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PAPER

Uncoupling proteins-2 and 3 influence obesity and inflammation in transgenic mice

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OBJECTIVE: To test the hypothesis that either uncoupling protein-2 UCP2 or UCP3 or both together influence obesity and inflammation in transgenic mice.
DESIGN: We generated 12 lines of transgenic mice for both human UCP2 and 3 using native promoters from a human bacterial artificial chromosome (BAC) clone. The BAC expresses no genes other than UCP2 and 3. Mice used for experiments are N4 or higher of backcross to C57BL/6J (B6). Each experiment used transgenic mice and their nontransgenic littermates.
RESULTS: Northern blots confirmed expression on human UCP2 in adipose and spleen, while human UCP3 expression was detectable in gastrocnemius muscle. Western blots demonstrated a four-fold increase of UCP2 protein in spleens of Line 32 transgenic animals. Heterozygous mice of four lines showing expression of human UCP2 in spleen were examined for obesity phenotypes. There were no significant differences between Lines 1 and 32, but female transgenics of both lines had significantly smaller femoral fat depots than the control (littermate) mice ($P=0.015$ and 0.005, respectively). In addition, total fat of transgenic females was significantly less in Line 1 ($P=0.05$) and almost significantly different in Line 32 ($P=0.06$). Male Line 1 mice were leaner ($P=0.04$) while male Line 32 mice were almost significantly leaner ($P=0.06$). Heterozygous mice of Lines 35 and 44 showed no significant differences from the nontransgenic littermate controls. Effects of the UCP2/UCP3 transgene on obesity in Line 32 mice were confirmed by crossing transgenic mice with the B6.Cg-*Ay* agouti obese mice. B6.Cg-*Ay* carrying the UCP2/UCP3 transgene from Line 32 were significantly leaner than nontransgenic B6.Cg-*Ay* mice.
Line 32 UCP2/UCP3 transgenics showed increased hypothalamic Neuropeptide (NPY) levels and food intake, with reduced spontaneous physical activity. Transgenic baseline interleukin4 (IL-4) and interleukin6 (IL-6) levels were low with lower or later increases after endotoxin injection compared to wild-type littermates. Endotoxin-induced fever was also diminished in transgenic male animals. Low-density lipoprotein (LDL) cholesterol levels were significantly higher in both Line 1 and 32 transgenics ($P=0.05$ and 0.001, respectively) after they had been placed on a moderate fat-defined diet containing 32% of calories from fat for 5 weeks.
CONCLUSION: Moderate overexpression of UCP2 and 3 reduced fat mass and increased LDL cholesterol in two independent lines of transgenic mice. Thus, the reduced fat mass cannot be due to insertional mutagenesis since virtually identical fat pad weights and masses were observed with the two independent lines. Line 32 mice also have altered inflammation and mitochondrial function. We conclude that UCP2 and/or 3 have small but significant effects on obesity in mice, and that their mechanism of action may include alterations of metabolic rate.
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Introduction

All known uncoupling proteins (UCPs) influence the mitochondrial proton gradient,[1–7] but little is understood about the physiological consequences of UCP function (other than UCP1 in brown fat. [1,2,8]) UCP2 is potentially

the most interesting of these proteins because it is widely but variably expressed in many tissues including adipose, muscle, spleen, brain, and pituitary,[3,9–11] and thus may influence many physiological processes. For example, obesity is increased in humans with UCP2 alleles that decrease UCP2 transcription.[12] Further, UCP2 is expressed very strongly in spleen tissue, which suggests a role in the immune response.

Many association studies of natural allele influences on obesity have shown that UCP2 and/or 3 are candidate obesity genes in humans.[13] However, studies of UCP phenotype effects in knockout mouse models have yielded contradictory results. For example, UCP2 and 3 knockout in mice did not affect food intake or adiposity,[14–17] yet adiposity was decreased and food intake was elevated in transgenic UCP3 mice hyperexpressing from the skeletal muscle actin promoter.[18] Thus, there is an apparent conflict between association studies in humans and studies of knockout mice. Since previous studies have also shown that knockout of UCP1 has no influence on obesity,[19] while transgenic mice overexpressing UCP1 are leaner,[20] we have tested the possibility that moderate overexpression of UCP2 and 3 in transgenic mice also influences obesity.

While UCP2 and 3 may have different functions, it is also possible that they both influence obesity. The likelihood that these two proteins have similar or overlapping functions is supported by recent genome sequence data from non-Mammalian species showing that both proteins have a long evolutionary history. While the data are still very incomplete, sequencing of *Danio rerio* (zebrafish) and *Xenopus lacvis* has identified separate orthologs (mutual best hits by BLAST) for both mammalian UCP2 and 3. The *D. rerio* UCP2 ortholog has accession number AJ243250, and the UCP3 ortholog has accession number AI497417. The *X. laevis* UCP2 ortholog has accession number BJ031744 and the UCP3 ortholog has accession number BG410087 (all data are from the NCBI homologene database: http://www.ncbi.nlm.nih.gov/HomoloGene/). The existence of separate orthologs for both UCP2 and 3 suggests that UCP2 and 3 have existed as separate proteins since before the development of mammals. It also suggests that the close linkage of UCP2 and 3 does not result from a recent duplication in the mammalian genome. Finally, the possible existence of separate and ancient orthologs leads to a new question—why are the UCP2 and 3 genes so very closely linked in mammals? Are they closely linked by an accident of duplication that has been conserved by chance during evolution, or, does their presence together indicate that some aspect of their combined actions requires proximity? While genome sequence data from *D. rerio* and *X. laevis* is not complete enough for us to determine whether or not the orthologs of UCP2 and 3 are also closely linked in zebrafish and Xenopus, this possibility cannot be ruled out. We conclude that evolutionary data strongly support the necessity to overexpress both UCP2 and 3 in a transgenic as a first step towards understanding their potential combined physiological roles.

