BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01134.x
www.brjpharmacol.org

RESEARCH PAPER

Lipoamide or lipoic acid stimulates mitochondrial biogenesis in 3T3-L1 adipocytes via the endothelial NO synthase-cGMP-protein kinase G signalling pathway

Weili Shen[1], Jiejie Hao[2], Zhihui Feng[3], Chuan Tian[3], Weijun Chen[4], Lester Packer[5], Xianglin Shi[6], Weijin Zang[7] and Jiankang Liu[4,8]

[1]*State Key Laboratory of Medical Genomics, Shanghai Key Laboratory of Vascular Biology and Department of Hypertension, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China,* [2]*School of Medicine and Pharmacy, Ocean University of China, Qingdao, China,* [3]*Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Graduate School of the Chinese Academy of Sciences, Shanghai, China,* [4]*Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wenchang, Hainan, China,* [5]*Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA, USA,* [6]*Graduate Center for Toxicology, University of Kentucky College of Medicine, Lexington, KY, USA,* [7]*Department of Pharmacology, Xi'an Jiaotong University College of Medicine, Xi'an, China, and* [8]*Institute of Mitochondrial Biology and Medicine, The Key Laboratory of Biomedical Information Engineering of Ministry of Education, Xi'an Jiaotong University School of Life Science and Technology, Xi'an, China*

Correspondence
Jiankang Liu, Institute of Mitochondrial Biology and Medicine, Jiaotong University School of Life Science and Technology, Xi'an 710049, China. E-mail: j.liu@mail.xjtu.edu.cn

Keywords
lipoic acid; lipoamide; mitochondria; peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α); mitochondrial transcription factor A (TFAM); nuclear respiratory factor 1 (NRF1)

Received
9 July 2010
Revised
30 September 2010
Accepted
28 October 2010

BACKGROUND AND PURPOSE

Metabolic dysfunction due to loss of mitochondria plays an important role in diabetes, and stimulation of mitochondrial biogenesis by anti-diabetic drugs improves mitochondrial function. In a search for potent stimulators of mitochondrial biogenesis, we examined the effects and mechanisms of lipoamide and α-lipoic acid (LA) in adipocytes.

EXPERIMENTAL APPROACH

Differentiated 3T3-L1 adipocytes were treated with lipoamide or LA. Mitochondrial biogenesis and possible signalling pathways were examined.

KEY RESULTS

Exposure of 3T3-L1 cells to lipoamide or LA for 24 h increased the number and mitochondrial mass per cell. Such treatment also increased mitochondrial DNA copy number, protein levels and expression of transcription factors involved in mitochondrial biogenesis, including PGC-1α, mitochondrial transcription factor A and nuclear respiratory factor 1. Lipoamide produced these effects at concentrations of 1 and 10 μmol·L^{-1}, whereas LA was most effective at 100 μmol·L^{-1}. At 10 μmol·L^{-1}, lipoamide, but not LA, stimulated mRNA expressions of PPAR-γ, PPAR-α and CPT-1α. The potency of lipoamide was 10–100-fold greater than that of LA. Lipoamide dose-dependently stimulated expression of endothelial nitric oxide synthase (eNOS) and formation of cGMP. Knockdown of eNOS (with small interfering RNA) prevented lipoamide-induced mitochondrial biogenesis, which was also blocked by the soluble guanylate cyclase inhibitor, ODQ and the protein kinase G (PKG) inhibitor, KT5823. Thus, stimulation of mitochondrial biogenesis by lipoamide involved signalling via the eNOS-cGMP-PKG pathway.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that lipoamide is a potent stimulator of mitochondrial biogenesis in adipocyte, and may have potential therapeutic application in obesity and diabetes.

Abbreviations

CPT-1α, carnitine palmitoyl transferase-1; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; LA, lipoic acid; L-NAME, N^G-nitro-L- arginine methyl ester; L-NMMA, N^G-methyl L-arginine; NRF1, nuclear related factor 1; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PGC-1α, PPAR-γ co-activator-1α; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TFAM, mitochondrial transcription factor A

Introduction

Mitochondria play a central role in the maintenance of energy stores, regulation of metabolism and pathways of cell death. Mitochondrial dysfunction has been suggested as a causal factor of insulin resistance and diabetes (Choi *et al.*, 2005). Anti-diabetic thiazolidinediones enhance insulin sensitivity and appear to exert their therapeutic effects by changing morphological features and protein profiles of mitochondria in 3T3-L1 adipocytes (Wilson-Fritch *et al.*, 2003; 2004). Thus, rosiglitazone treatment up-regulated decreased gene transcripts encoding mitochondrial proteins of *ob/ob* mice at the onset of obesity. This gene up-regulation by rosiglitazone was accompanied by an increase in mitochondrial mass and morphology, and also a markedly enhanced oxygen consumption and palmitate oxidation in adipocytes (Wilson-Fritch *et al.*, 2004).

α-Lipoic acid (LA) is a multifunctional antioxidant (Packer *et al.*, 1995; 1997a) and mitochondrial nutrient (Liu and Ames, 2005). LA improves insulin sensitivity in patients with type 2 diabetes (Evans and Goldfine, 2000), experimental diabetic retinopathy (Civitarese *et al.*, 2006), diabetic vascular complications (Da Ros *et al.*, 2005) and diabetic neuropathy (Stevens *et al.*, 2000; Bruckner *et al.*, 2002). It has been suggested that stimulating peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) could suppress reactive oxygen species (ROS) and mitochondrial dysfunction-related neurodegeneration (St-Pierre *et al.*, 2006). PGC-1α is known to be a key regulator of mitochondrial biogenesis and respiration. We therefore hypothesized that one of the mechanisms of the effects of LA on diabetic symptoms and complications is to stimulate mitochondrial biogenesis and remodelling during adipogenesis. In a previous study, we reported that LA plus acetyl-L-carnitine, both at 1 μmol·L^{-1} or 10 μmol·L^{-1}, significantly stimulated the expression of PGC-1α, and the mitochondrial transcription factors, nuclear related factor 1 (NRF1), NRF2 and mitochondrial transcription factor A (TFAM), and the mitochondrial electron transport complexes (ETCs), as well as the ETC activities and mitochondrial DNA (mtDNA) copy numbers. In contrast, LA or acetyl-L-carnitine alone at such concentrations hardly affected these parameters (Shen *et al.*, 2008). Further study found that 100 μmol·L^{-1} LA was required to fully stimulate mitochondrial biogenesis.

Lipoamide is the neutral amide of LA. Unlike LA, lipoamide does not occur naturally in either animals or plants. Lipoamode was first synthesized from LA based on the mixed carbonic-carboxylic anhydride method (Reed *et al.*, 1958). Lipoamide has been shown to be a better cofactor for α-oxo-acid dehydrogenase enzymes than free LA, and it is able to promote the recovery of post-ischemic myocardium in rats (Sumegi *et al.*, 1994). It also has been shown to be an antioxidant *in vitro* (Bisby and Parker, 1998) and prevents Fenton-type chemistry and resultant oxidative damage and apoptosis more efficiently than LA in a lysosomal membrane system (Persson *et al.*, 2001).

To find more effective stimulators of mitochondrial biogenesis and therapeutic agents for treating diabetes and/or its complications, in the present study, we investigated the stimulating effect of lipoamide compared with that of LA, on mitochondrial biogenesis.

Methods

Cell culture and adipocyte differentiation

Murine 3T3-L1 pre-adipocytes (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and allowed to reach confluence. Differentiation of pre-adipocytes was initiated with 1 μmol·L^{-1} insulin, 0.25 μmol·L^{-1} dexamethasone and 0.5 mmol·L^{-1} 3-isobutyl-1-methylxanthine in DMEM supplemented with 10% FBS. After 48 h, the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 μmol·L^{-1} insulin. The culture medium was changed every other day with DMEM containing 10% FBS. Cells were used 9–10 days following induction of differentiation, and when at least 90% exhibited the adipocyte phenotype. Cells were made quiescent by incubation in DMEM supplemented with 0.1% BSA for 24 h, then treated with LA or lipoamide for 24 h.

