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ARTICLE

R-α-Lipoic acid and acetyl-L-carnitine complementarily promote mitochondrial biogenesis in murine 3T3-L1 adipocytes

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Abstract
Aims/hypothesis The aim of the study was to address the importance of mitochondrial function in insulin resistance and type 2 diabetes, and also to identify effective agents for ameliorating insulin resistance in type 2 diabetes. We examined the effect of two mitochondrial nutrients, *R*-α-lipoic acid (LA) and acetyl-L-carnitine (ALC), as well as their combined effect, on mitochondrial biogenesis in 3T3-L1 adipocytes.
Methods Mitochondrial mass and oxygen consumption were determined in 3T3-L1 adipocytes cultured in the presence of LA and/or ALC for 24 h. Mitochondrial DNA and mRNA from peroxisome proliferator-activated receptor gamma and alpha (*Pparg* and *Ppara*) and carnitine palmitoyl transferase 1a (*Cpt1a*), as well as several transcription factors involved in mitochondrial biogenesis, were evaluated by real-time PCR or electrophoretic mobility shift (EMSA) assay. Mitochondrial complexes proteins were measured by western blot and fatty acid oxidation was measured by quantifying CO_2 production from [1-^{14}C] palmitate.
Results Treatments with the combination of LA and ALC at concentrations of 0.1, 1 and 10 μmol/l for 24 h significantly increased mitochondrial mass, expression of mitochondrial DNA, mitochondrial complexes, oxygen consumption and fatty acid oxidation in 3T3L1 adipocytes. These changes were accompanied by an increase in expression of *Pparg*, *Ppara* and *Cpt1a* mRNA, as well as increased expression of peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1 alpha (*Ppargc1a*), mitochondrial transcription factor A (*Tfam*) and nuclear respiratory factors 1 and 2 (*Nrf1* and *Nrf2*). However, the treatments with LA or ALC alone at the same concentrations showed little effect on mitochondrial function and biogenesis.
Conclusions/interpretation We conclude that the combination of LA and ALC may act as PPARG/A dual ligands to complementarily promote mitochondrial synthesis and adipocyte metabolism.

Keywords Mitochondrial complex · Mitochondrial transcription factor A · Nuclear respiratory factor 1 · Nuclear respiratory factor 2 · Peroxisome proliferator-activated receptor gamma · Peroxisome proliferator-activated receptor alpha · Peroxisome proliferator-activated receptor · Gamma coactivator 1 alpha

Abbreviations

ALC	acetyl-L-carnitine
EMSA	electrophoretic mobility shift

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KRH	Krebs Ringer solution buffered with HEPES
LA	*R*-α-lipoic acid
mtDNA	mitochondrial DNA
PPAR	peroxisome proliferator-activated receptor

Introduction

Mitochondrial dysfunction plays a central role in a wide range of age-associated disorders and various forms of cancer [1]. Mitochondrial glucose and fatty acid metabolism in muscle and adipocytes is impaired in patients with insulin resistance and type 2 diabetes [2], while mitochondrial loss in adipose tissue is correlated with the development of type 2 diabetes [3]. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily [4]. Each PPAR member displays a tissue-selective expression pattern and has distinct roles in lipid metabolism. *Pparg*, which is required for adipose tissue formation, has emerged as a transcriptional regulator of metabolism and plays an important role in diabetes and obesity [5]. *Ppara* is centrally involved in mitochondrial biogenesis and fatty acid oxidation [6]. Increased production of *Ppargc1a*, a key regulator of mitochondrial biogenesis, may be involved in obesity and the pre-diabetic state in skeletal muscle [7]. Therefore, regulating *Pparg/a* activity and adipocyte metabolism may improve insulin sensitivity and glucose disposal [2, 5, 8]. Promoting mitochondrial biogenesis by upregulation of the *Ppargc1a* pathway has been suggested as a strategy for preventing and reversing insulin resistance, obesity and diabetes [3, 9, 10]. In this regard, several drugs have been tested, such as metformin and 5-aminoimidazole-4-carboxamide ribonucleoside [11], the *Pparg* agonist pioglitazone/rosiglitazone and *Ppara* agonist WY-14,643 [12–14], as well as beta 3-adrenergic receptors agonist CL-316,243 [15] and oestrogen-related receptor α [16].

Dietary supplementation with micronutrients may complement pharmacological agents in the prevention and treatment of diabetes, with a particular focus on the prevention of diabetic complications [17]. *R*-α-Lipoic acid (LA) and acetyl-L-carnitine (ALC) have been found to be protective nutrients of mitochondria [18]. LA is an antioxidant and exogenously supplied LA can be NADH- and NADPH-dependently reduced in mitochondria and cytosol [19]. LA has been shown to act as an NADH oxidase inhibitor to block oxidant production and decrease phagocytosis of myelin by macrophages [20]. LA is involved in mitochondrial α-keto acid dehydrogenase complexes that catalyse both carbohydrate and amino acid metabolism. LA has also been found to be able to stimulate glucose uptake by activating the insulin signalling pathway in adipose and muscle cells [21]. However, LA also inhibits differentiation of 3T3-L1 pre-adipocytes induced by a hormonal mixture or troglitazone [22] and causes an increase in oxidants at relatively high concentrations (500–1,000 μmol/l) in 3T3-L1 adipocytes [23]. LA has been reported to function as a weak dual PPARG/A [24] and to promote weight loss, ameliorate insulin resistance and atherogenic dyslipidaemia, as well as to lower blood pressure. ALC is the acetyl derivative of L-carnitine, which is required for the transport of long-chain fatty acids into the mitochondria for β-oxidation, ATP production and for the removal of excess short- and medium-chain fatty acids [25]. LA and/or ALC improve mitochondrial function in ageing rats and their combination appears more potent owing to complementary effects [25–28]. LA [29] and ALC [30, 31] have been tested in several large clinical trials on prevention or treatment of diabetes and its complications. Both nutrients result in an improvement in insulin sensitivity, and evidence suggests beneficial effects on cardiovascular parameters associated with the metabolic syndrome and type 2 diabetes. The present study sought to determine whether treatment of adipocytes with LA and/or ALC affects mitochondrial mass and the expression of genes and proteins involved in mitochondrial biogenesis.

