Long-term high-fat feeding induces greater fat storage in mice lacking UCP3

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Costford SR, Chaudhry SN, Crawford SA, Salkhordeh M, Harper ME. Long-term high-fat feeding induces greater fat storage in mice lacking UCP3. Am J Physiol Endocrinol Metab 295: E1018–E1024, 2008. First published August 19, 2008; doi:10.1152/ajpendo.00779.2007.—Uncoupling protein-3 (UCP3) is a mitochondrial inner-membrane protein highly expressed in skeletal muscle. While UCP3’s function is still unknown, it has been hypothesized to act as a fatty acid (FA) anion exporter, protecting mitochondria against lipoperoxidation and/or facilitating FA oxidation. The aim of this study was to determine the effects of long-term feeding of a 45% fat diet on whole body indicators of muscle metabolism in congenic C57BL/6 mice that were either lacking UCP3 (Ucp3−/−) or had a transgenically induced approximately twofold increase in UCP3 levels (UCP3tg). Mice were fed the high-fat (HF) diet for a period of either 4 or 8 mo immediately following weaning. After long-term HF feeding, UCP3tg mice weighed an average of 15% less than wild-type mice (P < 0.05) and were 20% less metabolically efficient than both wild-type and Ucp3−/− mice (P < 0.01). Additionally, wild-type mice had 21% lower, whereas UCP3tg mice had 36% lower, levels of adiposity compared with Ucp3−/− mice (P < 0.05 and P < 0.001, respectively), indicating a protective effect of UCP3 against fat gain. No differences in whole body oxygen consumption were detected following long-term HF feeding. Glucose and insulin tolerance tests revealed that both the UCP3tg and Ucp3−/− mice were more glucose tolerant and insulin sensitive compared with wild-type mice after short-term HF feeding, but this protection was not maintained in the long term. Findings indicate that UCP3 is involved in protection from fat gain induced by long-term HF feeding, but not in protection from insulin resistance.

uncoupling protein-3; obesity; insulin resistance; type 2 diabetes mellitus; skeletal muscle

BECAUSE OF ITS HIGH SEQUENCE HOMOLOGY to the original uncoupling protein [uncoupling protein-1 (UCP1)] expressed in brown adipose tissue (BAT), UCP3 was first hypothesized to dissipate the proton gradient across the mitochondrial inner membrane in skeletal muscle, thereby uncoupling oxidative phosphorylation and releasing energy as heat. Since its discovery in 1997, several studies have shown that UCP3 is likely not responsible for basal proton leak in muscle (1, 6). In fact, the adenine nucleotide translocator is probably responsible for the majority of proton leak in this tissue (5). The novel uncoupling proteins (UCPs 2-5) have been studied over the last decade; however, their physiological functions are still unclear. UCP3 is expressed highly selectively in skeletal muscle, BAT, and at low levels in the heart (3, 35). Early studies of UCP3 overexpression in experimental systems ranging from yeast to mice were confounded by supraphysiological levels of expression (9, 14, 16, 31, 39). These high levels of protein expressed on the mitochondrial inner membrane induced artifactual proton leak (14, 31), making it difficult to draw conclusions about the true function of UCP3. UCP3 expression is increased in response to fasting (4, 20, 37) and acute exercise (28, 34), situations of energy deficit in which metabolic inefficiency would be disadvantageous. These data, together with the observation that UCP3 expression is upregulated in response to a high-fat (HF) diet (24, 26), led researchers to hypothesize that the function of UCP3 might be related to mitigating situations of HF influx to the mitochondria. Indeed, the content and/or activity of key proteins involved in fatty acid (FA) uptake and oxidation have been shown to be increased in UCP3tg muscle, supporting a role for UCP3 in FA metabolism (2). MacLellan et al. (19) also demonstrated that the approximately two- to threefold overexpression of UCP3 in L6 muscle cells resulted in increased FA oxidation and decreased reactive oxygen species production. To date, several hypotheses regarding the molecular function of UCP3 have emerged: 1) UCP3 facilitates β-oxidation by acting in conjunction with mitochondrial thioesterase-1 to export FA anions from the mitochondrial matrix to liberate coenzyme A so that it can participate in reactions of β-oxidation and the tricarboxylic acid cycle (15); 2) UCP3 protects against the accumulation of nonesterified FAs, leading to lipotoxicity by exporting FA anions from the mitochondrial matrix (29); and 3) UCP3 decreases reactive oxygen species production by mediating proton leak when activated by 4-hydroxynonenal (11).

