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# Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3-L1 adipocytes

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**Abstract**

Hydroxytyrosol (HT) in extra-virgin olive oil is considered one of the most important polyphenolic compounds responsible for the health benefits of the Mediterranean diet for lowering incidence of cardiovascular disease, the most common and most serious complication of diabetes. We propose that HT may prevent these diseases by a stimulation of mitochondrial biogenesis that leads to enhancement of mitochondrial function and cellular defense systems. In the present study, we investigated effects of HT that stimulate mitochondrial biogenesis and promote mitochondrial function in 3T3-L1 adipocytes. HT over the concentration range of 0.1–10 μmol/L stimulated the promoter transcriptional activation and protein expression of peroxisome proliferator-activated receptor (PPAR) coactivator 1 alpha (PPARGC1α, the central factor for mitochondrial biogenesis) and its downstream targets; these included nuclear respiration factors 1 and 2 and mitochondrial transcription factor A, which leads to an increase in mitochondrial DNA (mtDNA) and in the number of mitochondria. Knockdown of *Ppargc1α* by siRNA blocked HT's stimulating effect on Complex I expression and mtDNA copy number. The HT treatment resulted in an enhancement of mitochondrial function, including an increase in activity and protein expression of Mitochondrial Complexes I, II, III and V; increased oxygen consumption; and a decrease in free fatty acid contents in the adipocytes. The mechanistic study of the PPARGC1α activation signaling pathway demonstrated that HT is an activator of 5′AMP-activated protein kinase and also up-regulates gene expression of PPARα, CPT-1 and PPARγ. These data suggest that HT is able to promote mitochondrial function by stimulating mitochondrial biogenesis.


*Keywords:* 5′AMP-activated protein kinase (AMPK); Fatty acid oxidation; Mitochondrial transcription factor A (Tfam); Mitochondrial DNA (mtDNA); Nuclear respiration factors 1 and 2 (Nrf1 and Nrf2); Peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1α)

## 1. Introduction

Mitochondrial dysfunction plays a central role in a wide range of age-associated disorders and various forms of cancer [1], as well as type 2 diabetes [2]. Increasing evidence shows that mitochondrial metabolism and ATP synthesis decline in concert with a reduction of key factors regulating mitochondrial biogenesis in patients with insulin resistance, type 2 diabetes and obesity [3–6]. Key factors regulating this process include peroxisome proliferator-activated receptor (PPAR) coactivator 1 alpha (PPARGC1α) and the nuclear respiratory factors (Nrfs). It also has been shown that a reduction of mitochondrial DNA (mtDNA) copy number in adipose tissue from diabetic volunteers and treatment with thiazolidinedione (TZD), an insulin-sensitizing drug currently used in treating type 2 diabetes, restored diminished mtDNA content and expression of genes involved in mitochondrial biogenesis and fatty acid oxidation [6,7]. Therefore, with the emerging evidence that mitochondrial dysfunction is associated with various diseases, it has been suggested that promoting mitochondrial biogenesis, just as improving adipocyte metabolism, could be a strategy for preventing and reversing various diseases, including cardiovascular disease, cancer, insulin resistance, obesity and diabetes [2,8–10].

The Mediterranean diet has been associated with a lower incidence of certain cancers and of cardiovascular disease, which is the most common and serious complication of diabetes [11–13]. Olive oil is the principal source of fats in the Mediterranean diet, and hydroxytyrosol (HT), a polyphenolic constituent of extra-virgin olive oil, is considered to be one of the most potent determinants of its efficacy [14–17]. Studies on the mechanism of HT's action have focused on its antioxidant properties so far [14,18,19]. We have recently shown that HT protects retinal pigment epithelial cells from

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acrolein-induced oxidative damage and mitochondrial dysfunction by inducing detoxifying Phase II enzymes [20]. Based on our hypothesis and recent results [21,22] that mitochondrial nutrients can improve mitochondrial function through stimulating mitochondrial biogenesis, we hypothesize that the Mediterranean diet or supplementation with HT could stimulate mitochondrial function and prevent diabetes and obesity-related mitochondrial dysfunction, thus reducing the risk of cardiovascular disease. Therefore, in the present study, we determined whether treatment of 3T3-L1 adipocytes with HT could improve mitochondrial function by stimulating mitochondrial biogenesis.

It has been observed that the expression of regulatory factors for mitochondrial biogenesis was reduced in adipose tissues of diabetic and obese subjects [3,6]. Therefore, we used adipocytes as a model to study the effects of HT. In the present study, we first examined the effect of HT on the protein expression of PPARGC1α, the key regulator of mitochondrial biogenesis, and of its downstream targets, nuclear respiration factors 1 and 2 (Nrf1 and Nrf2) and mitochondrial transcription factor A (Tfam). Second, we examined mtDNA; protein expression of Mitochondrial Complexes I, II, III, IV and V; and mitochondrial mass/numbers. Third, we monitored the expression of proteins or genes related to fatty acid oxidation, adipogenesis and mitochondrial function, including activities of Mitochondrial Complexes I, II, III, IV and V; oxygen consumption; and free fatty acid (FFA) content. Fourth, we investigated the effects of HT on signaling pathways involving phosphorylation of 5′AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC).

## 2. Materials and methods

### 2.1. Materials

The AMPK activator, 5-amino-imidazole-4-carboxamide-riboside (AICAR), was from Sigma (St. Louis, MO); anti-phospho-(Thr 172)-AMPK (pAMPK), total AMPK (tAMPK), anti-phospho-ACC (Ser79) (pACC) and total ACC (tACC) were from Cell Signaling Technology, Inc. (Beverly, MA); anti-OxPhos Complexes I, II, III, IV and V were from Invitrogen (Carlsbad, CA); anti-α-tubulin and anti-β-actin were from Sigma; anti-PPARGC1α (Santa Cruz, Heidelberg, Germany); the Reverse Transcription System Kit was from Promega (Mannheim, Germany); HotStarTaq was from Takara (Otsu, Shiga, Japan); primers were synthesized by Bioasia Biotech (Shanghai, China); and TRIzol and other reagents for cell culture were from Invitrogen. HT was from DSM Nutritional Products Ltd., Switzerland, and used in all experiments.

### 2.2. Cell culture and differentiation

Cell culture and differentiation of 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 program 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 [23]. In the present study, murine 3T3-L1 pre-adipocytes (American Type Culture Collection, Manassas, VA) 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 induction of differentiation and when 90% exhibited the adipocyte phenotype.

### 2.3. Transient transfection and promoter activity assay

A 2-kb *Ppargc1α* promoter in pGL3-basic luciferase reporter construct was a gift from Dr. X. Ge (Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China). Cells were fully differentiated as described above and then seeded in 24-well plates at about 80% confluence and grown overnight. The cells were transiently transfected with pGL3-*Ppargc1α* or pGL3-basic plasmid using the Cell Line Nucleofector Kit from Amaxa (Gaithersburg, MD) following the manufacturer's instructions. The Renilla vector was used to monitor the transfection efficiency. The transfected cells were cultured for 18–20 h and then incubated with HT (1 μmol/L) for 24 h. Cells were lysed, and the reporter activity was measured by a luciferase assay kit (KenReal, Shanghai, China) with a luminometer (Berthold Technologies, Bad Wildbad, Germany). The relative Luc activity was calculated as the ratio of firefly Luc activity to Renilla luc activity. Transfections were performed in duplicate and repeated at least three times.

