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Regulation of the Third Member of the Uncoupling Protein Family, UCP3, by Cold and Thyroid Hormone

Sarah Larkin, Emily Mull, Wendy Miao, Richard Pittner, Keith Albrandt, Candace Moore, Andrew Young, Maurizio Denaro, and Kevin Beaumont[1]

Departments of Pharmacology and Physiology, Amylin Pharmaceuticals, Inc., 9373 Towne Centre Drive, San Diego, California 92121

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Uncoupling protein (UCP1) is a transmembrane proton transporter present in the mitochondria of brown adipose tissue (BAT), a specialized tissue which functions in temperature homeostasis and energy balance (Nicholls, D. G., and Locke, R. M. (1984) *Physiol. Rev.* 64, 2–40; Lowell, D. D., and Flier, J. S. (1997) *Annu. Rev. Med.*). UCP1 mediates the thermogenesis that is characteristic of BAT by uncoupling mitochondrial oxidation of substrates from ATP synthesis. Recently, two proteins related to UCP1 have been identified and designated UCP2 (Fleury, C., *et al.* (1997) *Nature Genetics* 15, 269–272) or UCP homolog (UCPH) (Gimeno, R. E., *et al.* (1997) *Diabetes* 46, 900–906) and UCP3 (Boss, O., *et al.* (1997) *FEBS Lett.* 408, 39–42; Vidal-Puig, A., *et al.* (1997) *Biochem. Biophys. Res. Commun.* 235, 79–82). We investigated the regulation in rats of UCP3, which is expressed primarily in skeletal muscle and BAT. Expression of rat UCP3 mRNA in BAT was upregulated by in vivo treatment with triiodothyronine (T_3) and by exposure to cold, suggesting that UCP3 is active in thermogenesis and energy expenditure. In skeletal muscle, UCP3 mRNA was also upregulated by T_3 but, surprisingly, not by cold exposure. A hypothesis is proposed to account for this differential regulation.

Thermogenesis in BAT is stimulated by cold exposure and overfeeding (7, 8), and is associated with an activation of UCP1 (9). These responses are mediated in part by adrenergic and thyroid hormone stimulation of BAT, which results in an acute increase in UCP1 activity as well as increases in UCP1 gene transcription rate, RNA stability and recruitment of precursor cells into the mature BAT population (10–15). The second member of the family, UCP2, by contrast, is found in many tissues and does not appear to be regulated by cold exposure or β_3 adrenergic stimulation, both potent inducers of UCP1 (3). However, UCP2 mRNA levels were increased in response to a high fat diet, and differences were found in the magnitude of this response in mouse strains that develop obesity or remain lean on this diet (3). UCP2 expression was also upregulated in adipose tissue in association with genetic obesity secondary to defective leptin signalling in both ob/ob and db/db mice (4). In addition, virally-mediated leptin over-expression increased UCP2 mRNA levels in pancreas islets and in adipose depots in Zucker diabetic fatty rats (16).

A third member of the uncoupling protein family, UCP3, has recently been reported (5, 6). We have also cloned UCP3 and report here on its regulation in rat skeletal muscle and BAT in response to cold, thyroid hormone treatment, and high fat feeding.

MATERIALS AND METHODS

Cloning of UCP3. Clone ID 628529 (GenBank Accession No. AA192136), available through the I.M.A.G.E. Consortium (17) was isolated from a human skeletal muscle library and purchased through Research Genetics (Huntsville, AL). This clone contains a ~1.5 kb insert encoding a portion of the UCP3 coding region followed by the 3′ untranslated (3′UT) region. Amplification of the entire UCP3 coding region was accomplished by a modified 5′-RACE protocol employing the Advantage cDNA PCR kit (Clontech, Palo Alto, CA), a human skeletal muscle Marathon cDNA library (Clontech), and antisense gene-specific primer A850 (5′-CTTCCATTCTTAACTGGTTTCGGAC-3′; positions 404-380, GenBank Accession No. AA192136) following the manufacturers protocol. Amplified product was subcloned into a plasmid vector (TA Cloning System, Invitrogen, San Diego, CA) following the manufacturer's instructions. DNA was sequenced on both strands by a modified dideoxy chain termination method utilizing internal gene-specific synthetic oligonucleotide primers and the dsDNA Cycle Sequencing System (Life Technologies, Inc., Grand Island, NY) per the manufacturer's protocol. Oligonucleotides were synthesized (Life Technologies, Inc. and Genosys, The

