Uncoupling Protein-3 Is a Mediator of Thermogenesis Regulated by Thyroid Hormone, β3-Adrenergic Agonists, and Leptin*

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Mitochondrial uncoupling proteins (UCPs) are transporters that are important for thermogenesis. The net result of their activity is the exothermic movement of protons through the inner mitochondrial membrane, uncoupled from ATP synthesis. We have cloned a third member of the UCP family, UCP3. UCP3 is expressed at high levels in muscle and rodent brown adipose tissue. Overexpression in yeast reduced the mitochondrial membrane potential, showing that UCP3 is a functional uncoupling protein. UCP3 mRNA levels are regulated by hormonal and dietary manipulations. In contrast, levels of UCP2, a widely expressed UCP family member, showed little hormonal regulation. In particular, muscle UCP3 levels were decreased 3-fold in hypothyroid rats and increased 6-fold in hyperthyroid rats. Thus UCP3 is a strong candidate to explain the effects of thyroid hormone on thermogenesis. White adipose UCP3 levels were greatly increased by treatment with the β3-adrenergic agonist, CL214613, suggesting another pathway for increasing thermogenesis. UCP3 mRNA levels were also regulated by dexamethasone, leptin, and starvation, albeit differently in muscle and brown adipose tissue. Starvation caused increased muscle and decreased BAT UCP3, suggesting that muscle assumes a larger role in thermoregulation during starvation. The UCP3 gene is located close to that encoding UCP2, in a chromosomal region implicated in previous linkage studies as contributing to obesity.

Changes in body weight result from the difference between energy intake and energy expenditure. It is presumed that each individual has a target weight that the body tries to maintain. Since weight remains relatively constant despite large variations in energy intake, energy expenditure must be regulated. For example, people who gain weight become metaboli
cally less efficient, whereas those who lose weight become more efficient (1).

The biochemical mechanisms responsible for the regulation of energy expenditure and the efficiency of energy usage are poorly understood. Possible ways to increase energy expenditure include increasing physical activity (2) and energy dissipation as heat by futile metabolic cycles (3–5). Thermogenesis is the major function of brown adipose tissue (BAT), which is found in small mammals and the young of larger mammals (6). The inner membrane of BAT mitochondria contains uncoupling protein (UCP1, previously UCP (7)), which is a member of the mitochondrial anion carrier family (8). UCPs are thought to transport fatty acid anions from the inner surface to the outer surface of the inner mitochondrial membrane (9–11). At the outer surface the anions become protonated and the neutral molecules return inside. The net result is the exothermic movement of protons from outside to inside the inner mitochondrial membrane, down their concentration gradient and uncoupled from ATP synthesis. (An alternate model posits that UCPs are proton transporters, using fatty acids as activating cofactors.)

UCP1 is encoded by a nuclear gene, which is expressed only in BAT (12, 13). The protein consists of six transmembrane domains with the C and N termini oriented toward the cytosol (14), and its activity is inhibited by purine nucleotides (15, 16). Transcription of UCP1 is induced by cold exposure, β-adrenergic stimulation, and thyroid hormone (17). Because mice lacking UCP1 are not obese (18) and because adult humans have little BAT, the importance of UCP1 in weight homeostasis is not clear. Recently, UCP2, a second member of the UCP family, was reported (19, 20). UCP2 mRNA is present in many tissues, and its expression levels are altered in obesity, suggesting that it may contribute to weight homeostasis. We report here a new UCP family member, UCP3, whose expression, unlike that of UCP2, is regulated by known thermogenic stimuli, such as thyroid hormone, β3-adrenergic agonists, and leptin.

EXPERIMENTAL PROCEDURES

Animals and Materials—Rats (male, CD strain, Charles River Laboratories) and C57BL/6J ob/ob and +/+ age-matched mice (Jackson Laboratories) were typically housed three per cage and acclimatized for 1 week before beginning experiments. Animals had free access to water and food pellets. Thyroidectomized 6–8-week-old male SD rats (Harlan Sprague-Dawley) were further treated with 6-propyl-2-thiouracil (0.25% in the drinking water) for 3 weeks. Severe hypothyroidism results from this treatment (not shown). Male ob/ob and +/+ control mice were injected daily with leptin (2 μg/g mouse intraperitoneally; R & D Systems) or saline vehicle for 4 days. The vehicle-treated ob/ob mice gained a mean of 1.5 g, whereas the leptin-treated group averaged a 1.3-g loss. Animals were sacrificed 6 h after the last leptin dose, and RNA was isolated from BAT and muscle. CL-316243 was a gift of J. D. Bloom (Wyeth-Ayerst Research, NY).

