Original Article

Physiological Increases in Uncoupling Protein 3 Augment Fatty Acid Oxidation and Decrease Reactive Oxygen Species Production Without Uncoupling Respiration in Muscle Cells

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Decreased uncoupling protein (UCP)3 is associated with insulin resistance in muscle of pre-diabetic and diabetic individuals, but the function of UCP3 remains unclear. Our goal was to elucidate mechanisms underlying the negative correlation between UCP3 and insulin resistance in muscle. We determined effects of physiologic UCP3 overexpression on glucose and fatty acid oxidation and on mitochondrial uncoupling and reactive oxygen species (ROS) production in L6 muscle cells. An adenoviral construct caused a 2.2- to 2.5-fold increase in UCP3 protein. Palmitate oxidation was increased in muscle cells incubated under normoglycemic or hyperglycemic conditions, whereas adenoviral green fluorescent protein infection or chronic low doses of the uncoupler dinitrophenol had no effect. Increased UCP3 did not affect glucose oxidation, whereas dinitrophenol and insulin treatments caused increases. Basal oxygen consumption, assessed in situ using self-referencing microelectrodes, was not significantly affected, whereas dinitrophenol caused increases. Mitochondrial membrane potential was decreased by dinitrophenol but was not affected by increased UCP3 expression. Finally, mitochondrial ROS production decreased significantly with increased UCP3 expression. Results are consistent with UCP3 functioning to facilitate fatty acid oxidation and minimize ROS production. As impaired fatty acid metabolism and ROS handling are important precursors in muscular insulin resistance, UCP3 is an important therapeutic target in type 2 diabetes. *Diabetes* 54: 2343–2350, 2005

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CPT, carnitine palmitoyl transferase; CoASH, uncombined CoA; DCFDA, 5-(and 6)-carboxy-2′,7′-dichlorohydofluorescein diacetate; GFP, green fluorescent protein; MTE1, mitochondrial thioesterase-1; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; UCP, uncoupling protein.

Insulin resistance in muscle is a primary component of type 2 diabetes, a disease with a prevalence that is increasing at an alarming rate in modern society. The importance of mitochondrial oxidative phosphorylation in the development of type 2 diabetes has recently been demonstrated through gene expression profiling studies in muscle of pre-diabetic, diabetic, and nondiabetic populations. Individuals with type 2 diabetes were shown to have reduced expression of genes for key proteins in oxidative metabolism and mitochondrial function in muscle (1). Furthermore, pre-diabetic individuals with insulin resistance but normal glucose tolerance display the same pattern of decreased expression of genes related to mitochondrial oxidative metabolism, consistent with the possibility that such characteristics are directly relevant to the origins and development of type 2 diabetes (2).

One of the mitochondrial proteins having decreased mRNA expression in pre-diabetic and diabetic subjects is uncoupling protein (UCP)3 (2). Not only are the levels of mRNA for UCP3 decreased but also are the levels of UCP3 protein. Schrauwen et al. (3) demonstrated decreased UCP3 protein in vastus lateralis of type 2 diabetic patients and no significant association between UCP3 protein and BMI of patients.

Mitochondrial UCPs belong to a large family of anion carrier proteins that facilitate the exchange of substrates across the mitochondrial inner membrane. Members of this family play essential roles in the trafficking of intermediary metabolites into and out of the mitochondrial matrix. The archetypal UCP, UCP1, is abundantly expressed in brown adipose tissue, where it dissipates the energy potential generated by respiration to serve both thermoregulatory and metaboregulatory roles (4–6). However, UCP1 knockout mice, although cold intolerant, are no more susceptible to obesity than their wild-type littermates (7) and in fact demonstrate some resistance to diet-induced obesity (8). The latter suggests that the compensatory mechanisms are energetically more costly than UCP1-mediated thermogenesis (8).

In 1997, genes for two additional anion carrier proteins

having ~56% sequence homology to UCP1 were identified and named UCP2 (9,10) and UCP3 (11,12). The corresponding proteins are both expressed in brown adipocytes, albeit at levels far less than that of UCP1. In other tissues, UCP2 protein is localized to areas of the pancreas, immune system, white adipose tissue, and the brain (13) and has been linked to insulin secretion and protection from reactive oxygen species (ROS) (14–16). Although UCP3 is expressed predominantly in skeletal muscle, it is also found in brown adipose tissue, cardiac muscle, and in certain areas of the brain (11,12,17). Originally it was thought that these additional UCPs functioned in a thermoregulatory manner similar to UCP1. However, expression patterns of UCP2 and UCP3 are not always consistent with changes in thermogenesis (18) or mitochondrial uncoupling (19,20). Moreover, in overexpression studies in which uncoupling has been observed, there is concern that uncoupling is an artifact of supraphysiological levels of the protein in the mitochondrial inner membrane (21,22).