[1] Correspondence should be addressed to K.B. Fax: (619) 552-2212. E-mail: kbeaumont@amylin.com

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Woodlands, TX) for use as primers in polymerase chain reaction (PCR) amplifications and sequencing.

Probe preparation. A UCP3 hybridization probe was prepared by *EcoRI*/*XhoI* excision of the partial hUCP3 insert from clone ID 628529. PCR amplification of this template was used to generate a 349bp probe radiolabelled to a specific activity of 5×10^9 cpm/μg, essentially as described (18). In brief, 4 ng of the gel purified UCP3 template was amplified in a 20μl reaction containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl2, 0.5μM of each of each primer (A844, sense strand, positions 591-612, Fig. 1; A845, antisense strand, positions 918-939, Fig. 1), 0.2mM dGTP, 0.2mM dTTP, 2.5mM [α-^{32}P]dCTP (3000Ci/mmole; 150uCi; Amersham). The reaction was heated for 3 minutes at 94°C at which time 1.25U AmpliTaq® DNA polymerase (Perkin-Elmer, Foster City, CA) was added and followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C and extension for 30 seconds at 72°C. A final 10 minute extension was performed at 72°C prior to the removal of unincorporated nucleotides by spin filtration (Chroma spin-100, Clontech) per the manufacturer's protocol.

In vivo regulation studies. 180-200g male HSD rats were fed standard rodent chow and kept in a 12 h light/dark cycle during the treatment period, with 5-6 animals per group. Animals in the "T_3" group received 3.3ug/ml triiodothyronine in their drinking water for 5 days, giving a final dosage of approximately 500ug/kg/day (19). The "Cold" treatment group were maintained at 4°C, individually caged, for 10 days. Animals in the "High Fat" group were allowed ad libitum access to a diet containing 36% fat (Bio-Serve, Frenchtown, NJ) for 10 days. Following the treatment period, animals were sacrificed by decapitation and tissues were harvested and immediately frozen in liquid nitrogen. Total RNA was isolated from skeletal muscle, heart, and both white and brown fat depots using TriReagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Twenty μg of RNA was loaded per lane onto 1% denaturing agarose gels and blotted to supported nitrocellulose membranes following standard protocols (20).

Northern analysis. Human and rat multiple tissue Northern blots were obtained from Clontech (containing 2ug of poly $(A)^+$ RNA per lane from 8 different human tissues: heart, brain, placenta, lung, liver skeletal muscle, kidney, and pancreas; or 8 rat tissues: heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testes) or from BioChain (San Leandro, CA; containing 20μg total RNA per lane from 16 different human tissues: heart, brain, kidney, liver, lung, pancreas, spleen, muscle, esophagus, stomach, intestine, colon, uterus, placenta, bladder, white adipose tissue). These Northern blots and the total RNA Northern blots prepared from rat treatment groups were prehybridized for 1 hour at 68°C in ExpressHyb hybridization solution (Clontech) prior to the addition of heat denatured ^{32}P-labeled hUCP3 DNA probe (described above) and heat-denatured, sheared salmon sperm DNA at a final concentration of 100μg/ml. Following 1 hour or overnight hybridization at 68°C, the blots were washed in 0.1 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0 - 0.1% sodium dodecyl sulfate) at 50°C (moderate stringency) or 68°C (high stringency) and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) with an intensifying screen at −80°C for 2-3 days. A human RNA Master blot (Clontech) containing poly A^+ RNA from 50 different human tissues and six different control RNAs and DNAs was hybridized with the ^{32}P-labeled hUCP3 probe essentially as described above and subsequently exposed to Kodak XAR film (Eastman Kodak) with an intensifying screen at −80°C for 6 days.