### 2.4. Western blot analysis

After treatment with HT, cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed in sample buffer (62.5 mmol/L Tris–Cl, pH 6.8, 2% SDS and 5 mmol/L dithiothreitol) at room temperature and vortexed. Cell lysates were then boiled for 5 min and cleared by centrifugation (13,000 rpm, 10 min at 4°C). Protein concentrations were determined using the Bio-Rad DC protein assay. The soluble lysates (10 μg per lane) were subjected to 10% SDS-PAGE; proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk/TBST for 1 h at room temperature. Membranes were incubated with primary antibodies directed against anti-α-tubulin (1:5000), PPARGC1α (1:1000), phospho-(Thr 172)-AMPK (1:1000), tAMPK (1:1000), phospho-ACC (Ser79) (1:1000), tACC (1:1000), anti-OxPhos Complex I (NADH ubiquinone oxidoreductase 39-kDa subunit, 1:2000), anti-OxPhos Complex II (succinate–ubiquinone oxidoreductase 70-kDa subunit, 1:2000), anti-OxPhos Complex III (ubiquinol–cytochrome *c* oxidoreductase core II 50 kDa, 1:2000) or anti-OxPhos Complex V (ATP synthase, 53 kDa) in 5% milk/TBST at 4°C overnight. 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 ECL (Roche, Mannheim, Germany) and quantified by scanning densitometry [24].

### 2.5. 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 oligodT15, 25 U AMV reverse transcriptase and 10× RT buffer to give 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*, *Cpt1a*, *Ppara*, *Pparg* and 18S rRNA mRNAs were the same as those published previously [22]. Quantitative PCR was performed using Mx3000P (see above). Each quantitative PCR was performed in triplicate. The mouse 18S rRNA 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 $2^{CT}$ (using CT as a base 2 exponent) for each target gene was normalized to that for 18S rRNA, followed by comparison with the relative value in control cells. Final results are presented as percentage of control.

### 2.6. RT-PCR for mtDNA

Total DNA and mtDNA were 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 the mitochondrial D-loop as an mtDNA target [22]. 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′-GACTAATGATTCTTCACCGT; 18S rRNA: forward, 5′-CATTCGAACGTCTGCCCTATC-3′; reverse, 5′-CCTGCTGCCTTCCTTGGA-3′. The mouse 18S rRNA gene served as an endogenous reference gene. Melting curves were performed 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.

### 2.7. Cell respiration test

Oxygen consumption by intact cells was measured as an indication of mitochondrial respiration activity [22]. The BD Oxygen Biosensor System utilizes 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 media. Oxygen quenches the dye in a concentration-dependent manner. The fluorescence correlates directly to oxygen consumption in the well. This unique technology allows homogenous instantaneous detection of oxygen levels. After treatment, adipocytes were washed in Krebs–Ringer solution buffered with HEPES (KRH) buffer plus 0.1% bovine serum albumin (BSA). Cells from each condition were divided into aliquots in a BD Oxygen Biosensor System plate (BD Biosciences) in triplicate. The number of cells contained in equal volumes did not differ statistically among samples treated with various nutrients and nutrient concentrations. Plates were sealed and "read" on a fluorescence spectrometer (Molecular Probes, Sunnyvale, CA) at 1-min intervals for 60 min at an excitation of

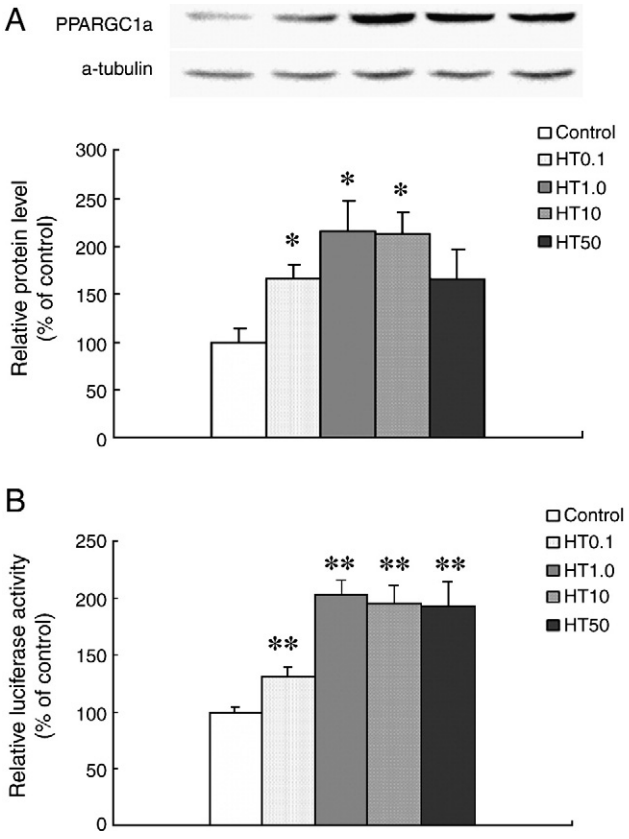


Fig. 1. Effect of HT treatments on protein expression and transcriptional activation of *PPARGC1α*. 3T3-L1 adipocytes were treated for 48 h with HT at concentrations of 0.1, 1, 10 and 50 μmol/L. Cells were subsequently solubilized into SDS sample buffer and analyzed by Western blotting with antibodies against α-tubulin and PPARGC1α. (A) Upper panel: Immunoblots for representative samples of steady-state levels of proteins; lower panel: Quantitative values (in percentage) were tabulated for PPARGC1α:α-tubulin ratios determined by densitometry. (B) Relative luciferase reporter activity. Values are mean±S.E. of the results from four independent experiments. *$P$≤.05 versus control (without HT treatment).

485 nm and emission of 630 nm [25]. Results are expressed as the slope of time-varying fluorescence intensity.

### 2.8. Mitochondrial mass analysis

The fluorescent probe MitoTracker Green FM (Molecular Probes, Eugene, OR) was used to determine the mitochondrial mass of adipocytes [22]. In brief, adipocytes treated with HT for 48 h were trypsinized and centrifuged at 3000×*g* at 4°C for 5 min, resuspended in KRH buffer 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 3000×*g* at 4°C for 5 min and resuspended in 400 μl of fresh KRH buffer. Fluorescence was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA).

