Uncoupling Protein-3 Expression in Rodent Skeletal Muscle Is Modulated by Food Intake but Not by Changes in Environmental Temperature*

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A new member of the uncoupling protein (UCP) family called UCP3 has recently been cloned and shown to be highly expressed in skeletal muscle of rodents and humans. In the present study, UCP3 was overexpressed in C2C12 myoblasts where it acts as an uncoupling protein. Changes in UCP3 mRNA expression were examined in rodent muscles under conditions known to modulate thermogenesis in brown adipose tissue. In skeletal muscle, UCP3 expression did not change in response to 48 h of cold exposure (6 °C), whereas it was decreased by 81% or increased 5.6-fold by 1 week of 50% food restriction or fasting, respectively. It was also decreased by 36% in soleus muscle of obese (fa/fa) as compared with lean Zucker rats. The unexpected rise of UCP3 mRNA level induced by fasting did not change in vitro muscle basal heat production rate but decreased by 31% the capacity to produce heat in response to the uncoupler carbonyl-cyanide p-trifluoromethoxyphenylhydrazone. This decrease may reflect underlying uncoupling by UCP3. Up-regulation of UCP3 mRNA after a 24-h fast was still observed in mice exposed at thermoneutrality. These results show that the increase in UCP3 expression induced by fasting is associated with the maintenance of thermogenesis measured in muscle in vivo and is not modulated by environmental temperature. The notion that UCP3 expression is modulated by food intake is of importance to better understand the pathophysiology of obesity in humans.

The uncoupling protein-1 (UCP1) gene encodes a mitochondrial protein carrier that stimulates heat production by uncoupling respiration from ATP synthesis in brown adipose tissue (BAT) and plays an important role in nonshivering and diet-induced thermogenesis in rodents (1). Recently, new proteins sharing 55 and 56% amino acid identities with UCP1 have been identified and called UCP2 and UCP3 (2–4). The tissue distributions of UCP2 and UCP3 were very different from that of UCP1, which is BAT-specific, being either ubiquitous (UCP2) or specific to skeletal muscle and BAT (UCP3) (2–4). UCP2 was shown to be an uncoupling protein because in transfected yeast it partially uncouples mitochondrial respiration (2). The modulations of UCP2 expression have also been studied in various tissues of the rat. Cold exposure was found to affect UCP2 similarly to UCP1 in BAT (5). Fasting, however, was found to up-regulate UCP2 mRNA in the skeletal muscle (5).

The presence of UCP3 in skeletal muscle is of great interest because this tissue is an important site of catecholamine and diet-induced thermogenesis in rats (6) and in humans (7, 8). UCP3 might therefore play a major role in whole body thermogenesis.

The aims of this work were: (i) to develop a cell line of UCP3-transfected myoblasts and to examine the effects of UCP3 expression on mitochondrial membrane potential and (ii) to study in vivo modulations of UCP3 expression in BAT and in skeletal muscle under conditions known to affect UCP1 and UCP2. The results obtained show that UCP3 has an uncoupling activity in transfected myoblasts. They also demonstrate that UCP3 mRNA expression in skeletal muscle is modulated by food intake but not by changes in environmental temperature.

EXPERIMENTAL PROCEDURES

Cell Transfection—A 0.9-kilobase pair fragment containing the coding sequence of the human UCP3 was excised from pBluescript at EcoRI and HindIII sites (present in the multiple cloning site of the vector) and inserted into the EcoRI/HindIII sites of the expression vector, pcDNA 3.1 (Invitrogen, Leek, Netherlands). C2C12 myoblast cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and transfected with either the empty pcDNA 3.1 or the vector containing the human UCP3 using the calcium phosphate precipitation method. Stable transfectants were obtained by growth of the cells in culture medium containing 600 μg/ml G418 (Life Technologies, Inc.). Colonies derived from single cells were isolated and expanded. Cell lines were screened for the expression of UCP3 by Northern blot analysis.