It has also been proposed that UCP3 protects against ROS damage. This is supported both in vivo (increased ROS damage in muscle of UCP3 knockout mice) and in vitro (mitochondria from knockout versus control mice treated with superoxide or hydroxynonenal). ROS are normal by-products of mitochondrial respiration. However, ROS produced in excess of scavenging systems can mediate cellular damage, which is known to contribute to hyperglycemia-induced diabetes complications (23,24). Elevated mitochondrial membrane potential can be a direct cause of accelerated cellular ROS production, so one way to mitigate ROS-mediated damage is to increase the proton "leakiness" of the mitochondrial inner membrane (25). Because mitochondrial ROS production is exquisitely sensitive to membrane potential, even mild uncoupling is thought to be highly effective for reducing ROS at this source. Thus, an alternative explanation for UCP3-dependent protection against hyperglycemia-induced damage in muscle may be a reduction in ROS production through mild uncoupling of oxidative phosphorylation.

The goal of the research described herein was to study the mechanisms underlying the negative correlation between UCP3 expression and insulin resistance in muscle. Specifically the aim was to determine, in intact L6 muscle cells, the effects of physiological increases in UCP3 expression on glucose and fatty acid oxidation and on the interrelationship between ROS production and mitochondrial uncoupling. We demonstrate that moderate (2.2- to 2.5-fold) overexpression of UCP3 protein in L6 myotubes specifically increases fatty acid oxidation but is without effect on glucose oxidation. Furthermore, we demonstrate for the first time that UCP3 overexpression mitigates the production of ROS in a manner that is independent of mitochondrial uncoupling. The findings thus imply that the decreased expression of UCP3 in muscle of pre-diabetic and diabetic individuals may be specifically mediated through some impairment in fatty acid metabolism and/or by increasing ROS-mediated cellular damage.

RESEARCH DESIGN AND METHODS

Cell culture reagents, including α-minimum essential medium, fetal bovine serum, and antibiotics/antimycotics were purchased from Invitrogen-Gibco (Burlington, ON). Fluorescent probes were obtained from Molecular Probes (Eugene, OR). Radioactive [U-^{14}C]glucose and [1-^{14}C]palmitate were from New England Nuclear (Boston, MA). Affinity-purified polyclonal rabbit anti-UCP3, directed against a 19–amino acid sequence in the COOH-terminus was purchased from Chemicon (Temecula, CA). All other chemicals were purchased from Sigma (Oakville, ON).

Cell culture and treatment. L6 rat myoblasts were maintained in α-minimum Eagle's medium containing 10% (vol/vol) fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (growth medium). For experiments, myoblasts were reseeded into 6-cm^2 cell culture dishes, 10-cm^2 cell culture dishes, opaque 96-well plates, or 25-cm^2 flasks, depending on the experiment to be performed. Once cells reached confluency, differentiation was induced by lowering the serum concentration to 2% (differentiation medium). Differentiation medium was replaced every 48 h for 5 days, unless otherwise stated, allowing sufficient time for myotubes formation. All experiments were initiated 5 days' postdifferentiation.

Adenoviral transfection. Transfection of L6 cells with an adenoviral vector (1×10^7 pfu/ml) containing recombinant UCP3 was performed 2 days' postdifferentiation. The virus was removed after a 48-h incubation period, and fresh differentiation medium was added. The efficacy of infection was determined by transfecting parallel plates with an equivalent titer of green fluorescent protein (GFP). GFP-transfected myotubes were also used to verify that experimental outcomes were not adenoviral mediated. To simultaneously study the effects of chemical uncoupling, separate plates were treated with 50 μmol/l 2,4-dinitrophenol starting 2 days' postdifferentiation for 72 h with the dinitrophenol being replaced every 24 h. This concentration was used based on the results of our preliminary studies and on the concentrations used chronically with the same cell line by others (26). For acute uncoupling, higher concentrations ranging from 0.5 to 5 mmol/l can be used and effects observed within minutes. However, the use of such higher concentrations for longer periods of time will cause cell death. Control myotubes were left untreated.

Western blotting. Cells were removed from flasks following incubation with 0.25% trypsin, washed, and then total protein was isolated using Trizol. To increase the concentration of protein before Western blotting, cell extracts were passed through Microcon YM-10 centrifugal filters (Millipore, Etobicoke, ON, Canada) following manufacturer's guidelines. The filters were spun at 14,000*g* to exclude proteins having molecular weights <10 kDa and to concentrate protein for Western blotting. To prevent increases in the concentration of SDS during these procedures, concentrated protein was diluted in PBS and respun twice at 14,000*g*. Equal amounts of cellular protein (80 μg) were loaded into each lane of a BioRad Minigel (12% polyacrylamide) system. After transfer to nitrocellulose membranes, primary antibody (UCP3, AB-3046; Chemicon) was incubated at a 1:1,000 dilution overnight at 4°C. The secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, Santa Cruz, CA) and was incubated at a 1:500 dilution for 1 h at room temperature. As a negative control, muscle mitochondrial proteins (75 μg) from UCP3$^{-/-}$ mice were used. As a positive control, recombinant murine UCP3 (prepared in our laboratory) was used. For detection, blots were processed using enhanced chemiluminescence kits (Amersham Pharmacia; Baie d'Urfe, Quebec, Canada).