### 2.9. Electron microscopic observation

3T3-L1 adipocytes on Day 8 of differentiation were seeded on glass coverslips. On Day 9, cells were treated with HT (1.0 μmol/L) for 48 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 ethanol series, they were washed with propylene oxide and embedded in Spurr's low viscosity resin. Silver to gold sections were cut and examined using a Philips CM 10 (Eindhoven, the Netherlands) transmission electron microscope at a 60-kV accelerating voltage [26]. Measurements were made on six individual adipocytes treated with or without HT. For each individual adipocyte in each image, the number of mitochondria and the total mitochondrial sectional area were determined. All electron microscopic photographs were analyzed by observers blind with respect to treatments [22].

### 2.10. 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 and 20 mmol/L HEPES, pH 7.2) and homogenized on ice with a 2-ml glass homogenizer (Dounce, Fisher Scientific, Pittsburgh, PA). The mitochondria were then purified by differential centrifugation at 1300×*g* for 5 min to pellet unbroken cells and the nuclei. The supernatant was then centrifuged at 13,000×*g* for 10 min to pellet the mitochondria. The pellet was resuspended in EGTA-free isolation buffer [22].

### 2.11. Assays for activities of Mitochondrial Complexes I, II, III, IV and V

Adipocytes were cultured in 100-mm plates, washed in PBS, resuspended in an appropriate isotonic buffer (0.25 M sucrose, 5 mM Tris–HCl, pH 7.5, and 0.1 mM phenylmethylsulfonyl fluoride) and homogenized. Mitochondria were isolated by differential centrifugation of the cell homogenates. NADH–CoQ oxidoreductase (Complex I) activity was tested by monitoring the reduction of 2,6-dichlorophenol indophenol at 600 nm upon addition of assay buffer (finally, 0.05 M Tris–HCl, pH 8.1, 0.1% BSA (w/v), 1 mM antimycin A, 0.2 mM $NaN_3$ and 0.05 mM coenzyme Q1) [27]. Assays of succinate–CoQ oxidoreductase (Complex II), CoQ–cytochrome *c* reductase (Complex III) and cytochrome *c* oxidase (Complex IV) were performed spectrometrically using conventional assays [27] with minor modifications. Complex V (ATP synthase) activity was measured as oligomycin-sensitive, $Mg^{2+}$-ATPase activity [27].

### 2.12. Determination of FFA and glycerol content in the cell culture supernatant

Adipocytes cultured in six-well plates were stimulated with 1.0 μM HT for 72 h; then, the FFA content of the supernatant was estimated by a commercially available FFA kit (Jiancheng Biochemical Inc.). The test is based on the reaction of FFAs with $Cu^{2+}$ to form copper salt, which is detected photometrically by absorbance at 440 nm. The glycerol content of the supernatant was determined by a free glycerol reagent kit from Sigma (Cat #F6428). In brief, 10-μl samples were added to wells of 96-well plates, 200 μl of glycerol reagent was pipetted into each well and all samples were incubated

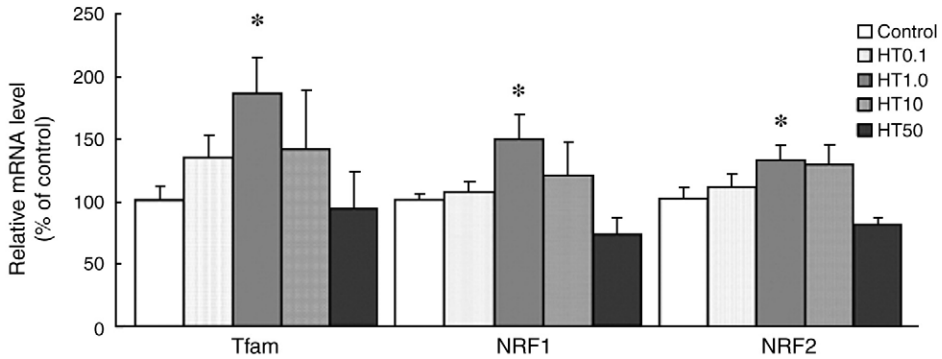


Fig. 2. Effect of HT treatments on mRNA levels of *Tfam*, *Nrf1* and *Nrf2* in adipocytes. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L. mRNA levels of *Tfam*, *Nrf1* and *Nrf2* were analyzed by quantitative RT-PCR with gene-specific oligonucleotide probes in adipocytes. The cycle number at which the various transcripts were detectable was compared with that of 18S rRNA as an internal control. Results were expressed as percentage of untreated control cells. Values are mean±S.E. of the results from at least four independent experiments. *$P$≤.05 versus control cells without HT treatment.

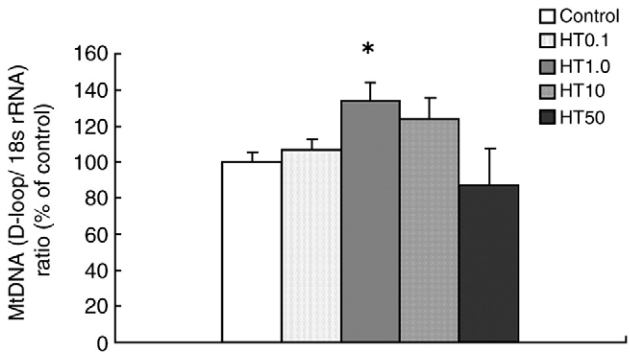


Fig. 3. Effect of HT treatments on expression of mtDNA. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L. DNA was isolated and PCR products were quantified using SYBR Green fluorescence. Quantitative values (in percentage) were tabulated for D-loop:18S rRNA ratios. Values are mean±S.E. of the results from seven independent experiments. *$P$<.05 versus control without HT taken as 100%.

for 5 min at 37°C. The absorbance of each sample was spectrophotometrically measured at 540 nm, with water as reference, with three samples per condition.

### 2.13. Determination of triglycerides using Oil Red O staining

After the induction of differentiation, adipocytes cultured in 24-well plates were stimulated with HT (1.0 μM) for 72 h. Cells were washed twice with PBS and fixed with 10% formalin in PBS for 1 h. After being washed three times with PBS, cells were stained with Oil Red O (six parts of 0.6% Oil Red O dye in isopropanol and four parts of water) for 1 h, and the excess of the stain was removed by washing with water. Then, stained cells were put in a fume cabinet until they are dried, and the stained oil droplets were dissolved in isopropanol containing 4% Nonidet P-40 overnight. The triglycerides in adipocytes were quantified by measuring the absorbance at 520 nm.

### 2.14. Construction of RNAi adenovirus and transient transfection

The siRNA targeting of *Ppargc1α* has been described previously [28]. The selected sequence was screened using a BLAST search to ensure that only the *Ppargc1α* gene was targeted. The double-strand oligonucleotides used in the present study were as follows: sense, 5′-GATCCGGTGGATTGAAGTGGTGTAGttcaagagaCTACACCACTTCAATCCACCTTTTTCTGCAGG-3′; antisense, 5′-AATTCCTGCAGAAAAAAGGTGGATTGAAGTGGTGTAGtctcttgaaCTACACCACT TCAATCCACCG-3′. 3T3-L1 adipocytes were seeded into six-well plates at about 80% confluence the day before virus infection. Cells were then incubated with recombinant virus (Ad-siRNA-*Ppargc1α* or Ad-siRNA-control) at a concentration of $2\times10^4$ virus particles per cell. After incubation for 2 h, fresh growth medium was added and cells were further cultured for 4 h and then stimulated with HT (1.0 μmol/L) for 48 h.