Methods

Materials Anti-β-actin was from Sigma (St Louis, MO, USA), anti-oxphos complex I, II, III and IV from Invitrogen (Carlsbad, CA, USA), the reverse transcription system kit from Promega (Mannheim, Germany) and HotStarTaq from Takara (Otsu, Shiga, Japan). *Nrf1*, *Nrf2*, *Ppargc1a*, 18S rRNA and β-actin primers were synthesised by Bioasia Biotech (Shanghai, China). ALC (hydrochloride salt) was from Sigma Tau (Pomezia, Italy) and LA (tris salt) was a gift from K. Wessel, Viatris, Bad Homburg, Germany. TRIzol and other reagents for cell culture were from Invitrogen.

Cell culture and differentiation 3T3-L1 cells have been extensively used as a model of adipogenic differentiation and insulin action. 3T3-L1 cells undergo growth arrest and initiate a programme of differentiation manifested by large lipid droplet accumulation upon hormonal stimulation. In parallel, these cells become sensitive to insulin, express *Glut4* and display insulin-induced activation of glucose uptake comparable to that seen in primary adipose cells [12]. In the present study, murine 3T3-L1 pre-adipocytes (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 10% (*v*/*v*) fetal bovine serum and allowed to reach confluence. Differentiation of pre-adipocytes was initiated with 1.0 μmol/l insulin,

0.25 μmol/l dexamethasone and 0.5 mmol/l 3-isobutyl-1-methylxanthine in DMEM supplemented with 10% (*v*/*v*) fetal bovine serum. After 48 h, the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1.0 μmol/l insulin. The culture medium was changed every other day with DMEM containing 10% (*v*/*v*) fetal bovine serum. Cells were used at 9 to 10 days following differentiation induction when exhibiting 90% adipocyte phenotype.

Mitochondrial mass A fluorescent probe (Mito-Tracker Green FM; Molecular Probes, Eugene, OR, USA) was used to determine the mitochondrial mass of adipocytes, i.e. more accurately: the fractional volume of that part of an adipocyte that is occupied by mitochondria. Adipocytes treated with LA and/or ALC for 24 h were trypsinised and centrifuged at 3,000×*g* at 4°C for 5 min, resuspended in Krebs Ringer solution buffered with HEPES (KRH) and 0.1% BSA (*w*/*v*) and then incubated with 0.1 μmol/l MitoTracker Green FM in KRH buffer for 30 min at 37°C. Cells were centrifuged at 3,000×*g* at 4°C for 5 min and resuspended in 400 μl fresh KRH buffer. Fluorescence was analysed by FACS Calibur (Becton Dickinson, Mountain View, CA, USA).

Electron microscopy 3T3-L1 adipocytes at day 8 of differentiation were seeded on glass coverslips. On day 9, cells were treated with LA (10 μmol/l) and/or ALC (10 μmol/l) for 24 h. On day 10, adipocytes were fixed overnight with 2.5% (*v*/*v*) glutaraldehyde in 0.1 mol/l sodium phosphate buffer (pH 7.3). They were postfixed with 2% (*w*/*v*) OsO_4 in the same buffer, followed by block staining with 1% (*w*/*v*) uranyl acetate. After dehydration with a graded series of ethanol, they were washed by propylene oxide and embedded in Spurr's low viscosity resin. Silver to gold sections were cut and examined using a transmission electron microscope (CM 10; Philips, Eindhoven, the Netherlands) at a 60 kV accelerating voltage [32]. Measurements were made on ten individual adipocytes treated with or without LA and/or ALC. For each individual adipocyte profile in the area, the number of mitochondria and the total mitochondrial section area were determined. All electron microscopic photographs were analysed blind with regard to treatments.

Mitochondrial DNA Total DNA was extracted using a kit (QIAamp DNA Mini kit; Qiagen, Hilden, Germany) and quantitative PCR was done using 18S rRNA primers for a nuclear target sequence and primers for mitochondrial DNA target using mitochondrial D-loop. Quantitative PCR was performed using a real-time PCR system (Mx3000P; Stratagene, Amsterdam, the Netherlands). Reactions were performed with 12.5 μl SYBR-Green Master Mix (ABI, Warrington, UK), 0.5 μl of each primer (10 μmol/l) and 100 ng template (DNA) or no template (NTC), with RNase-free water being added to a final volume of 25 μl. The cycling conditions were as follows: 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s. Each quantitative PCR was performed in triplicate. The following primers were used: mitochondrial D-loop forward, 5′-AATCTACCATCCTCCGTG-3′, reverse 5′-GACTAAT GATTCTTCACCGT; 18S rRNA forward: 5′-CATTC GAACGTCTGCCCTATC-3′ and reverse: 5′-CCTGCT GCCTTCCTTGGA-3′. The mouse 18S rRNA gene served as the endogenous reference gene. The melting curve was done to ensure specific amplification. The standard curve method was used for relative quantification. The ratio of mitochondrial D-loop to 18S rRNA was then calculated. Final results are presented as percentage of control.

Cell respiration Oxygen consumption by intact cells was measured as an indication of mitochondrial respiration activity. We used the BD Oxygen Biosensor System (BD Biosciences, San Diego, CA, USA), an oxygen-sensitive fluorescent compound (Tris 1,7-diphenyl-1,10 phenanthroline ruthenium [II] chloride) embedded in a gas-permeable and hydrophobic matrix permanently attached to the bottom of a multiwell plate. The concentration of oxygen in the vicinity of the dye is in equilibrium with that in the liquid medium. Oxygen quenches the dye in a concentration-dependent manner. The fluorescence correlates directly to oxygen consumption in the well. The unique technology allows homogeneous instantaneous detection of oxygen levels. After treatment, adipocytes were washed in KRH buffer plus 0.1% (*w*/*v*) BSA. Cells from each condition were divided into aliquots in triplicate in a BD Oxygen Biosensor System plate (BD Biosciences). The number of cells contained in equal volumes was not statistically significant in response to various nutrient treatments and concentrations. Plates were sealed and 'read' on a fluorescence spectrometer (Molecular Devices, Sunnyvale, CA, USA) at 1 min intervals for 60 min at an excitation of 485 nm and emission of 630 nm [13]. Results are expressed as the slope of fluorescence intensity.