The clear association between mitochondrial fat influx and UCP3 expression has led to the assessment of rodents overexpressing UCP3 in response to HF feeding: Son et al. (30) reported less weight gain and epididymal white adipose tissue (EWAT) as well as higher oxygen consumption in mice overexpressing UCP3 mRNA (18-fold compared with control mice) when fed a HF diet for 4 wk (30); Choi et al. (8) showed that mice overexpressing UCP3 protein 60-fold were protected from the development of insulin resistance after 10-day HF feeding; and Tiraby et al. (33) showed that female (but not male) mice having twofold (glycolytic muscle-specific) UCP3 protein overexpression were more glucose tolerant than controls after 14 wk of HF feeding. Our laboratory published the only study in the literature to have compared congeneric mice overexpressing UCP3 (~2-fold at the protein level), UCP3 knockout mice, and controls fed a HF diet for 8 mo (10). The only HF feeding measurements reported on in this previous study were the lower and higher levels of intramuscular triglyceride (IMTG) seen in UCP3 overexpressor and UCP3

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knockout mice, respectively, compared with wild-type mice. Because MTG levels are highly correlated with insulin resistance (17, 21, 22, 32, 38), this result, combined with other reports in the literature linking UCP3 to protection from insulin resistance, suggested that UCP3 might protect against the development of insulin resistance and type 2 diabetes mellitus (T2DM) during long-term HF feeding.

Our aim in the current study was to assess the effect of long-term (4 or 8 mo) HF feeding on Ucp3−/− mice, wild-type control mice, and in mice with an approximately twofold increase in levels of UCP3 (UCP3tg). Here we report the effects of UCP3 on development of T2DM, whole body energetics, and fuel storage.

MATERIALS AND METHODS

Animal models. Male Ucp3−/−, wild-type, and UCP3tg C57BL/6 mice (n = 20/genotype) backcrossed 10 generations were housed individually in clear polycarbonate cages from weaning and fed a HF rodent diet (D12451; Research Diets, New Brunswick, NJ) ad libitum. The D12451 diet consisted of 20% protein (casein and l-cystine), 35% carbohydrate (corn starch, maltodextrin, and sucrose) and 45% fat (soybean oil and lard) by kilocalorie. Mice were housed at 23°C for 4 or 8 mo with light from 0600 to 1800. The F1 generation of the UCP3tg mice has been previously described (9); these mice were provided originally by Dr. John Clapham at Glaxo-Smith-Kline. The overexpressor mice used in this study express two- to threefold the UCP3 protein levels of wild-type mice in skeletal muscle. The Ucp3−/− mice have also been previously described, but on a mixed genetic background (12). Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council). The study was approved by the Animal Care Committee of the University of Ottawa.