### 2.15. Statistical analysis

All data are representative of at least three independent experiments. Data are presented as means±S.E.M. Statistical significance was calculated by SPSS 10.0 software using one-way ANOVA, with $P$ values <.05 considered significant.

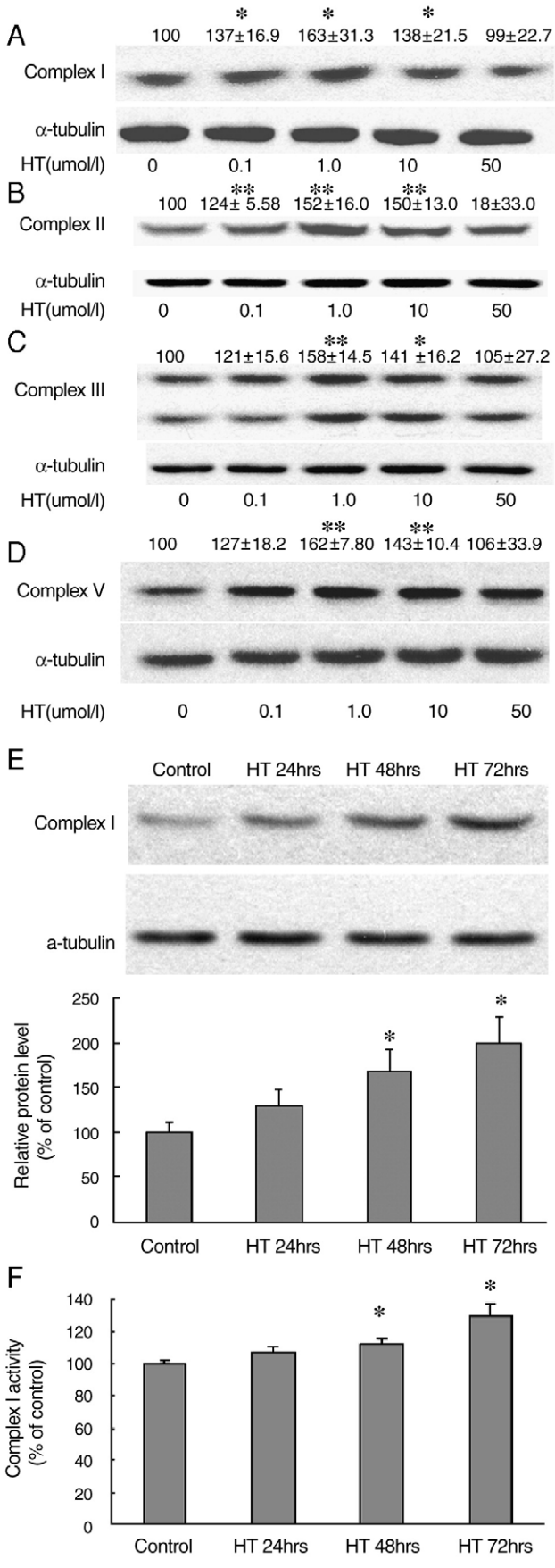


Fig. 4. Effect of HT treatments on protein expression of mitochondrial complexes in adipocytes. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L. Cells were subsequently solubilized into SDS sample buffer and analyzed by Western blotting with antibodies against α-tubulin and complex antibodies. (A) Anti-OxPhos Complex I (NADH ubiquinone oxidoreductase 39-kDa subunit, 1:2000); (B) anti-OxPhos Complex II (succinate–ubiquinone oxidoreductase 70-kDa subunit, 1:2000); (C) anti-OxPhos Complex III (ubiquinol–cytochrome *c* oxidoreductase core II 50 kDa, 1:2000); (D) anti-OxPhos Complex V (ATP synthase, 53 kDa). Representative immunoblots of steady-state levels of proteins are shown for Complexes I, II, III and V. Quantitative values (in percentage) were tabulated for complex:α-tubulin ratios determined by densitometry for Complexes I (A), II (B), III (C) and V (D). (E) Time-dependent effect of HT at 1 μmol/L on Complex I expression. (F) Time-dependent effect of HT at 1 μmol/L on Complex I activity. Values are mean±S.E. of the results from four independent experiments. *$P$<.05 and **$P$<.01 versus control cells without HT treatment.

## 3. Results

### 3.1. HT stimulated transcriptional activity and protein expression of PPARGC1α

PPARGC1α is a key factor that drives mitochondrial biogenesis, which also plays an important stimulatory role in thermogenesis and fatty acid oxidation in muscle and adipose tissues [29–31]. Treatment of adipocytes with HT at 0.1–50 μmol/L resulted in a dose-dependent curve of stimulation of expression of PPARGC1α by Western blot and its promoter transcription by luciferase reporter assay. Both assays showed that the most significant stimulation occurred at 0.1 and 10 μmol/L (Fig. 1A and B).

### 3.2. HT up-regulated genes involved in mitochondrial biogenesis and fatty acid oxidation

*Ppargc1α* autoregulates its gene expression, along with the expression of *Nrf1* and *Nrf2*, which are mitochondrial transcription factors encoded by nuclear genes. Nrf1 also induces the expression of *Tfam* [29–31]. Therefore, we examined the effects of HT on the mRNA expression of *Nrf1*, *Nrf2* and *Tfam*. Treatment of HT at 0.1–50 μmol/L resulted in bell-shaped response curves of mRNA expression of *Nrf1*, *Nrf2* and *Tfam*, similar to that of PPARGC1α expression. However, the increase was significant only at 1 μmol/L of HT for all three factors (Fig. 2).

### 3.3. HT treatment increased mtDNA

mtDNA content decreases age-dependently and may be one of the causal factors in age-related type 2 diabetes [32]. *Tfam* is involved in regulating expression of nuclear genes encoding some major mitochondrial proteins that regulate mtDNA transcription and replication. Its level is proportional to that of mtDNA [29–31]. Because HT stimulated the mRNA expression of *Tfam*, it is expected that mtDNA copy number should be increased. mtDNA expression was quantified by real-time PCR measuring the ratio of D-loop to 18s rRNA levels. The D-loop region is known as the major site of transcription initiation on both the heavy and light strands of mtDNA. As shown in Fig. 3, the HT treatment at 1 μmol/L resulted in a significant increase in the ratio of mitochondrial D-loop/18s rRNA.