Cloning—The 5’-end of hUCP3 cDNA was obtained by 5’-rapid amplification of cDNA ends (Life Technologies Inc.) using skeletal muscle poly(A)+ RNA (CLONTECH). Oligonucleotides 5’-TGGGGCACCACCTCTTTATCATA- CAG and 5’-TGAGATCAGGCGGTCGGGAGGTGAGG were used to prime reverse transcription and PCR, respectively.

Northern Blots—RNA was prepared with Trizol (Life Technologies Inc.), and unless noted otherwise, 15 μg of total RNA was used per lane and ethidium bromide staining confirmed equal RNA loading for all

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF011449.

1 The abbreviations used are: BAT, brown adipose tissue; UCP, uncoupling protein; rUCP, rat UCP; hUCP, human UCP; WAT, white adipose tissue; T3, triiodothyronine; DoOC5, 3,3’-dihydroxylacarbocyca-nine iodide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PCR, polymerase chain reaction; RT, reverse transcription; kb, kilobase(s).

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blots. Probes were random-labeled (Life Technologies Inc.). Hybridization was carried out at 65 °C in Rapid-hyb buffer (Amersham Corp.), and blots were washed twice with 0.5 × SSC/1% SDS at 65 °C. The hUCP3 probe corresponds to bases 1–861 in GenBank™ AF011449. The rUCP3 probe was isolated by RT-PCR using degenerate UCP3-specific primers. A 963-base pair hUCP2 probe was obtained by RT-PCR from human muscle RNA (CLONTECH) using primers 5'-GTACAGGAATTCGACATGGTGTTGGAATC and 5'-AGCAGCTTCTAGGCCCGAGAGGCCG. The rUCP2 probe was a PCR fragment from muscle cDNA obtained using UCP2-specific degenerate primers 5'-TTGGTACCAACAGGAATTCGACATGGTGTTGGAATC and 5'-AGCAGCTTCTAGGCCCGAGAGGCCG. The rUCP1 probe (nucleotides 84–1154 in M11814) was made by RT-PCR using BAT RNA (invitrogen) clones were selected on SC medium lacking uracil and containing 2% galactose for 6 h prior to assay. Mitochondrial membrane potential was measured with the potential-sensitive dye, 3,3'-dihexyloxycarbocyanine iodide (DiOC₆). Mitochondria were isolated by centrifugation and resuspended in 2% D-2 O in Rapid-hyb buffer and incubated with the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (CCCP; final concentration, 100 μM; Sigma) was used to hyperpolarize the mitochondria. Cells were counted (10,000 events) ~50 min after addition of the dye using a FACScan (Becton Dickinson). Dead cells (<1%) were identified by propidium iodide (final concentration, 5 μg/ml; Sigma) staining or by side scatter. Results are presented un gated; gating different subpopulations produced similar results.

Expression in Saccharomyces cerevisiae—The coding region of the human UCP3 was cloned into pYES2 (a high copy 2-micron vector, Invitrogen) between the HindIII and XbaI sites creating plasmid p2024. Similarly, rUCP1 was cloned between the EcoRI and XbaI sites, producing p2025. Transformed yeast (INVSc1, Invitrogen) clones were selected on ura plates. Clones were grown on SC medium lacking uracil and containing 2% raffinose overnight. Aliquots were then cultured in 96-well plates. Clones were grown on SC medium lacking uracil and containing 2% galactose for 6 h prior to assay. Mitochondrial membrane potential was measured with the potential-sensitive dye, 3,3'-dihexyloxycarbocyanine iodide (DiOC₆). All probes were verified by sequencing. Hybridization signals were quantitated by PhosphorImager analysis and expressed normalized to the control animals.

RESULTS

Identification of UCP3—We searched for UCP homologs in the EST section of GenBank™ and found a single EST (AA192136) from a human muscle library corresponding to the 3'-end of a novel UCP gene, now designated UCP3. 5'-rapid amplification of cDNA ends was used to obtain the rest of the coding region. The deduced 312-amino acid hUCP3 sequence (GenBank™ accession number AF011449) is 58% identical to hUCP1 and 73% identical to hUCP2. Sequence dissimilarities are concentrated in the regions between putative helices 1 and 2 and helices 3 and 4, the same regions that show the most variability among UCP1s from different species. The murine and rat UCP3 cDNAs were also obtained and will be reported elsewhere.

