 EGTA, and 0.2 phenylmethylsulfonyl fluoride, pH 7.1, with a Tekmar Tissumizer. After two centrifugations for 15 and 20 min at 750 and 1000 g, respectively, mitochondria were pelleted by centrifugation for 12 min at 10,000 g. The pellets were washed of loosely packed material by swirling with 5 ml of homogenization buffer. After the pellets were resuspended in 30 ml of buffer, the mitochondria were pelleted and washed as before. The final pellet was resuspended in the same buffer lacking EDTA. Once the tissues were removed, all procedures were carried out at 0–5°C.

Nonphosphorylating respiration of 0.2–0.7 mg liver mitochondrial protein or 0.12–0.28 mg skeletal muscle mitochondrial protein was measured at 37°C with a Clark type electrode in air-saturated buffer containing (final concentration) 120 mM KCl, 5 mM NaCl, 5 mM KH₂PO₄/K₂HPO₄, 5 mM MgCl₂, 2 mM EGTA, 0.1 mM Mg acetate, 50 mM HEPES, 5 µM rotenone, and 3.3 µg/ml oligomycin B (final pH 6.9). We found no correlation between the amount of protein used to measure respiration rates and the rates of oxygen consumption expressed per milligram of protein. Thus the preparation-to-preparation variation in the oligomycin-to-protein ratio had no effect on the measured rates of nonphosphorylating respiration. Oligomycin B was added to inhibit ATP synthesis; rotenone was added to inhibit oxidation of NADH via complex I of the respiratory chain. Respiration was initiated.
by addition of succinate to 5 mM. Nonphosphorylating respiration was measured over 5–10 min. Aliquots of each preparation were saved for quantification of total protein with the bicinchoninic acid assay (Pierce, Rockford, IL) with BSA as a standard.

Northern blot analysis. Total RNA was extracted from liver, gastrocnemius, soleus, and heart with Trizol reagent (Gibco, Gaithersburg, MD) according to the instructions of the manufacturer. Excluding soleus samples, 20 μg of total RNA from each tissue were fractionated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde. Preliminary experiments indicated that the amount of RNA obtained from each soleus was generally insufficient for individual determinations of transcript levels, so the RNA samples within a group were pooled in pairs. The final soleus RNA preparations from T x + vehicle mice (where n = 5) were the exception, consisting of two pooled and one individual sample. Ethidium bromide was added to each electrophoresed sample so that RNA integrity and loading could be verified. On a given gel, three samples from each treatment group were present. RNA was transferred to nylon membranes (Duralon-UV; Stratagene, LaJolla, CA) by overnight capillary transfer and then ultraviolet cross-linked.

Mouse UCP-2 and UCP-3 cDNA clones were obtained from the IMAGE consortium (GenBank access no. W71569 for the IMAGE consortium; GenBank accession no. W71569 for the IMAGE consortium). Clones were excised by NotI digestion and gel purified with the Qiaquick gel extraction kit (Qiagen, Santa Clarita, CA). Labeled probes were generated by random priming (Rediprime kit, Amersham). Unincorporated nucleotides were removed with NucTrap columns (Stratagene).

Blots were prehybridized 30 min at 68°C in Express Hyb solution (Clontech, Palo Alto, CA) and then hybridized for 60 min in the same solution containing labeled cDNA at 2 × 106 counts·min−1·ml−1. After the blots were washed [twice at room temperature in 2× saline sodium citrate (SSC)/0.05% SDS; twice at 60–65°C in 1× SSC/0.1% SDS], they were exposed to a storage Phosphor screen (Eastman Kodak, Rochester, NY) overnight; signal intensities were quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Quantification of 18S ribosomal RNA (DECAprobe template, Ambion; Austin, TX) was performed in a similar manner to control for variations in the total amount of RNA loaded into each lane. The data are expressed as the ratio of UCP-2 or UCP-3 signal to 18S signal. Two blots were made to accommodate all samples from a given tissue; these blots were exposed to the same hybridization buffer to avoid variances due to differences in probe specific activity.