### 3.4. HT promoted the protein expression of OxPhos Complexes I, II, III and V

Tfam, along with other nuclear-encoded mitochondrial proteins, is imported into mitochondria by the protein import machinery and regulates the expression of the 13 mtDNA-encoded proteins, which are components of Respiratory Chain Complexes I (ND1–6 and 4L) and III (Cyt *b* — cytochrome *b*) and ATP synthase (A8 and A6). The nuclear DNA-encoded mitochondrial proteins and the mtDNA-encoded proteins are assembled to form multisubunit enzyme complexes required for oxygen consumption and ATP synthesis [29–31]. As shown in Fig. 4, treatment of HT (48 h) significantly increased the expression of Complex I (Fig. 4A) and Complex II (Fig. 4B) at 0.1, 1 and 10 μmol/L and Complex III (Fig. 4C) and Complex V (Fig. 4D) at 1 and 10 μmol/L, respectively. The increases in protein expression were about 1.5- to 1.7-fold compared with control samples. The rationale for choosing the 48-h treatment for all experiments was based on the time-dependent effects of HT treatment: as shown in Fig. 4E and F, HT treatment effected a dose-dependent increase of Complex I expression and activity after periods varying from 24 to 72 h, with significant stimulation beginning at 48 h.

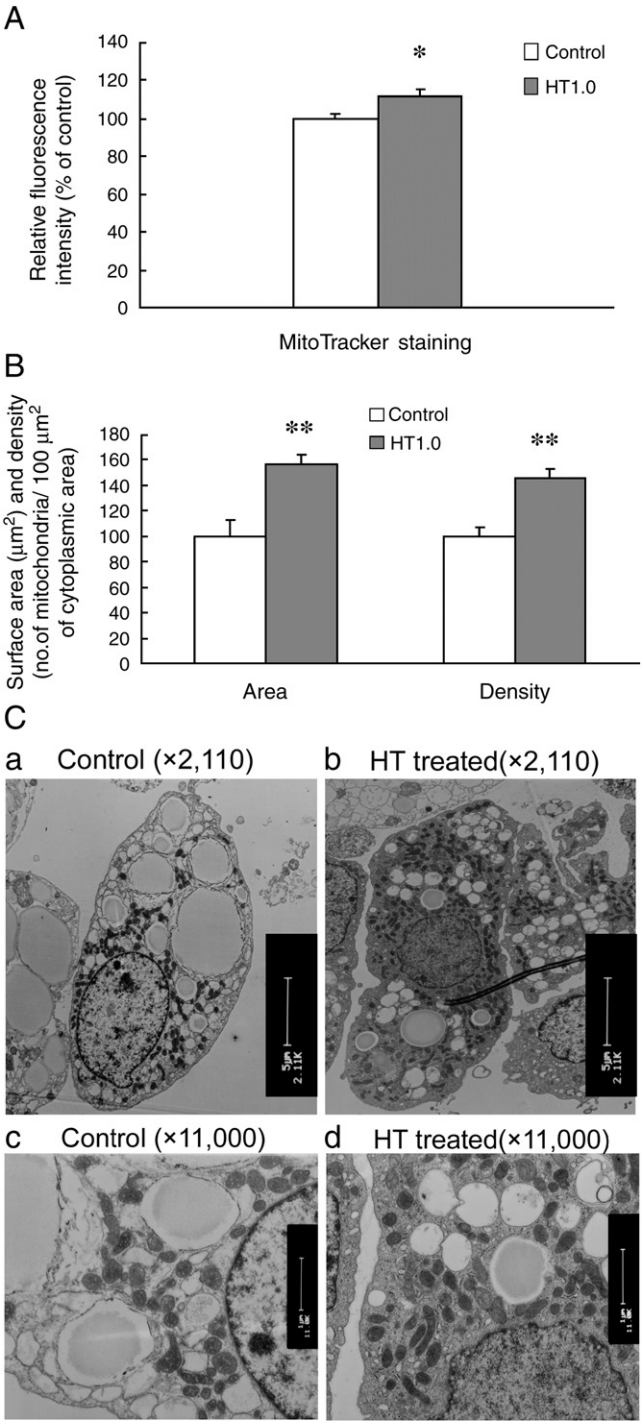


Fig. 5. Effect of HT treatments on mitochondrial mass and ultrastructural changes under the electron microscope. Adipocytes were stimulated with HT at 1.0 μM for 48 h. (A) Mitochondrial mass was estimated by MitoTracker (100 nmol/L) staining with flow cytometry. Results were expressed as fold increase of the fluorescence intensity over untreated control cells. Values are mean±S.E. of the results from four independent experiments. *$P$<.05 versus control cells without HT treatment. (B) Morphometric analysis of surface area and density of mitochondria under the electron microscope. Values are means±S.E. of data from six cells. **$P$<.01 versus control cells without HT treatment. (C) Representative illustrations of mitochondrial profiles under the electron microscope (magnification, ×2110 and ×11,000).

### 3.5. HT increased adipocyte mitochondrial mass

Mitochondrial formation is dependent on the assembly of large hetero-oligomeric complexes, and this assembly requires coordination between the nuclear and mitochondrial genomes [29–31]. We examined whether HT-induced activation of PPARGC1α and its downstream signaling leads to an increase in mitochondrial numbers. First, we used a specific dye, MitoTracker Green FM, which accumulates inside mitochondria, to label and quantify mitochondria in cells. As shown in Fig. 5A, treatment of HT at 1.0 μmol/L resulted in a significant increase in fluorescence intensity, suggesting an increase in mitochondrial mass. Mitochondrial morphology was also examined under the electron microscope. As shown in Fig. 5B, quantitative analysis (six cells were analyzed) demonstrated that the treatment with HT at 1.0 μmol/L for 48 h significantly increased mitochondrial section area and density. A representative control adipocyte and a 1.0-μmol/L HT-treated adipocyte are shown in Fig. 5C.

### 3.6. HT augmented oxygen consumption and activities of Mitochondrial Complexes I, II, III, IV and V

An increase in mitochondrial formation should be accompanied by an increase in mitochondrial function [29–31]. We examined oxygen consumption and activities of Mitochondrial Complexes I, II, III, IV and V. As shown in Fig. 6A and B, the basal rate of oxygen consumption was significantly increased in adipocytes by treatment with HT at concentrations between 1 and 10 μmol/L; the optimal increase was found to be at 1 μmol/L.

As shown in Fig. 7, treatment with HT (48 h) significantly increased the activities of Complex I (Fig. 7A) at 1 μmol/L HT; Complex II (Fig. 7A) at 0.1, 1 and 10 μmol/L; Complex III (Fig. 7B) at 1 and 10 μmol/L; and Complex IV (Fig. 7B) and Complex V (Fig. 7B) at 1 and 10 μmol/L.

### 3.7. HT up-regulated fatty-acid-oxidation-related expressions of Ppara, Cpt1 and Pparg genes

PPARα is an important regulator of mitochondrial biogenesis and β-oxidation. CPT-1 is the gatekeeper of mitochondrial fatty acid oxidation because it regulates long-chain fatty acid transport across the mitochondrial membrane by converting acyl-CoA into acylcarnitine. PPARγ plays an important role not only in adipogenesis but also in regulating lipid metabolism in mature adipocytes. To study the effects and mechanism of HT on mitochondrial biogenesis and fatty acid oxidation, the effects of HT on *Ppara*, *Cpt1* and *Pparg* were studied by quantitative RT-PCR. As shown in Fig. 8, HT showed dose-dependent increases in mRNA expression of Cpt1, Ppara and Pparg with significant increases at 1.0 μmol/L HT for *Cpt1* and *Ppara* and at 1.0 and 10 μmol/L HT for *Pparg*. However, HT did not increase PPAR induction of the reporter gene in PPARα or PPARγ transactivation assays (data not shown).