UCP3 Functions as an Uncoupling Protein—The evolutionary conservation and sequence similarity among the UCPs are consistent with the hypothesis that UCP3, like UCP1 and UCP2, is an uncoupler of oxidation from ATP production. To test this, we measured the fluorescence of DiOC₆, a dye sensitive to the mitochondrial membrane potential, in S. cerevisiae cells expressing UCP3 (Fig. 1). For reference, we compared the fluorescence of cells treated with CCCP (to uncouple the mitochondria) or with valinomycin (to hyperpolarize the cells) with that of cells treated with DiOC₆ alone. As expected, yeast expressing rUCP1 had a decreased fluorescence intensity relative to control cells. Yeast expressing hUCP3 had an even lower intensity, demonstrating that hUCP3 functions as an uncoupling protein.

UCP3 Is Expressed in Muscle and Adipose Tissue—We next examined the expression pattern of UCP3 by Northern analysis. In rats, the ~2.5-kb UCP3 mRNA was expressed at high level in skeletal muscle and BAT but was also present at low levels in heart and WAT (Fig. 2). In humans, UCP3 was found predominantly in skeletal muscle, with smaller amounts in heart and WAT (Fig. 2 and data not shown); BAT was not examined. We confirmed the distribution of the ~1.6-kb UCP2 transcript (19, 20), with high levels in WAT, muscle, lung, spleen, thymus, and leukocytes (Fig. 2).

UCP3 Is Regulated by Thyroid Hormone—Thyroid hormone (T₃) is a major regulator of energy homeostasis, with hyperthyroidism increasing basal metabolic rate and body temperature and hypothyroidism decreasing basal metabolic rate and temperature (5). We measured UCP mRNA in rats with alterations in their thyroid status (Fig. 3). In skeletal muscle, hyperthyroidism decreased the UCP3 RNA levels to 10–20% of control values (p = 0.05). Treatment of either hypothyroid or euthyroid rats with a single dose of T₃ produced UCP3 mRNA levels of 624 ± 90 and 473 ± 140% of the euthyroid controls, respectively. In BAT, the effects of T₃ on UCP3 were greatly blunted, with no drop in hypothyroid rats and no increase in euthyroid rats given a single dose of T₃, although an increase in hypothyroid rats treated with T₃ (354 ± 63%; p = 0.006) was observed. Rats made hyperthyroid by daily T₃ injections for 4 days showed muscle and BAT UCP3 expression patterns similar to the single-dose experiment (not shown). In contrast, no change in UCP2 expression was observed with any of these manipulations (not shown). These experiments demonstrate that UCP3 is regulated differently in muscle as compared with BAT and
suggest that thyroid hormone is a physiologic regulator of UCP3 but not of UCP2.

**UCP3 Is Also Regulated by β3-Adrenergic Stimulation, starvation, and Glucocorticoids—**β3-Adrenergic stimulation of adipose cells causes lipolysis and thermogenesis. This is mediated largely by β3-adrenergic receptors (23). Treatment of rats with a selective β3-adrenergic agonist, CL214613, greatly increased UCP3 mRNA levels in WAT (Fig. 4). A slight increase in WAT UCP1 (as expected (24)) and UCP2 mRNA amounts was also observed. In BAT, the β3 agonist increased UCP1 (also as expected) but not UCP2 or UCP3 mRNA levels. The induction of UCP3 in WAT by the CL-316243 is consistent with other observations that WAT assumes some BAT-like characteristics upon β-adrenergic stimulation (25). Thus under strong β-adrenergic stimulation, WAT may make a greater contribution to thermogenesis.

Because one of the adaptations to the fasting state is a reduction in energy expenditure, we examined the response of UCPs to fasting and refeeding (Fig. 5). In BAT, UCP3 and UCP1 levels dropped with fasting and rebounded upon refeeding. Thus, in BAT, UCP3 and UCP1 are regulated in a manner consistent with the predicted energy-conserving response to fasting. The regulation of UCP3 in muscle was quite different. UCP3 levels increased with fasting and dropped below the initial level with 24 h of refeeding (Fig. 5). In a replicate of this experiment, UCP3 levels increased 8.5-fold with 48 h of fasting and dropped to 30% of the initial level with 24 h of refeeding (data not shown). In contrast to UCP3, the UCP2 mRNA levels remained unchanged.