Western blot analysis

Cell lysates (10 μg protein per lane) were subjected to 10% SDS-PAGE, then transferred to nitrocellulose membranes and blocked with 5% non-fat milk/Tris-buffered saline with Tween (TBST) for 1 h at room temperature. Membranes were incubated with primary antibodies directed against PGC-1α (1:1000), α-tubulin (1:10 000), OxPhos Complex I (NADH ubiquinol oxidoreductase 39-kDA subunit 1:2000), OxPhos Complex II (succinate-ubiquinone oxidoreductase 70-kDA subunit 1:2000), OxPhos Complex III (ubiquinol-cytochrome c oxidoreductase core II 1:2000) or endothelial NO synthase (eNOS; 1:1000) in 5% milk/TBST at 4°C overnight. After washing with TBST three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed

using ECL (Roche Mannheim, Germany) and quantified by scanning densitometry (Boudina *et al.*, 2005). Relative protein expression of complexes and PGC-1α by using Western blotting normalized to tubulin expression. Results were presented as percent control.

DNA isolation and real-time PCR

Total DNA was extracted using a kit (QIAamp DNA Mini kit; Qiagen, Germany), and quantitative PCR was done using 18S rRNA primers for a nuclear target sequence and primers for a mitochondrial DNA D-loop target. The following primers were used: mitochondrial D-loop forward: 5′-AATCTACCAT CCTCCGTG-3′, reverse: 5′-GACTAATGATTCTTCACCGT; 18SrRNA forward: 5′-CATTCGAACGTCTGCCCTATC-3′ and reverse: 5′-CCTGCTGCCTTCCTTGGA-3′. Quantitative PCR was performed in Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA).

The ratio of mitochondrial D-loop to 18S was then calculated. Results were presented as percent control (Shen *et al.*, 2008).

Electron microscopy

On day 9, cells were treated with LA (10 μmol·L^{-1}) or lipoamide (10 μmol·L^{-1}) for 24 h. For each individual adipocyte profile in the image area, the number of mitochondria and the total mitochondrial section area were determined by transmission electron microscopy (Wilson-Fritch *et al.*, 2004). Measurements were made on 10 individual adipocytes treated with or without LA or lipoamide. These 10 cells were from the same experiments, but each experiment was repeated three times. 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 without knowledge of the treatments.

RNA isolation and reverse transcription PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To synthesize first-strand cDNA, 1 μg of RNA was reverse transcribed, and the synthesized cDNA were amplified in triplicate using specific primers. The primers used are shown in Table 1. Quantitative PCR was performed in Mx3000P real-time PCR system (Stratagene). Reactions were performed with 12.5 μL SYBR-Green Master Mix (ABI, Warrington, UK), 0.5 μL of each primer (10 μmol·L^{-1}), 1 μL template (cDNA) or no template (NTC), and RNase-free water was 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 evaluation of relative differences of PCR product amounts among the treatment groups was carried out using the $^{\Delta\Delta}$CT method. The reciprocal of 2CT for each target gene was normalized to that for 18S rRNA, followed by the comparison with the relative value in control cells. Results were presented as percent control.

Mitochondrial respiration

Oxygen consumption by intact cells was measured as described (Wilson-Fritch *et al.*, 2004). After treatment, adipocytes were washed in KRH buffer plus 1% BSA. Cells from each condition were divided into triplicate aliquots and measured in a BD Oxygen Biosensor System plate (BD Biosciences). Plates were sealed and 'read' on a fluorescence spectrometer (Molecular Probes, Eugene, OR, USA) at 1 min intervals for 60 min at an excitation wavelength of 485 nm and emission wavelength of 630 nm. We have used 2×10^5 cells in the assay. The oxygen consumption rate of cells generally follows Michaelis-Menten kinetics with respect to oxygen concentration. V_{max} is the maximum consumption rate.

Activity of mitochondrial complexes I, II and III

Adipocytes were cultured in 100 mm plates and washed in PBS. 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^{-1} mannitol, 75 mmol·L^{-1} sucrose, 0.1% BSA, 1 mmol·L^{-1} EGTA, 20 mmol·L^{-1} HEPES, pH 7.2) and homogenized on ice with a 2 mL glass homogenizer (Dounce, Fisher Scientific, Pittsburgh, PA, USA). The mitochondria were then purified by differential centrifugation at 1300 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 (Kanazawa *et al.*, 2002).

Briefly, complex I activity was assayed by monitoring the decrease of NADH at 340 nm. Final concentration of mitochondrial protein was 30 μg·mL^{-1}. Reaction was started by

Table 1

Primers for quantitation of mRNA by real-time quantitative PCR

Gene	Forward	Reverse
Nrf1	5′-CGCAGCACCTTTGGAGAA-3′	5′-CCCGACCTGTGGAATACTTG-3′
Tfam	5′-GGAATGTGGAGCGTGCTAAAA-3′	5′-TGCTGGAAAAACACTTCGGAATA-3′
Ppar-α	5′-TCACACAATGCAATCCGTTT-3′	5′-GGCCTTGACCTTGTTCATGT-3′
Ppar-g	5′-CACTCGCATTCCTTTGAC- 3′	5′-CCCACAGACTCGGCACTC-3′
Cpt-1	5′-TGTCCAAGTATCTGGCAGTCG -3′	5′-CATAGCCGTCATCAGCAACC-3′