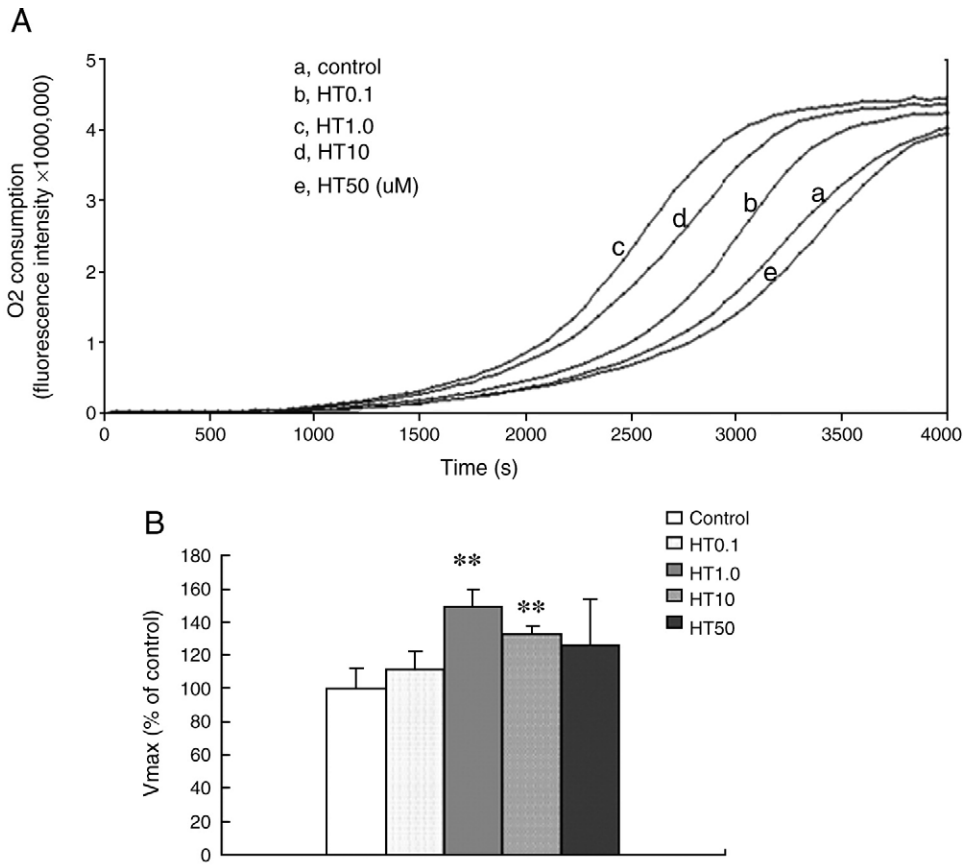


Fig. 6. Effect of HT treatments on oxygen consumption in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated for 48 h with various concentrations of HT as indicated. (A) Representative oxygen consumption curves. Cells were trypsinized and equal volumes of cells were separated into aliquots in wells of a 96-well BD Oxygen Biosensor plate. Plates were covered and fluorescence in each well was recorded over time with a fluorescence microplate spectrophotometer. (B) Quantitative changes in the respiratory rate of adipocytes under each condition were calculated by determining the kinetic parameters. $V_{max}$=maximum oxygen consumption rate. Final results are presented as percentage of control. Values are mean±S. E. of the results from three independent experiments. $^{**}P<.01$ versus control cells without HT treatment taken as 100%.

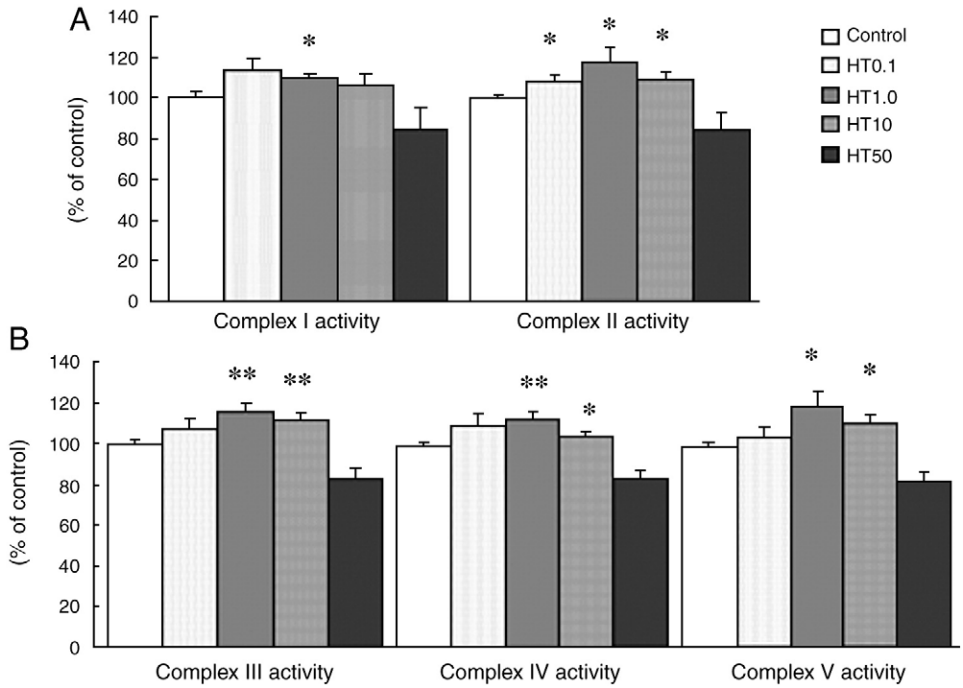


Fig. 7. Effect of HT treatments on activities of mitochondrial complexes in adipocytes. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L. Cells were then washed; mitochondria were isolated, and the complex activities were assayed. (A) Complexes I and II; (B) Complexes III, IV and V. Final results are presented as percentage of control. Values are mean±S.E. of the results from at least four independent experiments. *$P$<.05 and **$P$<.01 versus control cells without HT treatment.

### 3.8. HT decreased the FFA content in the supernatant of treated adipocytes

A chronic, high concentration of plasma FFAs is one of the factors that contributes to the underlying pathophysiology of type 2 diabetes, including development of insulin resistance. FFA treatment impaired insulin-receptor-mediated signal transduction and decreased insulin-stimulated GLUT4 translocation and glucose transport. In one study, FFAs activated the stress/inflammatory kinases JNK, IKKb and SOCS-3; increased secretion of the inflammatory cytokine TNF-α; and decreased secretion of adiponectin into the medium [33]. Pharmacological agents that effectively lower FFA (such as TZD) are likely to have a significant effect in reducing fasting plasma glucose. Therefore, reducing FFA might be a target for treating obesity and type 2 diabetes. Therefore, we examined whether HT can target FFA levels. As shown in Fig. 9, treatment of adipocytes with HT (1.0 μmol/L) for 72 h significantly reduced the FFA content of the supernatant ($P$<.01), although the glycerol and triglyceride content did not show distinct change compared with nontreated controls.