RESULTS

We detected a UCP2-related mRNA in rat skeletal muscle Northern blots while investigating UCP2 expression, and subsequently isolated a novel cDNA clone from a human skeletal muscle library (for details see Materials and Methods). The sequence of this clone is identical to the long form UCP3 (Genbank ID# U84763) reported independently by Boss et al. (5) and by Vidal-Puig et al. (6).

The size and distribution of UCP3 transcripts was examined by Northern blot analysis of panels of human tissues from two different commercial sources. Under high stringency hybridization and wash conditions, an approximately 2.5 kb transcript was observed in human skeletal muscle, but not in lung, heart, placenta, kidney, pancreas, brain or liver (Fig. 1a). Moderate levels of expression was detected in white adipose tissue and lower levels were seen in esophagus (Fig. 1b). Both the tissue-specific expression and the relative abundance of the UCP3 gene transcript were also determined using a human RNA dot blot normalized to the poly $(A)^+$ RNA expression levels of eight different housekeeping genes. Of the 50 human tissues examined, UCP3 transcripts were observed almost exclusively in skeletal muscle (Fig. 2c, d). The UCP3 probe hybridized weakly (<5% of skeletal muscle level) to thyroid and immune system tissues. Northern analysis of rat tissue transcripts hybridizing with the human UCP3 probe revealed a similarly limited pattern of distribution, with UCP3 RNA transcripts detectable in rat skeletal muscle but not in heart, lung, liver, spleen, testes, brain or kidney (Fig. 1c). We also detected high levels of UCP3 expression in brown adipose depots of normal untreated rats, and approximately 4 to 10 fold lower expression in rat white adipose tissue than in BAT and skeletal muscle respectively (see below; Fig. 3).

We next determined whether UCP3 expression in rats was altered by exposure to cold (4°C), thyroid hormone administration or high fat feeding, conditions that lead to thermogenesis and alterations in fat metabolism. UCP3 transcript levels in skeletal muscle, WAT and BAT were increased approximately 5-fold in rats administered T_3 for 5 days (Fig. 3a). A similar increase was observed in rats administered T_3 for 10 days (data not shown).

UCP3 transcript levels were also increased approximately 2-fold in BAT from rats cold acclimated for 10 days (Fig. 3a, d). However, UCP3 levels were not increased in skeletal muscle of cold-exposed animals. High fat feeding did not appear to alter UCP3 expression in skeletal muscle or WAT but a modest increase in UCP3 transcript level was seen in BAT (Fig. 3a, d). Similar results were observed for cold acclimation and high fat feeding in these tissues in a second independent experiment (data not shown).

DISCUSSION

BAT is the principal site of thermogenesis induced by cold and by food intake (21)(22)(8) and in rodents