Substrate oxidation. Rates of palmitate and glucose oxidation were measured in L6 myotubes cultured in 25-cm^2 flasks using a method similar to that described by Roduit et al. (27). Briefly, myotubes were washed once and incubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer containing 10 mmol/l HEPES (pH 7.4), 0.5% (wt/vol) defatted BSA, and 5 mmol/l glucose. For palmitate oxidation, myotubes were incubated for 1 h at 37°C in Krebs-Ringer bicarbonate HEPES buffer containing 0.2 mmol/l palmitate (bound to BSA at 0.5% [wt/vol]), 0.2 μCi/ml [1-^{14}C]palmitate, 1 mmol/l carnitine, and either 5.0 or 16.0 mmol/l glucose (oxidation medium). Glucose oxidation was measured under identical conditions but in the presence of 16 mmol/l glucose and 0.2 μCi/ml [U-^{14}C]glucose, 1 mmol/l carnitine, and either 0.05 or 0.2 mmol/l palmitate. To collect liberated $^{14}CO_2$, Whatman GF/B paper (one-third of a 25-mm circle) was soaked in 150 μl of 5% KOH and placed in the lumen of a 3-cm piece of polyvinyl chloride tubing (internal diameter 4.7 mm). The tube was then inserted into a rubber stopper, and the stopper was used to seal the flask with the tube facing inward. At the end of the incubation period, 200 μl of 40% (vol/vol) perchloric acid was injected into the flasks through the rubber stopper. The following day, the filter paper and 100 μl oxidation medium from each flask were collected and placed in scintillation vials containing 4 ml scintillation fluid. The vials were then left overnight before the radioactivity was measured by scintillation counting. As a positive control to verify that the assay was measuring glucose and palmitate oxidation, 100 nmol/l insulin was added into separate flasks during the 15-min preincubation period to influence their respective oxidation rates.

Oxygen uptake. Rates of oxygen consumption from individual L6 myotubes were determined using a self-referencing microelectrode system (28) and methods described by Porterfield et al. (29). Oxygen microelectrodes were

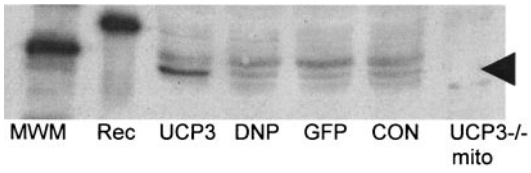

FIG. 1. Adenoviral-mediated increases in UCP3 protein expression. Representative Western blot for UCP3 expressed in infected and noninfected L6 myotubes. *Lane 1:* Molecular weight markers (MWM). The 34-kDa marker is the prominent band. *Lane 2*: Recombinant mouse UCP3 (Rec) migrates at a corresponding molecular weight of 39 kDa rather than 34 kDa, due to a 5-kDa fusion peptide. *Lane 3*: UCP3-infected L6 myotubes. *Lane 4*: Dinitrophenol (DNP)-treated cells. *Lane 5*: GFP-infected cells. *Lane 6*: Control (CON) (untreated) cells. *Lane 7*: Mitochondria (mito) (75 μg protein) isolated from hindlimb skeletal muscle of UCP3$^{-/-}$ mice. For *lanes 3–7*, 80-μg cellular proteins were loaded per lane. Densitometric analyses indicated that UCP3-infected cells expressed ~2.2- to 2.5-fold higher levels of UCP3 than cells infected with GFP or uninfected control cells. Note that the antibody reacts with two unknown proteins having slightly higher and lower molecular weights than UCP3; these nonspecific bands are present in mitochondria from UCP3$^{-/-}$ mice.

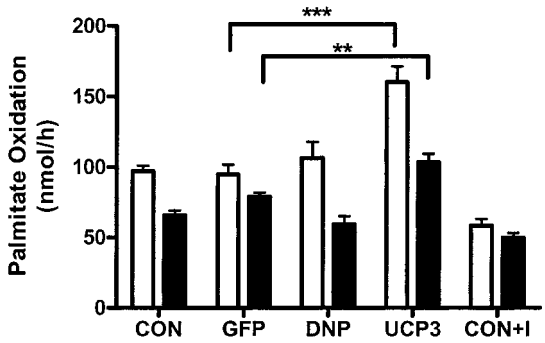

FIG. 2. Palmitate oxidation is increased by moderate overexpression of UCP3 but is unaffected by mild chemical uncoupling. Palmitate oxidation was measured in L6 myotubes incubated at normal (5 mmol/l; □) and high (16 mmol/l; ■) concentrations of glucose in the presence of 0.2 mmol/l palmitate. From left to right, results are shown for control (CON) (untreated) cells, GFP-infected cells, dinitrophenol (DNP)-treated cells, UCP3-infected cells, and control cells that were treated acutely with insulin (100 nmol/l). n = 8–16 for each treatment condition. **$P < 0.01$; ***$P < 0.001$.