RNA isolation and reverse transcription-polymerase chain reaction After incubation, cells were washed twice with ice-cold PBS. Total RNA was isolated using the single-step TRI reagent and 1 μg RNA was reverse-transcribed into cDNA. In brief, the isolated RNA was dissolved in sterile water and 2.5 mmol/l Mg^{2+}, 1 mmol/l dNTPs, 0.5 μg oligodT$_{15}$, 25 U AMV reverse transcriptase, 10× RT buffer, giving a final volume of 20 μl. The sample was incubated at 25°C (10 min), 42°C (60 min) and 99°C (5 min). cDNA was diluted in DNase-free water (1:25) before quantification by real-time PCR.

The primers for quantification of mRNA by real-time quantitative PCR for *Nrf1*, *Nrf2*, *Tfam*, *Ppara*, *Pparg*, *Ppargc1a*, *Cpt1a* and β-actin mRNAs are listed in the Electronic supplementary materials (ESM Table 1). Quantitative PCR was performed using Mx3000P (see above). Each quantitative PCR was performed in triplicate. The mouse β-actin gene served as the endogenous reference gene. The evaluation of relative differences of PCR product among the treatment groups was carried out using the ΔΔCT method. The reciprocal of 2CT (used CT as an exponent for the base 2) for each target gene was normalised to that for β-actin, followed by comparison with the relative value in control cells. Final results are presented as percentage of control.

Western blot analysis After treatment with either or both LA and ALC, cells were washed twice with ice-cold PBS, lysed in sample buffer [62.5 mmol/l Tris–Cl pH 6.8, 2% (*w*/*v*) SDS, 5 mmol/l dithiothreitol (DTT)] at room temperature and vortexed. Cell lysates were then boiled for 5 min and cleared by centrifugation (13,000×*g*, 10 min at 4°C). Protein concentration was determined using a protein assay (Bio-Rad DC; Hercules, CA, USA). The soluble lysates (10 μg per lane) were subjected to 10% (*w*/*v*) SDS-PAGE; proteins were then transferred to nitrocellulose membranes and blocked with 5% (*w*/*v*) non-fat milk/Tris-buffered saline Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies directed against β-actin (1:5,000), anti-OxPhos Complex I (NADH ubiquinol oxidoreductase 39 kDa subunit, 1:2,000), anti-OxPhos Complex II (succinate-ubiquinone oxidoreductase 70 kDa subunit, 1:2000) and anti-OxPhos Complex III (ubiquinol–cytochrome c oxidoreductase core II, 1:2,000) in 5% (*w*/*v*) milk/TBST. After washing membranes with TBST three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using electrochemoluminescence (Roche, Mannheim, Germany) and quantified by scanning densitometry [33].

