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UCP4 overexpression improves fatty acid oxidation and insulin sensitivity in L6 myocytes

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Abstract Obesity, which is caused by energy uptake being greater than energy expenditure, is widely prevalent today. Currently, only a limited number of efficient interventional strategies are available for the prevention of obesity. Previous studies have shown that UCP4 transcription occurs at a considerable level in mouse skeletal muscle; however, the exact functions of UCP4 remain unclear. In this study, we investigated the effect of UCP4 on mitochondrial function and insulin sensitivity in mature L6 myocytes. UCP4 overexpression in L6 myocytes induced increased mitochondrial carnitine palmitoyltransferase 1A (CPT1A) and decreased citrate synthase (CS) mRNA in the basal condition (i.e., in the absence of insulin). UCP4 overexpression significantly improved insulin sensitivity, increased tyrosine phosphorylation of IRS-1 in the presence of insulin, and significantly reduced intracellular triglyceride (TG). Additionally, intracellular ATP content and mitochondrial membrane potential were downregulated. We also observed that intracellular ROS, mitochondrial morphology, and mitochondrial mtDNA copy number were maintained upon UCP4 expression, with no change in mitochondrial fusion and fission. In summary, our findings provide evidence to show that UCP4 overexpression reduced the insulin sensitivity and mitochondrial fatty acid oxidation of L6 myocytes. These findings support the notion that UCPs are ideal targets for treatment of insulin resistance.

Keywords Uncoupling proteins (UCPs) · Carnitine palmitoyltransferase 1A (CPT1A) · Citrate synthase (CS) · Mitochondrial · Metabolic flexibility · Triglyceride · Insulin resistance

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Introduction

Obesity is prevalent worldwide and is caused by more energy uptake than energy expenditure. Therapeutic strategies, including dietary intervention (Hodgson et al. 2010), alteration in physical activity (Colley et al. 2008), and pharmacological intervention (Pi-Sunyer et al. 2006), have been adopted to prevent obesity. However, only a few of these strategies can efficiently prevent or treat obesity. Recently, a number of investigators have shown interest in a superfamily of five uncoupling proteins, which have been found to be closely associated with mitochondrial energy metabolism. Since the discovery of the first of these proteins, UCP1 (González-Barroso et al. 1999), these proteins have been studied extensively and are believed to have specific functions,

including thermogenesis, attenuation of reactive oxidative species (ROS) levels, regulation of mitochondrial calcium concentration, and mediation of cell differentiation and apoptosis (Chan et al. 2006; Echtay 2007; Zhang et al. 2006). Previous studies showed that UCP4 is mainly expressed in the brain and also in mouse skeletal muscle, where it is expressed at levels greater than those of UCP2 (Alán et al. 2009), However, the exact function of UCP4 remains unclear. Higher UCP4 levels in aged female rats have been found to be associated with better maintenance of oxidative homeostasis (Guevara et al. 2009). UCP4 overexpression also protected neurons against cell death induced by mitochondrial membrane potential, via maintenance of the adenosine triphosphatase (ATP) levels and MMP and the suppression of oxidative stress (Chu et al. 2009). It is well known that obesity is the result of a chronically positive energy balance and that one approach for improving bodily energy balance is to increase expenditure at the cellular level. Additionally, mitochondrial uncoupling represents an effective target for the treatment of obesity (Harper et al. 2001).

Skeletal muscle is involved in the utilization of a large proportion (80%) of whole body glucose, and several studies have shown that increased skeletal muscle triglyceride (TG) accumulation is associated with reduced insulin sensitivity (Zhang et al. 2009; Guo 2008). Hence, the aim of this study was to determine the effect of UCP4 overexpression on the energy metabolism and insulin sensitivity of cells of the rat myoblast (L6) line.

We found that surprisingly, overexpression of UCP4 in L6 myocytes upregulated carnitine palmitoyltransferase 1A (CPT1A) mRNA and downregulated citrate synthase (CS) mRNA to a great extent. Additionally, we observed that increased levels of UCP2 mRNA expression and myocyte insulin sensitivity had little effect on mitochondrial morphology, biogenesis, mtDNA copy number, and intracellular ROS. As expected, mitochondrial membrane potential and intracellular ATP levels decreased. Furthermore, we found significant reduction of intracellular levels of TG, but not nonesterified fatty acids (NEFAs).

Materials and methods

Antibodies The phospho-specific and total polyclonal antibody against IRS-1 (Tyr612), PI3K, and Akt were obtained from Biosource (Camarillo, CA, USA). Anti-β-actin antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Primary polyclonal PGC-1α and PGC-1β antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated AffiniPure goat anti-rabbit secondary antibodies were obtained from Zhongshan Gold Bridge Biotechnology (Beijing, China).

Cell culture and UCP4 transfection The line of L6 (ATCC, Manassas, VA, USA) myocytes was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Amresco, OH, USA), in an atmosphere of 5% CO_2 at 37 °C. Full-length rat UCP4 cDNAs were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using mRNAs isolated from white adipose tissues. The forward and reverse primer sequences were 5′-CGC GGA TCC GCC ACC ATG CCT ATC GCG AG-3′ and 5′-CCC AAG CTTAAATGG GCT GAC TCC-3′ and contained the *Bam*HI and *Hin*dIII sites, respectively. The PCR products were then subcloned into pcDNA3.1a(−)/His expression vectors. The UCP4 constructs and pcDNA3.1a(−)/His vectors were each transfected separately into L6 cells by using LIPOFACTIN 2000 (Invitrogen, Paisley, UK). After 24 h, the cells were cultured in a selective medium containing 800 μg/mL G418 (Sigma, St. Louis, MO, USA) for the selection of resistant colonies. Cells were fed with the selective medium every 2 days until resistant colonies could be identified. These resistant foci were selected, expanded, tested for expression, and frozen for future experiments.