We report here the first experiments with transgenic mice moderately overexpressing (four-fold for UCP2 in spleen) both UCP2 and 3. We provide new evidence that these genes affect obesity. Although we cannot distinguish between effects of UCP2 or UCP3 on obesity, the results are consistent with the hypothesis that one or both of these genes influences obesity in mammals.

Methods

Production of transgenic mice

We used an 80 kb bacterial artificial chromosome (BAC) containing the human UCP2 and 3 genes to construct an overexpressing transgenic mouse line. The transgene comprised the UCP2 and 3 genes, which are adjacent on chromosome 11, with their native promoters and *cis*-acting elements (Figure 1a). Sequence analysis of subclones from the BAC revealed that it contains 8 kb of sequence distal to exon 8 of UCP2. PCR with primers specific to the first and last exons of UCP2 and 3 confirmed the presence of both genes in the BAC. Since UCP3 is proximal to UCP2, and since the sizes and distance between these genes are known, then there is also approximately 30 kb of genomic sequence proximal to exon 1 of UCP3.

Founder transgenic mice (produced by Chrysalis DNX, Princeton, NJ, USA) were backcrossed onto the C57BL/6J background to minimize phenotype variability due to mouse strain. Transmission of the transgene follows a simple Mendelian pattern. Presence of the transgene was confirmed by PCR amplification of a conserved region of hUCP2 exon 4 in genomic DNA. We used heterozygous mice of N4 or higher backcross generation, so the phenotypes reported are from mice that are predominantly derived from the C57BL/6J strain. Nontransgenic (wild type) littermates were used as controls for all experiments.

All animal experiments were approved by Institutional Animal Use and Care Committees. Mice were housed singly or in groups of five and fed standard mouse chow or defined moderate- and low-fat experimental diets, with water and food *ad lib*. Mice were maintained on a 14- h light/10- h dark cycle (lights on at 06.00 PST). For circadian rhythm studies in thermoneutral conditions animals were kept under a 12 h light/12 h dark regimen.

Production of agouti A^y mice expressing the UCP2/3 transgene

Yellow agouti obese mice (B6.Cg-A^y spontaneously obese mice) on the C57BL/6J background (formerly named C57BL/6J-A^y mice) were purchased from The Jackson Laboratory. These mice express the normal agouti protein ectopically throughout the body, leading to obesity. They were bred to UCP2/UCP3 transgenic mice at the N9 generation of backcross to C57BL/6J. F1 mice were genotyped for the transgene as described above. Male and female mice were maintained on a low fat chow diet until killing at 5 months of age.

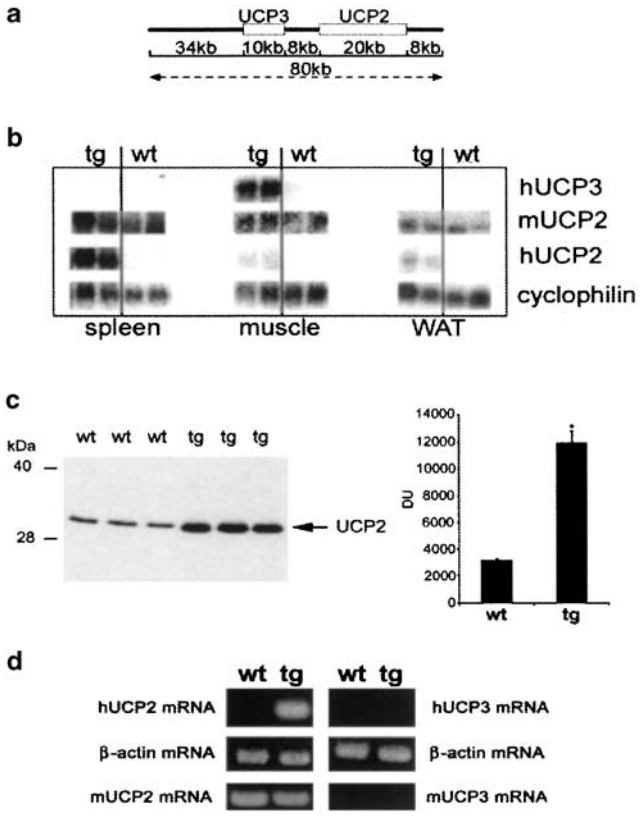

Figure 1 Transgene expression patterns: (a) Schematic drawing of the human UCP2/UCP3 DNA construct used for the generation of transgenic mice. (b) Northern blot analysis of the expression of human UCP3 (hUCP3), human UCP2 (hUCP2), mouse UCP2 (mUCP2), and cyclophilin mRNAs in spleen, muscle and white adipose tissue (WAT) for from transgenic (tg) and wild-type (wt) animals of Line 32. Lines 1, 35, and 44 are not shown, but expressed human UCP2 in spleen. (c) Western blot analyses revealed increased UCP2 protein expression in the spleen of transgenic Line 32 animals compared to wild-type littermates and densitometric analyses revealed an approximate 3.8-fold higher level of total UCP2 levels in the spleen of transgenic mice compared to wild-type littermates ($P=0.0015$). (d) RT-PCR analysis of hypothalamic samples revealed robust expression of human UCP2 (hUCP2) but not human UCP3 (hUCP3) mRNAs in the brain of Line 32 animals. Endogenous mouse UCP2 (mUCP2) mRNA levels did not seem to be affected by the presence of the transgene and endogenous mouse UCP3 (mUCP3) was not detected in the brain.