We next studied the effects of the glucocorticoids on UCP3 RNA levels (data are the means ± S.D.; n = 3, normalized to the untreated mice). A single dose of dexamethasone (3.7 μg/g body weight intraperitoneally 18 h before sacrifice) increased muscle UCP3 expression (201 ± 45%, p = 0.07) and decreased it in BAT (45 ± 8%, p = 0.04). After dexamethasone the levels of UCP2 mRNA were 89 ± 5% in muscle and 56 ± 14% in BAT. These data demonstrate that glucocorticoids regulate UCP3 expression and suggest that stress, with its attendant hypercortisolism, may regulate UCP3 mRNA levels.

**Leptin Regulation of UCP3—**Leptin regulates metabolic efficiency, energy expenditure, food intake, and adiposity (26–28). To see if UCP3 is regulated by leptin, we examined UCP mRNA levels in ob/ob mice (which lack functional leptin), ob/+ controls (data are the means ± S.D.; n = 3, normalized to the +/+ mice). Muscle UCP3
levels were unchanged in ob/ob mice (87 ± 55%), but leptin treatment of ob/ob mice caused an increase (193 ± 12%, p = 0.003) in UCP3 mRNA levels. Muscle UCP2 levels were constant in the three groups. In BAT, both UCP3 and UCP1 were low in ob/ob mice (37 ± 9 and 66 ± 14%, respectively) and increased with leptin treatment (to 54 ± 6%, p = 0.05, and 180 ± 7%, p = 0.0003, respectively). BAT UCP2 was elevated in ob/ob mice (319 ± 22%, p = 0.0001) and decreased slightly (260 ± 21%, p = 0.03) on leptin treatment. This induction of UCP3 by leptin in muscle and BAT presumably explains some of leptin’s thermogenic effect.

**UCP3 and UCP2 Are Adjacent Genes**—We used radiation hybrids (29) to map UCP3 to human chromosome 11q13. The most likely positions determined with the GeneBridge 4 panel (0.6 cR×000 from WI-6189) and the Stanford G3 panel (LOD = 6.47 at D11S4347) are concordant. Thus, UCP3 maps to the same position as UCP2 (19), which is clearly different from UCP1 (located at chromosome 4q31 (30)). We have recently determined that UCP3 and UCP2 are <100 kb apart,4 so these genes appear to be part of a gene cluster. Gene clusters are prone to duplications, deletions, gene conversions, and acquisition of new expression patterns. Further analysis is needed to determine if individual control elements affect the expression of both UCP3 and UCP2.

**Conclusions**—In summary, UCP3 mRNA is highly expressed in muscle and rodent brown fat. Independently, others have recently reported the existence of UCP3, although not its function or hormonal regulation (31, 32). We show by expression in yeast that UCP3 encodes a functioning uncoupling protein. UCP3 mRNA levels are regulated by a number of hormones, often in different ways in muscle than in BAT. Particularly striking are the changes with starvation, an ∼8-fold increase in muscle but a ∼3-fold decrease in BAT. Starvation also illustrates the differential regulation of UCP2 and UCP3; unlike UCP3, UCP2 levels do not change in either muscle or BAT. The basis for the divergent regulation of UCP3 and UCP2 will presumably be elucidated when the transcriptional regulatory elements have been characterized.

The impressive increase in muscle UCP3 mRNA upon starvation and decrease upon refeeding were unexpected observations; because energy expenditure is decreased in starvation, we had expected UCP3 levels to drop. Two explanations seem possible. One is that muscle, even in the starving animal, needs to be maintained above a certain temperature (perhaps in preparation for flight or flight) and UCP3 fulfills this role. An alternate explanation is that during starvation muscle assumes a more important role in whole body thermoregulation. On refeeding after starvation, the drop in muscle UCP3 below control levels would both facilitate efficient replenishment of muscle mass and transfer more responsibility for thermogenesis back to BAT.

Because UCP3 mRNA levels are increased by elevation of T3 levels and decreased by reduction of T3 levels, physiologic variation in T3 appears to be regulating UCP3. These observations suggest that UCP3 is a significant contributor to and possibly the major actor in T3-induced thermogenesis. This contrasts with the role of UCP1 in thyroid thermogenesis. Although T3 stimulates UCP1 expression (33), BAT is actually less active in hyperthyroidism (5). This is presumably a compensatory mechanism, adjusting for the extra heat production from other tissues.

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4 Y. He, D.-W. Gong, and M. Reitman, unpublished observations.