Confocal Microscopy—Stable transfected C2C12 myoblast cells were grown on 25-mm round glass coverslips in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells were washed with phosphate-buffered saline and incubated for 30 min at 37 °C with 10 mM tetramethylrhodamine ethyl ester (TMRE) in a balanced salt solution containing 5.5 mM glucose, 120 mM NaCl, 4 mM KCl, 1 mM KH2PO4, 1 mM MgSO4, 1.3 mM CaCl2, and 10 mM HEPES buffered adjusted to pH 7.4. Confocal images were acquired with an invert laser scan microscope (LSM 410 invert, Carl Zeiss, Germany). The cells were excited with a HeNe 543 nm laser and observed at emission above 590 nm. All images were acquired with the same parameters and analyzed with LSM-PC software (Carl Zeiss, Germany).

Analysis of Mitochondrial Membrane Potential by Flow Cytometry—Subconfluent C2C12 myoblast cells were loaded with TMRE (10 nM) in the presence or the absence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) (75 μM). After 30 min of incubation at 37 °C, the cells were washed, trypsinized, and resuspended in the balanced salt solution with TMRE (10 nM). Flow cytometry was performed with a FACStar™ Plus cell sorter (Becton Dickinson, Bedford, MA) using the 514 nm line of an argon laser to assay the mitochondrial membrane potential. Fluorescent light was directed to a photomultiplier tube equipped with...
with a filter selecting above 590 nm. A minimum of 10,000 cells/sample were acquired and analyzed with Lysis II software.

**Animal Treatments**—7-week old Sprague-Dawley male rats or 9-week old female lean (Fa/) and obese (fa/fa) Zucker rats fed ad libitum standard laboratory chow were maintained under a 12-h light-dark cycle at 23 °C. All animals were caged individually during the experimental periods. Rats were either exposed to cold (6 °C) or fasted with free access to water for 48 h. Studies were also performed with adult C57BL male mice, which were fed the same diet as the rats. They were divided into three groups: group 1 was kept at 23 °C for 1 week, group 2 was kept at thermoneutrality (33 °C) for 1 week, and group 3 was kept at 23 °C and pair-fed with the mice at 33 °C for 1 week. 24 h before sacrifice, half of the mice in each group were fasted with free access to water containing 4.5 g/liter of NaCl. The animals were killed by decapitation, and soleus and tibialis anterior muscles, as well as interscapular BAT carefully trimmed from white adipose tissue, connective tissue, and muscle, were excised, immediately frozen in liquid nitrogen, and stored at −80 °C.

**Northern Blot Analyses**—Total RNA was purified by the method of Chomczynski and Sacchi (9), and 12–20 μg were electrophoresed in a 1.2% agarose gel containing formaldehyde, as described by Lehrach et al. (10) and transferred to Electran Nylon Blotting membranes (BDH Laboratory Supplies, Poole, UK) by vacuum blotting. The probes used were a full-length rat UCP3 cDNA (GenBank™ accession number U92069). The UCP3 signal in rat and mouse was a doublet with sizes of 2.5 and 2.8 kilobases as already described (3). The probe was labeled by random priming with [α-32P]dCTP (Amersham) to a specific radioactivity of approximately 1 × 10^6 dpm/μg DNA. Northern blots were hybridized for 2 h at 65 °C in QuikHyb solution (Stratagene, La Jolla, CA) and then washed in a solution of 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate at 50 °C for 5 min. Blots were exposed to Hyperfilm ECL films (Amersham) at −80 °C with intensifying screens. Size estimates for the RNA species were established by comparison with an RNA Ladder (Life Technologies, Inc.). The signals on the autoradiograms were quantified by scanning photodensitometry using ImageQuant Software version 3.3 (Molecular Dynamics, Sunnyvale, CA). Hybridization of the blots with a γ-32P-labeled synthetic oligonucleotide specific for the 18S rRNA subunit was used to correct for differences in the amounts of RNA loaded onto the gel. Student’s unpaired t test was used to determine statistical significance.