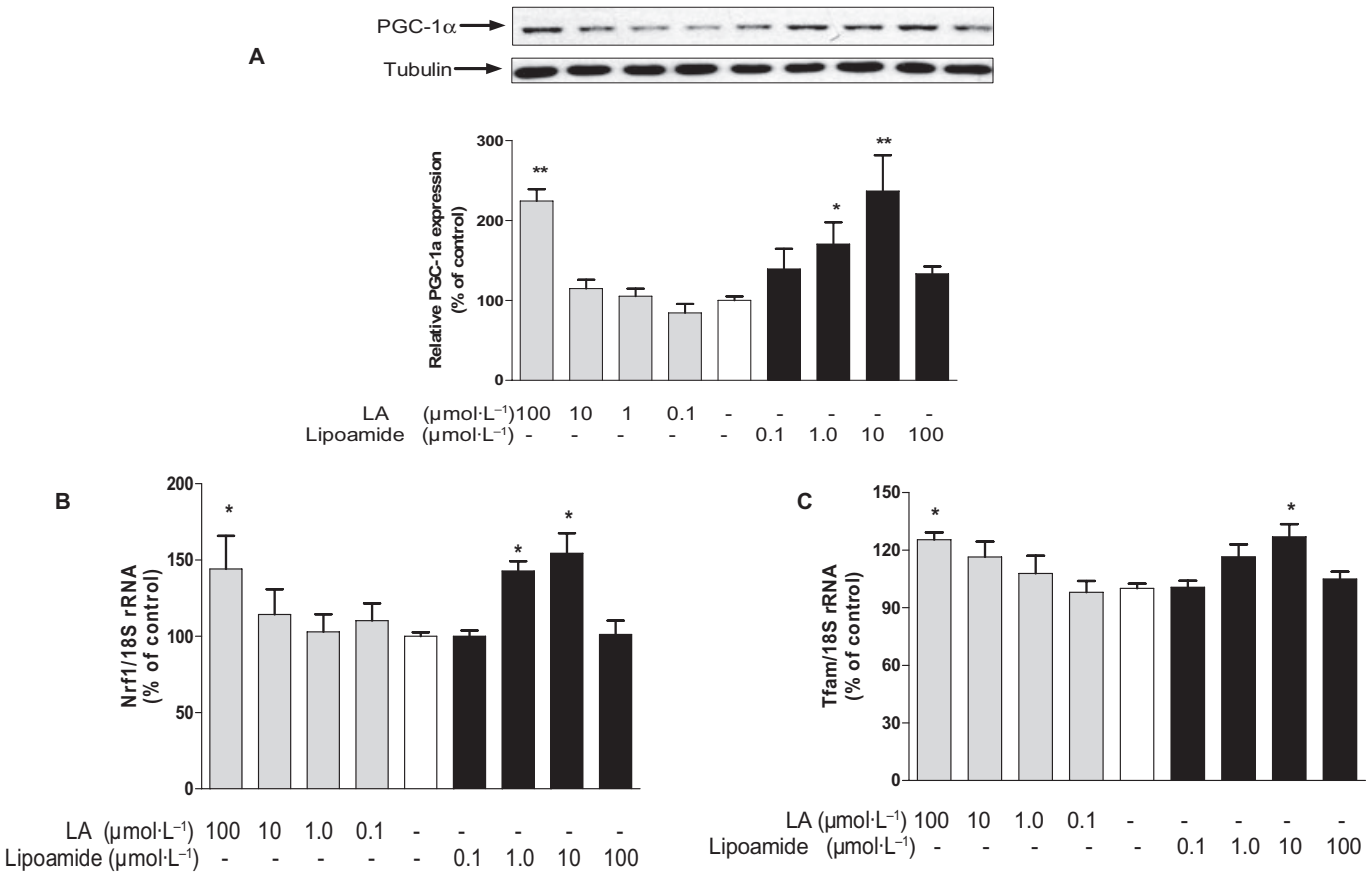

Figure 1

Effects of LA or lipoamide on the expression of PGC-1α, NRF1 and TFAM in adipocytes. (A) Protein expression of PGC-1α. Upper: representative Western blot image; below: quantification of PGC-1α protein expression of adipocytes incubated with LA or lipoamide for 24 h. Values are mean ± SEM from three independent experiments. *$P < 0.05$ versus control; **$P < 0.01$ versus control. (B) mRNA abundance of NRF1 and (C) mRNA abundance of TFAM. Total RNA was analyzed by quantitative RT-PCR with gene-specific oligonucleotide probes in adipocytes. The cycle number at which the various transcripts were detectable was compared to that of 18S rRNA as an internal control. Results are presented relative to values in untreated control cells (100%). All values are mean ± SEM of four independent experiments. *$P < 0.05$ versus control; **$P < 0.01$ versus control. LA, lipoic acid; NRF1, nuclear related factor 1; PGC-1α, PPAR-γ co-activator-1α; PPAR, peroxisome proliferator-activated receptor; TFAM, mitochondrial transcription factor A.

adding 200 μmol·L^{-1} NADH and scanned at 340 nm for 3 min. Rotenone (3 μmol·L^{-1}) was added into the reaction system as blank control. Complex II was assayed with mitochondria (final concentration 30 μg·mL^{-1}), and the reaction was started with 10 mmol·L^{-1} succinate and scanned at 600 nm for 2 min. Complex III activity was measured in a mixture containing 250 mmol·L^{-1} sucrose, 1 mmol·L^{-1} EDTA, 50 mmol·L^{-1} K phosphate, pH adjusted to 6.5 to reduce autooxidation of reduced coenzyme Q_1 (CoQ_1), 2 mmol·L^{-1} KCN, 50 μmol·L^{-1} cytochrome C, 0.1% BSA, and the reaction was initiated by 20 μg·mL^{-1} mitochondria and 50 μmol·L^{-1} reduced CoQ1, recording the increase of absorption at 550 nm for 2 min (Sun *et al.*, 2006).

cGMP immunoassays

Adipocytes were pretreated 1 h with 100 μmol·L^{-1} L-NAME, and then stimulated with lipoamide for 24 h. cGMP was measured using a cGMP immunoassay (R&D Systems Inc., Minneapolis, MN, USA) according to the supplier's protocol.

Transfection of siRNA

On day 6 after 3T3-L1 differentiation, the media of 3T3-L1 cells in six-well plates were changed to siRNA Transfection Medium (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the cells were transfected with eNOS- and control-siRNA (Santa Cruz Biotechnology) using siRNA Transfection Reagent (Santa Cruz Biotechnology) according to the instructions provided by the manufacturer. On day 7, media of 3T3-L1 adipocytes were changed to DMEM, and 3T3-L1 adipocytes were treated for 24 h with 10 μmol·L^{-1} lipoamide (Tedesco *et al.*, 2008; Cheng *et al.*, 2009).

Statistics

All values are expressed as means ± s.e.mean. 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 $P < 0.05$.

Materials

Anti-rabbit PGC-1α;, eNOS siRNA and control siRNA were purchased from Santa Cruz Biotechnology, anti-α-tubulin from Sigma (St. Louis, MO, USA), anti-OxPhos complexes I, II and III from Invitrogen, SYBR™GREEN PCR Master Mix from ABI, BD Oxygen Biosensor System plates from BD Biosciences (CA, USA), cGMP immunoassay was from R&D Systems; R-α-lipoic acid (Tris salt) was a gift from Dr K. Wessel (Viatris, Germany); DL-α-lipoamide from Fluka (Buchs, Switzerland); mitochondrial D-loop, NRF1, TFAM, 18S rRNA, PPAR-γ, PPAR-α and CPT-1 primers were synthesized by Bioasia Biotech (Shanghai, China); other reagents for cell culture were from Invitrogen. N^G-nitro-L- arginine methyl ester (L-NAME), N^G-methyl L-arginine (L-NMMA) and KT5823 were purchased from Sigma. The drug and molecular target nomenclature follows Alexander *et al.* (2009).

Results

Expression of mitochondrial biogenesis genes

The PGC-1α; protein is a co-activator that promotes mitochondrial biogenesis and mitochondrial fatty acid oxidation. The effect of LA on PGC-1α protein expression in adipocytes was dose-dependent (Figure 1A), and increased significantly at 100 μmol·L^{-1} LA. However, over the range of 0.1–100 μmol·L^{-1}, lipoamide produced a bell-shaped effect on PGC-1α, with a maximum protein expression at 10 μmol·L^{-1} lipoamide (Figure 1A).

The transcription factors NRF1 and TFAM are involved in regulating expression of nuclear genes encoding major mitochondrial proteins that regulate mtDNA transcription and replication. TFAM is identified as a D-loop DNA binding protein (Choi *et al.*, 2005). The relative abundance of mRNA transcripts encoding NRF1 and TFAM were examined by QRT-PCR. Treatment with LA at 0.1–100 μmol·L^{-1} resulted in a trend toward a dose-dependent increase in expression of NRF1 and TFAM mRNA with a significant increase at 100 μmol·L^{-1} LA. However, over the range of 0.1–100 μmol·L^{-1}, lipoamide produced a bell-shaped dose-related effect on both NRF1 (Figure 1B) and TFAM (Figure 1C) mRNA expression.

Mitochondrial protein and DNA

A decrease of mtDNA content is found in tissues of diabetic patients. The D-loop is known as the major site of transcription initiation of both the heavy and light strands of mtDNA. We examined *in vitro* whether LA or lipoamide could increase mtDNA expression (Figure 2). Lipoamide significantly increased the ratio of mtD-loop/18S rRNA at concentrations of 1.0 and 10 μmol·L^{-1}, and LA significantly increased the ratio of mtD-loop/18S rRNA at 100 μmol·L^{-1}.

Western blotting was used to obtain an estimate of the actual increase in mitochondrial complexes caused by LA or lipoamide treatment. Lipoamide showed an increase of mitochondrial electron transport complex I protein, complex II protein and complex III protein at 10 μmol·L^{-1}. However, LA