Mitochondrial isolation and Western blotting. Mice fed the 45% fat-defined diet or a 10% defined control diet for 8 mo following weaning were killed via decapitation. Hindlimb skeletal muscle was dissected and transferred in ice-cold Basic Medium (in mM: 140 KCl, 20 HEPES, 5 MgCl2, and 1 EGTA, pH 7.0). Muscle was then minced with a razor blade and then incubated for 2 min on ice in Homogenization Medium (140 mM KCl, 20 mM HEPES, 5 mM MgCl2, 2 mM EGTA, 1 mM ATP, and 1% BSA, pH 7.0) supplemented with 2 U/mg muscle subtilisin A. The mixture was then diluted sixfold with Homogenization Medium and centrifuged for 10 min at 9,681 g (high speed, HS). Pellets were resuspended in Homogenization Medium and centrifuged using a Potter-Elvehjem-type homogenizer. Homogenates were centrifuged for 10 min at 482 x g (low speed); supernatants were then centrifuged for 10 min at HS. Pellets were washed one time in Wash Medium (Basic Medium supplemented with 1% BSA) and one time in Basic Medium, flash-frozen in liquid nitrogen, and stored at −80°C. Mitochondrial protein content was determined via bicinechonic acid assay. Mitochondrial lysates were separated on a 10% bis-Tris polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane. Membranes were incubated with 1:1,000 rabbit anti-UCP3 (ab-3477; Abcam) and 1:2,000 mouse anti-succinate dehydrogenase (SDH) (sc-59687; Santa Cruz Biotechnology) primary antibodies for 16 h at 4°C. Goat anti-rabbit horse radish peroxidase (HRP) (sc-2030; Santa Cruz Biotechnology) and goat anti-mouse HRP (sc-2031; Santa Cruz Biotechnology) were used as the secondary antibodies at dilutions of 1:1,000 and 1:5,000, respectively, for 1 h at 25°C. Visualization was accomplished using an Enhanced Chemi-Luminescence kit (Amersham Pharmacia). Spot densitometry was performed using an Alpha multi-image light camera and Alpha imaging software. Values represent the integrated density value (IDV) of UCP3 divided by the IDV of SDH.

Whole body analyses. Body weight and food intake were determined two times per week for the duration of the study. Food intake is expressed as the total amount of energy ingested over the study period divided by the number of days in the study period (mean kcal ingested/day). Metabolic efficiency was calculated as total body weight gained divided by total amount of energy consumed during the study period (mg body weight gained/kcal ingested).

Glucose and insulin tolerance tests. Mice were assessed for glucose and insulin tolerance after 1, 2, 3, 4, and 8 mo on the diet. Tests were carried out at 1-wk intervals to allow recovery time. Saphenous blood was used to measure blood glucose using a glucometer (One Touch Basic; LifeScan, Burnaby, BC, Canada). Before the tests, fasting blood glucose was measured, and then mice were intraperitoneally injected with 1 mg glucose (10% dextrose in sterile saline) or 1 x 10−4 units human insulin (in sterile saline; Humalog Rapid Acting; Eli Lilly) per gram body weight. Blood glucose was assessed again at 10, 20, 30, and 120 min postinjection.

Indirect calorimetry. A customized four-chamber Oxymax system (Columbus Instruments, Columbus, OH) was used to measure oxygen consumption and carbon dioxide production in the mice. The system was programmed to maintain chamber temperature at 24°C with lights on from 0600 to 1800. Flow rate was set at 0.5 l/min and the sample line purge at 2 min. Mice were housed individually in the 2.5-liter calorimetry chambers and given ad libitum access to water and the diet. Measurements were taken every 12 min for 60 s over a period of 24 h after 4 or 8 mo of HF feeding. Energy expenditure was expressed as percent relative cumulative frequencies of all points collected, as described previously (23). Respiratory exchange ratios (RER) were calculated as the ratio of carbon dioxide production divided by oxygen consumption.

Analysis of 2-deoxy-[1-3H]glucose 6-phosphate in tissues. This procedure was adapted from Wang et al. (36) with minor modifications and has been described previously (10). At 4 mo, mice fasted overnight were injected intraperitoneally with 0.5 μCi 2-deoxy-[1-3H]glucose mixed with 10% dextrose to provide a fixed specific activity of 1 g glucose/kg body weight. Postinjection (70 min), quadriceps, gastrocnemius, diaphragm, interscapular brown adipose tissue (IBAT), EWAT, liver, pancreas, and heart were flash-frozen in liquid nitrogen and stored at −80°C until use. Mitochondria were isolated as described previously. Mitochondrial lysates were separated on a 10% bis-Tris polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane. Membranes were incubated with 1:1,000 rabbit anti-UCP3 (ab-3477; Abcam) and 1:2,000 mouse anti-succinate dehydrogenase (SDH) (sc-59687; Santa Cruz Biotechnology) primary antibodies for 16 h at 4°C. Goat anti-rabbit horse radish peroxidase (HRP) (sc-2030; Santa Cruz Biotechnology) and goat anti-mouse HRP (sc-2031; Santa Cruz Biotechnology) were used as the secondary antibodies at dilutions of 1:1,000 and 1:5,000, respectively, for 1 h at 25°C. Visualization was accomplished using an Enhanced Chemi-Luminescence kit (Amersham Pharmacia). Spot densitometry was performed using an Alpha multi-image light camera and Alpha imaging software. Values represent the integrated density value (IDV) of UCP3 divided by the IDV of SDH.