**Microcalorimetric Studies**—Soleus muscle obtained from adult mice kept at 23 °C either fed ad libitum or fasted for 24 h were mounted on a rigid frame made of thin stainless steel thread and introduced in the test chamber of a twin microcalorimeter (Secfroid, S.A., Lausanne, Switzerland) as described previously (11). Both test and reference chamber of a twin microcalorimeter (Secfroid, S.A., Lausanne, Switzerland) as described previously (11). Both test and reference chambers were perfused with the same standard, Krebs–Ringer bicarbonate buffer (pH 7.4) as described (12). Before calorimetric studies were performed using mouse soleus muscle. It was found that 24 h of fasting increased UCP3 expression by 3.5-fold (p < 0.001; results not shown). As seen in Fig. 3A, it had no effect on UCP3 expression in rat tibialis anterior muscle. A 48-h fast was found to decrease UCP3 expression by 74% in rat BAT (p < 0.005, n = 5; results not shown); however, as seen in Fig. 3A, it increased UCP3 expression 5.6-fold in rat tibialis anterior muscle.

To determine whether the up-regulation of UCP3 mRNA induced by fasting in the skeletal muscle was reflected by changes in muscle heat production, direct microcalorimetric studies were performed using mouse soleus muscle. It was shown in Fig. 2, UCP3 transfection resulted in a shift of the fluorescence peak to the left, which reflected a decrease in the level of fluorescence per cell. The mean values of 24.2 ± 0.7 (n = 3) and 18.8 ± 0.7 (n = 5) fluorescence arbitrary units in control and UCP3-transfected cells, respectively, differ significantly from each other (p < 0.0025). Addition of CCCP induced a large shift to the left in both populations of cells (p < 0.001). In fully uncoupled cells, the fluorescence peaks of control and UCP3 transfected cells were superimposed. The effect of CCCP was therefore weaker in the transfected cells than in control cells (p < 0.001), confirming the lower basal mitochondrial membrane potential in the former.

**In Vivo Modulations of UCP3 Expression**—Conditions known to modulate UCP1 expression were chosen to examine the possible changes of UCP3 mRNA expression in vivo, i.e., cold exposure and fasting. Cold exposure was found to increase UCP3 expression 1.5-fold in rat BAT (p < 0.05, n = 5; results not shown). As seen in Fig. 3A, it had no effect on UCP3 expression in rat tibialis anterior muscle. A 48-h fast was found to decrease UCP3 expression by 74% in rat BAT (p < 0.005, n = 5; results not shown); however, as seen in Fig. 3A, it increased UCP3 expression 5.6-fold in rat tibialis anterior muscle.

**RESULTS**

**Effect of UCP3 Expression in Myoblasts**—C2C12 cells, which expressed low amounts of UCP2 mRNA at the myoblast stage of differentiation and no UCP3 mRNA, were transfected with either an empty vector (control) or a vector containing the human UCP3 cDNA. A cell clone was chosen in which the level of expression of UCP3 mRNA, as measured by Northern blot analysis, was as high as in human skeletal muscle. Control and transfected cells were incubated with TMRE, a fluorescent dye that is sensitive to the mitochondrial potential, i.e., to the electrochemical gradient across the mitochondrial inner membrane. Confocal fluorescence studies confirmed that the fluorescence is specifically localized in mitochondria as already described (12). As expected, the specific mitochondrial uncoupler CCCP (75 μM) strongly decreased the fluorescence (Fig. 1).

To evaluate the effect of UCP3 on mitochondrial membrane potential, fluorescence intensities were measured using flow cytometry in UCP3-transfected and control C2C12 cells. As shown in Fig. 2, UCP3 transfection resulted in a shift of the fluorescence peak to the left, which reflected a decrease in the level of fluorescence per cell. The mean values of 24.2 ± 0.7 (n = 3) and 18.8 ± 0.7 (n = 5) fluorescence arbitrary units in control and UCP3-transfected cells, respectively, differed significantly from each other (p < 0.0025). Addition of CCCP induced a large shift to the left in both populations of cells (p < 0.001). In fully uncoupled cells, the fluorescence peaks of control and UCP3 transfected cells were superimposed. The effect of CCCP was therefore weaker in the transfected cells than in control cells (p < 0.001), confirming the lower basal mitochondrial membrane potential in the former.