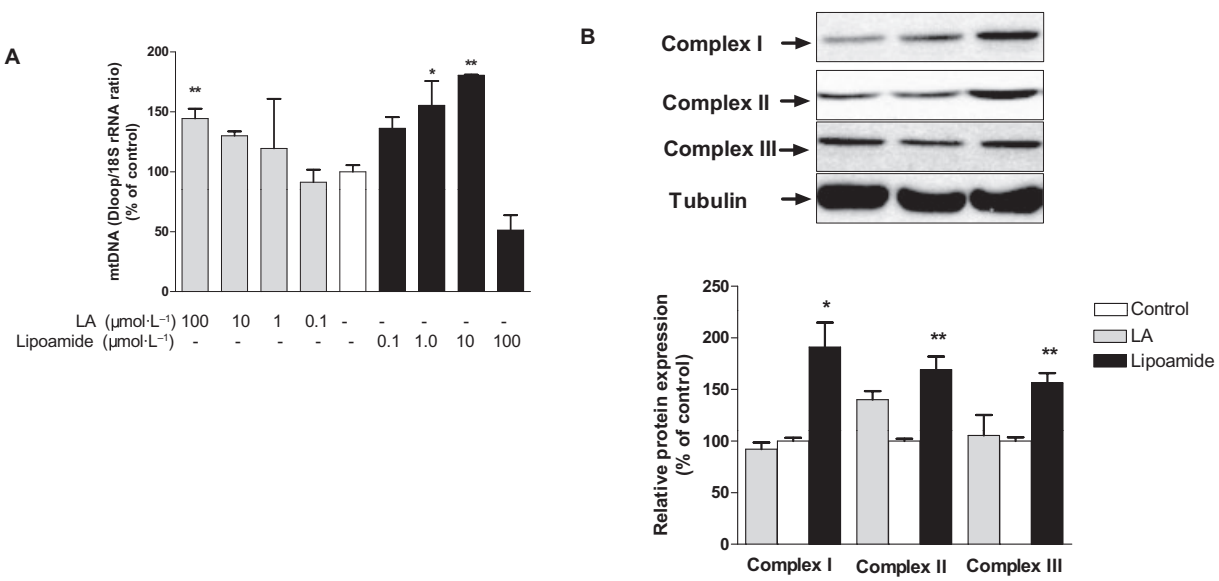

Figure 2

Effects of LA and lipoamide on the expression of mitochondrial DNA and proteins. (A) Mitochondrial DNA. 3T3-L1 adipocytes were treated with LA or lipoamide for 24 h. Total DNA was isolated from synchronized cells. The mtDNA contents were determined by real-time PCR. The DNA contents of mtDNA and of the 18S rRNA gene (18S rDNA) were calculated, and the relative ratios of mtDNA contents against 18S rRNA gene levels were determined in five independent experiments. (B) Expressions of mitochondrial complexes I, II and III. 3T3-L1 adipocytes were treated with LA or lipoamide for 24 h; cells were subsequently solubilized in SDS sample buffer and analyzed by Western blotting with antibodies against α-tubulin, and mitochondrial electron transport complex I, complex II and complex III. The quantitative analyses of the bands by densitometry are shown for mitochondrial complexes I, II and III, respectively. Upper: representative Western blot image; below: quantification of protein expression of complexes I, II and III. Results are presented relative to values in untreated control cells (100%). Data are mean ± SEM of four independent experiments. *P < 0.05 versus control; **P < 0.01 versus control. LA, lipoic acid A.

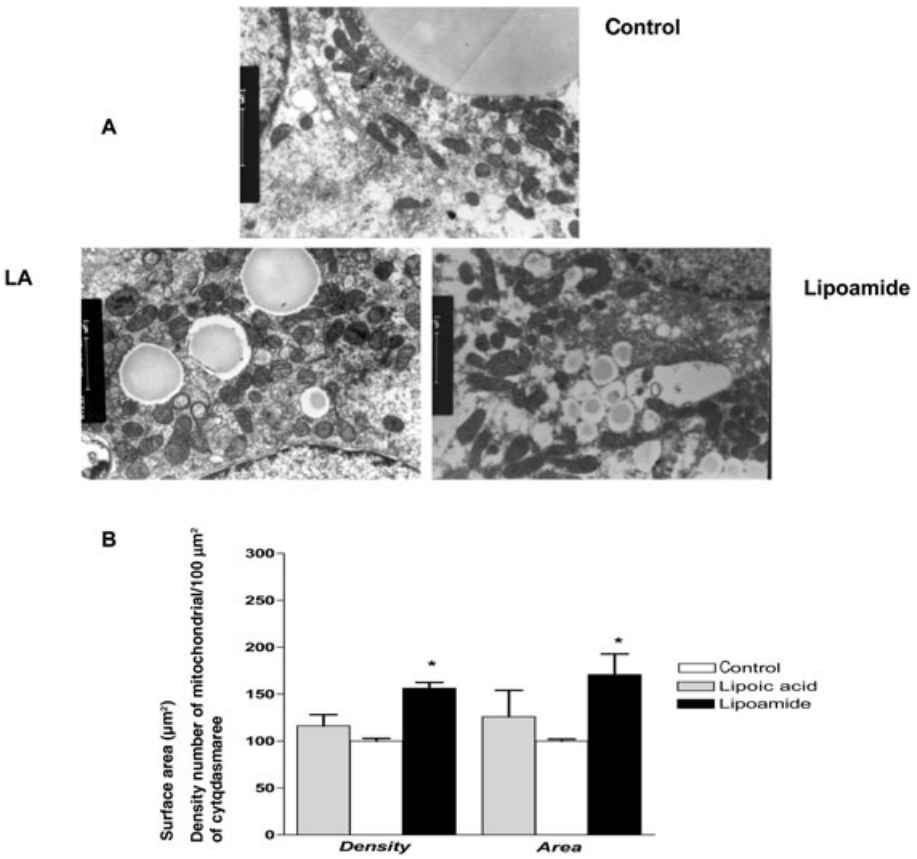

Figure 3

Electron microscopy of mitochondria from 3T3-L1 adipocytes treated with LA or lipoamide. (A) Mitochondrial morphometry in adipocytes (magnification, ×10 000): images of whole-cell profiles and mitochondrial profiles surrounding a lipid droplet by arrows. On day 8 of differentiation, 3T3-L1 adipocytes were seeded onto coverslips. On day 9, cells were treated with LA (10 μmol·L^{-1}) or lipoamide (10 μmol·L^{-1}). (B) Morphometric analyses of surface area of the mitochondria and mitochondrial density were performed. Results are presented relative to values in untreated control cells (100%). Values are means ± SEM of 10 cells. $^{**}P < 0.01$ versus control. LA, lipoic acid.

at 10 μmol·L^{-1} had no effect on the expression of mitochondrial complex proteins (Figure 2B).

Mitochondrial morphology

LA (10 μmol·L^{-1}) or lipoamide (10 μmol·L^{-1}) treatment altered both the size of individual mitochondria and their structure (Figure 3). Quantitative analysis showed that LA did not affect mitochondrial area and number, while lipoamide increased the total mitochondrial section area to 170 ± 22% of control, and also increased mitochondrial numbers to 156 ± 6.2% of control (both $P < 0.05$). The average numbers of mitochondrial profiles per cell were counted in a double-blinded fashion in 10 cells containing whole-cell profiles sectioned through the middle of the nucleus.

Oxygen consumption

To determine if increased mitochondrial biogenesis was accompanied by changes in oxygen consumption, cells were treated with LA or lipoamide at 10 μmol·L^{-1}. As shown in Figure 4, the basal rate of oxygen consumption was increased in adipocytes treated with lipoamide (10 μmol·L^{-1}), whereas LA at the same concentration had no effect.

Activity of mitochondrial complexes I, II and III

As shown in Figure 5, lipoamide increased the activity of mitochondrial complex I and of complex II at 1.0 and at 10 μmol·L^{-1}, but it increased the activity of mitochondrial complex III only at the higher concentration (10 μmol·L^{-1}). LA produced a significant increase in the activity of complex II at 100 μmol·L^{-1}, but had no effect on mitochondrial complex I and III activities.

mRNA abundance of PPAR-γ, PPAR-α and CPT-1α

The relative abundance of mRNA transcripts encoding for PPAR-γ, PPAR-α and CPT-1α was examined by quantitative RT-PCR (Figure 6). Lipoamide treatment at 10 μmol·L^{-1} increased the mRNA for all three proteins PPAR-γ PPAR-α and CPT-1α. LA had no effect at the same concentration (10 μmol·L^{-1}).

eNOS expression and cGMP levels

To investigate the role of endogenous NO, eNOS protein level was measured in adipocytes incubated with LA or lipoamide

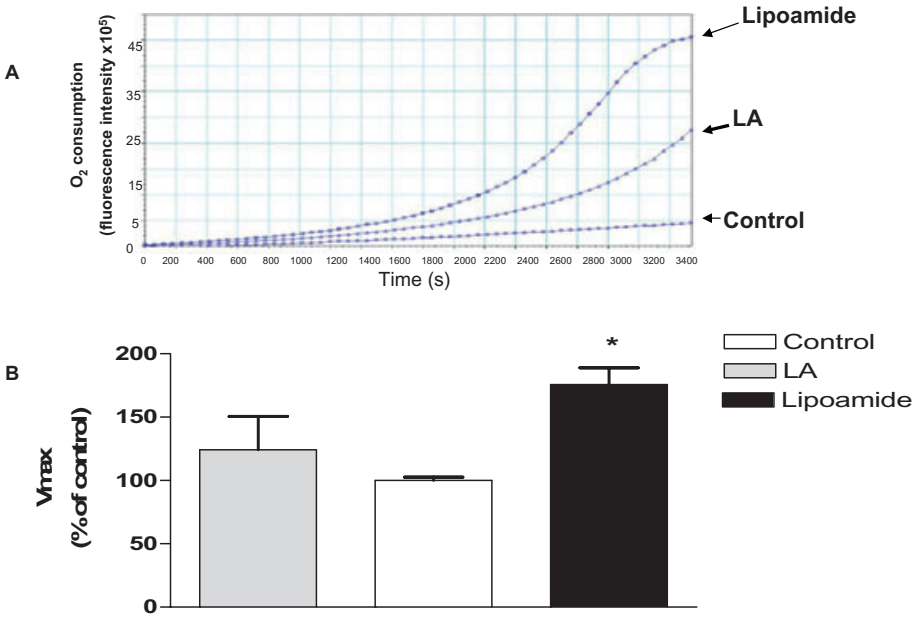

Figure 4

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 to yield representative oxygen consumption curves. (A) Representative oxygen consumption curves. (B) Quantitative changes in the respiratory rate of adipocytes during each condition were calculated by kinetic measurements. V_{max} is the maxium consumption rate. Values are mean ± SEM from three independent experiments; results are presented relative to values in untreated control cells (100%). *$P < 0.05$ versus control. LA, lipoic acid.