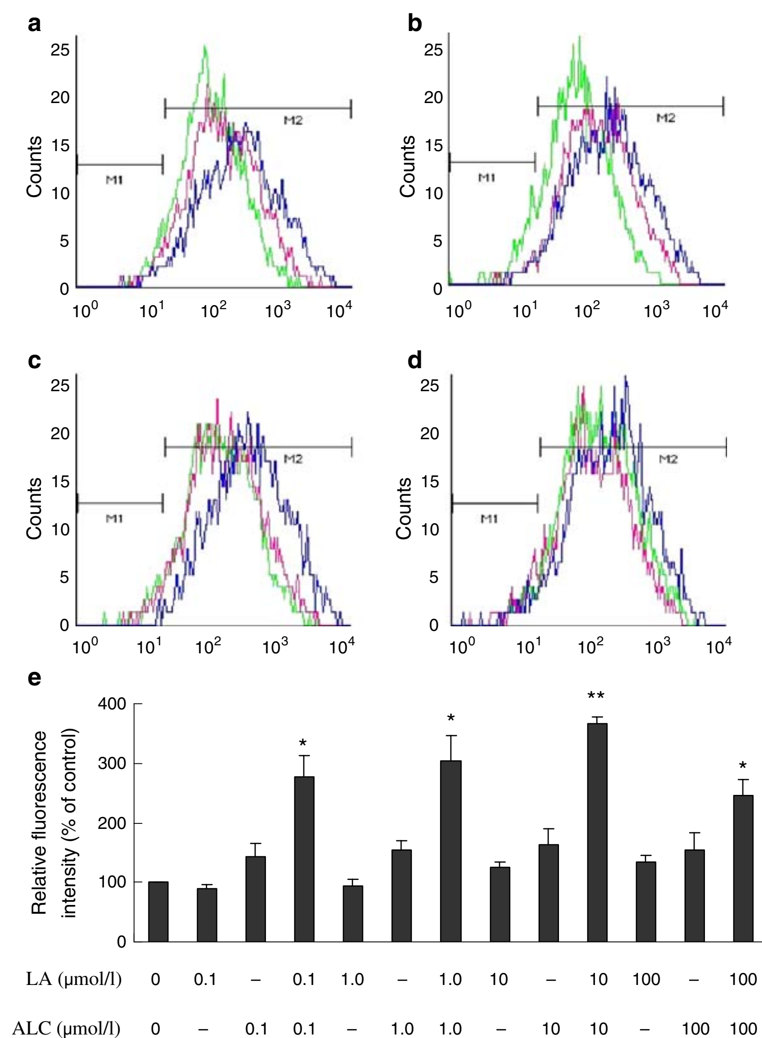

Fig. 1 Increase of mitochondrial mass in adipocytes treated with LA and/or ALC. Adipocytes were stimulated with LA and/or ALC from 0.1 to 100 μmol/l for 24 h and mitochondrial mass was estimated by MitoTracker (100 nmol/l) staining using flow cytometry. The rightward shift in the curve represents an increase in mitochondrial mass. The curves were generated using Becton Dickinson FACS Calibur using Cell Quest software. The curves were modified with smooth function of the software. M1 and M2 indicate the boundaries between cells that were negative (M1) or positive (M2) for Mito-Tracker Green FM; these were arbitrarily set on cells without stain (not shown) and with stain for Mito-Tracker Green FM samples. **a** LA and/or ALC 0.1 μmol/l; **b** LA and/or ALC 1.0 μmol/l; **c** LA and/or ALC 10 μmol/l; **d** LA and/or ALC 100 μmol/l. Green, LA treatment; red, ALC treatment; blue, LA+ALC treatment. **e** 3T3-L1 adipocytes were incubated with LA and/or ALC at the indicated concentrations. Results are presented as percentage of untreated control cells. Values are mean±SEM of the results from four independent experiments. *$p<0.05$ and **$p<0.01$ vs control

Mitochondrial isolation Following addition of trypsin, the cells were pelleted by centrifugation at 300×*g* for 5 min at 4°C. All of the subsequent steps were performed on ice. The resulting pellet was then resuspended in 0.5 ml of mitochondrial isolation buffer (215 mmol/l mannitol, 75 mmol/l sucrose, 0.1% BSA, 1 mmol/l EGTA, 20 mmol/l HEPES, pH 7.2) and homogenised on ice with a 2 ml glass homogeniser (Dounce, Fisher Scientific, Pittsburgh, PA, USA). The mitochondria were then purified by differential centrifugation at 1,300×*g* for 5 min to pellet unbroken cells and the nuclei. The supernatant fraction was then centrifuged at 13,000×*g* for 10 min to pellet the mitochondria. The pellet was resuspended in EGTA-free isolation buffer [34].

Electrophoretic mobility shift assay Binding activity of mitochondrial transcription factor A (*Tfam*) was assessed by electrophoretic mobility shift assay (EMSA) according to Kanazawa et al. [34]. Briefly, a radioactive probe containing the nucleotide sequence of the heavy-strand promoter of *Tfam* was prepared by annealing paired oligonucleotides with the sequences 5′-TTTCCTCCTAAC TAAACCCTCTTTAC-3′ and 5′-GTAGGCAAGTAAA GAGGGTTTAGTTA-3′ and was labelled using γ-^{32}P-labelled ATP (1.11×10^{14} Bq/mmol; Amersham Biosciences, Buckinghamshire, UK) and T4 polynucleotide kinase (Promega, Mannheim, Germany). The protein–DNA binding protein reaction was performed at room temperature for 20 min in a volume of 20 µl. The reaction mixture contained 10 µg mitochondrial protein, 100 µg/ml poly dI–dC, 10 mmol/l Tris–HCl (pH 7.5), 50 mmol/l NaCl, 0.5 mmol/l EDTA, 0.5 mmol/l dithiothreitol, 1 mmol/l $MgCl_2$, 4% glycerol (*v*/*v*) and 100,000 cpm-labelled nucleotides. Protein–DNA complexes were resolved by electrophoresis on a 6% (*w*/*v*) acrylamide gel and subjected to autoradiography. For competition assays, nonlabelled oligonucleotides were added at 50-fold molar excess to the reaction mixture before the addition of the mitochondrial protein extract.

Fatty acid oxidation Following incubation, $^{14}CO_2$ was measured according to the method of Thupari et al. [35] with some modifications. Adipocytes were preincubated for 30 min with 1.5 ml of the following buffer: 114 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 11 mmol/l glucose. After preincubation, 200 µl of assay buffer was added containing 114 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 11 mmol/l glucose and 2.5 mmol/l palmitate (containing 0.37 MBq [1-^{14}C] palmitate) bound to albumin, and the cells were incubated at 37°C for 2 h. After incubation, the plate was clamped and sealed, and perchloric acid was injected into the medium through the holes in the lid, driving CO_2 through the tunnel into an adjacent well, where it was trapped in 1 mol/l NaOH. After trapping, aliquots of NaOH and medium were transferred into scintillation vials and radioactivity was measured on a multipurpose scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA, USA). Cells were collected into 0.3 ml 0.05% (*w*/*v*) SDS for subsequent protein measurement. All assays were performed in duplicate and data were normalised to protein content. Blanks were prepared by adding 500 µl 6% (*w*/*v*) perchloric acid to the cells before incubation with the assay buffer for 2 h.

Statistical analysis All data are representative of at least three independent experiments. Data are presented as means±SE. Statistical significance was determined by using one-way ANOVA with Bonferroni's post hoc tests between the two groups. The criterion for significance was set at $p<0.05$.