**FIG. 1. Effect of CCCP on TMRE fluorescence in C2C12 myoblasts.** Control myoblasts (transfected with the empty vector) were incubated in 10 nM TMRE in a balanced salt solution (see "Experimental Procedures") and then treated without (left) or with the chemical uncoupler CCCP (75 μM) (right) for 30 min at 37 °C. Confocal fluorescence images were acquired with an invert laser scan microscope. White scale bar, 25 μm. Inset, fluorescence localization of TMRE in mitochondria of C2C12 myoblasts. Black scale bar, 5 μm.
respectively. Of the lean fa/ fa control values in BAT and soleus muscle, rats, UCP3 mRNA expression was shown to be decreased in is a decrease in thermogenesis (1) has been studied. In these pressed as percentages as described under “Experimental Procedures.” The results are expressed as means ± S.E. of the mean respective control values number of experiments was nine to eleven. *, p < 0.05; **, p < 0.005 versus respective controls.

Under the metabolic conditions studied, i.e. cold exposure or fasting, the level of UCP3 mRNA in BAT was modulated in the same way as that of UCP1 (results not shown). Indeed, in this tissue, it has been reported that cold exposure increases (1, 6) and fasting decreases (5, 15) UCP1 expression. The striking parallelism found between UCP1 and UCP3 modulations in BAT is consistent with a role of UCP3 as an uncoupler of oxidative phosphorylation in this thermogenic tissue.

A model of genetically obese Zucker fa/ fa rats in which there is a decrease in thermogenesis (1) has been studied. In these rats, UCP3 mRNA expression was shown to be decreased in obese fa/ fa to 58 ± 5 (p < 0.01) or 59 ± 2% (p < 0.05) of that of the lean Fa/ control values in BAT and soleus muscle, respectively.

**DISCUSSION**

Previously reported UCP1 and UCP2 transfection studies have been performed in yeast (2, 13). Because UCP3 is highly expressed in skeletal muscle (4), the putative uncoupling ac-

**Fig. 2. Human UCP3 expressed in C2C12 myoblasts decreases the mitochondrial membrane potential.** Cells transfected with the empty vector (Cont.) or with the human UCP3 cDNA (UCP3) were incubated with the mitochondrial potential-sensitive dye TMRE and analyzed by flow cytometry. The chemical uncoupler CCCP (75 μM) was used as a positive control. One experiment representative of three to five is shown, with a minimum of 10,000 events/sample.

**Fig. 4. In vitro heat production is maintained in soleus muscle after 24 h of fasting.** Basal heat production (basal) and the change in heat production induced by FCCP in soleus muscle of control mice (C, white columns) or mice fasted for 24 h (F, hatched columns). The measurements were performed in a microcalorimeter, and the results are expressed as means ± S.E. in μW × (mg tissue wet weight)⁻¹. The number of experiments was nine to eleven. *, p < 0.05; **, p < 0.005 versus respective controls.

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observation that the mean stimulation of heat production induced by FCCP was significantly lower in fasted than in control mouse soleus muscle.

In mice kept at thermoneutrality, the increase in tibialis anterior muscle UCP3 expression induced by fasting was similar to that of control mice kept at 23 °C (Fig. 3B). This result does not support the notion that changes in muscle UCP3 level might play a role in the maintenance of body temperature.

These results indicate that the increase in UCP3 expression induced by fasting is associated with the maintenance of thermogenesis measured in muscle in vitro and is not modified by changes in the environmental temperature. They also show that different mechanisms are responsible for the control of UCP3 expression in BAT and in skeletal muscle. In BAT, the effects of cold exposure or fasting on UCP3 expression, like those on UCP1, should be mediated by an increase or a decrease, respectively, of the sympathetic nervous system activity. In muscle UCP3 expression seems not to be controlled by the same system but probably by changes in food intake among other factors.

Among the metabolic changes induced by fasting, the increase in circulating fatty acids (17, 18) or the increase in plasma glucocorticoids (17) with no change in catecholamines (18) reported in rats fasted for 48 h could be responsible for the increase in muscle UCP3 expression. Further experiments are underway in our laboratory to elucidate this mechanism.

UCP3, being highly expressed in human skeletal muscle (3), might be an important effector of thermogenesis in humans. It is conceivable that disregulations of UCP3 activity or expression could favor body weight gain and obesity. The notion that UCP3 expression is dependent on food intake is of importance to better understand the biochemical events relating energy intake to energy expenditure.

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