RESULTS

Before beginning injections (and 5–8 wk after surgery), sham mice weighed significantly more than did T x mice (24.66 ± 0.31 vs. 22.82 ± 0.49 g; P < 0.01 by t-test). T 3 treatment significantly increased body weight gain (T x + T 3: 1.01 ± 0.44 g and sham + T 3: 1.34 ± 0.47 g vs. T x + vehicle: 0.33 ± 0.52 g and sham + vehicle: 0.11 ± 0.19 g; P = 0.037 for main effect of treatment). T x mice tended to have lower colonic temperatures (P = 0.07) despite no effect of thyroidectomy on RMR (Table 1). Mice treated with T 3 had significantly greater RMR than those treated with vehicle (Table 1). Although T 3 treatment did not have a significant effect on colonic temperatures measured at the beginning of the experiment (0700, 1 h after lights on), the treatment significantly attenuated the fall in rectal temperature observed in all mice over the 7–8 h they were in the metabolic chambers (Table 1). Final colonic temperatures at this time positively correlated with RMR (Fig. 1).

Nonphosphorylating respiratory rates of hindlimb skeletal muscle mitochondria isolated from T x mice were significantly lower than those from sham mice; liver mitochondrial respiratory rates from T x mice tended to be lower than sham mice, but this was not significant (Table 1). Mitochondrial nonphosphorylating respiratory rates from both tissues were unaffected by T 3 treatment. Surprisingly, liver mitochondrial respiration negatively correlated with RMR (Fig. 2), whereas skeletal muscle mitochondrial respiration did not correlate with RMR (r 2 = 0.054, P = 0.43; data not shown).

To determine if expression of UCP-2 and UCP-3 was correlated with mitochondrial proton leak, we quanti-
fied steady-state mRNA levels in the same tissues from which mitochondrial respiration measurements were made. T₃ treatment significantly increased gastrocnemius UCP-2 and UCP-3 steady-state mRNA levels (Figs. 3 and 4), with the effect being greater in Tx mice (63 and 60% for UCP-2 and UCP-3, respectively) than in sham mice (27 and 46% for UCP-2 and UCP-3, respectively), although the surgery × treatment interaction was not significant. Gastrocnemius UCP-2 and UCP-3 mRNA levels significantly correlated with RMR (Fig. 5) but did not significantly correlate with muscle mitochondrial nonphosphorylating respiration (UCP-2: $r^2 = 0.058$, $P = 0.41$; UCP-3: $r^2 = 0.119$, $P = 0.23$; data not shown). Soleus UCP-2 and UCP-3 levels were unaffected by surgery or treatment (data not shown). We were unable to determine if soleus UCP-2 and UCP-3 levels were correlated with RMR or mitochondrial respiration because soleus samples were pooled for Northern analysis.

Liver UCP-2 mRNA levels were affected by surgery and treatment (Fig. 6); the levels were stimulated by T₃ to a similar extent in Tₓ (60%) and sham (71%) mice compared with their respective controls. However, UCP-2 levels did not significantly correlate with muscle mitochondrial nonphosphorylating respiration (UCP-2: $r^2 = 0.058$, $P = 0.41$; UCP-3: $r^2 = 0.119$, $P = 0.23$; data not shown). Soleus UCP-2 and UCP-3 levels were unaffected by surgery or treatment (data not shown). We found no significant correlation between liver mitochondrial nonphosphorylating respiration and UCP-2 levels ($r^2 = 0.022$, $P = 0.51$; data not shown). Heart UCP-2 was unaffected by surgery or treatment (Fig. 7A), although Tₓ mice given T₃ had levels 39% greater than controls.
Similarly, T3 treatment tended to upregulate heart UCP-3, with this effect just failing to reach statistical significance (Fig. 7B; P = 0.06). As was the case with gastrocnemius UCP levels, T3 tended to stimulate heart UCP-3 expression more in Tx (57%) than in sham (27%) mice. Heart UCP-3 levels significantly correlated with RMR, whereas the levels of UCP-2 were positively, but not significantly, correlated with RMR (Fig. 8).