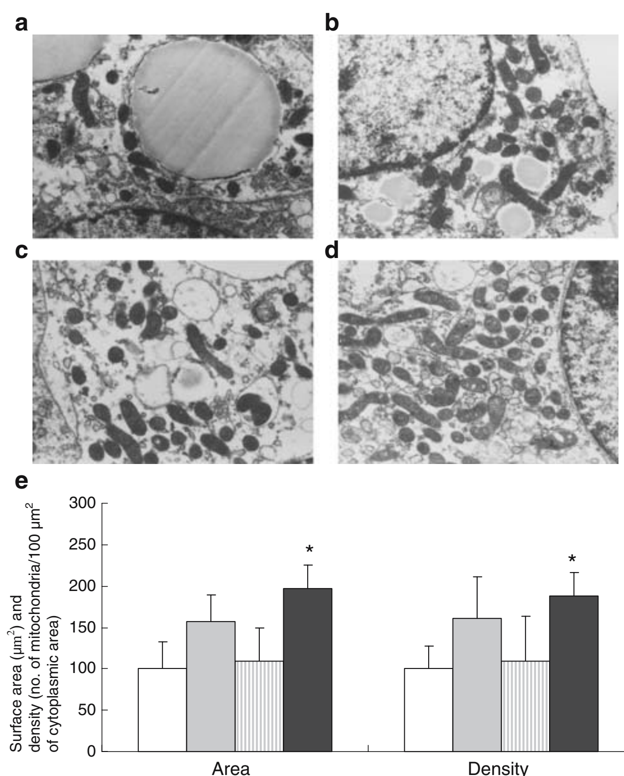

Fig. 2 Electron microscopy of LA and/or ALC-treated 3T3-L1 adipocytes. **a**–**d** Mitochondrial morphometry in adipocytes (magnification ×10,000). Illustrations of whole-cell profiles, with mitochondrial profiles surrounding a lipid droplet indicated by arrows. On day 8 of differentiation, 3T3-L1 adipocytes were seeded on to coverslips. On day 9, cells (**b**–**d**) were treated for 24 h with LA 10 µmol/l, ALC 10 µmol/l and LA 10 µmol/l+ALC 10 µmol/l or left untreated (**a**). **e** Morphometric analysis of surface area of the mitochondria and mitochondrial density was performed. Values are means±SEM. *$p<0.05$ vs control. White bars, control; grey bars, LA; striped bars, ALC; black bars, LA+ALC

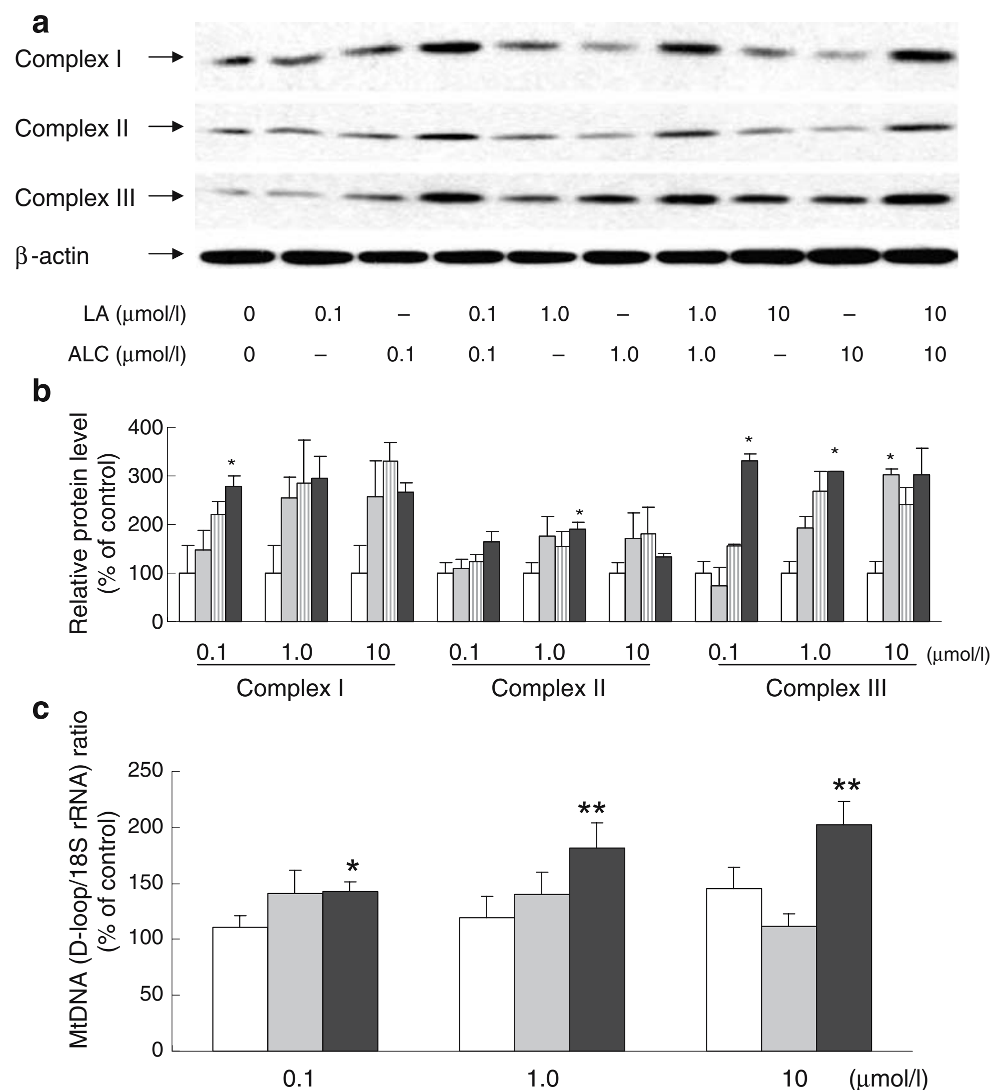

Fig. 3 Effect of LA and/or ALC on levels of mitochondrial protein and DNA. **a** 3T3-L1 adipocytes were treated for 24 h with LA and/or ALC. Cells were subsequently solubilised into SDS sample buffer and analysed by western blotting with antibodies against β-actin and mitochondrial electron transport complex I, II and III. Immunoblots are representative of steady-state levels of proteins. **b** Quantitative analyses of the bands for mitochondrial complex I, II and III by densitometry. Results are presented as percentage of untreated control cells. Values are mean±SEM from four independent experiments. *$p<0.05$ vs control. White bars, control; grey bars, LA; striped bars, ALC; black bars, LA+ALC. **c** 3T3-L1 adipocytes were treated for 24 h with LA and/or ALC. PCR products were quantified for fluorescence using SYBR Green. Quantitative values were tabulated for D-loop: 18S rRNA ratio. Results are presented as percentage of untreated control cells. Data are mean±SEM ($n=5$). *$p<0.05$ vs control taken as 100; **$p<0.01$ vs control taken as 100. White bars, LA; grey bars, ALC; black bars, LA+ALC

Results

LA and ALC increase adipocyte mitochondrial mass To determine whether LA and ALC lead to a modification of mitochondrial mass, the dye, MitoTracker Green FM, which accumulates in mitochondria, was used to label and quantify mitochondria in adipocytes. Seven days after differentiation was initiated, 3T3-L1 adipocytes were exposed to LA and/or ALC for 24 h. Treatment with either LA or ALC at 0.1 to 100 μmol/l resulted in a trend towards a dose-dependent increase in fluorescence intensity, but these increases were not significant. However, the combination of LA and ALC statistically significantly increased the relative fluorescence intensity in the concentration range of 0.1 to 100 μmol/l, these increases being higher than those found with LA or ALC alone (Fig. 1).