manufactured using the methods of Jung et al. (30). Myotubes, cultured in a 6-cm^2 dish, were placed in an Earle's basic salt solution containing 117 mmol/l NaCl, 5.4 KCl, 1.5 $CaCl_2$, 0.8 $MgSO_4$, 0.9 mmol/l NaH_2PO_4, 10 mmol/l HEPES, and 5.6 mmol/l glucose. The dish was then placed under an Axiovert 200 (Zeiss) inverted microscope that is housed within an insulated Faraday box equipped with temperature control equipment that allowed the internal environment to be maintained at 37°C. The tip of an oxygen microelectrode was placed ~2 μm from the surface of a myotube and was oscillated at a frequency of 0.3 Hz to collect alternating oxygen flux and reference measurements. Translational movement of the microelectrode was accomplished with a translational motion control system mounted onto the head stage of the microscope. Movement was driven by IonView software developed, along with motion controllers and amplifiers, at the Biocurrents Research Center (MBL, Woods Hole, MA). The differential current outputs (in fA) obtained from three separate myotubes per plate were averaged and converted to oxygen flux using the Fick equation: $J = -D\ (\Delta C/\Delta r)$, where J is the flux rate, D is the diffusion coefficient of oxygen in water, ΔC is the oxygen concentration difference between the flux and reference positions, and Δr is the distance of measurement. ΔC was determined by multiplying the differential current obtained with the slope of an independently determined electrode calibration curve.

Mitochondrial membrane potential. The mitochondrial membrane potential of myotubes cultured in opaque 96-well plates was determined using tetramethylrhodamine ethyl ester (TMRE). Myotubes were incubated in differentiation medium containing 100 μmol/l TMRE for 15 min in the dark at 37°C. Fresh differentiation medium was then added after washing the myotubes twice with PBS to remove extracellular TMRE. Fluorescence intensity was detected with excitation and emission wavelengths of 544 and 590 nm, respectively.

ROS production. Myotubes cultured in opaque 96-well plates were incubated in PBS containing 1 μmol/l carboxy-H_2[5-(and-6)-carboxy-2′,7′-dichlorohydofluorescein diacetate] (DCFDA) for 10 min in the dark. After addition of 16.0 mmol/l glucose, fluorescence emission was followed for 1 h with measurements collected every 2 min (excitation = 490 nm; emission = 516 nm).

Statistical analysis. Statistical analysis was performed using ANOVA and Bonferroni post hoc tests. Results are presented as means ± SE, unless otherwise stated. Analyses were conducted using GraphPad Prism 4 (San Diego, CA) software.

RESULTS

Induction of UCP3 protein expression through adenoviral infection. Western blots of cellular homogenates indicated that the level of UCP3 protein expression in UCP3-infected L6 myotubes was ~2.2- to 2.5-fold higher than that in uninfected control cells, cells that were infected with GFP, or cells that were treated with the chemical uncoupler dinitrophenol (Fig. 1). As cross-reactivity is a common (although not widely appreciated) problem with the polyclonal UCP3 antibodies that are currently available, we used mouse recombinant UCP3 fusion protein as a positive control. It migrates at a molecular weight of 39 kDa due to a 5-kDa fusion peptide. As a negative control, protein isolated from purified mitochondria of muscle from UCP3 knockout mice was used. The immunoblotted proteins from muscle mitochondria of UCP3 knockout mice consistently showed that the middle band of three bands was absent, indicating that this band was indeed UCP3. This band is increased in the cells infected with UCP3. The identities of the two other bands are unknown but both appeared in the immunoblots of cellular and muscle mitochondrial proteins.

Moderate overexpression of UCP3 increases palmitate oxidation. Myotubes that were left untreated (i.e., uninfected control) and GFP-infected myotubes showed similar rates of palmitate oxidation at normal (5.0 mmol/l) and high (16.0 mmol/l) glucose concentrations (Fig. 2). Thus, adenoviral treatment itself had no effect on fatty acid oxidation. However, UCP3-infected myotubes oxidized 68% more palmitate in the presence of 5.0 mmol/l ($P < 0.001$) and 32% more palmitate in the presence of 16.0 mmol/l glucose ($P < 0.01$) compared with corresponding GFP-infected myotubes.

To test for any effect of chemical uncoupling of mitochondrial oxidative phosphorylation on fatty acid oxidation, cells were treated chronically with low concentrations of dinitrophenol (50 μmol/l for 72 h). Results showed that dinitrophenol had no effect on palmitate oxidation at either normal or high concentrations of glucose ($P > 0.05$). The effect of high glucose concentrations on the oxidation of this fatty acid was also assessed. Incubation in the presence of high concentrations of glucose (16 mmol/l) led to statistically significant decreases in palmitate oxidation in control cells, dinitrophenol-treated cells, and UCP3-infected cells compared with oxidation rates under normoglycemic (5 mmol/l) conditions ($P < 0.05$).