Northern blot analysis

RNA was extracted by the TRIZOL reagent method (Life Technologies, Rockville, MD, USA) and species specific probes were used for Northern blots.

All blots were quantified with a Storm phosphorimager and normalized *vs* cyclophilin mRNA levels.

Western blot of UCP2 protein

Mitochondria were prepared from spleens of wild-type ($n=3$) and transgenic mice ($n=3$). A total of 30 μg of protein were run on SDS-PAGE. Western blot analysis for total UCP2 protein was performed with Santa Cruz SC C-20 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). This antibody reacts with both mouse and human UCP2. The specificity of this antibody was confirmed by Western blot of spleen mitochondria from UCP2 knockout mice that revealed no reactivity with the Santa Cruz antibody (data not shown). Antibody was detected with a HRP conjugate and visualized with Lumiglo.

Measurement of daily food intake

Mice were singly housed in wire mesh cages, maintained on the Purina Stock Chow 5001 diet until 8 weeks of age, and then started on the experimental diet. Food intake is the initial daily ration minus spilled and leftover food.

Measurement of resting metabolic rate

We measured resting metabolic rates as described previously.[21] The resting O_2 consumption rate for each transgenic and wild-type mouse was measured at thermoneutral (29–31°C) and vivarium (21–23°C) temperatures. The values presented are the average of the rates measured on two trials separated by 48 h.

Measurement of spontaneous movement

Spontaneous physical activity was measured in a Digiscan optical animal activity monitoring standard system (Accuscan Instruments, Columbus, OH, USA). Six each of male and female transgenic and wild-type mice were measured for 10 consecutive days. Reported results are the means of the last 6 days for each animal.

Body temperature measurements and fever study

Female mice were implanted with radio transmitters (E-mitters, MiniMitter, OR, USA) to record core body temperature and horizontal motor activity as previously described.[22] Male mice were implanted with HR-series transmitters to record heart rate, core body temperature, and horizontal motor activity in parallel. Data were collected every 60 s with Vitalview 4000 software and averaged every 10 min. For fever studies, animals were acclimated for 1 week at 30°C after the surgery and then given intraperitoneal injections with LPS 50 μg/ kg ip (from *E coli* 026:B6 Sigma L-2654 Lot 107H4091) between 09.00 and 11.00 h.

Mitochondrial isolation and determination of mitochondrial respiration rate

Oxygen consumption in states 3 and 4 was measured in homogenate of hypothalamus and isolated mitochondria from liver and skeletal muscle as previously described.[10,23]

Body composition

Body composition (percent of live weight that is fat or lean mass) was determined as previously described.[26] Adiposity index is summed fat pad weights (gonadal, retroperitoneal, femoral and mesenteric) divided by live weight. Internal organs and muscle of transgenic mice and wild-type

littermates (controls) were dissected and weighed immediately after euthanasia. Female mice were euthanized in the morning of metestrus.

Immunocytochemistry for NPY

Hypothalamic NPY levels were assessed by previously described quantitative immunocytochemical methods[24] with antisera against NPY previously tested in brain tissue[11,25].

Immunological analysis

The number and proportion of splenic and thymic T, B, and macrophage cells were determined by flow cytometry by staining with antibodies specific for CD4, CD8, B220, and Mac-1 antigens.

Transgenic ($n=4$) and wild-type ($n=4$) females were challenged with intraperitoneal administration of LPS (15 g/kg in PBS, Sigma L-2654, 026:B6, lot107H4091), and serum samples from animals were collected retro-orbitally and analyzed by ELISA for circulating levels of interleukin4 (IL-4) and interleukin6 (IL-6) at 0, 1, 2, 4, 9, 24, and 48 h after LPS injection (50 μg/kg).

Statistical analysis

Significance of linkage between phenotype and genotype was determined by analysis of variance (ANOVA) with StatView Version 5.0.1. Simple comparisons between two means were assessed by Student's *t*-test and Fisher's PLSD.

Results

Transgenic mice overexpress only human UCP2 and 3

We have used an 80 kb human BAC clone containing the natural genes and promoters to produce transgenic mice that slightly overexpress UCP2 and 3. We have sequenced the BAC clone and searched for adjacent genes to determine if genes other than UCP2 and 3 are expressed from the BAC clone in the transgenic mice The Celera human genome database reveals the uncharacterized gene DKFZP586P0123 19KB upstream of UCP3, at least partially within the BAC clone transgene (Figure 1a). We made primers to amplify DKFZP586P0123 exons 1–2 in the transgenic mouse genomic DNA. We obtained specific bands of the predicted size only in the transgenics. Then we used a sequence-verified I.M.A.G.E. clone (2343676) for human DKFZ586P0123 to probe a 75-tissue human mRNA expression array (Clontech 7776-1). The probe revealed that all of the included human tissues express DKFZ586P0123, but yeast and *E. coli* controls do not. Finally, we probed Northern blots of transgenic and wild-type mouse spleen, gastrocnemius, and adipose RNA with the same I.M.A.G.E. clone. After 3 days of exposure and analysis with a STORM Phosphorimager system, we detected no signal in the transgenics or wild-type mouse RNA, even though the same probe bound strongly to human spleen and muscle RNA on the expression array. Thus, even though the original construct includes part of the DKFZ586P0123 gene, that gene is not expressed in our transgenic mice, which specifically overexpress only hUCP2 and 3.