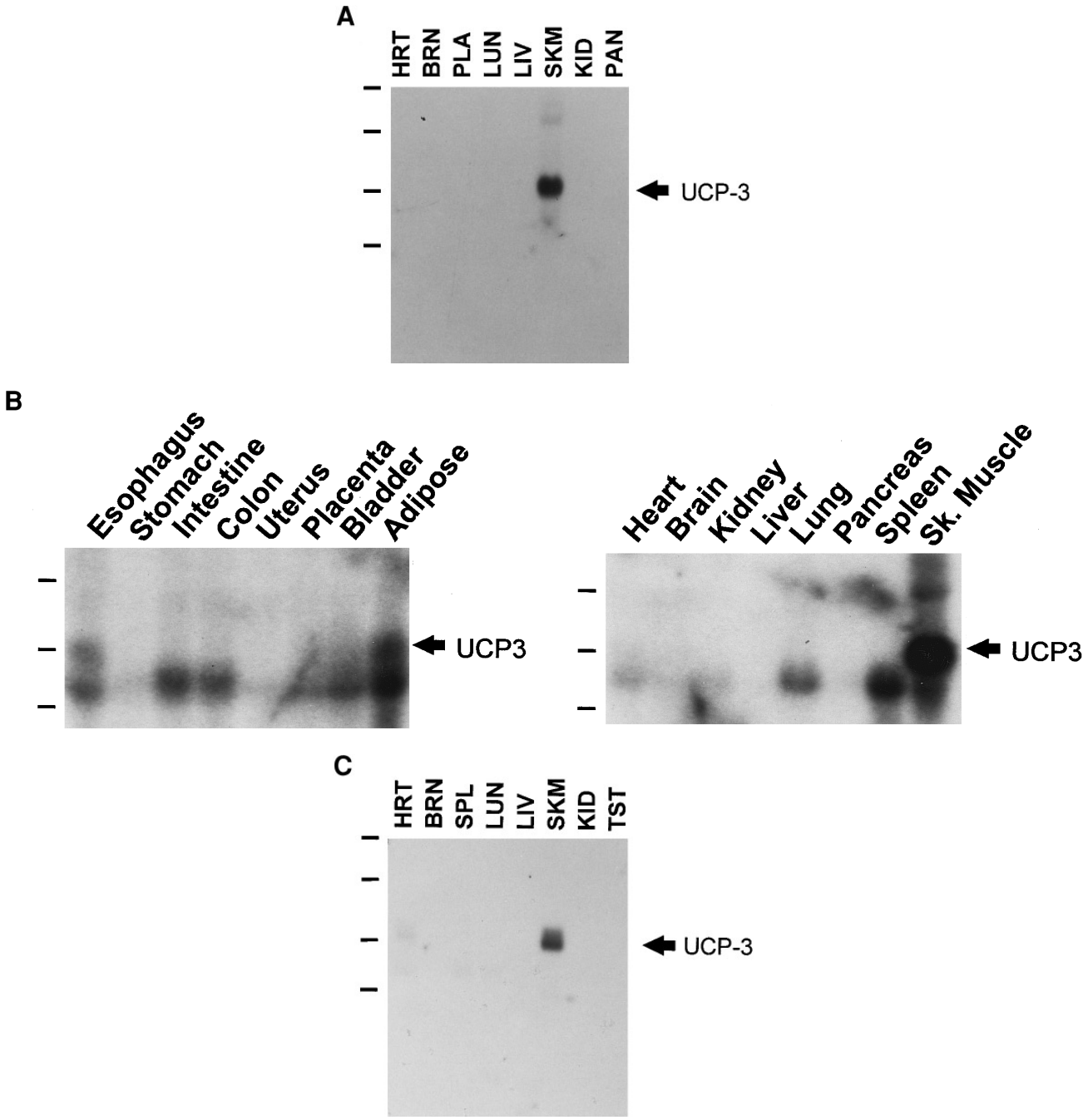
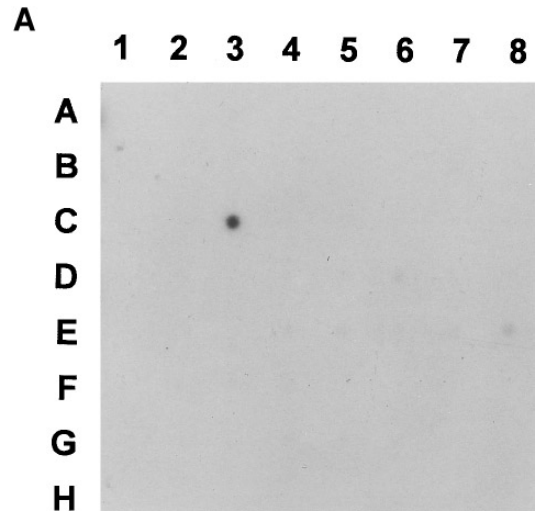