Glucose oxidation is unaltered by moderate overexpression of UCP3 but is increased by dinitrophenol-mediated uncoupling. Uninfected control myotubes, GFP-infected myotubes, and UCP3-infected myotubes demonstrated similar rates of glucose oxidation in the presence of 0.05 and 0.2 mmol/l palmitate (Fig. 3). How-

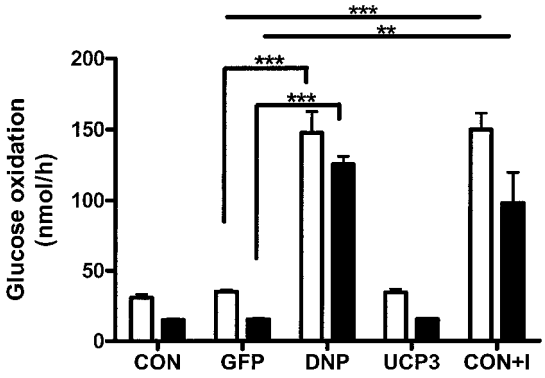

FIG. 3. Glucose oxidation is unaltered by moderate overexpression of UCP3 but is increased by mild chemical uncoupling. Glucose oxidation was measured in L6 myotubes incubated at low (0.05 mmol/l; □) and high (0.20 mmol/l; ■) concentrations of palmitate in the presence of 16 mmol/l glucose. From left to right, results are shown for control (CON) (untreated) cells, GFP-infected cells, dinitrophenol (DNP)-treated cells, UCP3-infected cells, and control cells that were treated acutely with insulin (100 nmol/l). n = 5–15 for each treatment condition. **P < 0.01; ***P < 0.001.

ever, treatment with the chemical uncoupler dinitrophenol (50 μmol/l) dramatically increased glucose oxidation in the presence of either 0.05 or 0.2 mmol/l palmitate compared with control myotubes and GFP-infected myotubes (all $P < 0.01$). As expected, insulin (100 nmol/l) increased glucose oxidation in the presence of 0.05 or 0.2 mmol/l palmitate compared with either control myotubes or GFP-infected myotubes (all $P < 0.01$).

Moderate overexpression of UCP3 does not significantly increase basal cellular oxygen consumption. Basal cellular oxygen consumption of cells was assessed in situ using self-referencing microelectrodes (see above). The oxygen consumption of myotubes infected with GFP or UCP3 was similar to that of uninfected control myotubes (Fig. 4). However, oxygen uptake of myotubes treated chronically with low concentrations of dinitrophenol was significantly elevated compared with uninfected controls (80% increase; $P < 0.001$) and GFP-infected cells (64% increase; $P < 0.01$).

Mitochondrial membrane potential is unaffected by moderate overexpression of UCP3. To determine whether infection with UCP3 was associated with any inherent decreases in mitochondrial membrane potential, the latter was assessed using TMRE fluorescence. During these measurements, the incubation medium was the one in which cells were maintained (differentiation medium; see above). Results in Fig. 5 demonstrate that dinitrophenol (chronic treatment, as above) significantly lowered mitochondrial membrane potential ($P < 0.001$). However, L6 myotubes infected with UCP3 had membrane potentials that were not significantly different from uninfected control cells and GFP-infected cells ($P > 0.05$).

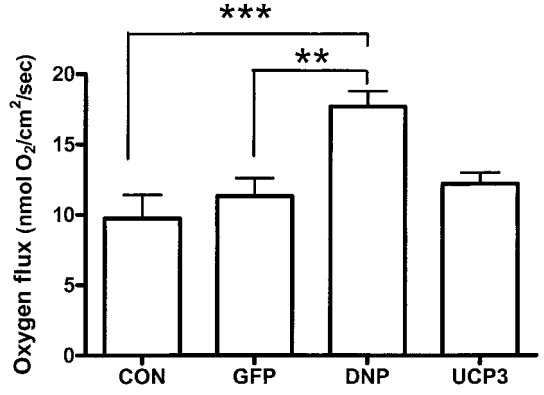

FIG. 4. L6 cellular oxygen consumption is not significantly increased by moderate overexpression of UCP3 but is increased by mild chemical uncoupling. In situ oxygen consumption of L6 cells was measured using oxygen-sensitive microelectrodes as described in the text. From left to right, results are shown for control (CON) (untreated) cells, GFP-infected cells, dinitrophenol (DNP)-treated cells, and UCP3-infected cells. n = 8–10 for each treatment condition. **P < 0.01; ***P < 0.001.

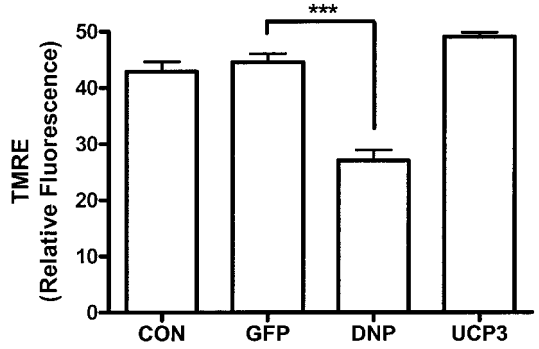

FIG. 5. Mitochondrial membrane potential is unaffected by moderate overexpression of UCP3. Mitochondrial membrane potential was assessed in intact L6 myotubes using the fluorescent marker TMRE as described in the text. From left to right, results are shown for control (CON) (untreated) cells, GFP-infected cells, dinitrophenol (DNP)-treated cells, and UCP3-infected cells. n = 5 for each treatment condition. **P < 0.01; ***P < 0.001.

Cellular ROS production is decreased by moderate overexpression of UCP3. Increased expression of UCP3 in L6 myotubes decreased the rate of cellular ROS production, as assessed by DCFDA fluorescence, compared with that in uninfected control cells and GFP-infected cells ($P < 0.001$ and < 0.01, respectively) (Fig. 6). Chronic treatment with low concentrations of dinitrophenol also significantly reduced ROS production in L6 myotubes compared with uninfected control cells and GFP-infected cells ($P < 0.001$ for both).