Differentiation of myoblasts L6 rat myoblasts were maintained in DMEM supplemented with 10% FBS, and their differentiation into myotubes was induced by exposure to DMEM supplemented with 2% FBS. Myogenic differentiation to myocytes was confirmed morphologically and biochemically by using a previously described method (el-Naggar et al. 2004).

Glucose uptake 2-Deoxy-D-[^{3}H] glucose (CIC, Beijing, China) uptake was assayed using a previously described method with minor modifications (Gao et al. 2010a, b). The cells were cultured in six-well plates and induced to differentiate into mature myocytes. Before the experiment, the cells were serum-starved for 3 h in DMEM containing 0.5% FBS. The cells were then washed twice with phosphate-buffered saline (PBS) and incubated in Krebs-Ringer-phosphate- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (KRP-HEPES) buffer (30 mM HEPES [pH 7.4], 10 mM NaHCO3, 120 mM NaCl, 4 Mm KH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$) in the presence or absence of 100 nM insulin for 30 min at 37 °C. Labeled 2-deoxy-D-[^{3}H] glucose was added to a final concentration of 2 μCi/mL. After incubation for 10 min at 37 °C, the reaction was terminated by washing the cells 3 times with ice-cold PBS supplemented with 10 mM D-glucose. The cells were solubilized by the addition of 200 μL of 1 M NaOH to each well, and aliquots of the cell lysates were transferred to scintillation vials for measuring radioactivity. The remainder of the lysate was used for the protein assay.

Real-time quantitative PCR for mitochondrial DNA Relative amounts of nuclear DNA and mtDNA were determined by quantitative real-time PCR. The ratio of mtDNA to nuclear DNA reflects the tissue concentration of mitochondria per cell. For this purpose, a 220-nt-long mtDNA fragment within the gene *ND1* was selected for the quantification of mtDNA according to the method described by Ylikallio E et al. (Ylikallio et al. 2010).

Quantitative real-time PCR Total RNA was extracted using Trizol reagent (Invitrogen). First-strand cDNA was generated with random primers by using a reverse transcription kit (Invitrogen). Real-time analysis was performed on an ABI 7500 RT-PCR system (Foster City, CA, USA) with the SYBR Green kit (Applied Biosystems). Each sample was assayed in duplicate, with a 25-μL reaction volume containing 1 μL cDNA (or DNA), 12.5 μL SYBR Green master mix (Applied Biosystems), and 0.58 μmol/L of each primer. Negative controls (no template DNA or selected untranscribed RNA) were run to ensure the absence of contamination. Analysis was performed according to the ΔC_t method using β-actin as the housekeeping gene. Specific primers for each gene were designed to amplify each PCR product (see Table 1), and the identity of the products were confirmed by regular PCR and dissociation curve analysis after real-time PCR. The primer sequences are available upon request.

Electromicrograph morphometry The myocytes were collected after trypsin digestion, dissected, and fixed in a mixture of 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were then washed in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide/1.5% potassium ferrocyanide for 1 h, washed in water, and stained in 1% aqueous uranyl acetate for 30 min, and then dehydrated in a graded alcohol series (5 min in 70% alcohol, 5 min in 90% alcohol, and 5 min in 100% alcohol). The samples were then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (approximately 60-nm thick) were cut on a Reichert Ultracut-S microtome, placed onto copper grids stained with uranyl acetate and lead citrate, and examined under a JEOL 1200EX microscope.

Table 1 Primers used for Real time-PCR quantitation of mRNA and mtDNA levels

Gene	Forward prime r(5′-3′)	Reverse primer(5′-3′)
CPT1A	Agtccatcacctttgttgtctt	tgtcccattgtagccttgtg
CPT1B	Ggtgatggcactgggtatg	ttgaagaagcgacctttgtg
CS	Gccatcacagccctcaac	gcaatcaggtccatacagtcttc
CYCS	Ggctgctggattctcttacac	tgccctttctcccttcttct
ACC2	Gcacgggattgcttttct	tccgctccagggtagagtt
ND2	Ccttggaatgatgtggatgtt	gtgtaggagcccgatgtgag
COX4	Caggctctcacttcttccattc	atgttcttcatcggcttcac
ATPsyn	Tgtccaggtctacggcatc	cactcgcagcaactccttt
Mfn1	Tttgtcgcctgtctgttttg	ttgctgagattgaagaatggag
Mfn2	Agatgtccctgctcttttctc	ttctgtattcctgtgggtgtctt
Fis1	Gcctgccgttacttcttctac	ttcatcccttaccacgcaac
Opa1	Cctttgtcggagaggttttt	cagcgtgttggcagtgatag
Drp1	Ctgccttatcctggctgtgt	gagcatctgtgccctcgt
Ucp4	Cacggcttatccagtttgtgt	gccctcttccttgtttgtctc
Ucp2	Gttctggactggtggcttct	gccctcttccttgtttgtct
ND1	Caatggtggttttaggcagtt	agcagtttgggtgggttctt
28 s	Gatgcttgcgatttccttct	cctacgaacacacccaacct
B-Actin	Aaagaaagggtgtaaaacgca	tcaggtcatcactatcggcaat

Western blotting The cells were washed with ice-cold PBS and lysed in protein lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 200 mM sodium fluoride, and 4 mM sodium orthovanadate-containing protease inhibitors; pH 7.5) for 1 h on ice. Protein concentration was measured by the Bradford method. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20). The membrane was incubated overnight at 4 °C in 5% BSA in TBST containing one of the following primary antibodies: PI3K, 1:500; AKT, 1:500; IRS1, 1:1000; β-actin, 1:1000, PGC-1β, 1:200; and PGC-1α, 1:200. The membrane was washed five times with PBST, with 5 min for each wash. After washing, the membrane was incubated with secondary antibodies for 1 h at room temperature, washed with PBST, and developed using an enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA).