Electron microscopic analysis of adipocyte mitochondria LA (10 μmol/l) and/or ALC (10 μmol/l) treatment altered the size of individual mitochondria and their structure (Fig. 2). A quantitative analysis (ten cells analysed) showed that ALC (Fig. 2c) did not affect mitochondrial area and number, while LA (Fig. 2b) showed a trend towards increasing mitochondrial area and number, although this was not statistically significant. However, the combination of LA and ALC treatment (Fig. 2d) caused a significant increase in the total mitochondrial section area ($p<0.05$) and also an increase (187.8±24%) in the average number of mitochondrial profiles per cell, counted in a double-blinded fashion in five images containing whole-cell profiles sectioned through the middle of the nucleus.

Production of OxPhos complex I, II, III and IV proteins and mitochondrial DNA Because LA and/or ALC treatment

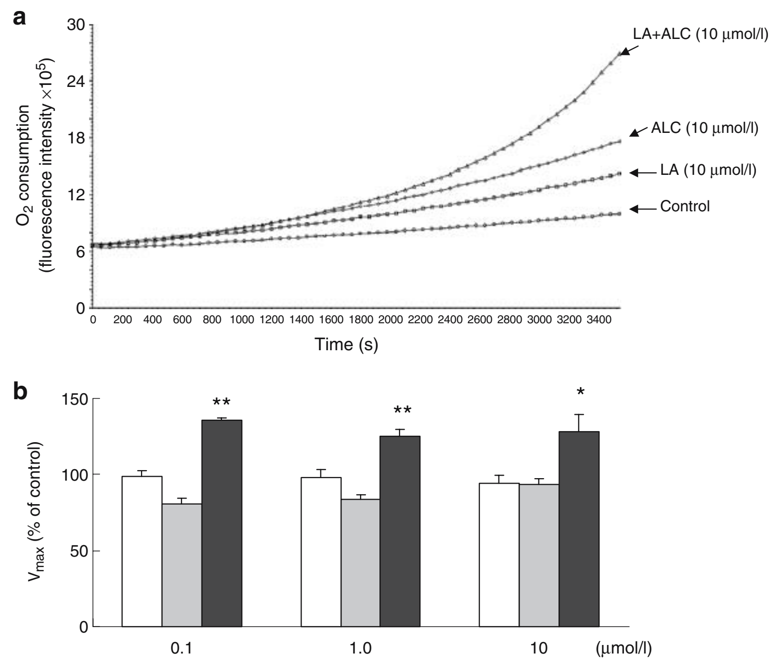

Fig. 4 Oxygen consumption in 3T3-L1 adipocytes. Equal volumes of cells were separated into aliquots in wells of a 96-well BD Oxygen Biosensor plate. Plates were covered and fluorescence in each well was recorded over time with a fluorescence microplate spectrophotometer. **a** Representative oxygen consumption curves. **b** Quantitative changes in the respiratory rate of adipocytes during each condition were calculated by determining the kinetic measurements. V_{max} is the maximum oxygen consumption rate. Values are mean±SEM; results are presented as percentage of untreated control cells from three independent experiments. *$p<0.05$ vs controls taken as 100; **$p<0.01$ vs control taken as 100. White bars, LA; grey bars, ALC; black bars, LA+ALC

altered the size of individual mitochondria and their apparent number, western blotting was used to estimate the actual increase in mitochondrial complexes caused by LA and/or ALC treatment. LA showed an increase in mitochondrial OxPhos Complex III protein at 10 μmol/l (303±10.2%). The combination treatment of equal concentrations of LA and ALC showed a significant increase ($p<0.05$ vs control) in the levels of OxPhos Complex I at 0.1+0.1 μmol/l (279±21.8%), OxPhos Complex II at 1.0+1.0 μmol/l (190±13.9%) and OxPhos Complex III at both 0.1+0.1 μmol/l (329±16.1%) and 1.0+1.0 μmol/l (310±0.23%; Fig. 3b). LA and/or ALC showed no effect on the expression of mitochondrial OxPhos complex IV in adipocytes (data not shown).

As D-loop is known to be the major site of transcription initiation on both the heavy and light strands of mitochondrial DNA (mtDNA), we examined in vitro whether LA and/or ALC could increase mtDNA expression. As shown in Fig. 3c, the combination of LA and ALC increased the ratio of mitochondrial D-loop/18S rRNA in the concentration range of 0.1 to 10 μmol/l, with all increases statistically significantly higher than those found with either LA or ALC alone.

Oxygen consumption To determine whether increased mitochondrial biogenesis is accompanied by changes in oxygen consumption, cells were treated with LA and/or ALC at 0.1 to 10 μmol/l. As shown in Fig. 4, the basal rate of oxygen consumption was statistically significantly increased in adipocytes treated with the combination of LA and ALC in the concentration range of 0.1 to 10 μmol/l, all increases being significantly higher than those found with either LA or ALC alone.

Expression of mitochondrial biogenesis genes Ppargc1a is a coactivator that promotes mitochondrial biogenesis and mitochondrial fatty acid oxidation. The relative abundance of mRNA transcripts encoding for *Ppargc1a*, *Nrf1* and *Nrf2* were examined by quantitative RT-PCR. Treatment of LA at 0.1 to 10 μmol/l resulted in a trend towards a dose-dependent increase in expression of transcripts encoding for *Ppargc1a*, but ALC did not affect expression of transcripts encoding for *Ppargc1a* and *Nrf1* and *Nrf2*. However, the combination of LA and ALC increased abundance of transcripts encoding for *Ppargc1a* and *Nrf1* and *Nrf2* significantly in the concentration range of 0.1 to 10 μmol/l; all increases were statistically significantly higher than those found with either LA or ALC alone (Fig. 5a–c).