DISCUSSION

The upregulation of UCP3 in skeletal muscle in response to fasting, a period characterized by energy conservation

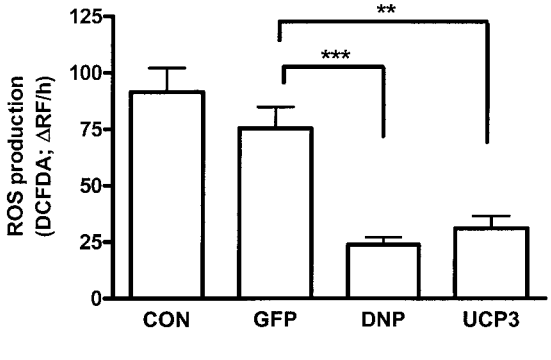

FIG. 6. L6 cellular ROS production is decreased by moderate overexpression of UCP3 and by mild chemical uncoupling. ROS production was assessed in intact L6 myotubes using the fluorescent marker, carboxy-H_2-DCFDA, as described in the text. From left to right, results are shown for control (CON) (untreated) cells, GFP-infected cells, dinitrophenol (DNP)-treated cells, and UCP3-infected cells. n = 10–13 for each treatment condition. RF, relative fluorescence. **P < 0.01; ***P < 0.001.

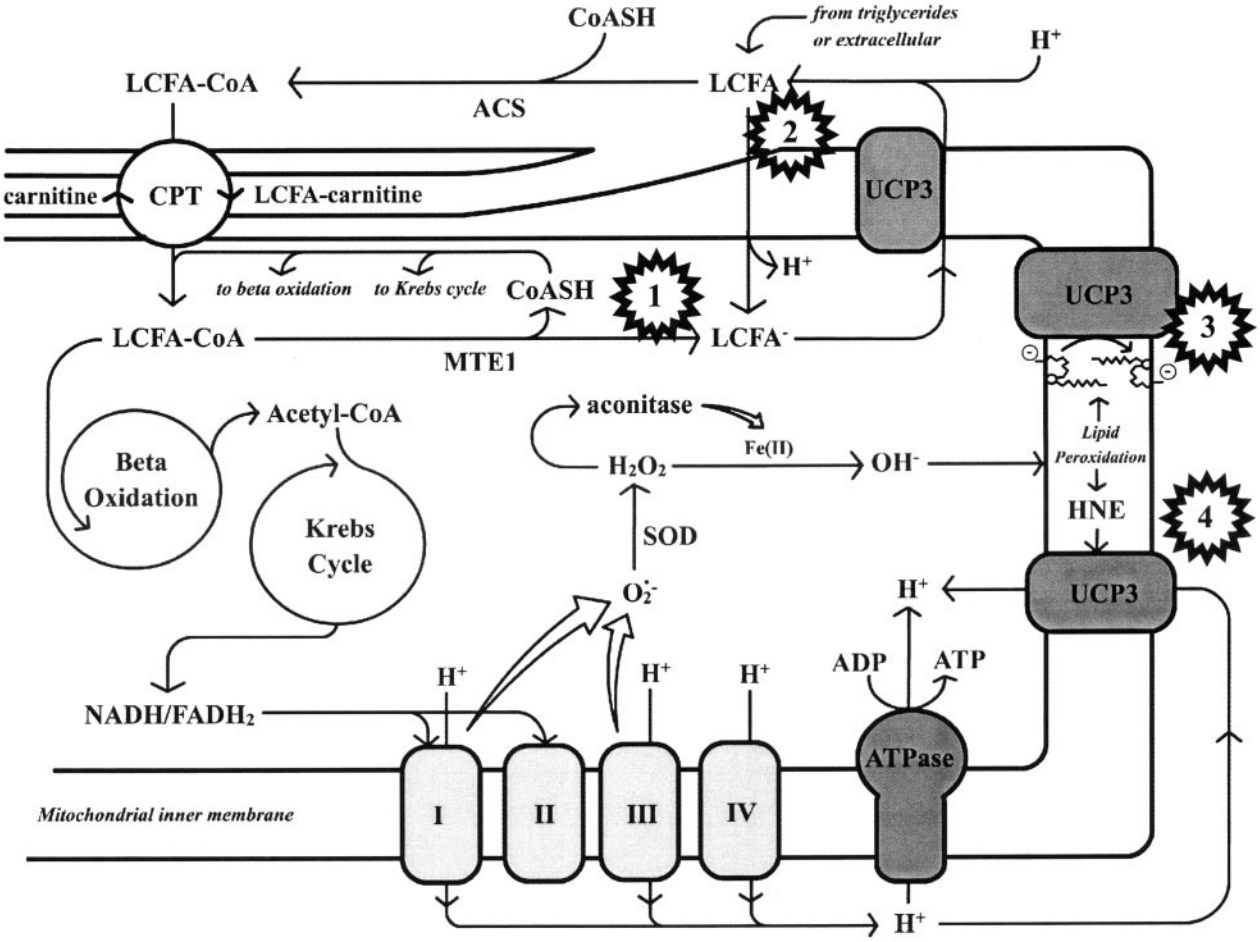

FIG. 7. Hypothesized functions of UCP3. The function of UCP3 is as yet unresolved, but four functions have been proposed and are presented in their chronological order of publication. *1*) UCP3 removes long-chain fatty acids (LCFA) produced by MTE1; the latter liberates mitochondrial matrix CoASH, a rate-limiting coenzyme for β-oxidation and the Krebs cycle. Long-chain fatty acids are exported from the matrix by UCP3 for reactivation by acyl-CoA synthase in the intermembrane space. Thus, MTE1 and UCP3 are proposed to function in tandem to facilitate fatty acid oxidation (35). *2*) UCP3 removes excess long-chain fatty acids that have entered the mitochondrial matrix independently of the CPT system. This would serve to remove potentially damaging fatty acid anions from the matrix (40). *3*) UCP3 translocates lipid peroxide anions, generated from the interaction of matrix ROS with unsaturated fatty acids of the inner leaflet, from the inner to the outer leaflets of the mitochondrial inner membrane (44). This would remove fatty acid peroxides and prevent damage of mitochondrial DNA, aconitase, and other matrix components. *4*) 4-hydroxynonenal (HNE), a lipid by-product of mitochondrial superoxide (SOD) production, is proposed to activate a UCP3-mediated proton leak (43). It is proposed that this decreases mitochondrial membrane potential and hence decreases ROS production.

and increased fatty acid oxidation rates, strongly suggests that the physiological function of UCP3 (unlike that of UCP1) is not thermoregulation. Free fatty acids are reported as being obligatory for UCP3 activity (31) and are known agonists of peroxisome proliferator–activated receptors, which may activate UCP3 gene expression through the putative peroxisome proliferator response elements in the promoter region of the UCP3 gene (32). There is a high correlation between UCP3 expression patterns and conditions in which free fatty acids are the predominant fuel source, such as fasting, free fatty acid infusion, and high-fat feeding (33,34). The physiological function of UCP3 is as yet unknown, but its association with enhanced fatty acid metabolism and increased insulin responsiveness in muscle lead to the possibility that it may be involved in protection from lipotoxicity in muscle.