ATP production The ATP content of the myocytes was measured using ATP lite-glo, a luciferase-based luminescence assay kit (PerkinElmer). Briefly, the treated cells were mixed with the detection reagent for 5 min, and luminescence was measured using a VERITASTM Microplate Luminometer (Turner Biosystems).

MMP Myocytes were incubated with 150 nM of Mitotracker, a red mitochondria-specific cationic fluorescent dye (Molecular Probes, Invitrogen) for 30 min at 37 °C and then washed 3 times with pre-warmed PBS. The spectral characteristics of the harvested cells were analyzed by fluorescence-assisted cell sorting (FACS; excitation at 579 nm, emission at 644 nm). The fluorescence intensity reflects the MMP.

Measurement of intracellular ROS content Intracellular ROS content was assessed using 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (H2-DCFDA) as described previously (Park et al. 2006). Cells were washed twice in KRP buffer,

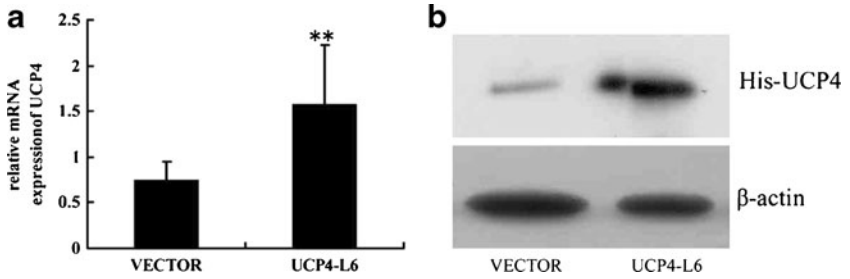

Fig. 1 Expression of UCP4 in L6 cells transfected with pcDNA3.1-UCP4. **a** Expression of UCP4 mRNA. Total RNA was extracted from stable cell lines transfected with pcDNA3.1 or pcDNA3.1-UCP4 constructs. Real-time PCR was performed using the primers indicated in Table 1. **b** Expression of UCP4 protein. Total protein was isolated from stable cell lines and analyzed by western blotting using a mouse anti-His antibody. Values represented are the means ± SD from 3 independent experiments. $^{**}P<0.01$ vs. vector myocytes

incubated in pre-warmed KRP containing 25 mM glucose and 5 μM H2-DCFDA (Sigma), and incubated at 37 °C. After 30 min, the cells were washed twice with KRP, and fluorescence was immediately measured in a plate reader using FACS (excitation at 488 nm, emission at 525 nm).

TG and NEFAs analyses Intracellular TG and NEFA levels were measured using commercial kits (Triacylglycerol Kit and NEFA Kit, Jiancheng, Nanjing, China).

Statistical analysis All data are expressed as means ± SEM. Statistical analysis was performed using Student's *t*-test or one-way ANOVA with the SPSS 10.0 statistical software package (SPSS Inc., Chicago, IL, USA). The threshold of significance was defined as $P<0.05$.

Results

Stable expression of UCP4

An L6 cell line stably expressing a transfected UCP4 plasmid was established and maintained in DMEM containing 200 μg/mL G418. Expression of UCP4 was verified by real-time PCR (Fig. 1a) and western blot analysis (Fig. 1b).