The transcription factor *Tfam* is involved in regulating expression of nuclear genes encoding major mitochondrial proteins that regulate mtDNA transcription and replication. In the ESMA assay, a competition reaction was performed by preincubating a 50-fold molar excess of unlabelled oligonucleotide representing the *Tfam* binding site with the isolated mitochondria. The specific of *Tfam* binding of activity was confirmed by the absence of *Tfam* complex band in the negative control (mutant *Tfam* probe). A dose-dependent increase in *Tfam* binding was found with LA and ALC, respectively; the combinations of LA and ALC also showed a significant stimulation at concentrations of 0.1 to 10 μmol/l (Fig. 5d).

mRNA of Pparg, Ppara, Cpt1a *and fatty acid oxidation* The relative abundance of mRNA transcripts encoding for *Pparg*, *Ppara* and *Cpt1a* were examined by quantitative RT-PCR. Treatment of LA at 10 μmol/l resulted in increase

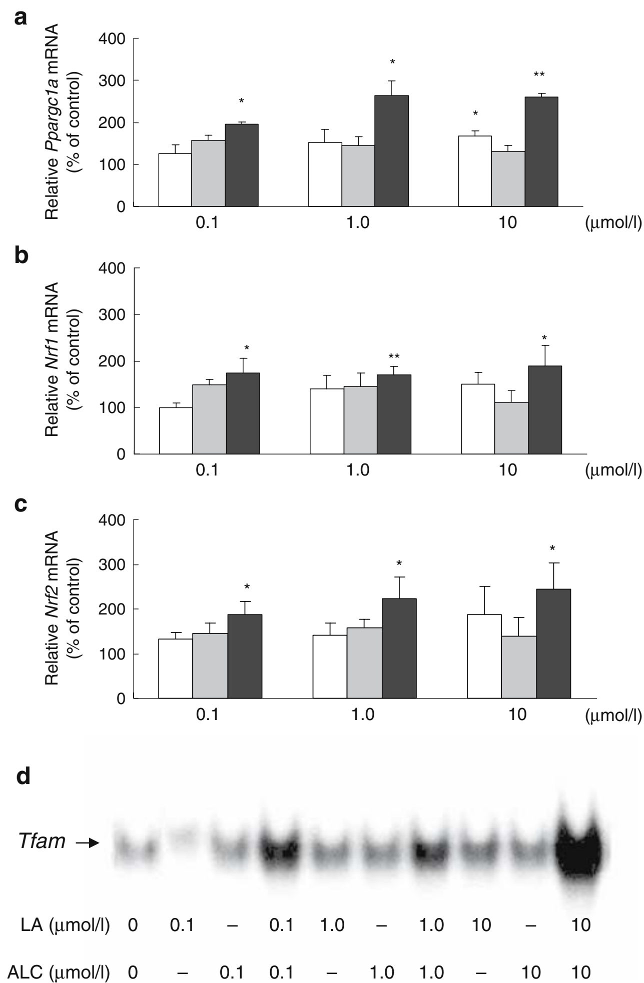

Fig. 5 Effect of treatment with LA and/or ALC on expression of *Ppargc1a*, *Nrf1*, *Nrf2* and *Tfam* mRNA in adipocytes. Adipocytes were incubated with LA and/or ALC for 24 h and total RNA was isolated. *Ppargc1a* , *Nrf1* and *Nrf2* mRNA was analysed by quantitative RT-PCR with gene-specific oligonucleotide probes in adipocytes. **a** Effect of LA and/or ALC on expression of *Ppargc1a*; **b** effect of LA and/or ALC on expression of on *Nrf1*; **c** effect of LA and/or ALC on expression of on *Nrf2*. The cycle number at which the various transcripts were detectable was compared with that of β-actin as an internal control; results are expressed as percentage of untreated control cells. All values are mean±SEM of four independent experiments. $^*p<0.05$ vs controls taken as 100; $^{**}p<0.01$ vs controls taken as 100. White bars, LA; grey bars, ALC; black bars, LA+ALC. **d** Analysis of the binding activity of mitochondrial proteins to an *Tfam* probe. Autoradiograph of EMSA performed with a ^{32}P-labelled mutant *Tfam* nucleotide and mitochondrial protein extract isolated from LA and/or ALC at the indicated concentrations

in expression of *Pparg*, *Ppara* and *Cpt1a*. ALC also increased the expression of *Ppara* and *Cpt1a* significantly at 10 μmol/l, but showed no effect on *Pparg*. However, the combination of LA and ALC statistically significantly increased the abundance of *Ppara* and *Cpt1a* at a concentration of 10 μmol/l, all increases being significantly higher than those found with LA or ALC alone (Fig. 6a). As shown in Fig. 6b, ALC increased fatty acid oxidation by 223% ($p<$ 0.05) at 10 μmol/l, but LA had no effect at the same concentration. However, the combination of LA and ALC significantly increased fatty acid oxidation by 323% ($p<0.01$).

Discussion

White adipose tissue is an important endocrine organ involved in the control of whole-body metabolism and insulin sensitivity. Thus, mitochondrial biogenesis could in part underlie the central role of adipose tissue in the control of whole-body metabolism and the actions of some insulin sensitisers [12]. Indeed, it has been reported that mitochondrial dysfunction might be an important contributing factor in insulin resistance and type 2 diabetes [2], while mitochondrial loss in adipose tissue is correlated with the development of type 2 diabetes [3]. Hence it is possible that stimulation of mitochondrial biogenesis may reduce the effects of mitochondrial loss of function. The combination of relatively low doses of LA and ALC improved mitochondrial function and may provide a possible therapeutic intervention for preventing and treating insulin resistance and type 2 diabetes.