Two hypotheses have been forwarded to outline mechanistic roles for UCP3 in fatty acid metabolism (mechanisms 1 and 2 in Fig. 7). The first proposed that UCP3 and mitochondrial thioesterase 1 (MTE1) function in tandem to facilitate high rates of fatty acid oxidation (35). During periods of elevated fatty acid oxidation, when ~90% of mitochondrial uncombined CoA (CoASH) is bound to free fatty acids (36,37), MTE1 liberates CoASH to support fatty acid oxidation. CoASH-requiring steps include fatty acid entry into the mitochondria through carnitine palmitoyl transferase (CPT)2, α-ketoglutarate dehydrogenase in the Krebs cycle, and β-ketothiolase in the β-oxidation pathway. The expression of MTE1, like that of UCP3, is regulated by peroxisome proliferator–activated receptors (37), and recent studies show that expression patterns of the two genes are similar (38,39). In mechanism 1, UCP3 is proposed to export the MTE-liberated fatty acid anions, which cannot be reesterified to CoASH in the matrix due to the absence of acyl-CoA synthetase. Thus, UCP3 is proposed to export the potentially detrimental fatty acid anions to the intermembrane space and cytosol where they can be reesterified to CoASH for subsequent use in other pathways (e.g., phospholipids, triacylglycerol synthesis, or reentry into the matrix for oxidation).

The second mechanism also involves mitochondrial fatty acid handling and is schematically depicted in Fig. 7.

Schrauwen et al. (40) hypothesized that when fatty acid supply exceeds oxidation, fatty acids spontaneously enter the mitochondria independent of CPT mechanisms and accumulate as nonesterified fatty acid anions in the matrix. UCP3, according to this second hypothesis, also translocates free fatty acid anions out of the matrix. The latter hypothesis thus proposes a mechanism for efflux of potentially detrimental fatty acid anions but does not implicate UCP3 in facilitating increased fatty acid oxidation.

The results of this study support the first hypothesized mechanism, as we observed increased fatty acid oxidation due to increased UCP3 expression. The latter was not related to any effects of the adenoviral infection process itself as there was no change in fatty acid oxidation in GFP-infected cells. Previous studies involving supraphysiological expression of the UCPs have been criticized for the artifactual uncoupling of oxidative phosphorylation that results (21). By inducing UCP3 protein expression within the physiological range, we aimed to avoid any artifactual uncoupling. As the 2.2- to 2.5-fold induction of UCP3 expression resulted in no inherent decreases in mitochondrial membrane potential (Fig. 5) or any significant increases in basal oxygen consumption (Fig. 4) of cells, it is clear that artifactual uncoupling was not an issue. Although there was a trend for increased oxygen consumption with increased UCP3 expression, this however did not coincide with any decrease in mitochondria membrane potential (Fig. 5). It is possible that with substantially increased sample sizes, a statistically significant increase might be detected. If there were actually an increase in coupled oxygen consumption, then it would presumably be driven by increased ATP demands (e.g., acyl-CoA synthase activity). Our findings indicate that uncoupled respiration does not necessarily follow overexpression, an important advance in our understanding of this mitochondrial anion carrier protein.