**DISCUSSION**

A major finding of this study is that T3 stimulates RMR but not isolated liver and skeletal muscle mitochondrial nonphosphorylating respiration. Because nonphosphorylating respiration is an index of proton leak activity (2), we conclude that the stimulatory effect of T3 on RMR is not accompanied by greater proton leak of isolated liver and skeletal muscle mitochondria. This conclusion, although requiring further scrutiny by determining the proton leak kinetics as described by Brand (2), suggests that either liver and skeletal muscle mitochondrial proton leak do not contribute to the stimulatory effect of T3 on RMR or the leak in vitro does not reflect that which occurs in vivo. We believe the latter alternative is important to consider given studies reporting that liver and skeletal muscle mitochondrial proton leak accounts for 20% of rat RMR (53, 54) as well as those suggesting that differences in proton leak partly explain the differences in mass-specific metabolic rates of animals having different body masses (50–52).

Another finding of this study is that T3 stimulates expression of liver UCP-2 and gastrocnemius and heart UCP-2 and UCP-3. Moreover, gastrocnemius UCP-2 and UCP-3 expression, and to a lesser extent, liver UCP-2 expression, positively correlate with RMR but not with isolated skeletal muscle and liver mitochondrial nonphosphorylating respiration. Heart UCP-2 and UCP-3 also positively correlate with RMR (we did not measure heart mitochondrial respiration because of limited amounts of this tissue). There are several possible reasons to account for the discordance between UCP-2 and UCP-3 expression and in vitro mitochondrial proton leak activity. These include 1) T3 stimu-
lates the total leak capacity (i.e., the leak per mitochondrion) but not the specific leak activity (i.e., the leak per protein); 2) T3 stimulates UCP-2 and UCP-3 expression, but a required cofactor or allosteric activator (whether constitutively present or upregulated by T3) is removed during mitochondrial isolation; 3) UCP-2 and UCP-3 mRNA levels do not reflect functional mitochondrial protein levels; and 4) UCP-2 and UCP-3 do not facilitate proton leak. There is insufficient evidence in the literature to negate any of these possibilities.

The possibility that T3 stimulates the total leak but not the specific leak requires that T3 1) stimulates UCP-2 and UCP-3 expression to the same extent as other mitochondrial proteins while having no effect on their specific activities or 2) increases the total area of the inner mitochondrial membrane without affecting the surface area-to-protein mass ratio. Regardless of the mechanism, this effect would result in a greater total leak activity in mitochondrial fractions from T3-treated mice. Two-way ANOVA of our data revealed no main effect of T3 treatment on total leak activity of the mitochondrial fractions (data not shown). Assuming equivalent recoveries of mitochondria from T3- and vehicle-treated mice, our data do not support the hypothesis that T3 stimulates the total leak per mitochondrion.

Allosteric regulation of UCP-2 and UCP-3 is a reasonable possibility given that the activity of UCP-1 is activated by fatty acids and inhibited by purine nucleotide di- and triphosphates (47). The COOH-terminal region of UCP-1 thought to interact with fatty acids is not highly conserved in UCP-2 (6 of 18 amino acids conserved) or UCP-3 (7 of 18 amino acids conserved), which indicates that a putative activator may be something other than a fatty acid. Amino acids comprising the purine nucleotide-binding domain in UCP-1 are well-conserved in UCP-2 (12 of 16 conserved) and UCP-3 (13 of 16 conserved), although contradictory evidence has been reported on whether mitochondrial proton leak is inhibited by purine nucleotide di- and triphosphates (46 vs. 10, 34, and 44).