Expression of hUCP2 and 3

In transgenic mice, hUCP2 mRNA expression levels were highest in spleen and brain, lower in white adipose tissue, and lowest in skeletal muscle. Human UCP3 mRNA was expressed only in skeletal muscle (Figure 1b). Constitutive mouse UCP2 and UCP3 message levels were not changed in the same tissues. UCP2 protein expression was four-fold higher in transgenic over wild-type littermates, confirming that these mice moderately overexpress UCP2 (Figure 1c).

Effect of transgene expression on mitochondrial uncoupling

To verify if overexpression of UCP2 and 3 gene products affected mitochondrial uncoupling activity, we measured oxygen consumption at states 3 and 4, respectively, in homogenates or mitochondria from tissues of transgenic and wild-type animals (Table 1). We analyzed respiratory control ratio (RCR) in brain (hUCP2 mRNA), skeletal muscle (hUCP3 mRNA predominantly), and liver (no hUCP2 and 3 expression). In all tissues, oxygen consumption during state 3 (via phosphorylating mitochondria) was similar in wild-type and transgenic animals. However, state 4-oxygen consumption was significantly elevated in hypothalamic homogenates and skeletal muscle mitochondria of transgenic animals compared to wild-type littermates. No such differences were seen in the liver. Since only hUCP2 was overexpressed in the brain (Figure 1d), and muscle hUCP3 mRNA expression greatly exceeded hUCP2 mRNA, the RCR data are consistent with increased mitochondrial uncoupling associated with the overexpression of human UCP2 and 3 genes as previously shown *in vitro* and *in vivo*.[1–8]

Transgenic mice overexpressing UCP2 and 3 are lean

We produced 12 independent lines of transgenic mice from the founding chimeras which were on a mixed strain background. The lines were maintained by backcrossing to C57BL/6J (B6). Each line produced approximately half transgenic progeny and half nontransgenic littermates. Four lines expressing human UCP2 in the spleen were chosen for phenotyping of obesity traits in heterozygous N4–N5 mice. The mice were killed for measurement of fat pad weights between 109 and 125 days. Control mice were littermates from the transgenic breeding. Thus, the various control mice have a B6 background together with a congenic region surrounding the inserted transgene. We chose Lines 1, 32, 35 and 44 for further study because their hUCPx expression as determined by Northern blot was by far the highest of any of

Table 1 State 3 and state 4 respiration and respiratory control ratio (RCR) in different tissues of wild-type and transgenic animals (females)

	Wild-type hypothalamus (n=*7*)	*Transgenic hypothalamus* (n=*7*)	*Wild-type skeletal muscle* (n=*5*)	*Transgenic skeletal muscle* (n=*5*)	*Wild-type Liver* (n=*5*)	*Transgenic liver (parantheses)* (n=*5*)
State 4	13.9±0.5	22.7±1.7*	59.9±8.4	84.7±5.8*	44.9±5.9	45.4±3.1
State 3	57.1±3.5	53.7±4.2	231±22	199±17.5	267.3±32	334.2±43.3
RCR	4.1±0.3	2.4±0.2*	3.5±0.2	2.4±0.3*	6.1±0.3	7.3±0.7

Respiration rates are expressed as *n*Atoms O/min mg proteins. Data were analyzed by Student's *t*-test, and *P*-values less than 0.05 were considered significant (*). Owing to small amounts of mitochondria in brain samples of mice, hypothalamic respiration rates were measured on whole tissue homogenates. Skeletal muscle and liver respiration rates were measured on isolated mitochondria.

Table 2 Obesity phenotyping data in transgenic lines 1 and 32

	Males			*Females*		
	Control (*n*=26)	*Line 1* (*n*=9)	*Line 32* (*n*=17)	*Control* (*n*=18)	*Line 1* (*n*=10)	*Line 32* (*n*=10)
Femoral	0.17±0.012	0.15±0.015	0.15±0.010	0.14±0.012	0.10±0.008 (*P*=0.015)	0.095±0.011 (*P*=0.005)
Total fat	0.64±0.059	0.43±0.065 (*P*=0.04)	0.48±0.046 (*P*=0.06)	0.38±0.039	0.26±0.022 (*P*=0.05)	0.27±0.044 (*P*=0.06)
Adiposity Index	0.025±0.002	0.019±0.002	0.02±0.002	0.020±0.002	0.015±0.001 (*P*=0.07)	0.014±0.002 (*P*=0.02)

P-values are for comparisons of Line 1 or 32 *vs* the male or female controls using Fisher's PLSD. *P*-values are not shown if both lines had *P*>0.05. In all cases, transgenics are leaner than wild type. Furthermore, there are no significant differences between Lines 1 and 32 — values for each line are very similar. We show *P*-values for both lines if one comparison is significant, to further indicate the trend that both lines are exhibiting essentially the same obesity phenotypes. Ages at killing were male controls 117±7.0, male line 1 121±8.0, and male line 32 109±3.8 days±s.d. Female controls were 117±11, female line 1 125±7.0 and female line 32 110±7.2 days ±s.d. Thus, Line 1 mice averaged 4–8 days older than controls, while Line 32 mice were 7–8 days younger than controls. Regression of age *vs* phenotypes revealed no significant effects of these small age differences on any phenotypes.