Fig. 1. Uncoupling protein-3 (UCP3) protein levels in skeletal muscle mitochondria after 8 mo of high-fat (HF) or low-fat (LF) feeding. Integrated densitometry value (IDV) of UCP3 normalized to IDV of succinate dehydrogenase (SDH); n = 4 mice; means ± SE, 1-way ANOVA, Tukey’s posttest. A: *P < 0.05 and **P < 0.01. B: representative Western blot.
liquid nitrogen. Thawed tissues homogenized in 2% HClO4 were incubated at 4°C overnight and then centrifuged for 10 min at 2,000 g. Total 3H radioactivity was determined in 50% of the neutralized supernatant, and the other 50% was applied to an anion exchange resin-filled column (Ag1-X8; Bio-Rad, Mississauga, ON, Canada). 3H radioactivity was measured in free 2-deoxyglucose and 2-deoxyglucose-6-phosphate eluants. Tissue glucose uptake was determined by dividing tissue 2-deoxy-[3H]glucose-6-phosphate activity by the glucose specific activity.

Analysis of 2-deoxy-[1-3H]glucose-6-phosphate in liver glycogen. This procedure was adapted from Wang et al. (36) with minor modifications and has been described previously (10). Preparation of liver samples was carried out as described above in the analysis of tissue 2-deoxy-[1-3H]glucose-6-phosphate. Oyster glycogen (2.5%) and 0.2% Na2SO4 were added to the neutralized supernatant before glycogen precipitation with ethanol. The resulting pellet was washed one time and resuspended in distilled water before 3H radioactivity counting.

Statistical methods. A one-way ANOVA with Tukey’s posttest was used to assess statistical significance for densitometry, body weight, food intake, naso-anal length, metabolic efficiency, tissue weights, oxygen consumption, RER, 2-deoxy-[1-3H]glucose tissue uptake, and incorporation of 2-deoxy-[1-3H]glucose in liver glycogen (Figs. 1–3; Tables 1 and 2). A two-way ANOVA with Bonferroni correction was used to assess statistical significance for glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) (Figs. 4 and 5).

RESULTS

UCP3 protein expression. UCP3 protein expression was examined in mitochondria isolated from skeletal muscle of wild-type mice after 8 mo of HF feeding and compared with mitochondria isolated from skeletal muscle of wild-type mice fed a low-fat (10% by kcal) defined diet for 8 mo following weaning. UCP3tg mice fed the HF diet had a 2.2-fold higher expression of UCP3 compared with wild-type mice (P < 0.01), whereas UCP3tg mice fed the LF control diet had a similar 2.5-fold higher expression of UCP3 compared with wild types (P < 0.05) (Fig. 1A). HF feeding induced a 1.8-fold increase in UCP3 expression UCP3tg mice (P < 0.01) and a 2.0-fold increase in UCP3 expression in wild-type mice (trend did not reach statistical significance) (Fig. 1A). No UCP3 expression was detected in mitochondria from Ucp3−/− mice fed either diet (Fig. 1B).

Whole body analysis. There were no differences in body weight after 4 mo of HF feeding (Fig. 2A); however, after 8 mo on the diet, UCP3tg mice weighed 15% less than wild-type mice (Fig. 2B). There was a trend for UCP3tg mice to have lower body weights compared with Ucp3−/− mice at 8 mo, although this did not reach statistical significance. UCP3tg mice did, however, have 20% lower metabolic efficiency compared with both wild-type and Ucp3−/− mice after 8 mo of HF feeding, but not after 4 mo (Fig. 2, C and D). There were no differences in naso-anal length between the genotypes, but there was a trend for Ucp3−/− mice to have higher food intake compared with wild-type and UCP3tg mice at both 4 and 8 mo, but this did not reach statistical significance (Table 1).

