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Overexpression of mitochondrial uncoupling protein-3 does not decrease production of the reactive oxygen species, elevated by palmitate in skeletal muscle cells

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Abstract **Fatty acids induced an increase in reactive oxygen species (ROS) and enhanced NF-κB activation in L6 myotubes differentiated in culture. Palmitate proved more effective than oleate in eliciting these effects. The induction of uncoupling protein-3 (UCP3) at levels similar to those occurring in vivo, attained through the use of an adenoviral vector, led to a reduction of mitochondrial membrane potential in L6 myotubes. However, the capacity of palmitate to increase ROS was not reduced but, quite the opposite, it was moderately enhanced due to the presence of UCP3. The presence of UCP3 in mitochondria did not modify the expression of genes encoding ROS-related enzymes, either in basal conditions or in the presence of palmitate. However, in the presence of UCP3, UCP2 mRNA expression was down-regulated in response to palmitate. We conclude that UCP3 does not act as a protective agent against palmitate-dependent induction of ROS production in differentiated skeletal muscle cells.**

Keywords: Uncoupling protein; Fatty acid; Skeletal muscle; Reactive oxygen species

1. Introduction

Uncoupling protein-3 is a member of the uncoupling protein (UCP) family expressed preferentially in skeletal muscle [1]. Like UCP1, the founder member of the family, UCP3 can uncouple respiratory chain from oxidative phosphorylation [2]. Over-expression of UCP3 in several cell lines and tissue systems reduces mitochondrial membrane potential, whereas mitochondria from UCP3 gene-null mice exhibit high mitochondrial membrane potential [3–5]. Increasing evidence indicates that fatty acids directly activate the proton conductance of UCP3, while GDP's capacity to inhibit it has been demonstrated in reconstituted systems or in permeabilized cells expressing UCP3, but not in heterologous yeast expression systems [6–9]. From a physiological point of view, however, it appears that UCP3 is not particularly involved in mediating cold-induced or diet-induced thermogenesis [4,5] and its involvement in heat production has only been demonstrated in relation to drug-induced hyperthermia [10].

The transcription of the UCP3 gene is extremely sensitive to the availability of free fatty acids to skeletal muscle. Thus, in multiple physiological or pathological situations (fasting, high-fat diets, lactation, exercise, etc.), UCP3 mRNA and protein levels are dramatically modulated depending on changes in circulating free fatty acid levels, whatever their source (diet or lipolysis in white fat depots) [11,12]. Although these observations did not provide any direct information on the physiological role of UCP3, they suggested that UCP3 function might be related to fatty acid metabolism in skeletal muscle. Several hypothesis have been proposed in relation to the physiological role of UCP3 in relation to fatty acids. UCP3 has been proposed to favor fatty acid oxidation in cells and, for instance, an increase in fatty acid versus glucose oxidation has been reported in skeletal muscle cells expressing high levels of UCP3 [13,14]. Indeed, some authors have hypothesized that UCP3 could favour the export of fatty acids from mitochondria when the former's oxidation capacity is exceeded by fatty acid availability [15,16]. Another hypothesis propose that UCP3 may have protective effects by translocating fatty acid peroxides from the inner to the outer membrane leaflet in mitochondria, and this mechanism would also account for uncoupling activity [17,18]. On the other hand, data on the biochemical behaviour of UCP3 has led to the proposal that its uncoupling activity leads to a reduction in reactive oxygen species (ROS) production by mitochondria, similarly to what has been proposed for UCP2 [19–21]. Thus, UCP3-null mice exhibited enhanced ROS production in skeletal muscle mitochondria and accumulation of products resulting from oxidative stress in this tissue [5,22]. These findings would be consistent with prior bioenergetic studies in mitochondria that led to the conclusion that "mild-uncoupling" is associated with protection against ROS production [23]. Moreover, ROS or

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Abbreviations: ROS, reactive oxygen species; UCP, uncoupling protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; GPX-1, glutathione peroxidase-1; SOD, superoxide dismutase

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their derivatives like hydroxynonenal, activate UCP3 activity [19,24]. However, other studies have questioned this role for UCP3 and it has been highlighted that evidence for a significant physiological role of UCP2 or UCP3 in protection against ROS is still circumstantial [25].