Table 3 UCP2/UCP3 Transgenic female A^y mice are leaner than wild-type littermates

Trait examined	*Transgenic* n=*8*	*Wild type* n=*7*	P-*value*
Mesenteric fat	0.58±0.76 (s.e.)	1.12±0.21	*P*=0.024
Gonadal fat pad	2.2±0.2	2.9±0.25	*P*=0.037
Total fat pads	4.2±0.43	5.8±0.58	*P*=0.036

Transgenic mice at 19 of backcross to C57BL/6J were bred to B6.Cg-A^y mice. A^y obese yellow F1 progeny were genotyped for the UCP2/3 transgenic. They were maintained and killed as described in Materials and methods.

the expressing lines. Lines 1 and 32 transgenics were significantly leaner than littermate controls (Table 2), and control mice from those lines did not differ significantly from each other. Line 35 transgenic homozygotes were viable and healthy.

Lean transgenic phenotype confirmed in A^y mice

We sought to confirm that the lean phenotype of the UCP2/3 transgenic mice by combining the transgene with the agouti A^y mutation since A^y produces a mild slow onset autosomal dominant obesity. F1 mice were produced by breeding heterozygous Line 32 transgenic mice at the N9 generation of backcross to B6, with heterozygous B6.Cg-A^y mice. Progeny were genotyped for the transgene. A^y genotype was determined by yellow coat color. The mice were maintained on a low-fat-defined diet with 12% of calories from fat from weaning until killing. Adiposity index was significantly lower in male and female transgenic mice. Values for female transgenic mice were 0.13±0.027 (s.d., $n=8$, mean age 145±27), while nontransgenic littermates were 0.16±0.023 ($n=7$, mean age 157±33). Adiposity index of male transgenics was 0.099±0.021 ($n=4$, mean age 148±41), while nontransgenic littermates were 0.109±0.005 ($n=9$, mean age = 157±31) ($P=0.0047$ transgenic *vs* nontransgenic after adjusting for age differences). Effects of the transgene expression on Mesenteric and gonadal fat pad weights and total fat pads in females are shown in Table 3. In all cases, transgenics were leaner than non-transgenic (wild-type) littermates. There was no difference for males.

Metabolic rate and core temperature

Resting metabolic rate (RMR) differed significantly in female transgenics *vs* wild-type female littermates. At thermoneutral temperatures (29–30°C), mean RMR was 9.6±1.1 ml O_2/(min × kg body mass$^{2/3}$) in the transgenics ($n=15$), and

8.6 ± 0.96 ml O_2/(min × kg body mass$^{2/3}$) in the wild types ($n = 10$) ($P = 0.026$). At vivarium temperature (21–23°C) transgenics had a mean RMR of 14.5 ± 1.87 ($n = 15$) and wild-type RMR averaged 12.5 ± 2.77 ($n = 10$) ml O_2/(min × kg body mass$^{2/3}$), ($P = 0.05$). RMR for male transgenic mice on the low-fat diet did not differ from that of the wildtypes at either temperature.

Ambient temperature influences food intake in mice. We exposed single caged wild-type and transgenic female mice ($n = 6$) for 2 weeks to three different ambient temperatures (25–30°C, then 21°C). Changes in food intake with increased (30°C) or decreased (21°C) temperature were not significantly different for transgenic and wild-type mice (data not shown). Thus, thermoregulatory feeding behavior is normal in UCP2/UCP3 overexpressing mice.

Core body temperatures were similar between wild-type and transgenic mice maintained at thermoneutrality (30°C) or at 4°C. Circadian analysis of core body temperature, horizontal motor activity, and heart rate in thermoneutral conditions suggested normal hypothalamic control of both circadian rhythms and core body temperature set point. However, cold exposure experiments showed that transgenics may maintain core body temperature better than wild-type controls in extreme conditions. Colonic temperatures decreased 2.45 ± 1.02°C in the transgenics and 3.5 ± 1.65°C in the wild-type mice, respectively ($P = 0.058$) during the 6 measurements.

Mice with no adipose tissue (A-ZIP/F-1) or with impaired leptin functionality (*ob*/*ob* mice) can use torpor as adaptive mechanism during fasting.[26] We carried out 24 h-fasting experiments in thermoneutral conditions (30°C) to study possible metabolic differences in the adipose tissue of transgenic female mice ($n = 6$ in each experimental group). All animals showed similar core body temperature reduction only during the dark period with no evidence of periods of torpor.