(Figure 7). Lipoamide over the concentration range of 0.1 to 100 μmol·L^{-1} increased eNOS expression, whereas LA had no effect on eNOS expression.

Figure 8A shows that eNOS expression decreased by 80% after transfection of adipocytes with eNOS siRNA. Transfection with scrambled siRNA, used as control siRNA, did not decrease the normal expression of eNOS in adipocytes (Figure 8A). Further, in cells with eNOS knocked down with siRNA, the effects of lipoamide (10 μmol·L^{-1}) on mtDNA content (Figure 8B) and expression of biogenesis factors PGC-1α (Figure 8C), NRF1 and TFAM (Figure 8D), were clearly reduced compared with its effects in cells after transfection with control siRNA or in untreated cells.

eNOS-cGMP-PKG signaling

Nitric oxide generated by eNOS increases mitochondrial biogenesis and enhances respiration and ATP content in various mammalian cells by acting through its second messenger, cGMP (Nisoli *et al.*, 2003; 2004). To investigate whether eNOS and cGMP play a role in the mitochondrial biogenesis induced by lipoamide, we first tested its effects on cGMP. As shown in Figure 9, lipoamide (10 μmol·L^{-1}) increased cGMP levels in adipocytes after 24 h incubation, and this increase was abolished in the presence of the eNOS inhibitor L-NAME.

Pre-treatment with L-NAME, another NOS inhibitor L-NMMA or the selective guanylate cyclase inhibitor ODQ significantly inhibited lipoamide-induced mtDNA content (Figure 10A), PGC-1α; protein (Figure 10B), and mRNA for NRF1 and TFAM (Figure 10C). Next, to determine if the cGMP-dependent protein kinase G (PKG) was mediating these effects of lipoamide on mitochondrial biogenesis, we pre-treated cells with the PKG inhibitor KT5823 (1 μmol·L^{-1}; Borniquel *et al.*, 2006) before exposure to lipoamide. KT5823 treatment blocked the lipoamide-mediated up-regulation of mtDNA, PGC-1α; and expression of NRF1 and TFAM.

Discussion

Oxidative stress and mitochondrial loss are believed to form the foundation for the induction of multiple cellular pathways that can ultimately lead to both the onset and subsequent complications of type 2 diabetes (Mootha *et al.*, 2003; Maiese *et al.*, 2007a,b). In the present study, we have shown that lipoamide stimulates mitochondrial biogenesis through the activation of a PGC-1α-dependent pathway in adipocytes. The biological activity of LA has commonly been attributed to the antioxidant properties of LA, because the reduced form of LA, dihydrolipoic acid, is a powerful mitochondrial antioxidant (Packer *et al.*, 1995; 1997a,b; Cho *et al.*, 2003), as it recycles other cellular antioxidants, including CoQ, vitamins C and E, and glutathione. Furthermore, dihydrolipoic acid is an effective transition metal chelator (Ou *et al.*, 1995), notably of iron and copper (Packer *et al.*, 1995; 1997a,b; Cho *et al.*, 2003), and LA functions as a redox regulator of proteins such as myoglobin, prolactin, thioredoxin and the transcription factor NF-kappa B (Packer *et al.*, 1995; Fuchs *et al.*, 1997; Bustamante *et al.*, 1998). Finally, LA is an inducer of phase 2 detoxifying enzymes (Suh *et al.*, 2004; Liu, 2008).

Another mechanism of LA action may be the regulation of the acetyl-CoA/CoA ratio. A high acetyl-CoA/CoA ratio in

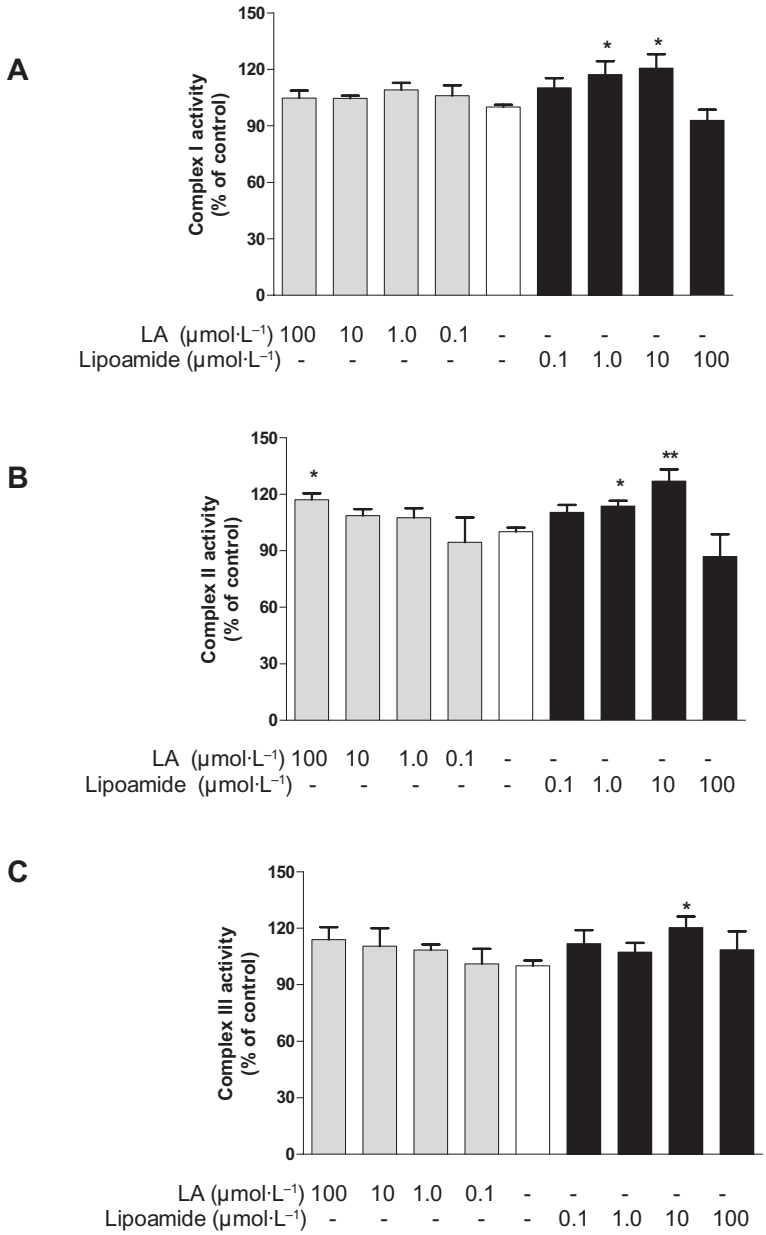

Figure 5

Effect of treatment with LA or lipoamide on activities of ETC complexes in adipocytes. (A) Complex I, (B) complex II and (C) complex III. Adipocytes were treated with different concentrations of LA or lipoamide for 24 h. Results are presented relative to values in untreated control cells (100%). Values are mean ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.01$ versus control. LA, lipoic acid.

mitochondria causes inhibition of the pyruvate dehydrogenase complex by activating pyruvate dehydrogenase kinase. LA acts as a cofactor when covalently bound to lysine residues in the second component of the α-oxo-acid dehydrogenase complex, and is known to act as an acceptor of short-chain acyl groups from acyl-CoA *in vitro* (Sumegi *et al.*, 1994).

The present study shows that LA is a promoter of mitochondrial biogenesis. This is a new mechanism for the protective effects of LA in improving complications of type 2 diabetes. From our results, lipoamide was about 10 times more effective than LA in stimulating mitochondrial biogenesis in adipocytes. Lipoamide should share the same mechanism as LA, because these two low molecular weight thiols have similar structures and the same dithiol group.