A likely hypothesis for the role of UCP3 in skeletal muscle may be that UCP3 gene expression is induced as a protective mechanism, when high free fatty acids are available, without excess ROS production [26]. Such a hypothesis would link events such as fatty acid-dependent regulation of UCP3 gene to the control of ROS production. However, direct evidence for this scenario is lacking. First, there is no experimental data regarding the effects of fatty acids on ROS production specifically in skeletal muscle cells and the effects of fatty acid on cellular ROS are known to be highly variable, ranging from induction to inhibition depending on the cell type [27–30]. Second, it remains unknown whether UCP3 levels influence ROS production in skeletal muscle cells specifically in response to high fatty acid levels.

In the present study we show that fatty acids induce ROS production and NF-κB activity, a sensor of cellular oxidative stress and mediator of several cellular ROS effects, in differentiated L6 muscle cells. UCP3, while lowering mitochondrial membrane potential, does not act as a protective agent against these overall effects.

2. Materials and methods

L6 (L6-E9 cells, kindly supplied by Dr. A. Zorzano) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). To induce differentiation, L6 cells at 80% confluence were changed to DMEM containing 2% FBS. Subsequently, L6 were maintained for 5 days under these culture conditions to acquire myotube morphology. After 3 days of differentiation, L6 myotubes were infected with adenoviral vectors at a multiplicity of infection rate of 200. This treatment led to a transduction efficiency of approximately 90%. Analyses (ROS levels, NF-κB activation, real-time PCR, and western blot) were performed 2 days after transduction. Recombinant adenoviruses expressing the human UCP3 cDNA (AdCMV-UCP3) or *Escherichia coli* β-galactosidase (AdCMV-LacZ), under the control of the cytomegalovirus promoter were obtained and handled as previously reported [13].

Oleate and palmitate salts were prepared immediately before use by dissolving the fatty acid in deionized water containing 1.2 equiv. of NaOH at 70 and 95 °C, respectively, until an optically clear dispersion was obtained. Fatty acid salt solution was immediately added to DMEM medium containing fatty acid free bovine serum albumin (BSA) (Sigma) with continuous agitation to avoid precipitation. Following 1 h of conjugation at 37 °C, this medium was added to the cells. The fatty acid:BSA molar ratio was 5:1.

The probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl-ester (H_2-DCFDA; DCF, 2′,7′-dichlorodihydrofluorescein, Molecular Probes, USA) was used to estimate the generation of intracellular ROS as previously reported [31]. $\Delta\Psi_m$ was estimated by using the JC-1 probe (Molecular Probes, USA) in accordance with supplier's instructions. After being transduced with the adenoviral vectors, L6 myotubes were rinsed in serum-free medium and incubated with 5 μM of JC-1 at 37 °C for 15 min in the dark. After rinsing the cells with serum-free culture medium, the cell plates were analyzed with a confocal microscope. The ratio of the reading at 590 nm to that at 527 nm (590:527 ratios) was considered as the relative $\Delta\Psi_m$ value. The chemical uncoupler CCCP (5 μM, 40 min) was used as a positive control for $\Delta\Psi_m$ decrease. Caspase-3 activity was measured using the fluorimetric CaspACE Assay System (Promega) whereas caspase-9 activation, was determined by immunoblotting of proteins from homogenates probed with a rabbit polyclonal antibody specific for the cleaved, active form, of caspase-9 (Cell Signaling technology 9505, 1:1000), as reported elsewhere [9].