Food intake and NPY levels

Transgenic mice consumed more food than wild-type littermates, despite decreased fat accumulation: females 4.44 ± 0.048 *vs* 4.20 ± 0.1 (g/day ± s.e.m., $P = 0.04$); males 4.72 ± 0.19 *vs* 4.42 ± 0.04 (g/day ± s.e.m., $P = 0.12$) (Figure 2a). Over 2 weeks female transgenics consumed about 3.3 g more than the wild-type females, and the male transgenics consumed about 4.2 g more than the wild-type males. Since increased food intake may be due to increased appetite, we assessed the levels of the most potent appetite inducer, hypothalamic neuropeptide Y (NPY). In the arcuate nucleus, the surface density of NPY immunolabeling was about 50% in transgenics and wild types (NS, data not shown). In the paraventricular nucleus, a major site of NPY-induced feeding,[27] the surface density of NPY immunolabeling was almost 60% higher ($P = 0.0065$) in transgenic animals (76.7 % ± 5.2 s.e.m.) than in wild-type controls (48.5 % ± 3.7 s.e.m.) (Figure 2b). Thus, unchanged body

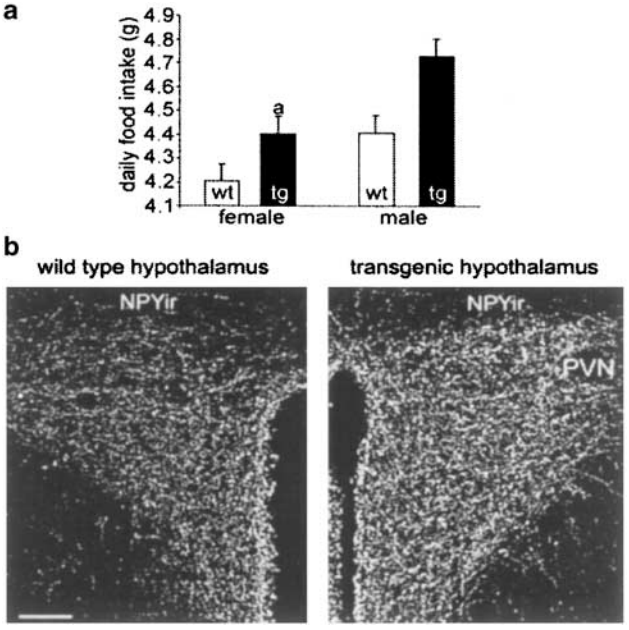

Figure 2 Decreased adiposity but increased food intake and hypothalamic NPY levels in transgenic animals: (a) Daily food intake of wild-type (empty bars) and transgenic (filled bars) female and male Line 32 mice. Significant differences ($P < 0.05$). *P* transgenic *vs* wild type for all animals = 0.017; $n = 9$–10 in all groups. (b) Immunocytochemical analysis of NPY revealed increased level of NPY protein ($P < 0.05$) in the hypothalamic paraventricular nucleus of transgenic animals. Bar scale represents 100 mm.

weight of transgenic animals may also result from small increases in food intake correlated with increased hypothalamic NPY expression. The increased hypothalamic NPY levels and consequent increased feeding may be directly related to increased hypothalamic UCP2 expression or to increased peripheral energy expenditure. Although the expression of NPY is increased only 60% in the paraventricular nucleus, the fact that a statistically significant difference was not observed in the arcuate nucleus shows a specificity that supports the hypothesis that the increase has physiological impact—the paraventricular nucleus is the key site for induction of food intake by NPY. However, there are other possible mechanisms that might provide the underlying mechanism responsible for the modest increase of food intake of the transgenic mice.

Transgenic animals of Lines 1 and 32 have increased LDL plus VLDL cholesterol

We have examined the effects of transgene expression on plasma cholesterol levels. Transgenic mice and their non-transgenic littermate controls, at the N4 generation of backcross to C57BL/6J, were placed on a moderate fat defined diet containing 32% of calories from fat at 3 months of age and were bled 5 weeks later after an overnight fast. They were analyzed for total triglycerides, total cholesterol and high-density lipoprotein (HDL) cholesterol as described

previously.[28] Low-density lipoprotein (LDL) plus very low-density lipoprotein (VLDL) cholesterol was calculated as the difference between total and HDL cholesterol. Both transgenic lines had significantly higher levels of LDL plus VLDL cholesterol than controls. Values in mg cholesterol/dl (mean $\pm$ s.e.) for Line 32 were 22 ± 4.5 (males $n=4$) and 14.6 ± 2 mg cholesterol/de (females $n=5$), while values for Line 1 transgenics were 13 ± 5.2 (males $n=4$) and 14.6 ± 1.5 mg cholesterol/de (females $n=5$). Controls were 7.2 ± 1.9 (males $n=8$) and 10.3 ± 1.2 mg cholesterol/dl (females $n=8$). Line 32 transgenics differed highly significantly from controls ($P=0.001$, Fisher's PLSD), but Line 1 transgenics differed marginally significantly from controls ($P=0.05$, Fisher's PLSD). Neither transgenic line differed significantly from controls for triglycerides, total, and HDL cholesterol. There were no significant differences between Line 1 and 32 transgenics and littermate controls for any of these traits for mice maintained on a defined low-fat diet with 12% of calories from fat. Thus, overexpression of UCP2 and 3 apparently influences plasma LDL plus VLDL cholesterol levels in mice maintained on a moderate fat diet. We do not know if the transgenes influence only LDL or only VLDL or both.