The possibility that UCP-2 and UCP-3 mRNA levels do not reflect functional mitochondrial protein levels is of interest because UCP-1 exists in inactive and active forms. Brown fat mitochondria from rodents acutely cold exposed or aroused from hibernation show greater GDP binding than do controls (14, 15, 30). Because the time frame is too short for an increase in the amount of
UCP-1 via transcription and translation, the increase in GDP binding has been thought to reflect the unmasking/activation of preexisting inactive UCP-1 proteins (14, 15); this effect is primarily mediated by catecholamines (14, 15). As shown here and elsewhere (20, 38, 39, 42), UCP-2 and UCP-3 expression and presumably protein levels are stimulated by T3 (although the assumption of parallel changes in expression and protein levels is not true for UCP-1 under certain conditions; see Ref. 36). However, changes in one or more additional factors may be required to activate the preexisting and newly synthesized proteins. Whatever factors might be involved, this possibility, as with the previous one, suggests that UCP-2 and UCP-3 are posttranslationally regulated.

The possibility that UCP-2 and UCP-3 do not facilitate proton leak in mammalian cells needs further evaluation. The primary evidence supporting an uncoupling effect of UCP-2 and UCP-3 derives from yeast transfection experiments (17, 19, 20, 41). These experiments show that yeast expressing UCP-2 and UCP-3 have a greater proportion of mitochondria with lower membrane potentials than those transfected with vector alone. Recent evidence (57) suggests that this is not a nonspecific effect of a protein being inserted into the inner mitochondrial membrane because yeast transfected with the oxoglutarate carrier do not exhibit reduced membrane potentials. Because UCP-1 is a known uncoupler in mammalian mitochondria and has effects in yeast similar to those of UCP-2 and UCP-3, the evidence to date indicates that UCP-2 and UCP-3 are likely to be uncouplers in mammalian mitochondria. Whether or not they uncouple by directly facilitating proton leak or as a consequence of some other activity remains to be determined.

Many experiments investigating the relationship between thyroid hormone status and proton leak have focused on the liver because it is rich in thyroid hormone receptors, an important contributor to basal metabolism, and easily homogenized for preparation of mitochondria. Yet, the liver is not an ideal tissue to examine the relationship between UCP-2 expression and proton leak because UCP-2 mRNA is normally present at low levels in whole liver RNA preparations (11, 29, 40); presumably, the protein is also present at low levels in mitochondria isolated from whole liver preparations. This is one explanation to account for our finding that liver UCP-2 expression did not correlate with mitochondrial respiration (P = 0.51). However, because we found that liver UCP-2 expression positively correlates, albeit not significantly (P = 0.09), with RMR, it is possible that low expression of UCP-2 contributes to hepatic and thus resting metabolism.

Skeletal muscle mitochondria were isolated from the gastrocnemius and soleus of one leg along with most of the other muscle groups from both legs. Our results show that T3 regulation of UCP-2 and UCP-3 expression (determined from the remaining gastrocnemius and soleus) is dependent on fiber type and/or muscle group. Expression in the soleus (slow-red oxidative) was insensitive to T3, whereas expression in the gastrocnemius (a mixed muscle, but predominantly fast-white glycolytic) was induced by T3. Despite the fact that the gastrocnemius is a mixed muscle, we cannot state with certainty that the mitochondria isolated from most of the hindlimb muscles would be representative of the mitochondria isolated from the gastrocnemius. This could be a potential source of variation leading to our observation that gastrocnemius UCP-2 and UCP-3 mRNA levels are not correlated with mitochondrial respiration from a mixed hindlimb preparation.

Lanni et al. (37) recently reported that state 4 skeletal muscle mitochondrial respiration correlated with UCP-3 expression in rats of different thyroid hormone status. Several possible reasons for the discrepancy with our data are noted. First, their correlation involved three points (the means for hypo-, eu-, and hyperthyroid conditions) due to the fact that UCP-3 expression and mitochondrial respiration were not measured in the same animals. Thus their data do not reflect the individual variability occurring among as
well as within the groups. Second, the physiological responses to thyroid state apparently differ between rats and mice (see below). Third, hyperthyroidism was induced with a sixfold higher concentration of T3 than we used (see below). Fourth, hypothyroidism was induced over 3 wk by injections of propylthiouracil and iodoacetic acid, which inhibit not only thyroid hormone synthesis but also peripheral deiodinases; thus peripheral metabolism and clearance of thyroid hormones are markedly different in propylthiouracil-iodoacetic acid-treated animals compared with those that have undergone thyroidectomy.