Wet weight tissue analysis revealed no differences in muscle mass (gastrocnemius, soleus, quadriceps, diaphragm), IBAT,
or organ (pancreas, liver, heart, spleen) weights (Table 2). There were no differences in adiposity as measured by EWAT depots compared with wild-type mice (Fig. 2); however, after 8 mo of HF feeding, UCP3tg mice had 36% smaller EWAT depots compared with Ucp3−/− mice, and Ucp3−/− mice had 21% higher EWAT depots compared with wild-type mice (Fig. 2).

**DISCUSSION**

Given the hypothesized roles for UCP3 in skeletal muscle energy expenditure and lipid handling, our aim overall was to assess the impact of the long-term feeding of a HF diet. Several reports in the literature have linked UCP3 to protection from the development of insulin resistance and T2DM. In rats, higher UCP3 mRNA levels have been associated with increased glucose tolerance (25), and, in humans, decreased UCP3 protein expression in skeletal muscle has been reported in subjects with T2DM (27). In fact, F1 generation mice overexpressing UCP3 protein in skeletal muscle at 20-fold higher than wild-type mice had lower fasting glucose and insulin levels as well as increased glucose tolerance (7, 9). We previously published a study in which we assessed the absence and the approximately twofold transgenic overexpression of UCP3 in congenic C57BL/6 mice fed a 10% fat-defined diet (10). Here we confirmed that the UCP3tg mice overexpress UCP3 two- to threefold in skeletal muscle (Fig. 1), as previously published (2). Although UCP3tg mice fed the 10% fat diet were protected from obesity and the development of insulin resistance, Ucp3−/− mice were also protected from...
insulin resistance. However, when challenged with a HF (45%) diet, Ucp3−/− showed increased accumulation of IMTG compared with wild types, whereas UCP3tg mice showed decreased IMTG content (10). Because accumulation of IMTG is known to be highly correlated with development of insulin resistance (17 and reviewed in Ref. 13), and UCP3 has been shown to be upregulated in response to HF feeding (24, 26), it seemed possible that the physiological role of UCP3 had been masked during low-fat feeding studies and that the true phenotype of this set of complementary congenic mice might be revealed by a HF challenge. Indeed, Choi and colleagues (8) demonstrated that UCP3tg mice were protected from the development of insulin resistance after short-term (10 day) HF feeding, and the latter finding was recently corroborated by Tiraby et al. (33) in female, but not male, mice. However, neither of these reports included analyses of Ucp3−/− mice.