Transgenic animals have reduced inflammatory response

Since endogenous and transgenic UCP2 were expressed in the spleen (Figure 1b), a tissue not normally associated with metabolic regulation, we assessed innate immune system alterations in the transgenic mice. Flow cytometric analysis of the spleen and thymus showed no significant differences in the number of various subsets of lymphocytes and macrophages (data not shown). Transgenic ($n=4$) and wild-type ($n=4$) female mice were challenged by intraperitoneal administration of lipopolysaccharide (LPS; 15 μg/kg in PBS) and serum samples were collected and analyzed for circulating levels of cytokines at 0, 1, 2, 4, 9, 24 and 48 h after LPS injection (Figure 3a). IL-4 was nearly undetectable at 0 h in transgenics and the peak levels of IL-4 at 4–8 h in the transgenic animals hardly reached the baseline (0 h) levels of control animals. Peak levels of circulating IL-6 were also delayed and reduced significantly in the transgenics (Figure 3a). The proinflammatory cytokine IL-6 plays an obligatory role in triggering fever response in the presence of both exogenous and endogenous pyrogens.[22] Systemic administration of LPS (50 μg/kg) induced significant elevations in core body temperature (fever) of control male mice, but fever response was shorter and significantly lower in transgenic male animals (Figure 3b). Female transgenic mice mounted a normal fever response when challenged with LPS 50 μg/kg i.p. (data not shown).

The long-term effects of suppressed inflammatory responses in transgenic animals probably depend on the type and extent of infection. For example, suppressed inflammatory response would be detrimental during infection by aggressive pathogens such as *Toxoplasma gondii*.[14] On the other hand, lesser inflammatory response might be beneficial during frequent mild infections or autoimmune mechanisms widespread among mammals, with long-term effects on longevity. In a pilot experiment, we have recorded three and two spontaneous deaths over 26 months in wild-type males ($n=6$) and females ($n=6$) fed regular mouse chow. However, no transgenic littermate males ($n=6$) and females ($n=6$) died spontaneously during that period. Further, although three of five wild-type males on the moderate-fat diet died spontaneously over 24 months, only one of four transgenic males died. Thus, overexpression of UCP2 and 3 may affect longevity.

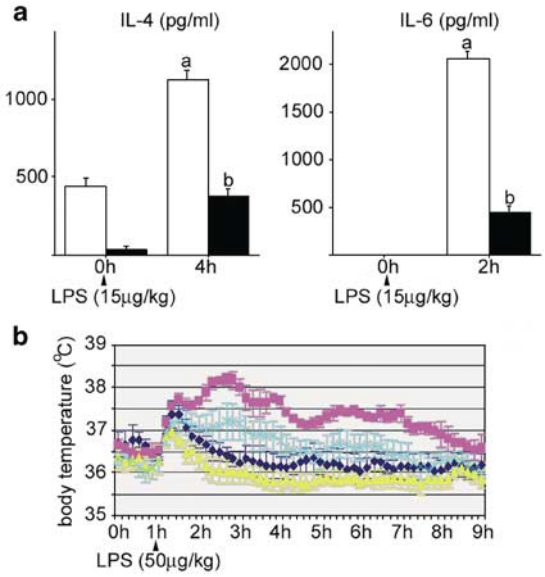

Figure 3 Suppressed inflammatory and fever responses in transgenic animals. (a) Systemic injection of lipopolysaccharide (LPS) induced lower elevation of IL-4 in transgenic Line 32 animals compared to wild-type littermates (left graph, open bars = wild type, filled bars = transgenics) 4 h after LPS infusion. IL-6 induction was also lower in transgenics 2 h following systemic injection of LPS (right graph); a, significant differences ($P<0.05$). (b) Systemic LPS injection induced fever in wild-type (control) male mice (pink squares), which was significantly ($P<0.05$) diminished in transgenic animals (turquoise ''x''). Control (blue diamonds) and transgenic animals (yellow triangles) that received saline injection only had no significant elevations of core body temperature.

Heart rate and spontaneous activity

Heart rate, core body temperature, and horizontal motor activity were measured simultaneously in freely moving male transgenic and wild-type mice implanted with radio transmitters, in thermoneutrality. Heart rate seemed unaffected by hUCP2/3 overexpression. Both experimental groups maintained maximal heart rates of about 500 beats/min at the beginning of the nocturnal period, when food intake, core body temperature and motor activity are maximal. Heart rate was lowest (330 ± 30 beats/min $\pm$ s.e.)

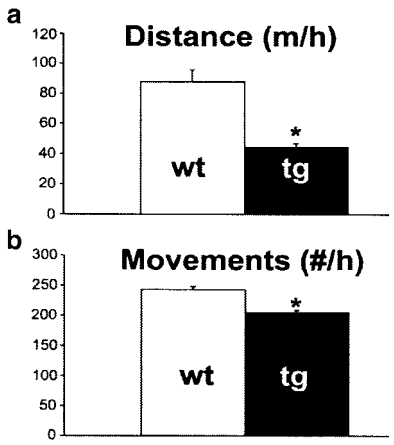

Figure 4 Decreased spontaneous activity of transgenic animals. Analysis of spontaneous physical activity of wild-type and transgenic Line 32 animals revealed that transgenic animals (tg; black columns) traveled shorter distances per hour (a) and engaged in less number of movements per hour (b) than their wild-type littermates (wt; white columns). *P-values lower than 0.05.

and core body temperature was lowest in transgenic and wild-type littermates during the light period ($n=4$, wild-type mice and $n=6$, transgenic mice). Handling stress and intraperitoneal saline injection raised heart rate from 350 to about 550 beats/min rapidly in both groups.