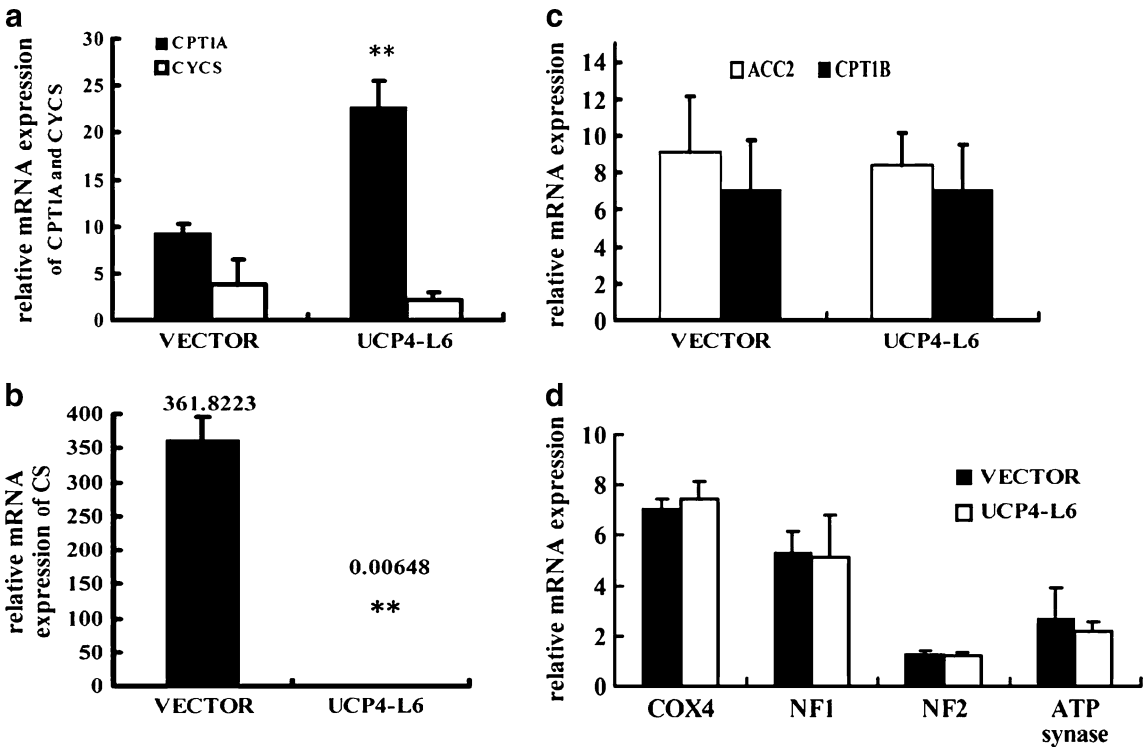

Fig. 2 Upregulation of CPT1A but not CPT1B and marked down-regulation of CS in UCP4-L6 myotubes. Differentiation of L6 cells transfected with UCP4 or the empty vector (pcDNA3.1Myc/His B) was induced. RNA was extracted and relative expressions of CPT1A, CYCS (**a**), CPT1B, ACC2 (**b**), CS (**c**), COX4, ND1, ND2, and ATP synthetase (**d**) mRNA were examined by real-time qPCR. β-actin mRNA was used as an internal control. Values shown are mean ± S.D. of triplicate experiments. $^{**}P<0.01$ vs. vector myocytes

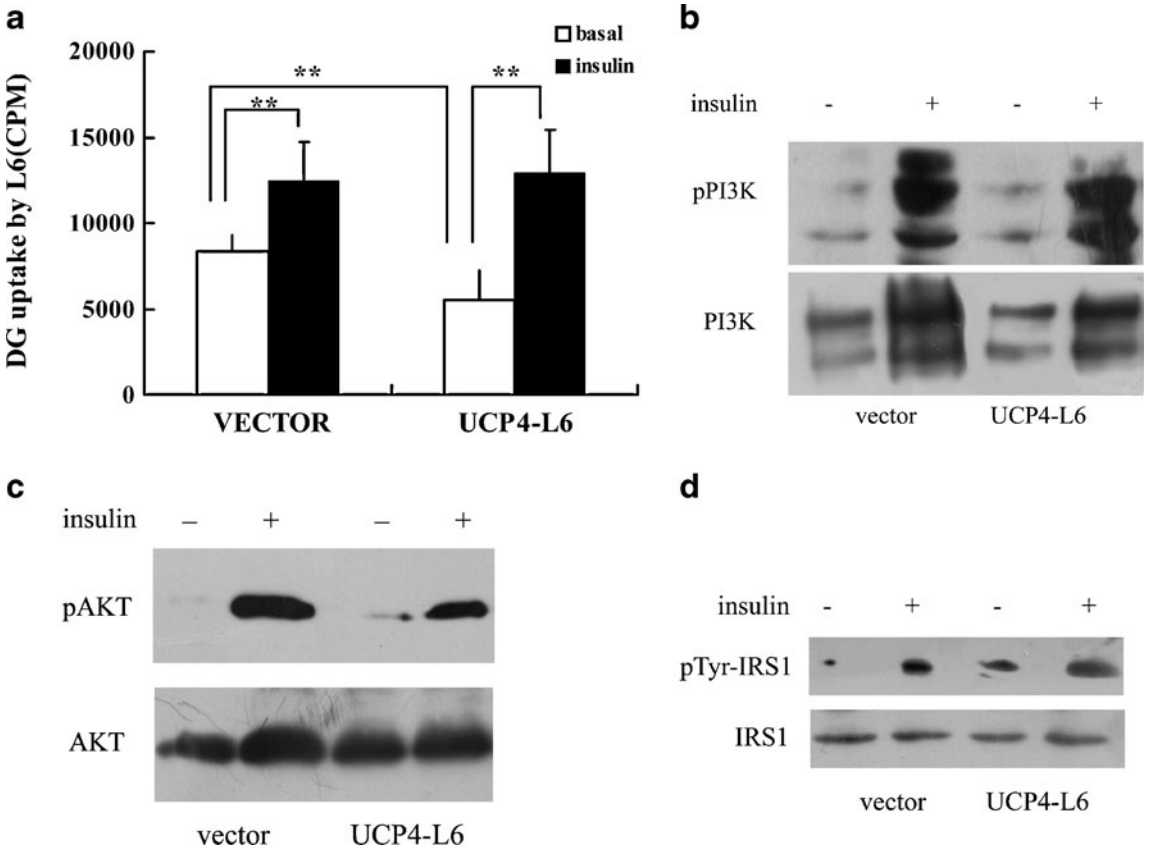

Fig. 3 Effect of UCP4 on glucose uptake and insulin signaling pathway. **a** Effect of UCP4 on glucose uptake. Differentiation of L6 cells transfected with UCP4 or empty vector (pcDNA3.1Myc/His B) was induced. After serum starvation for 3 h, the cells were incubated with (*black columns*) or without (*white columns*) 100 nmol/L insulin for 30 min, and 2-deoxy-D-[^{3}H] glucose uptake was measured. Values represent the means ± SD from 3 independent experiments. $^{**}P<0.01$. **b**–**d** Effect of UCP4 on insulin signaling transduction. Differentiation of L6 cells transfected with UCP4 or the empty vector (pcDNA3.1Myc/His B) was induced. After incubation with or without 100 nmol/L insulin for 30 min, the cell lysates were analyzed by SDS-PAGE, blotted onto a membrane, and then probed with antibodies against molecules involved in the insulin signaling pathway. The results are representative of those obtained from 3 independent experiments. Panels **b**–**d** represent the total protein concentration and phosphorylation level of PI3K (**b**), AKT (**c**), and IRS-1 (**d**)