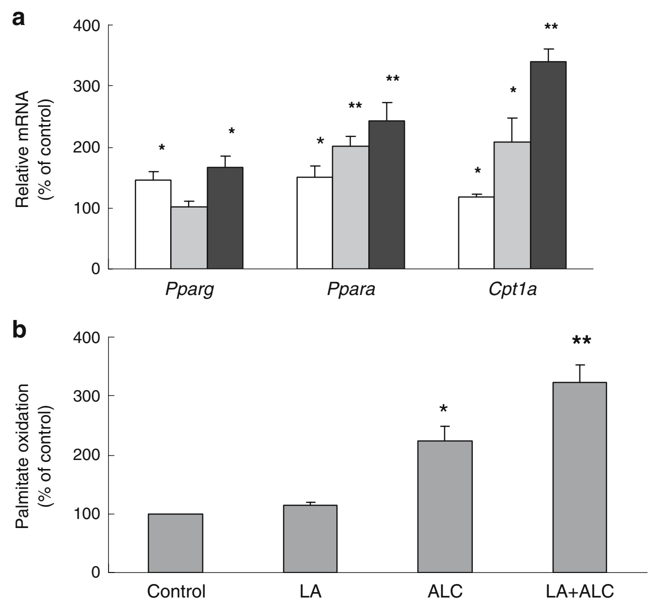

Fig. 6 Effect of LA and/or ALC on expression of *Pparg*, *Ppara* and *Cpt1a* mRNA, and on fatty acid oxidation in adipocytes. **a** Adipocytes were incubated with LA and/or ALC at 10 μmol/l for 24 h. *Pparg*, *Pparα* and *Cpt1a* mRNA were analysed by quantitative RT-PCR with gene-specific oligonucleotide probes of adipocytes. The cycle number at which the various transcripts were detectable was compared with that of β-actin as an internal control and results expressed as percentage of untreated control cells. All values are mean±SEM of four independent experiments. $^*p<0.05$ vs controls taken as 100; $^{**}p<0.01$ vs controls taken as 100. White bars, LA; grey bars, ALC; black bars, LA+ALC **b** Adipocytes were incubated with LA and/or ALC for 24 h and [^{14}-C] palmitate oxidation was measured. Values are mean±SEM; results are presented as percentage of untreated control cells from three independent experiments. $^*p<0.05$ vs controls; $^{**}p<0.01$ vs controls

Pparg plays an important role not only in adipogenesis, but also in regulating lipid metabolism in mature adipocytes [12]. PPARG activity can be modulated by direct binding of low molecular weight ligands, some of which are clinically effective glucose-lowering agents, albeit with adverse side effects that limit their utility [5]. Activation of *Pparg* by glucose-lowering agents such as thiazolidinedione, a high-affinity agonist ligand for *Pparg*, led to a net flux of fatty acids from the circulation and other tissues into adipocytes [5]. Interestingly, increased fat storage did not increase the size of adipocytes, but rather led to smaller adipocytes, possibly due to increased adipocyte differentiation and activation of *Ppargc1a*, which promotes mitochondrial biogenesis. *Ppara* is also known to be an important regulator of mitochondrial biogenesis and β-oxidation in tissues like heart and liver [4, 36]. As shown in our experiments, *Ppara* and *Pparg* levels were upregulated by LA and ALC treatment in 3T3L1 adipocytes. This upregulation closely correlates with the stimulation of mitochondrial biogenesis and induction of *CPT1a* involved in fatty acid oxidation, suggesting these nutrients may act as *PPARG/A* ligands to increase fatty acid uptake, increase adipocyte differentiation, and activate *Ppargc1a* to promote mitochondrial biogenesis in 3T3L1 adipocytes. Since LA and ALC are nutrients without apparent side effects, they might exert better PPARg/a ligand stimulation than glucose-lowering agents or other ligands. The *Pparg* agonist pioglitazone and *Ppara* agonist WY-14,643 were able to increase *Ppargc1a* expression and mtDNA copy number, as well as enhancing the oxidative capacity of white adipose tissue leading to insulin sensitisation [12, 13, 37].

Mitochondrial biogenesis and remodelling in white adipocyte tissue enhances fatty acid uptake and oxidation by increased oxygen consumption. Consistent with the morphological data, oxygen consumption in adipocytes was increased when adipocytes were treated with LA and ALC, indicating that adipocytes treated with a combination of LA and ALC have a greater mitochondrial mass than cells treated with LA or ALC alone. In vivo, an increase in fatty-acid oxidation may protect against adipocyte hypertrophy under conditions where increased uptake of fatty acids occurs from the circulation. Thus, the effect of LA and ALC may contribute directly and indirectly to changes in whole-body energy metabolism and insulin sensitivity.

The mechanisms of the protective effects of the combination of LA and ALC are not clear, but might include [18, 38, 39]: (1) protection of mitochondria from oxidative damage and thus slowing down of the loss of mitochondria; (2) stimulation of repair of less damaged mitochondria; (3) stimulation of degradation of more damaged mitochondria (lysosomes); and (4) stimulation of de novo mitochondrial biogenesis. The complementary effect of LA and ALC on cognitive and mitochondrial dysfunction has been shown in ageing rats [27, 28, 40]. One reason is that LA+ALC act on different pathways necessary for mitochondria: LA is a mitochondrial antioxidant and cofactor of pyruvate dehydrogenase, while ALC is an energy enhancer [25, 41]. Another possibility is that ALC, although stimulating mitochondrial function, may cause side effects of oxidative stress in mitochondria [42], while LA, an effective mitochondrial antioxidant, is able to ameliorate that side effect of ALC. The complementary effect may also come from the different functions of LA and ALC on the four various aspects.

In conclusion, the strong synergistic effect of the combination of LA and ALC in 3T3L1 adipocytes suggests that these two nutrients complement each other's function in mitochondrial biogenesis. As a next step administration of combinations of LA and ALC should be tested in animal models of insulin resistance to determine whether such combinations might be an effective nutrient intervention for ameliorating mitochondrial dysfunction in vivo.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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