There are reports that increased UCP3 expression increases glucose metabolism. Mice that overexpress UCP3 protein in muscle at levels ~20-fold normal have improved glucose homeostasis (41) and protection against hyperglycemic-induced damage (17). Further, Huppertz et al. (26) demonstrated that substantial UCP3 overexpression using adenovirus gene transfer in L6 myotubes increases GLUT4 translocation and glucose uptake. The relative levels of UCP3 expression in the latter study were much higher than those herein. Although a very pronounced band for UCP3 was detected in Western blots of infected cells, no UCP3 was detected in the uninfected control cells. The latter findings may reflect artifactual uncoupling (e.g., due to supraphysiologic UCP3 levels). For example, the chronic treatment of the L6 cells with doses of dinitrophenol similar to those used herein (10–50 μmol/l) resulted in increased glucose uptake and metabolism through a phosphatidylinositol-3-kinase mechanism, similar to the effects observed for UCP3 infection. Although these authors did not assess either mitochondrial membrane potential or oxygen consumption, they did demonstrate increased lactate production in UCP3-infected cells, consistent with the possibility that mitochondria were uncoupled (i.e., cells would need to rely exclusively on glycolytic ATP production). Our results showing dinitrophenol-activated glucose oxidation but no effect of UCP3 infection thus suggest that the effects on glucose metabolism in the latter study resulted from secondary uncoupling due to supraphysiological levels of UCP3.

A number of other investigations into UCP3 function have identified a role for the protein in the protection from ROS. The UCP3 knockout mouse provided the first evidence of such a role (42). Elegant in vitro studies conducted thereafter identified a potential role for byproducts of ROS, such as the lipid peroxide 4-hydroxynonenal, as activators of UCP3 (43). Hydroxynonenal was shown to activate UCP3 as evidenced by mitochondrial uncoupling. This was interpreted as a mechanism to lower membrane potential and minimize further ROS production. This mechanism for UCP3 activation is depicted as mechanism 4 in Fig. 7.

It has also been proposed that UCP3 functions to remove lipid peroxides from mitochondria and thus protect cells from potentially damaging ROS and their byproducts (mechanism 3 in Fig. 7) (44). Based on the latter two proposed mechanisms, we investigated ROS production in our treated and untreated L6 muscle cells.

Our findings demonstrate that moderate increases in UCP3 protein and chronic dinitrophenol treatment both decrease ROS production. However, classic evidence of mitochondrial uncoupling was evident only in the case of dinitrophenol treatment, which caused increased cellular oxygen consumption and a corresponding reduction in membrane potential. This is consistent with earlier observations indicating that mild uncoupling, by reducing membrane potential, can diminish ROS production at complex I of the electron transport chain (25). It has been hypothesized that UCPs (including UCP3) function within a negative-feedback pathway that limits mitochondrial ROS production through mild uncoupling. In vitro evidence for this hypothesis comes from the observation that superoxide-dependent activation of UCP3 causes mild uncoupling in mitochondria (45). In vivo overexpression of UCP1 completely reverses hyperglycemia-induced mitochondrial ROS overproduction in aortic endothelial cells (46). However, we found that UCP3 overexpression was not accompanied by changes in either cellular oxygen consumption or membrane potential, despite its significant negative impact on ROS production. This implies that diminished ROS production in myotubes, although UCP3 dependent, is not mediated by uncoupling of oxidative phosphorylation.

Mitochondrial ROS has been implicated as a central mediator of hyperglycemia-induced diabetes complications in endothelial cells (23). This, together with our observation that UCP3 mitigates ROS production in myotubes and a growing body of evidence that UCP3 overexpression improves glucose homeostasis (17,26,41), underscores the central role and potential importance of UCP3 function in the development treatment of diabetes.

In conclusion, our results demonstrate that moderate physiological induction of UCP3 protein expression in muscle cells results in increased fatty acid oxidation in the absence of uncoupling. The results are consistent with the clinical findings of Argyropoulos et al. (47) who demonstrated decreased fat oxidation by indirect calorimetry in a population of Gullah-speaking African Americans with an exon six-splice donor single nucleotide polymorphism in the UCP3 gene. The latter single nucleotide polymorphism

was not, however, associated with decreased fat oxidation in the Maywood African-American population (48), in whom the degree of genetic admixture was greater than that in the Gullah-speaking population. Decreased fat oxidation has also been documented through indirect calorimetry in UCP3$^{-/-}$ mice (20). In recent studies of skeletal muscle of UCP3tg mice with a ~twofold increase in UCP3 expression (49), there was an increased capacity for fat oxidation (e.g., increased carnitine palmitoyltransferase I and β-hydroxyacyl-CoA dehydrogenase activities) in the absence of significant increases in thermogenesis. Thus, our current findings support and extend the latter and provide a potential mechanism for the detrimental effects of decreased UCP3 expression in muscle with regard to the development of lipotoxicity and insulin resistance in muscle.

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