Upregulation of CPT1A, and not CPT1B, and marked downregulation of CS in UCP4-L6 myotubes

Because UCP4 is a mitochondrial protein, we first examined mRNA expression of the factors related to mitochondrial metabolism. Three CPT1 isoforms are known to be key factors involved in FFA transport into the mitochondria for oxidation: CPT1A in the liver, CPT1B in muscle, and CPT1C in the brain. A previous study showed that CPT1A was highly expressed in L6 myocytes

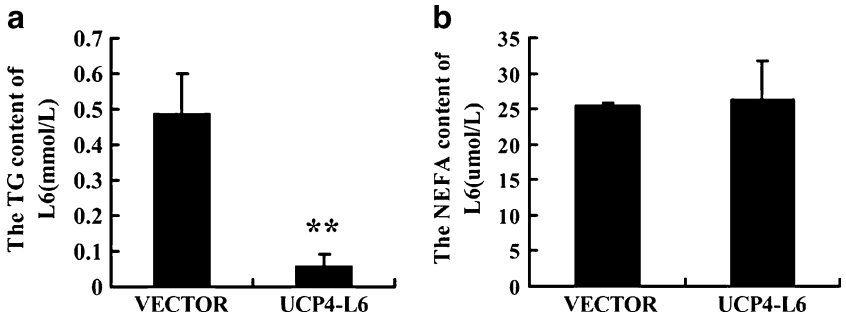

Fig. 4 Effect of UCP4 on intracellular TG and NEFA. Differentiation of L6 cells transfected with UCP4 or the empty vector (pcDNA3.1-Myc/His B) was induced. Intracellular levels of TG (**a**) and NEFA (**b**) were determined. Values represented the means ± SD from 3 independent experiments. $^{**}P<0.01$

(Perdomo et al. 2004). In our study, we found that CPT1A mRNA (Fig. 2a) levels increased, while those of CPT1B mRNA (Fig. 2b) remained unchanged. Next, we examined another key factor for FFA metabolism, ACC2, which catalyzes the synthesis of malonyl-CoA, a natural inhibitor of CPT1. Similar to CPT1B, ACC2 showed no change in mRNA expression. Unexpectedly, we found that CS (Fig. 2c), a key enzyme involved in the tricarboxylic acid (TCA) cycle, was greatly downregulated (to more than 1/60000). This implied that glucose oxidation was inhibited, while lipid oxidation improved.

Insulin sensitivity improved upon UCP4 overexpression

UCPs function as proton transporters and previous studies have shown that in vivo or in vitro expression of UCPs improved insulin resistance (Tiraby et al. 2007; Katterle et al. 2008; Neschen et al. 2008). To determine whether UCP4 overexpression has an effect on insulin sensitivity in L6 myotubes, we monitored the insulin sensitivity after inducing forced expression of UCP4 in these cells. As shown in Fig. 3a, basal 2-deoxy-D-[^{3}H] glucose uptake of UCP4-L6 was less than that of the vector control. However, in the presence of insulin, there was a considerable increase in the insulin-stimulated 2-deoxy-D-[^{3}H] glucose uptake, which then reached the same value as that observed in the vector cells.

To examine the effect of UCP4 overexpression on the insulin signaling pathway, we investigated the changes in the extent of insulin-stimulated phosphorylation of insulin signaling molecules involved in glucose uptake. UCP4 overexpression resulted in a significant increase in the extent of the tyrosine phosphorylation of IRS-1 and the inhibition of Akt serine phosphorylation induced by insulin, while PI3K serine phosphorylation remained unaffected. As shown in Fig. 3, there was no significant difference in the total protein content of these signaling molecules (Fig. 3b, c and d).

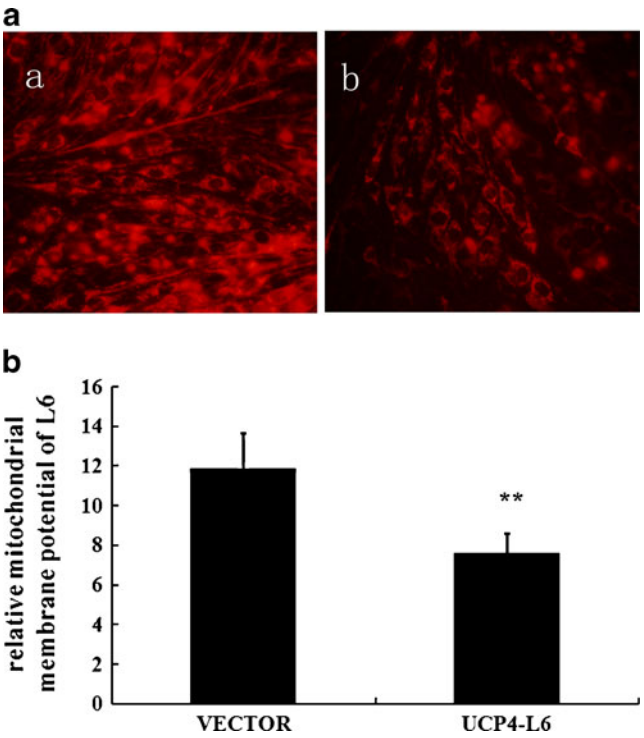

Fig. 5 Mitochondrial membrane potential. Differentiation of L6 cells transfected with UCP4 or empty vector (pcDNA3.1Myc/His B) was induced. Mitochondrial membrane potential was visualized using mitotracker-red fluorescence probe. **a** *a* vector; *b* UCP4. (*$P<0.05$ vs. vector myocytes). The mitochondrial membrane potential in myocytes was visualized by fluorescence microscopy (**b**). The values represented are the means ± SD from 3 independent experiments

UCP4 overexpression significantly reduced intracellular levels of TG but not those of the NEFAs

As a result of the increase in the CPT1A mRNA expression and insulin sensitivity of UCP4-L6 myocytes, the ectopic deposition of lipid in muscle played a critical role in muscle insulin resistance. We measured the levels of TG and NEFAs in these cells. We found a noticeable drop in the intracellular TG levels, while those of NEFAs were similar to the corresponding levels in the controls. This indirectly demonstrated that UCP4 improved lipid oxidation, because lipids are stored as TGs (Fig. 4a and b).