In contrast to our finding that UCP-2 and UCP-3 expression positively correlates with RMR, Schrauwen et al. (58) found that skeletal muscle (vastus lateralis) UCP-3, but not UCP-2, expression positively correlated with sleeping metabolic rate in Pima Indians, whereas Millet et al. (43) found no correlation between RMR of lean or obese Caucasians and vastus lateralis UCP-2 and UCP-3 expression or subcutaneous white adipose tissue UCP-2 expression. These differences indicate that species and/or genetics may influence the contribution of UCPs to RMR. Alternatively, these differing results could indicate that UCP-2 and UCP-3 expression is not causally connected to RMR.

Liver and skeletal muscle mitochondria from rats injected with higher concentrations of thyroid hormone (10 injections of 15 µg T3/100 g body mass over 10 days or 20 µg T3/100 g body mass over 21 days) exhibit greater proton leak and basal respiration than do mitochondria isolated from euthyroid and hypothyroid rats (21–23, 37, 60). This effect also occurs in mitochondria of isolated hepatocytes, demonstrating that changes in the leak of isolated mitochondria can qualitatively reflect the changes that occur in situ (22). For liver mitochondria, much of the change has been ascribed to two factors that could alter the nonspecific leak of protons across the inner mitochondrial membrane. One is the inner mitochondrial membrane surface area-to-protein mass ratio, and the other is the inherent permeability of the phospholipid bilayer (5). Hypothyroidism decreases and hyperthyroidism increases these two factors (5). Because we saw no effect of T3 on mitochondrial respiration, we conclude that the T3 treatment in this study did not affect either of these variables.

We offer two potential explanations to account for the fact that the surface area per mass protein and the inherent bilayer permeability were not affected by T3. First, the sixfold lower concentration of T3 used in our study vs. that of Hafner et al. (21), Brand et al. (5), and Lanni et al. (37) may be insufficient to induce these changes. [The fact that we used 6 injections over 6 days as opposed to 10 injections over 10 days (5, 21) is unlikely to have contributed to the absence of in vitro changes in mitochondrial respiration because Tata et al. (60) have shown that a single injection of T3 into rats at ~7–15 µg/100 g body mass (our estimation of their dose) will produce liver and skeletal muscle mitochondria with higher rates of basal respiration 48–72 h later.] The second factor that may be important in accounting for the difference in our results is the species used. Liver mitochondrial proton leak negatively correlates with body mass in mammalian species ranging from the mouse to the horse (51, 52). This phenomenon has primarily been attributed to smaller animals having liver mitochondria with greater inner mitochondrial membrane surface area (per vol matrix and per mass protein) (52). Morphologically, a greater inner mitochondrial membrane surface area is reflected in a greater number of invaginations of the inner mitochondrial membrane into cristae. This is an efficient manner of increasing surface area with minimal changes in volume, yet it imposes physical limitations on the surface area that can be achieved. Mouse mitochondria may be less responsive to induction of inner membrane surface area by T3 because of this physical limitation.

Thyroidectomy for 5–8 wk tended to reduce the specific and total proton leak activities of isolated liver and skeletal muscle mitochondria compared with mitochondria from sham-operated mice. Thus loss of physiological levels of thyroid hormone over a 5- to 8-wk period is sufficient to induce in vitro changes in mouse liver and skeletal muscle mitochondria. These changes were not accompanied by a reduction in UCP-2 and UCP-3 expression in the tissues studied, indicating that if UCP-2 and UCP-3 are involved in the leak, their depressed activities may be the result of changes in inner mitochondrial membrane phospholipid composition (an effect known to alter the activity of some integral membrane proteins). Furthermore, the data imply that chronic treatment of Tx mice with T3 at 2.5 µg/100 g body mass would cause in vitro changes to liver and skeletal muscle mitochondria. Therefore, long-term regulation of RMR by thyroid hormone-induced changes in proton leak may involve both changes in protein-mediated leak (via the amount and activities of UCP-2 and UCP-3) and changes in nonspecific leak (via the total area of the inner mitochondrial membrane).