To test whether muscle mass increase in transgenic mice is associated with increased physical activity, we analyzed spontaneous daily activity of transgenic mice. Female transgenic mice moved 44 ± 3 m/h$\pm$s.e.m., only half the distance of the wild-type mice ($P=0.05$) during their nocturnal awake cycle (Figure 4a). The mean number of movements was also significantly lower in transgenic mice compared to their wild-type littermates (205 ± 4 movements/h$\pm$s.e.m. for transgenics *vs* 244 ± 4.2 movements/h$\pm$s.e.m for wild-type mice; $P=0.008$; Figure 4b). Wild-type mice tended to move faster during individual motions than transgenic animals (15 ± 6.9 *vs* 11 ± 2.4 cm/s in the transgenics), but the difference was not statistically significant ($P=0.19$).

Discussion

Transgenic mice of two independent lines had slightly smaller, but statistically significant, fat masses and more LDL plus VLDL cholesterol than littermate controls. We confirmed the obesity phenotype by transferring the Line 32 transgene to A^y mice, which made heterozygous transgenics significantly leaner. Thus, the obesity phenotype of the UCP2/UCP3 transgenics cannot be due to an accident of insertional mutagenesis, but rather reflects the effects of a moderate overexpression of UCP2 and 3. We have also successfully produced homozygous Line 32 transgenics, demonstrating that the transgene insertion in this line does not knock out function of an essential recessive gene. We have chosen not to phenotype homozygous transgenics or to produce homozygous Line 1 transgenic, but instead to focus on a model of moderate overexpression in the heterozygotes of both lines. Our results may also reconcile studies that have shown that decreased UCP2 mRNA levels are correlated with human but not mouse obesity changes. Our observation that transgenic mice expressing increased UCP2 and 3 have decreased fat mass is consistent with many human association studies. Thus, UCP2 and UCP3 may be obesity genes in both humans and mice.

The transgenic mice exhibited many other differences from wild-type, some expected and some surprising. Mitochondrial membrane potential was decreased and resting metabolic rate was increased. Overexpression of human UCP2 and 3 in mice resulted in a unique metabolic phenotype in which increased food intake and decreased physical activity are accompanied by increased muscle mass. These studies also revealed reduced proinflammatory reaction in response to LPS in transgenic animals.

Our results are consistent with mouse knockout experiment reports that UCP2 influences mitochondrial uncoupling activity and innate immune response.[14–16] The diminished transgenic cytokine response to LPS suggests overall suppression of signaling abilities of immune cells by UCP overexpression. UCP2 knockout studies suggest that UCP2 control on macrophage free radical production might alter innate immune responses.[14] Further, although the altered cytokine responses of transgenics may alone explain their suppressed febrile response, hypothalamic hUCP2 expression may affect the neuronal component of thermoregulatory responses to inflammation. Thus, the physiological basis of UCP2's influence on the immune system remains unknown.

Although our results in the metabolic parameter studies support reports that UCP2 and 3 are human obesity genes[12] and that UCP3 transgenic mice are lean,[18] they seemingly contradict mouse UCP2 and 3 knockout study results that did not demonstrate any obesity phenotype.[14,15,17] Reportedly, UCP3 knockout does not influence mitochondrial respiration rates, respiratory control ratios and proton conductance compared to the wild type under a variety of assay conditions. Further, increased uncoupling in a transgenic mouse overexpressing UCP3 20-fold was not well-correlated to UCP3 concentration and was neither activated by superoxide nor inhibited by GDP.[29] These results suggest that our transgenic phenotypes are due primarily to the overexpression of UCP2. It would be tempting to explain the observed decrease in spontaneous physical activity of transgenic mice by the potential of both UCP2 and UCP3 to decrease ATP levels, as has been observed in pancreas, and thus impair skeletal muscle contraction. However, this might not be correct, since several studies have observed a positive correlation of UCP3 mRNA levels in muscle and ATP levels. Thus, the observed hypolocomotion of UCP2/UCP3 transgenic mice could instead be due to events not directly associated with high levels of UCP3 gene product in skeletal

muscles, such as central hUCP2 overexpression affecting motor functions.[9–11,30,31] The contradictory results for effects of UCP2 and 3 on ATP levels in pancreas and skeletal muscle present in the current literature suggest that effects of UCP2 and 3 on ATP levels in any one tissue cannot yet be predicted from effects in other tissues.

Increased cholesterol in the transgenics may be related to reduced physical activity, but studies in humans and mice have suggested that UCP2 or 3 influence plasma cholesterol levels directly. For example, a UCP3 promoter variant (−55 C/T) is associated with total and LDL cholesterol levels in 1155 people.[32] A study in mice also identified coincident loci for total cholesterol and percent body fat in a quantitative trait locus that includes the genes for UCP2 and 3.[33] Moreover, UCP3 transgenic mice[18] and UCP1 overexpressing mice (under the control of the myosin light-chain 2 promoter)[34] on a high-fat diet exhibited lower plasma total cholesterol levels but not triglyceride and nonesterified fatty acid levels.

The results of this study suggest that controlled elevation of mitochondrial uncoupling activity by hUCP2 and possibly by hUCP3 tends to have positive (reduced fat mass) and negative (increased LDL plus VLDL cholesterol) effects. The results also suggest that natural alleles of UCP2 and 3 are positional candidate obesity genes underlying obesity QTLs.

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