UCP4 overexpression decreased MMP and ATP production, while intracellular ROS levels were unchanged

As uncoupling proteins, the primary function of UCPs is the transport of protons, which is closely related to ATP production and ROS. In this study, we examined this function of UCPs. As expected, the MMP and ATP content of UCP4-L6 cells were significantly lower than those in the control, whereas the mRNA expression level of ATP synthetase showed no change compared to that in the control (Fig. 5a, b and Fig. 7a).

Additionally, the levels of intracellular ROS, which are important to the development of insulin resistance, remained unchanged (Fig. 6a and b). Further, we measured the mRNA expression levels of the redox-reaction-associated enzymes COX4, ND1, and ND2, but failed to find any difference between the UCP4-L6 and vector groups in this regard (Fig. 2d). UCP2, which is mainly expressed in white adipose tissue, showed noticeable upregulation in UCP4-L6 cells (Fig. 7b).

Mitochondrial morphology and mitochondrial biogenesis were not affected by overexpression of UCP4

To understand the above results, we examined the biogenesis and ultrastructure of mitochondria. We first analyzed the

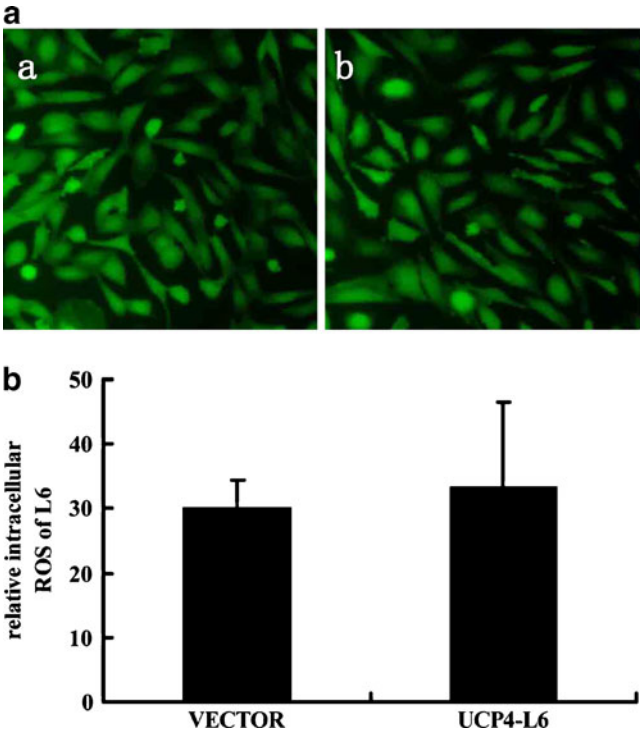

Fig. 6 Effects of UCP4 on ROS production. Differentiation of L6 cells transfected with UCP4 or empty vector (pcDNA3.1Myc/His B) was induced. Total cellular ROS levels were measured using a DCF fluorescence probe by FCM (**a**), and the ROS in myocytes were visualized using a fluorescence microscope (**b**). *a* vector; *b* UCP4. Values represented are the means ± SD from 3 independent experiments

mtDNA copy number, which reflects the mitochondrial number, and found no difference. Protein expression of PGC-1α and PGC-1β, key factors in mitochondrial biogenesis, was evaluated. UCP4 overexpression led to a significant increase in PGC-1β expression, but had no effect on PGC-1α expression. Mitochondria are membranous organelles and frequently undergo fusion and fission to facilitate effective exchange of power and content. Five factors, namely, mfn1, mfn2, Opa1, fis1, and drp1, mediate mitochondrial fusion and fission, and our results demonstrate that UCP4 expression had no effect on the fusion and fission process. Electron microscopic examination revealed that UCP4 overexpression did not alter the morphological characteristics of the mitochondria and the regular arrangement of the mitochondrial cristae and did not result in change in the size of the mitochondria (Fig. 8a, b, c and d).

Discussion

In the present study, we sought to clarify the following points: first, whether UCP4 overexpression in L6 myocytes increased mitochondrial CPT1a and decreased CS mRNA in basal conditions (in the absence of insulin); second, whether UCP4 overexpression significantly improved insulin sensitivity and caused increased tyrosine phosphorylation of IRS-1 in the presence of insulin; third, whether UCP4 overexpression significantly reduced intracellular TG levels; and finally, whether UCP4 overexpression impaired mitochondrial function when intracellular ATP content and MMP were downregulated.