Because of the artifactual uncoupling in early overexpression studies, concerns have arisen regarding the interpretation of UCP3 overexpressor mouse data, especially those mice of mixed genetic backgrounds. One of the strengths of the current study was the use of UCP3tg and Ucp3−/− mice, both backcrossed 10 generations into the C57BL/6 background. In addition to confirming the overexpression levels of UCP3 in the UCP3tg mice, we also showed an approximately twofold increase in UCP3 protein expression in both wild-type and transgenic mice in response to HF feeding (Fig. 1). As seen in the low-fat feeding study (10), Ucp3−/− and UCP3tg mice were paradoxically protected from the development of glucose intolerance and insulin resistance when fed the HF diet, but this moderate protection only lasted up to 3 mo (Fig. 4). UCP3tg and Ucp3−/− mice also showed moderate protection from insulin resistance, but not until 3 mo of HF feeding, and these differences had disappeared by 8 mo (Fig. 5). Unlike Ucp3−/− mice fed the 10% fat diet that showed increased 2-deoxy-[1-3H]glucose in BAT at 4 mo, there were no genotype differences in 2-deoxy-[1-3H]glucose incorporation in any of the muscles, fat depots, or organs examined or 2-deoxy-[1-3H]glucose incorporation in liver glycogen, consistent with the glucose tolerance data at 4 mo. These somewhat moderate short-term improvements in glucose and insulin tolerance seen in the UCP3tg mice are confounded by the observation of the same improvements in the Ucp3−/− mice. This, coupled with the lack of long-term protection from insulin resistance, calls into question whether UCP3 truly has a role in protection from the development of fat-induced insulin resistance.
There are many reports in the literature linking overexpression of UCP3 to leanness in laboratory animals (e.g., see Refs. 8–10 and 33); however, no association has been shown between UCP3 protein expression levels and body mass index in humans (27). Additionally, the Chow- or LF diet-fed Ucp3/−/− mouse is not obese (10, 12), has normal serum insulin, triglyceride, and leptin levels, and a trend for lower serum glucose and free FA levels (12). Consistent with what has been previously reported (12), we did not find increased Ucp3/−/− body weights when the mice were challenged with a HF diet (Fig. 2, A and B). Interestingly though, EWAT depots were significantly larger in Ucp3/−/− mice compared with both wild-type and UCP3tg mice (Fig. 2F) after long-term HF feeding, indicating a higher degree of adiposity when UCP3 was lacking (there was a trend for UCP3tg mice to have lower EWAT weights compared with wild-type mice, but this did not reach statistical significance). This result is difficult to reconcile with the lack of difference in body weight (Fig. 2B) or in metabolic efficiency (Fig. 2D) between Ucp3/−/− and wild-type mice after long-term HF feeding. There was a moderate trend for Ucp3/−/− mice to have increased food intake at both 4 and 8 mo (Table 1), but this did not reach statistical significance. This moderate increase in energy intake may have been compensated for by the increased energy expenditure observed in the Ucp3/−/− mice at 4 mo (Fig. 3A), resulting in no differences in body weight in the Ucp3/−/− mice. The increase in energy expenditure at the 4-mo time point was unexpected, although it could possibly be explained by spontaneous physical activity (not measured). Lower body weights and metabolic efficiencies were observed, however, in the UCP3tg mice after 8 mo of HF feeding (Fig. 2, B and D). Because there were no differences in food intake, the decrease in body weight must be explained by differences in energy expenditure. We were unable, however, to detect differences in energy expenditure after 8 mo of HF feeding (Fig. 3B); there were no statistical differences in oxygen consumption between UCP3tg mice and wild-type controls. Only a small increase in whole body energy expenditure would be required to incur a 15% lower body weight compared with controls over 8 mo. It can be difficult to detect such small differences in whole body energy expenditure via 24-h indirect calorimetry because of the ~5% error associated with this technique (18). Again, spontaneous physical activity was not assessed, although differences in this parameter might account for the oxygen consumption results at this early time point.

Therefore, the only tissues to be significantly affected by both the absence and approximately twofold overexpression of UCP3 in response to long-term HF feeding were skeletal muscle (10) and adipose tissue (Fig. 2F). Accumulation of IMTG is decreased in UCP3tg mice and increased in Ucp3/−/− mice, but this does not translate into a correlation with insulin resistance, since both UCP3tg and Ucp3/−/− mice are only transiently and moderately protected from glucose and insulin tolerance during long-term HF feeding. UCP3tg mice have decreased adiposity, as measured by EWAT weight when fed a LF diet (10), and showed a similar trend during long-term HF feeding (Fig. 2F), whereas Ucp3/−/− mice have increased EWAT depots. Taken together, these results suggest that UCP3 is playing a role in fuel storage but may not play as significant a role in whole body energy metabolism as previously thought.

In conclusion, our results support a role for UCP3 in the protection from fat gain when exposed to a HF diet over a prolonged period of time, but not a role for long-term protection against HF-induced insulin resistance. The physiological function of UCP3 requires further investigation, although the information we are able to obtain from whole body assessments of these UCP3 transgenic mice may be limited. The search for the molecular mechanism of UCP3 function will likely require more direct in vitro approaches.

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