Surprisingly, RMR was not depressed in T3 mice compared with sham-operated mice. This observation has been verified with a separate group of Tx and sham-operated mice whose RMR was measured at 26°C (n = 4/group, unpublished observations). The absence of an affect of thyroidectomy on RMR may be due to the possibility that 26–28°C is more of a cold stress to Tx mice than to sham-operated euthyroid mice (i.e., the thermoneutral zone for Tx mice is higher than that for sham-operated mice). We know of no studies addressing this specific issue in mice, but it is known that hypothyroid rats at 20–23°C exhibit enhanced sympathetic nervous system activity, as evidenced by studies reporting increased urinary catecholamines and tissue norepinephrine turnover (16, 59). Furthermore, Mory et al. (45) and Ikemoto et al. (33) found that brown adipose tissue of hypothyroid rats and mice at 20–25°C share some characteristics with brown adipose tissue of cold-exposed euthyroid animals (e.g., increased brown adipose tissue weight, protein content, and oxygen consumption). Thus, for a given temperature, hypothyroidism may be associated with a lower RMR, but this effect may be overridden by the cold stress in Tx mice.
roid rodents appear to be under a greater cold stress than are euthyroid rodents. Nonetheless, the thermogenetic mechanisms activated (e.g., brown adipose tissue) are not sufficient to raise the hypothyroid rat RMR to the level of its euthyroid control. Mice may differ from rats in their ability to recruit thyroid hormone-independent thermogenic mechanisms for maintenance of core temperature. If so, the RMR values we report for T₃ mice are overestimates of their true RMR.

In addition to the postulated effects on in vivo proton leak, T₃ regulation of RMR can partly be ascribed to activation of ATP-consuming pathways (24, 35). Thyroid hormone stimulation of protein synthesis and active ion transport is well established (13, 48). In our study, mice treated with T₃ gained more weight than those treated with vehicle. Although we did not determine body composition, this suggests that protein synthesis is an important component contributing to the elevated RMR of T₃-treated mice. The anabolic effect of T₃ indicates that our dose regimen did not induce pronounced hyperthyroidism, as this is typically associated with a reduction in body mass (e.g., Ref. 42).

In summary, administration of T₃ to Tx- and sham-operated mice at a concentration six times lower than that previously shown to stimulate in vitro rat mitochondrial proton leak had no such effect on isolated liver and skeletal muscle mitochondria despite significant effects on RMR. This indicates that T₃ did not alter the inner mitochondrial membrane surface area-to-protein ratio or the inherent lipid bilayer leakiness. Although correlations do not establish causality, the fact that gastrocnemius, heart, and liver UCP-2 and gastrocnemius and heart UCP-3 mRNA levels were correlated with RMR suggests that these proteins may contribute to RMR. If UCP-2 and UCP-3 function as leak pathways, the leak in vitro may have been unaffected because their in vivo activity requires the presence of an allosteric factor that is lost on isolation of mitochondria. The effect of T₃ on ATP-consuming reactions is also likely to be an important contributor to the increase in RMR.

NOTE ADDED IN PROOF

A recent study reported a discordance between UCP-3-expressing yeast respiration and respiration of the isolated mitochondria. This provides further support for the hypothesis that the activity of UCP-3 is subject to posttranslational regulation (Zhang, C.-Y., T. Hagen, U. K. Motha, L. J. Slieker, and B. Lowell. Assessment of uncoupling protein 3 using a yeast heterologous expression system. FEBS Lett. 449: 129–134, 1999).

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