PPAR-γ plays an important role not only in adipogenesis, but also in regulating lipid metabolism in mature adipocytes (Wilson-Fritch *et al.*, 2003). Activation of PPAR-γ by glucose-lowering agents such as thiazolidinediones, high-affinity agonist ligands for PPAR-γ, led to a net flux of fatty acids from the circulation and other tissues into adipocytes (Lehrke and Lazar, 2005). PPAR-α is also known to be an important regulator of mitochondrial biogenesis and β-oxidation in tissues like heart and liver (Gulick *et al.*, 1994; Kersten *et al.*, 2000). We have shown here that PPAR-α and PPAR-γ levels were

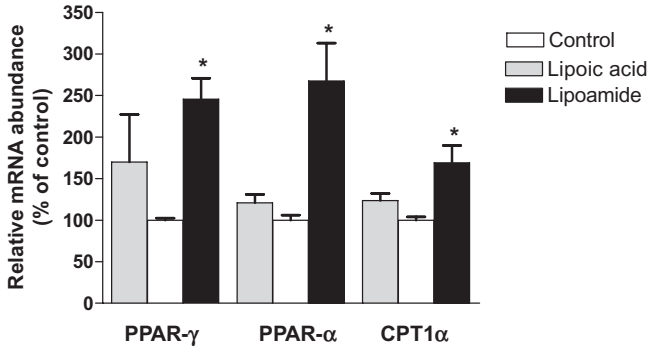

Figure 6

Effect of LA or lipoamide on expression of PPAR-γ, PPAR-α and CPT-1α mRNA in adipocytes. Adipocytes were incubated with LA or lipoamide at 10 μmol·L^{-1} for 24 h. PPPAR-γ, PPAR-α and CPT-1α 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 18S rRNA as an internal control and results expressed relative to values in untreated control cells (100%). All values are mean ± SEM of four independent experiments. *P < 0.05, **P < 0.01 versus control. LA, lipoic acid; PPAR, peroxisome proliferator-activated receptor.

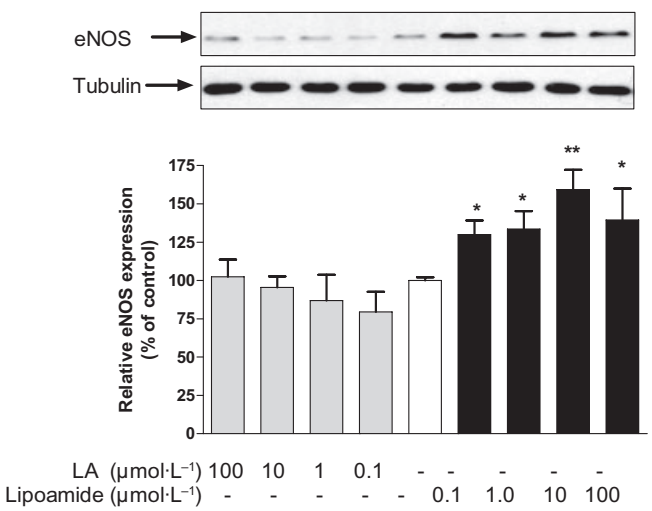

Figure 7

Effect of treatment with LA or lipoamide on eNOS expression. Representative Western blot image (upper) and quantification (below) of eNOS protein expression of adipocytes incubated with LA or lipoamide (10 μmol·L^{-1}) for 24 h. Results are presented relative to values in untreated control cells (100%). Values are mean ± SEM from three independent experiments. *P < 0.05 versus control. LA, lipoic acid.

up-regulated by lipoamide in 3T3-L1 adipocytes. This up-regulation closely correlates with the stimulation of mitochondrial biogenesis and induction of CPT-1α involved in fatty acid oxidation. In addition, PPAR-γ was well recognized as a key regulator of adipogenic differentiation. Because we found that lipoamide could increase PPAR-γ mRNA abundance, this suggests that lipoamide could increase adipocyte differentiation.

eNOS-dependent NO production induces mitochondrial biogenesis, with concomitant increases in PGC-1α, NRF1, and TFAM expression, oxygen consumption and ATP production in adipose and muscle cells (Nisoli *et al.*, 2003; 2004). We have demonstrated that inhibition of NO synthesis prevented lipoamide-induced mitochondrial biogenesis in adipocytes, suggesting that NO has an autocrine function in mediating the effects of lipoamide in adipocytes. Accordingly, we also found that genetic lack of eNOS prevented lipoamide-induced mitochondrial biogenesis and the up-regulation of mitochondrial biogenesis factors in differentiated 3T3-L1 adipocytes. The up-regulation of eNOS in adipocytes by lipoamide has been previously reported (Nisoli *et al.*, 2004; Borniquel *et al.*, 2006).

Once NO is produced in a cell, it initiates biological effects through the activation of soluble guanylate cyclase, leading to formation of cGMP. This in turn activates mechanisms involving cGMP-dependent protein kinases, thereby affecting the functions of target proteins. In our study, lipoamide was found to increase the cGMP levels in adipocytes. Therefore, it seems that NO-mediated mitochondrial biogenesis is dependent on cGMP. This assumption was examined by studying the effects of the selective guanylate cyclase inhibitor ODQ. Treatment with lipoamide was found to mimic the effects of NO, increased mtDNA content, mitochondrial ETC complex expression levels, PGC-1α protein levels, and NRF1 and TFAM mRNA levels. Pretreatment with the eNOS inhibitor L-NAME, the NOS inhibitor L-NMMA, the selective guanylate cyclase inhibitor ODQ, or the PKG inhibitor KT5823, significantly inhibited lipoamide-induced mtDNA content, and lowered levels of PGC-1α protein and NRF1 and TFAM mRNA. Thus, the stimulation of mitochondrial biogenesis by lipoamide may depend on the eNOS-cGMP-PKG pathway.

Another important finding is that lipoamide is about 10–100-fold more potent than LA for stimulating biogenesis and improving mitochondrial function. Lipoamide is a better substrate of lipoamide dehydrogenase than LA, and dihydrolipoamide is a much better substrate for the E2 component of the α-keto-acid dehydrogenase complexes than dihydrolipoic acid (Sumegi *et al.*, 1994). The greater protection provided by lipoamide compared with LA could possibly be attributed to the presence of –$CONH_2$ rather than –COOH. This substitution makes lipoamide relatively more stable than LA owing to the resonance stabilization of the CO-N, and much less acidic to match better to physiological pH. Its higher lipid solubility leads to better cellular penetration. Moreover, the amine group may have other possible physiological reactions, such as hydrolysis, Hofmann rearrangement to a primary amine, attack by thiol, hydroxyl, or amine that results in a thiacyclol, an oxacyclol or an azacyclol respectively.

It should be pointed out that the greater effectiveness of lipoamide over LA might be related to a greater toxicity than LA. As a therapeutic agent, the toxicity of lipoamide should be an important consideration, and low and safe doses should be used. Because lipoamide is about 10 times more effective than LA, a 10-fold lower dose than LA can be used to reduce its toxicity and guarantee its safety. Nevertheless, the toxicity of lipoamide should be further and carefully studied in the future.

In conclusion, we found that lipoamide was more effective than LA in stimulating mitochondrial biogenesis in