### 3.9. HT-activated phosphorylation of AMPK and ACC

One of the important pathways for activating PPARGC1α involves AMPK [34]. AMPK phosphorylates a number of targets, resulting in increases in glucose transport, fatty acid oxidation and gene transcription. One example of these targets is ACC. ACC is a biotin-dependent enzyme that catalyses the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA. The carboxylation inhibits β-oxidation, but when ACC is phosphorylated by AMPK, its activity is decreased [35]. AICAR, a pharmaceutical AMPK activator, has been shown to cause a time- and concentration-dependent increase in phosphorylation of AMPK and ACC [36]. As shown in Fig. 10C and D, HT affected ACC phosphorylation and AMPK phosphorylation

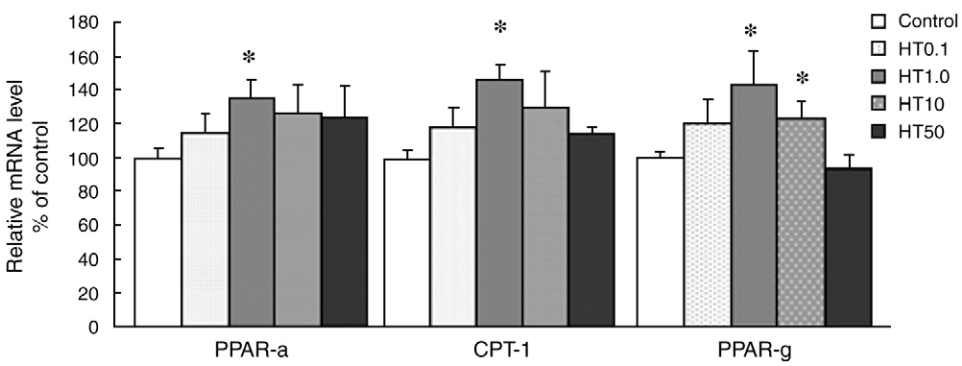


Fig. 8. Effect of HT treatments on expression of Cpt1a and Ppara mRNA. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L, and total RNA was isolated. PCR fluorescence products were quantified using SYBR Green. The cycle number at which the various transcripts were detectable was compared with that of 18S rRNA as an internal control. Results are expressed as percentage of control. Values are mean±S.E. of the results from at least four independent experiments. *$P$<.05 versus control without HT treatment.

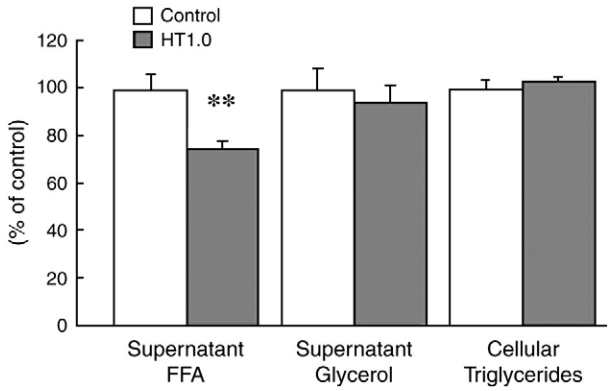


Fig. 9. Effect of HT treatments on levels of supernatant FFAs, glycerol and triglycerides in 3T3-L1 adipocytes. Adipocytes were stimulated with HT at 1.0 μmol/L for 72 h. FFA and glycerol contents in the supernatants of cells were detected. Results are expressed as a percentage fold increase of the metabolite concentration over untreated control cells. Values are mean±S.E. of the results from six independent experiments. $^{**}P<.01$ versus control without HT treatment.

similarly. We studied the effects of HT on the phosphorylation of AMPK and ACC, using AICAR as positive control. HT treatment (1.0 μmol/L) of 3T3-L1 adipocytes caused a time-dependent increase in the phosphorylation of AMPK (Fig. 10A and B) over the time range of 5, 15, 30 and 60 min, with the maximum increase at 30 min. Both HT (1.0 μmol/L) and AICAR (0.5 mmol/L) significantly affected AMPK phosphorylation (Fig. 10C and D). That HT, at about 1/500th the concentration, induced a similar degree of AMPK and ACC phosphorylation as AICAR suggests that HT is the more potent AMPK activator.

### 3.10. Knockdown of *Ppargc1α* blocked the effects of HT

To better determine how important *Ppargc1α* is in producing the stimulatory effects of HT on mitochondrial function and biogenesis in 3T3-L1 adipocytes, we knocked down *Ppargc1α* by siRNA. As shown in Fig. 11, introducing Ad-siRNA-*Ppargc1α* to the cells decreased PPARGC1α protein levels at the 24-h and 48-h time points. The Ad-siRNA-control did not affect PPARGC1α expression (Fig. 11A). Next, we determined the effects of *Ppargc1α* on the mtDNA quantity and Complex I expression. Knockdown of *Ppargc1α* suppressed them significantly (Fig. 11B), indicating the important role of *Ppargc1α*. These results also suggest that *Ppargc1α* is an important target in the process by which HT is able to stimulate mitochondrial function and biogenesis in adipocytes. No big difference was observed between control and Ad-siRNA-control groups, and HT stimulated both mtDNA and Complex I in Ad-siRNA-control cells, but for Ad-siRNA-*Ppargc1α* cells, mtDNA and Complex I were dramatically inhibited and HT treatment could not reverse this inhibition. These data suggest a crucial role of *Ppargc1α* in the effects of HT. Both mtDNA and Complex I levels tended to be higher in the Ad-siRNA-*Ppargc1α* cells treated with HT compared to untreated cells, but the increases were not significantly different.