CPT1 was the rate-limiting enzyme for the transport of fatty acids into the mitochondria for oxidation (Stephens et al. 2007). CPT1 activity is reduced in skeletal muscle of obese individuals and is likely to contribute to the suppressed rates of FA oxidation in obesity (Kim et al. 2000). CPT1 overexpression protected L6E9 muscle cells from fatty acid-induced insulin resistance (Sebastián et al. 2007). A recent report suggested that overexpression of CPT1 in skeletal muscle was sufficient to enhance fatty acid oxidation and improve insulin resistance induced by a high-fat diet (Bruce et al. 2009). The results of this study indicated that in the basal condition (i.e., in the absence of insulin), overexpression of UCP4 greatly promoted fatty acid oxidation in L6 myocytes (although we did not test enzyme activity,

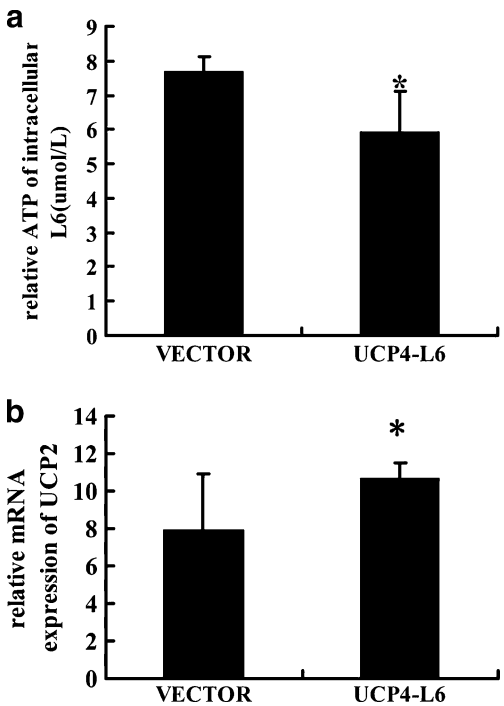

Fig. 7 Decrease of intracellular ATP and UCP2 mRNA levels in L6 myocytes upon forced expression of UCP4. Differentiation L6 cells transfected with UCP4 or empty vector (pcDNA3.1Myc/His B) were induced. Intracellular ATP content was determined by ATP lite-glo, a luciferase-based luminescence assay kit (PerkinElmer), according to the manufacturer's instructions (**a**). Expression of UCP2 mRNA (**b**). Total RNA was extracted from stable lines transfected with pcDNA3.1 or pcDNA3.1-UCP4 constructs. Real-time qPCR was performed using the primers indicated in Table 1. Values represented are the means ± SD from 3 independent experiments. *$P<0.05$

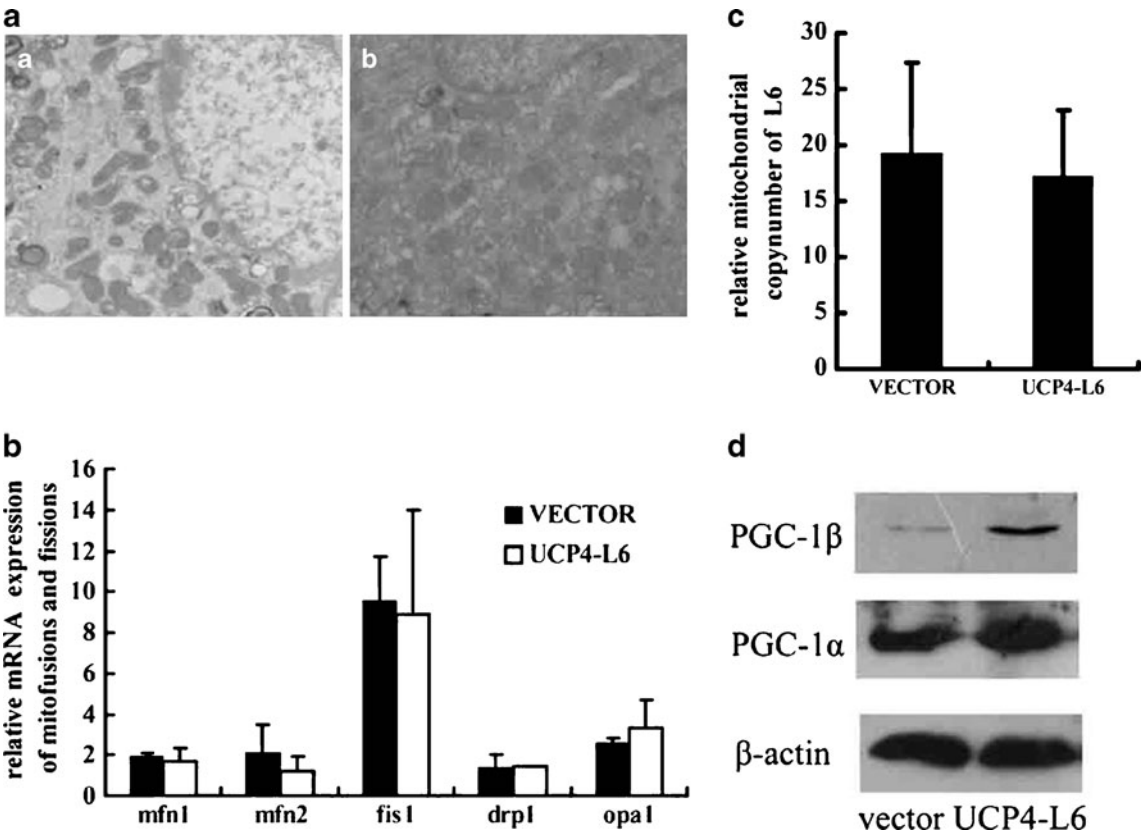

Fig. 8 Morphological features of mitochondria. Differentiation of L6 cells transfected with UCP4 or the empty vector (pcDNA3.1Myc/His B) was induced. Ultrastructure of the mitochondria was visualized by transmission electron microscopy (**a**) *a* vector and *b* UCP4 (under 20000×). RNA was extracted and relative mRNA expressions of mfn1, mfn2, opa1, fis1, and drp1 (**b**) were examined by real-time qPCR. β-actin mRNA was used as an internal control. Values are shown as mean ± S.D. of triplicate experiments. Total DNA was extracted and relative expression levels of mtDNA copy number (ND1) were determined by real-time qPCR; a 105-bp region of the nuclear gene for 28 S was used to normalize results. Total protein was isolated from stable cell lines and analyzed by western blot using antibodies against PGC1α and PGC1β. β-actin protein was used as an internal control (**d**)