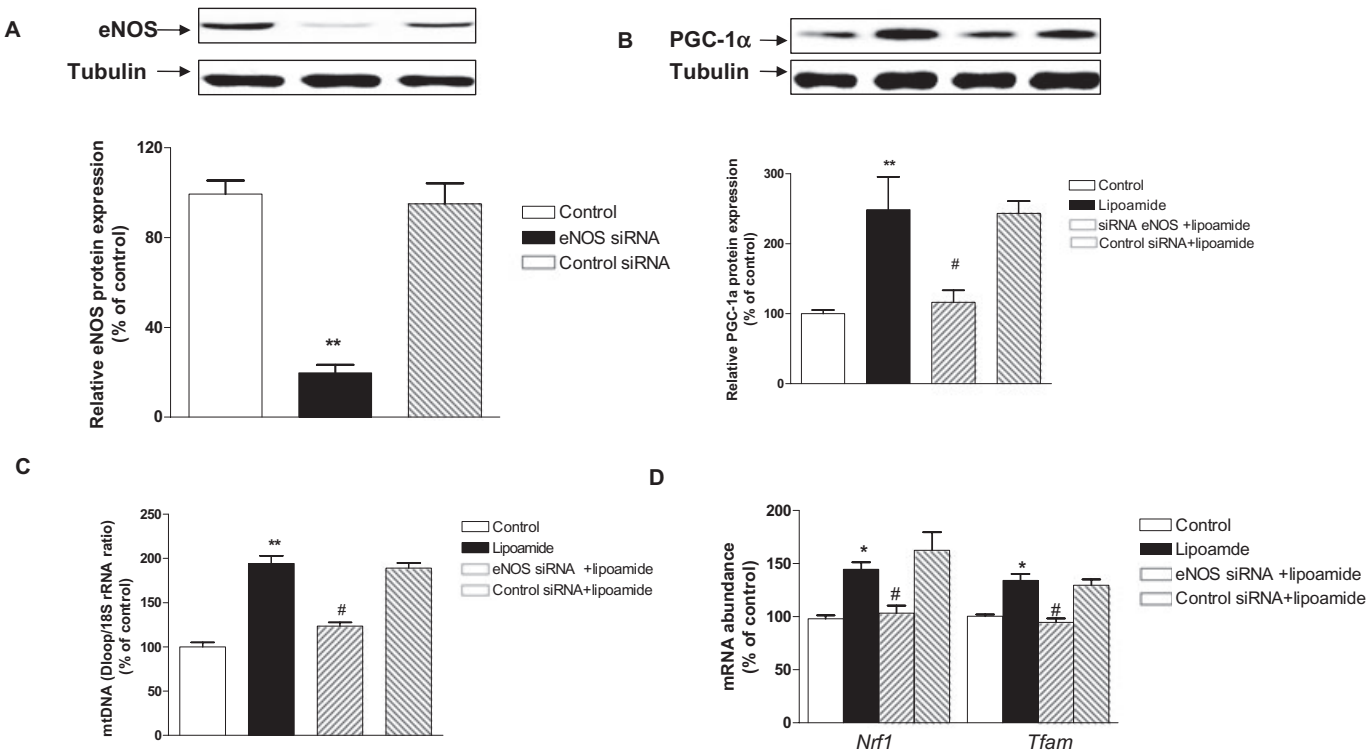

Figure 8

Knockdown of eNOS with siRNA prevented lipoamide-induced up-regulation of mitochondrial biogenesis factors. (A) 3T3-L1 adipocytes were transfected with control siRNA or eNOS siRNA. Representative Western blot image (upper) and quantification (lower) of eNOS protein expression of adipocytes after transfection for 48 h; (B) After transfection for 24 h, cells were incubated with lipoamide (10 μmol·L^{-1}) for 24 h. Representative Western blot image (upper) and quantification (below) of PGC-1α protein expression; (C) mtDNA contents and (D) Nrf1 and Tfam mRNA abundance. Values are mean ± SEM from three independent experiments. $^{**}P < 0.05$ and $^{**}P < 0.01$ versus control and $\#P < 0.05$ versus lipoamide alone. LA, lipoic acid; NRF1, nuclear related factor 1; PGC-1α, PPAR-γ co-activator-1α; PPAR, peroxisome proliferator-activated receptor; TFAM, mitochondrial transcription factor A.

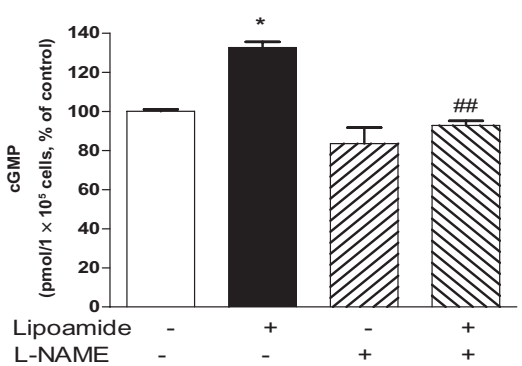

Figure 9

Effect of treatment with lipoamide on cGMP levels. Adipocytes were pre-incubated with L-NAME (100 μmol·L^{-1}), then stimulated with lipoamide (10 μmol·L^{-1}) for 24 h. Values represent mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus untreated cells, and $\#\#P < 0.01$ versus lipoamide alone or SNP alone. L-NAME, N^{G}-nitro-L- arginine methyl ester. LA, lipoic acid; L-NAME, N^{G}-nitro-L-arginine methyl ester; NRF1, nuclear related factor 1; PGC-1α, PPAR-γ co-activator-1α; PPAR, peroxisome proliferator-activated receptor; TFAM, mitochondrial transcription factor A.

adipocytes, and that the stimulation of mitochondrial biogenesis by lipoamide depended on the eNOS-cGMP-PKG pathway. These data suggest that lipoamide is a potent stimulator of mitochondrial biogenesis, and may have potential therapeutic application in obesity and diabetes.

Acknowledgements

The authors thank Lu Yang and Jinmin Ren for technical assistance, and Dr Edward Sharman for critical reading of this manuscript. This study was supported by Pujiang Talent Award (05PG14104) and a grant (074319105) from the Shanghai Science and Technology Committee, Shanghai, China, the National Natural Science Foundation of China (Key Program 30930105; General Program 30873058, 30770785, 30871002, and 81070325), the National Basic Research Program of China (973 Program 2007CB512005) and a support of 985 and 211 projects by Xi'an Jiaotong University.

Conflicts of interest

A patent has been filed on the compound (lipoamide) mentioned in this paper: Chinese patent application No.