Assessment of palmitate oxidation was performed as already described [13]. Briefly, following adenoviral vector infection, myotubes were starved in glucose-deprived DMEM for 1 h before the assay. Cells were incubated at 37 °C in DMEM containing 0.5 mM palmitate (9,10-[^{3}H]palmitate, 12 μCi/ml), BSA (fatty acid to BSA ratio of 5:1) and 3 mM glucose for 3 h. Palmitate oxidation was assessed by measuring ^3H_2O produced in the incubation medium.

Mitochondria were isolated from L6 myotubes as already reported [9]. Mitochondria-enriched extracts were also prepared from gastrocnemius muscles obtained from Wistar rats fed a high-fat diet (80% fat/20%protein) for three months, as previously described [9]. Protein concentrations of subcellular fractions were measured using the BCA Protein Assay Kit (Pierce). Mitochondria samples were separated by SDS–PAGE on 12.8% and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). These were incubated with antibodies against UCP3 (Chemicon AB3046 (1:1000)), voltage-dependent anion carrier (VDAC) (Calbiochem Anti-Porin 31HL (1:1000)). Antibody binding was detected with a horseradish peroxidase-coupled anti-mouse (Bio-Rad 170-6516 (1:3000)) or anti-rabbit (Santa Cruz sc-2004 (1:3000)) secondary antibody, as well as an enhanced chemiluminescence (ECL) detection kit (Amersham).

Isolation of nuclear protein extracts from L6 myotubes was performed as previously reported [32] and used in electrophoretic mobility shift assays (EMSA). A double-stranded oligonucleotide corresponding to a consensus NF-κB DNA binding site (5′-TCT AGA GTT GAG GGG ACT TTC CCA G-3′, obtained from Roche Applied Science) was end-labeled using [α-32P] dCTP and Klenow enzyme. NF-κB activity was estimated according to the appearance of a specific band in EMSA. Specificity was determined in competition tests (pre-incubation with a 100-fold molar excess of unlabeled doubled-stranded oligonucleotides before adding the DNA probe).

Total RNA was extracted from cells (Tripure Isolation Reagent, Boehringer Mannheim). Quantitative mRNA expression analysis was conducted via TaqMan RT-PCR. The reverse transcriptase reaction and further RT-PCR were performed on 1 μg RNA using TaqMan reagents (Applied Biosystems, USA) The Taqman gene expression probes used were: glutathione peroxidase-1 (GPx-1, Rn00577994), UCP2 (UCPRn00571166), UCP3 (Rn00565874), superoxide dismutase-2 (SOD-2, Rn00566942), and superoxide dismutase-1 (SOD1, Rn00584772). The quantity of the transcript for a gene under study was normalized to that of the housekeeping reference control (18S ribosomal RNA, Hs99999901), using a comparative ($2^{-\Delta CT}$) method.

Data, represented as the means ± S.E.M., were analyzed using the Student's *t*-test to determine any significant differences. *P* values less than 0.05 were considered significant.

3. Results

3.1. Gene transfer delivery of UCP3 to L6 myotubes. Effects on $\Delta\Psi_m$

Fig. 1A shows that the UCP3 protein was not detectable in differentiated L6 myotubes. This is consistent with the very low UCP3 mRNA expression levels found in cultured myogenic cells compared with those in skeletal muscle [33]. To obtain UCP3 levels similar to those occurring in skeletal muscle in vivo, a recombinant adenoviral vector containing the full-length cDNA for the human UCP3 was employed. We observed a dose-dependent increase in the presence of UCP3 in mitochondria from myotubes treated with increasing amounts of AdCMV-UCP3, as shown by the appearance of a single band of approximately 34 kDa under immunoblot analysis. Parallel assays were performed using mitochondrial fractions from gastrocnemius muscles from rats fed a high-fat diet, a known inducer of UCP3 gene expression in muscle. Equal loading of mitochondrial proteins was assessed by parallel detection of the mitochondrial protein VDAC. Our results indicated that the induction of mitochondrial UCP3 protein expression using an m.o.i. 200 transduction dose of AdCMV-UCP3 fell within physiological ranges for muscles