decreased TG levels are indirect evidence of increased fatty acid oxidation), but markedly inhibited carbohydrate metabolism. However, in the presence of insulin, the UCP4-L6 myocytes showed improved glucose uptake, which was reflected by ^{3}H-D-glucose uptake and phosphorylation of IRS-1. Thus, UCP4-L6 monocytes showed better metabolic flexibility than control L6 myocytes. Metabolic flexibility is the characteristic of normal skeletal muscle whereby it has the ability to switch from predominantly lipid oxidation and high rates of fatty acid uptake during fasting conditions to the suppression of lipid oxidation and increased glucose uptake, oxidation, and storage under insulin-stimulated conditions (Kelley and Mandarino 2000; Storlien et al. 2004). Current evidence suggests that whole-body metabolic flexibility was lower in type 2 diabetic individuals compared to obesity-matched nondiabetic individuals and that metabolic inflexibility improved after weight loss or anti-diabetic treatment with thiazolidinediones (Mensink et al. 2007). Although CS is known to be another key enzyme of the TCA cycle, a previous study suggested that metabolic inflexibility to glucose in type 2 diabetic subjects is mostly related to defective glucose transport but not glucose oxidation (Galgani et al. 2008). Hence, we believe that decreased CS mRNA expression may have had only a minor impact on the metabolic inflexibility of L6 myocytes observed in the present study.

A state of metabolic inflexibility could contribute to the accumulation of intracellular TG and, thus, contribute to the development of insulin resistance and type 2 diabetes. UCP4 overexpression resulted in improved insulin sensitivity, and we believe that the following factors were closely associated with this improvement. First, we can attribute the enhanced insulin sensitivity to the improved metabolic flexibility, which has been shown to be positively correlated with insulin sensitivity (Ukropcova et al. 2005). Second, decreased intracellular TG might play a role in improving insulin sensitivity. It was well known that the insulin resistance of skeletal muscle is closely related with ectopic lipid deposition (Lee et al. 2006; Hegarty et al. 2003). Third, increased tyrosine phosphorylation of IRS-1 also contributed to improving insulin sensitivity. This can be inferred from the

fact that UCP4 expression had no effect on PI3K but decreased AKT phosphorylation. Together, these results indicate that UCP4 expression exerts, to some extent, a direct effect on the tyrosine phosphorylation of IRS-1. Lastly, the proper functioning and maintenance of the structure of the mitochondria may have contributed to the improvement of insulin sensitivity. Energy metabolism occurs in the mitochondria, and proper mitochondrial function is required for proper oxidative substrate utilization and fuel switching. Our findings showed that mitochondrial structure and mitochondrial mtDNA copy number, as well as mitochondrial fusion and fission, were maintained during UCP4 overexpression. UCP4 overexpression in L6 myocytes induced a mild change in the MMP, decreased the ATP content, and increased UCP2 mRNA expression. This was consistent with the primary function of uncoupling proteins and the findings of previous studies that showed that exposure to interleukin (IL) 6 enhanced fat oxidation in L6 myocytes via upregulation of UCP2 (Holmes et al. 2008). PGC-1β protein expression increased, although PGC-1α levels remained the same as those in the controls. PGC-1α and PGC-1β are known as key positive regulators of mitochondrial activity and biogenesis and showed different specific actions (Liesa et al. 2008). Other functions of PGC-1β in mitochondrial physiology remain to be elucidated. In the present study, PGC-1β was found to mediate the UCP4-induced changes in mitochondrial metabolism, thus improving the metabolic flexibility of L6 myocytes. This effect of PGC-1β merits further investigation.

In our previous study, UCP4 overexpression in 3 T3-L1 adipocytes was found to induce noticeable insulin resistance (Gao et al. 2010a, b). The discrepancy between this result and those of the present study can be explained as follows. First, although both adipocytes and skeletal muscles are insulin targets, they have different functions in the body: the former is used for energy storage, while the latter is involved in energy utilization. Lipids normally deposit in the adipocytes, while lipid deposition in muscle leads to insulin resistance. Expression of uncoupling proteins in adipocytes promotes ROS production and induces mitochondrial dysfunction, while in skeletal muscle, it decreases ectopic deposition and maintains ROS levels. Secondly, the mitochondria in different cells or tissues played different roles: in adipocytes, the mitochondria produce ATP to maintain cellular metabolism, but in myocytes, the mitochondria utilize glucose and FFA for muscle contraction. Thus the energy demand of mitochondria in myocytes greatly exceeds that of mitochondria in adipocytes. Thirdly, UCP4 has different effects on glucose uptake, depending on the cell type: UCP4 downregulates GLUT4 mRNA expression in adipocytes, but promotes IRS1 tyrosine phosphorylation in L6 myocytes. Lastly, UCP4 expression has varied effects on mitochondrial function, mitofusion, and mitofission. The discrepancy can also be attributed to the effects of UCP4 overexpression on the structure and functions of mitochondria.

In summary, our results provide evidence that UCP4 overexpression reduced the insulin sensitivity and mitochondrial fatty acid oxidation of L6 myocytes; these findings support the notion that UCPs can be considered as potential targets for the treatment of insulin resistance.

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