FIG. 1. Tissue distribution of UCP3 transcripts. Northern blot analysis of poly $(A)^+$ RNA from (A, B) human tissues and (C) rat tissues, indicating the source and position of the poly$(A)^+$ RNA and control RNA and DNA samples. Blots were hybridized with a hUCP3-specific probe and washed under high stringency (A and C) and moderate stringency (B) conditions. Abbreviations: HRT, heart; BRN, brain; PLA, placenta; SPL, spleen; LUN, lung; LIV, liver; SKM, skeletal muscle; KID, kidney; PAN, pancreas, TST, testes. Lower band in (B) is due to cross hybridization to UCP2 transcripts. Dashes to the left of each panel indicate the relative migration of RNA size markers: 7.46, 4.40, 2.37 and 1.35 kb (A and C) or 4.40, 2.37 and 1.35 kb (B).

expresses all three forms of uncoupling protein (3-6). However, skeletal muscle is also a significant site of regulated thermogenesis, occurring through unknown mechanisms (23-26). Recent studies have indicated that leak of protons across the inner mitochondrial membrane, potentially due to uncoupling protein activity, accounts for up to 50% of the resting metabolic rate of skeletal muscle (27). The distribu-



	1	2	3	4	5	6	7	8
A	whole brain	amygdals	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	sub-thalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA 100 ng	yeast tRNA 100 ng	E.coli rRNA 100ng	E.coli DNA 100 ng	poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

FIG. 2. Quantitative human RNA dot blot analysis of 50 tissues. (A) Blots were hybridized with the hUCP3-specific probe and washed under high stringency conditions as described under Materials and Methods. (B) Key to the human RNA dot blot containing polyA RNA from 50 human tissues as well as 8 control sourcesn (H1-H8). Loading was adjusted between tissues to normalize to the expression level of 8 different housekeeping genes.

tion of UCP3 transcripts almost exclusively in these thermogenically active tissues, skeletal muscle and BAT, is in marked contrast to the widespread expression of UCP2 (3, 4), suggesting a distinct physiological role in thermogenesis.

Like UCP1, UCP3 is upregulated in BAT by cold acclimation. The tissue-specific expression pattern of UCP3, its regulation in BAT by cold and its conservation with UCP1 suggest that UCP3 is similarly involved in mitochondrial uncoupling and thermogenesis. However, cold acclimation did not increase UCP3 expression in skeletal muscle under these experimental conditions. Moreover, a targeted disruption of UCP1 results in homozygous mutant mice that are apparently unable to maintain body temperature in cold conditions, despite a compensatory increase in UCP2 levels (28). Thus, at least in species such as rodents that have significant BAT depots, the other members of the