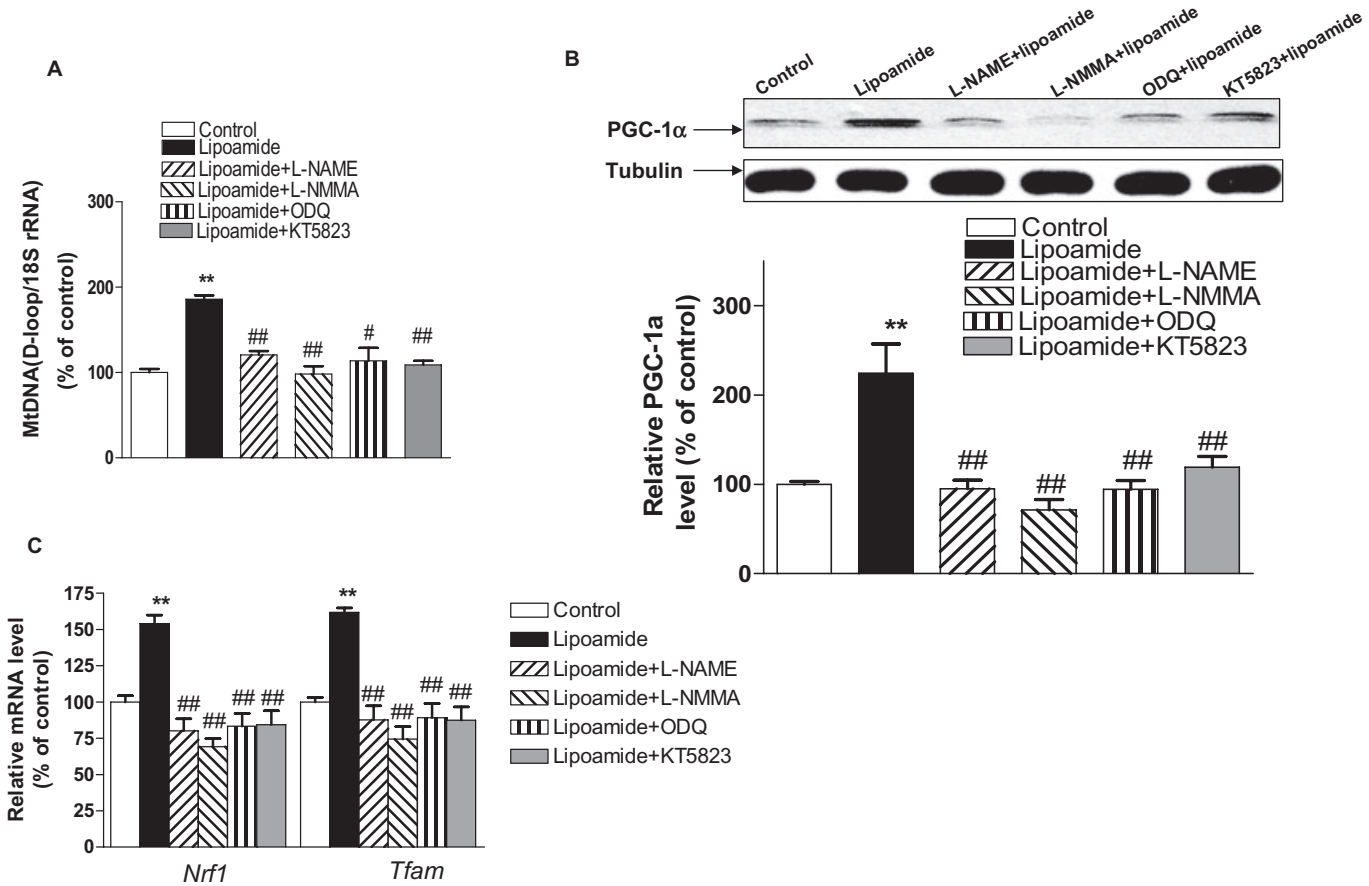

Figure 10

Lipoamide-regulated mitochondrial biogenesis through an eNOS-cGMP-PKG-dependent mechanism. Adipocytes were pre-incubated with L-NAME (100 μmol·L^{-1}), L-NMMA (100 μmol·L^{-1}), ODQ (10 μmol·L^{-1}) or KT5823 (1 μmol·L^{-1}) for 1 h, then stimulated with lipoamide (10 μmol·L^{-1}) for 24 h. (A) mtDNA contents. mtDNA and 18S rRNA gene (18S rRNA) levels were calculated from a standard curve, and the relative ratios of mtDNA contents against 18S rRNA gene levels were determined in five independent experiments. (B) PGC-1α expression. PGC-1α protein was analyzed by immunoblotting. Upper: representative Western blot image; below: quantification of the PGC-1α density against that of tubulin. (C) NRF1 and TFAM mRNA were measured by qRT-PCR. Results are presented relative to values in untreated control cells (100%). Data are mean ± SEM of three independent experiments. *$P < 0.05$ and **$P < 0.01$ versus control; #$P < 0.01$ versus lipoamide. LA, lipoic acid; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-methyl L-arginine; NRF1, nuclear related factor 1; PGC-1α, PPAR-γ co-activator-1α; PPAR, peroxisome proliferator-activated receptor; TFAM, mitochondrial transcription factor A.

200710046615.8. There are no other conflicts of interest to declare.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Bisby RH, Parker AW (1998). Antioxidant reactions of dihydrolipoic acid and lipoamide with triplet duroquinone. Biochem Biophys Res Commun 244: 263–267.

Borniquel S, Valle I, Cadenas S, Lamas S, Monsalve M (2006). Nitric oxide regulates mitochondrial oxidative stress protection via the transcriptional coactivator PGC-1alpha. Faseb J 20: 1889–1891.

Boudina S, Sena S, O'Neill BT, Tathireddy P, Young ME, Abel ED (2005). Reduced mitochondrial oxidative capacity and increased mitochondrial uncoupling impair myocardial energetics in obesity. Circulation 112: 2686–2695.

Bruckner I, Bustan C, Adamescu E, Dobjanschi C (2002). Diabetic neuropathy – choices of treatment. Rom J Intern Med 40: 53–60.

Bustamante J, Lodge JK, Marcocci L, Tritschler HJ, Packer L, Rihn BH (1998). Alpha-lipoic acid in liver metabolism and disease. Free Radic Biol Med 24: 1023–1039.

Cheng TH, Chen JJ, Shih NL, Lin JW, Liu JC, Chen YL *et al.* (2009). Mechanical stretch induces endothelial nitric oxide synthase gene expression in neonatal rat cardiomyocytes. Clin Exp Pharmacol Physiol 36: 559–566.

Cho KJ, Moon HE, Moini H, Packer L, Yoon DY, Chung AS (2003). Alpha-lipoic acid inhibits adipocyte differentiation by regulating pro-adipogenic transcription factors via mitogen-activated protein kinase pathways. J Biol Chem 278: 34823–34833.

Choi YS, Ryu BK, Min HK, Lee SW, Pak YK (2005). Analysis of proteome bound to d-loop region of mitochondrial DNA by DNA-linked affinity chromatography and reverse-phase liquid chromatography/tandem mass spectrometry. Ann N Y Acad Sci 1042: 88–100.

Civitarese AE, Ukropcova B, Carling S, Hulver M, DeFronzo RA, Mandarino L *et al*. (2006). Role of adiponectin in human skeletal muscle bioenergetics. Cell Metab 4: 75–87.

Da Ros R, Assaloni R, Ceriello A (2005). Molecular targets of diabetic vascular complications and potential new drugs. Curr Drug Targets 6: 503–509.

Evans JL, Goldfine ID (2000). Alpha-lipoic acid: a multifunctional antioxidant that improves insulin sensitivity in patients with type 2 diabetes. Diabetes Technol Ther 2: 401–413.

Fuchs J, Packer L, Zimmer G (1997). Lipoic Acid in Health and Disease. Marcel Dekker, Inc.: New York.

Gulick T, Cresci S, Caira T, Moore DD, Kelly DP (1994). The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. Proc Natl Acad Sci USA 91: 11012–11016.

Kanazawa A, Nishio Y, Kashiwagi A, Inagaki H, Kikkawa R, Horiike K (2002). Reduced activity of mtTFA decreases the transcription in mitochondria isolated from diabetic rat heart. Am J Physiol Endocrinol Metab 282: E778–E785.

Kersten S, Desvergne B, Wahli W (2000). Roles of PPARs in health and disease. Nature 405: 421–424.

Lehrke M, Lazar MA (2005). The many faces of PPARgamma. Cell 123: 993–999.

Liu J (2008). The effects and mechanisms of mitochondrial nutrient alpha-lipoic acid on improving age-associated mitochondrial and cognitive dysfunction: an overview. Neurochem Res 33: 194–203.

Liu J, Ames BN (2005). Reducing mitochondrial decay with mitochondrial nutrients to delay and treat cognitive dysfunction, Alzheimer's disease, and Parkinson's disease. Nutr Neurosci 8: 67–89.

Maiese K, Chong ZZ, Shang YC (2007a). Mechanistic insights into diabetes mellitus and oxidative stress. Curr Med Chem 14: 1729–1738.

Maiese K, Morhan SD, Chong ZZ (2007b). Oxidative stress biology and cell injury during type 1 and type 2 diabetes mellitus. Curr Neurovasc Res 4: 63–71.

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J *et al*. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 34: 267–273.

Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C *et al*. (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science 299: 896–899.

Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M *et al*. (2004). Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proc Natl Acad Sci USA 101: 16507–16512.

Ou P, Tritschler HJ, Wolff SP (1995). Thioctic (lipoic) acid: a therapeutic metal-chelating antioxidant? Biochem Pharmacol 50: 123–126.

Packer L, Witt EH, Tritschler HJ (1995). alpha-Lipoic acid as a biological antioxidant. Free Radic Biol Med 19: 227–250.

Packer L, Roy S, Sen CK (1997a). Alpha-lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. Adv Pharmacol 38: 79–101.

Packer L, Tritschler HJ, Wessel K (1997b). Neuroprotection by the metabolic antioxidant alpha-lipoic acid. Free Radic Biol Med 22: 359–378.

Persson HL, Svensson AI, Brunk UT (2001). Alpha-lipoic acid and alpha-lipoamide prevent oxidant-induced lysosomal rupture and apoptosis. Redox Rep 6: 327–334.

Reed LJ, Koike M, Levitch ME, Leach FR (1958). Studies on the nature and reactions of protein-bound lipoic acid. J Biol Chem 232: 143–158.

Shen W, Liu K, Tian C, Yang L, Li X, Ren J *et al*. (2008). R-alpha-Lipoic acid and acetyl-L: -carnitine complementarily promote mitochondrial biogenesis in murine 3T3-L1 adipocytes. Diabetologia 51: 165–174.

Stevens MJ, Obrosova I, Cao X, Van Huysen C, Greene DA (2000). Effects of DL-alpha-lipoic acid on peripheral nerve conduction, blood flow, energy metabolism, and oxidative stress in experimental diabetic neuropathy. Diabetes 49: 1006–1015.

St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S *et al*. (2006). Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 127: 397–408.

Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu RM *et al*. (2004). Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci USA 101: 3381–3386.

Sumegi B, Butwell NB, Malloy CR, Sherry AD (1994). Lipoamide influences substrate selection in post-ischaemic perfused rat hearts. Biochem J 297: 109–113.

Sun L, Luo C, Long J, Wei D, Liu J (2006). Acrolein is a mitochondrial toxin: effects on respiratory function and enzyme activities in isolated rat liver mitochondria. Mitochondrion 6: 136–142.

Tedesco L, Valerio A, Cervino C, Cardile A, Pagano C, Vettor R *et al*. (2008). Cannabinoid type 1 receptor blockade promotes mitochondrial biogenesis through endothelial nitric oxide synthase expression in white adipocytes. Diabetes 57: 2028–2036.

Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S *et al*. (2003). Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. Mol Cell Biol 23: 1085–1094.

Wilson-Fritch L, Nicoloro S, Chouinard M, Lazar MA, Chui PC, Leszyk J *et al*. (2004). Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone. J Clin Invest 1281–1289.