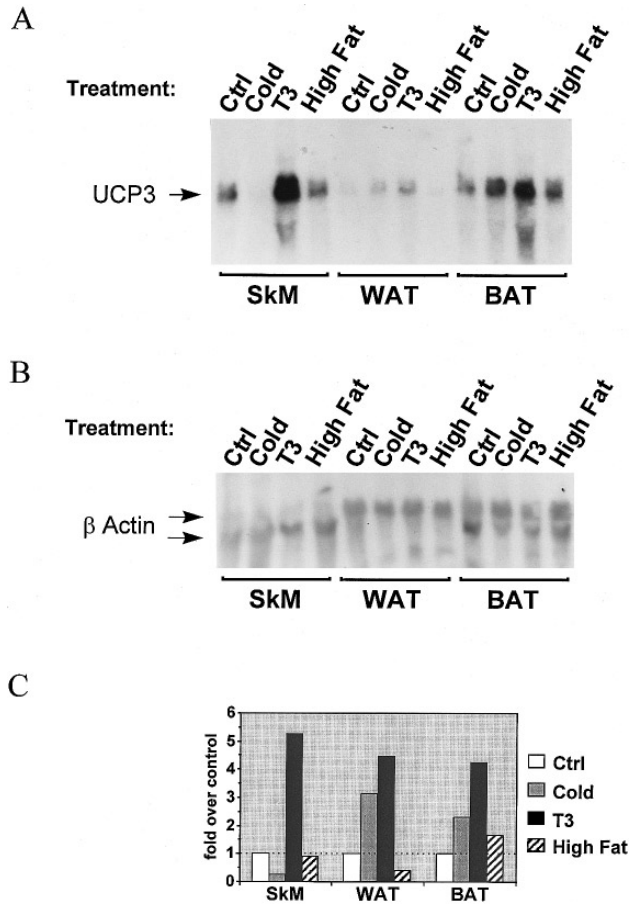


FIG. 3. In vivo regulation of UCP3 by T_3 treatment, cold acclimation or high fat diet. Northern analysis is shown of RNA extracted from skeletal muscle (SKM), white fat (WAT) and brown fat (BAT) pooled from rats (5-6 animals per group) treated as follows: no drug treatment and normal diet ("Control"), maintained at 4°C ("Cold"), treated with 3.3 μg/ml T_3 in their drinking water ("T_3"), or fed a high fat diet ("High Fat"). The Northern blot was probed with (A) UCP3 sequence and (B) β Actin, and (C) the hybridization signal for UCP3 was normalized to that of β Actin. Fold increases in UCP3 signal over the untreated control level were calculated for each treatment (Control = white bars, Cold = gray bars, T_3 = black bars, High Fat = hatched bars).

uncoupling protein family may have functions that are not related to cold-induced thermogenesis.

Treatment with T_3 increased UCP3 expression in both BAT and skeletal muscle. Thyroid hormone is a key regulator of thermogenesis and basal metabolic rate, working through mechanisms largely involving transcriptional regulation of gene expression (29). Hyperthyroidism is associated with an increase in mitochondrial proton permeability in hepatocytes (30, 31). UCP1 induction in response to cold exposure is dependent on thyroid hormone, which has a permissive effect on UCP1 expression through two thyroid hormone response elements located upstream of the UCP1 gene (12). Our studies indicate that UCP3 expression is also regulated in response to thyroid hormone status, and therefore altered UCP3 expression may contribute to T3-induced increases in oxygen consumption and thermogenesis. Further studies are necessary to determine whether the effects observed here are mediated by a direct effect of T3 on UCP3 transcription or occur in response to alterations in metabolism, such as enhanced lipid and protein turnover, that occur in the hyperthyroid state.

In these studies, UCP3 was differentially regulated by cold in BAT compared to skeletal muscle. In response to cold and cafeteria feeding, virtual saturation of nuclear thyroid hormone receptors is produced in BAT by local conversion of T_4 to its more active metabolite T_3 by type II thyroxine 5′ deiodinase (DII) (32-36). This enzyme is induced more than 17 fold by cold acclimation in BAT, stimulated by increased noradrenergic input to the tissue and by modest changes in serum thyroid hormone (32), resulting in a dramatic increase in local T_3 concentration. In rodents DII is expressed in BAT, pituitary and the brain but is absent from skeletal muscle and other tissues. We have shown here that UCP3 is upregulated in BAT, WAT and skeletal muscle by increasing serum T_3. Therefore the differential regulation of UCP3 by cold in BAT and skeletal muscle may possibly be due to the absence of this deiodinase in skeletal muscle compared to BAT. Interestingly, adult humans, that have relatively small BAT depots, appear to generate a thermogenic response mostly through skeletal muscle and white adipose tissue (25), and have been shown to express DII in skeletal muscle (white adipose tissue was not tested; (32)). Thus, in humans a cold stimulus may be capable of inducing UCP3 expression in muscle.

In summary, the emerging family of mitochondrial uncoupling proteins with distinct tissue distributions presents an explanation for the phenomena of mitochondrial proton leaks that have been described in a variety of organs (37). These leaks have been proposed to account for as much as 25% of resting metabolic rate in the rat (27). The activity of uncoupling proteins is therefore likely to exert a major influence on energy expenditure and body weight, and alterations of this metabolic checkpoint whether heritable or pharmacologically applied, may constitute a key variable in setting of body weight.

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