## 4. Discussion

Identifying the mitochondrial dysfunction mechanisms and developing mitochondrial targeting drugs/nutrients have formed a new discipline of mitochondrial medicine and opened up avenues for manipulating mitochondrial function and health [21,37–39]. It is well reported that 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 sensitizers [25] and that mitochondrial dysfunction might be an important contributing factor in type 2 diabetes [9]. Mitochondrial loss in adipose tissue is correlated with the development of type 2 diabetes [2]. Hence, it is possible that stimulating mitochondrial biogenesis may reduce

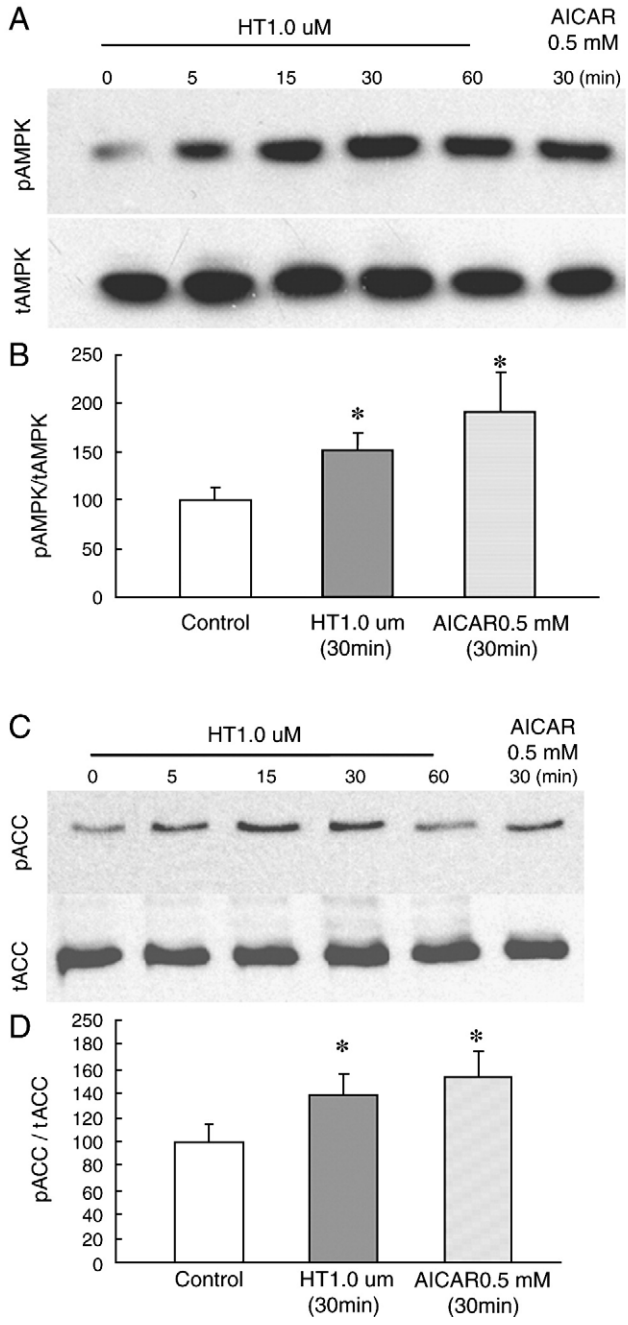


Fig. 10. Effect of HT treatments on AMPK and ACC phosphorylation in 3T3-L1 adipocytes. AMPK phosphorylation and ACC phosphorylation were determined by Western blotting using lysates of 3T3-L1 adipocytes that had been cultured for 5, 15, 30 or 60 min with 1.0 μM HT and for 30 min with AMPK activator AICAR at 0.5 mM. Proteins were prepared, applied (20 μg/lane) and separated. After transfer to a nitrocellulose membrane, polyclonal sera specific for phospho-AMPK (pAMPK), tAMPK, phospho-ACC (pACC) and tACC were applied overnight, and detection was carried out as described in Materials and Methods. (A) A representative image of AMPK phosphorylation. (B) Quantitative values (in percentage) are tabulated for pAMPK:tAMPK ratios. (C) A representative image of ACC phosphorylation. (D) Quantitative values (in percentage) are tabulated for pACC:tACC ratios. Values are mean±S.E. of the results from four independent experiments. $^{*}P<.05$ versus control cells without HT treatment.

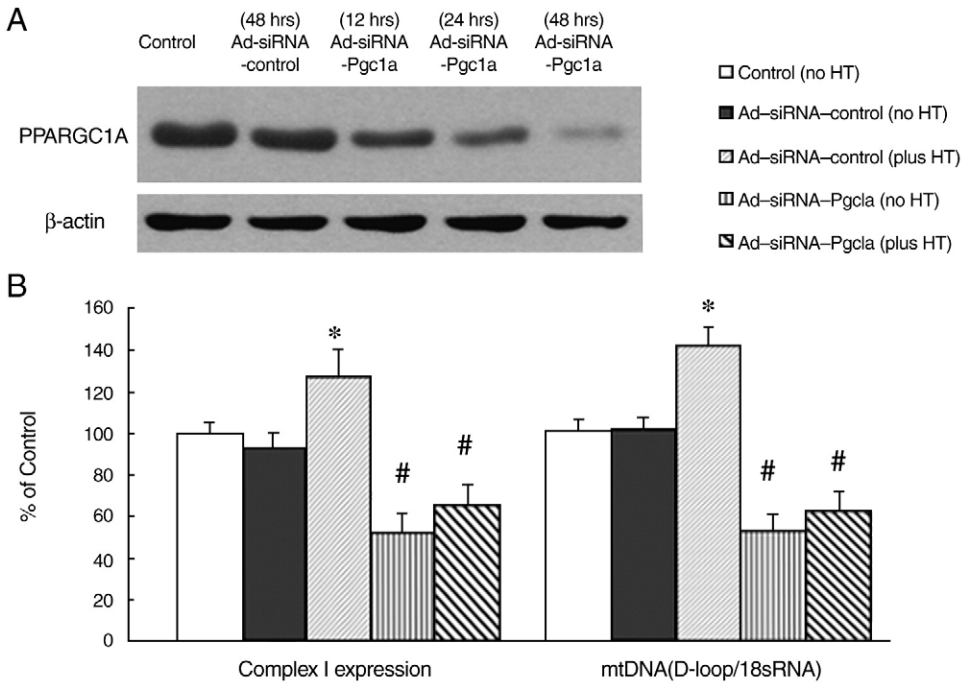


Fig. 11. Effect of PPARGC1a (Pgc1a) silencing on HT-stimulated mtDNA and Complex I expression. Cells were infected with Ad-siRNA-*Ppargc1α* or Ad-siRNA-control as described. DNA and protein were isolated and detected by RT-PCR and Western immunoblotting. (A) PPARGC1α protein expression after 12, 24 and 48 h of adenovirus infection. (B) Complex I expression in adipocytes treated with or without HT (1.0 μmol/L) and mtDNA quantity in adipocytes treated with or without HT (1.0 μmol/L). Values are mean±S.E. of the results from three independent experiments. *$P<.05$ versus Ad-siRNA-control without HT treatment.

the symptoms of metabolic syndrome. In the present study, we showed that HT over the concentration range of 0.1–10 μmol/L stimulated the protein expression of PPARGC1α — the central factor for mitochondrial biogenesis — and the mRNA of its downstream targets, *Nrf1*, *Nrf2* and *Tfam*. HT increased the quantity of mtDNA and the protein expression of Mitochondrial Complexes I, II, III and V; consequently, mitochondrial numbers increased. This increase in mitochondrial biogenesis was accompanied by an enhancement of mitochondrial function, including an increase in the activity and protein expression of Mitochondrial Complexes I, II, III and V and oxygen consumption, as well as a decrease in FFAs. Using siRNA, we further demonstrated that a key factor for HT to target is PPARGC1α. These data suggest that HT, as a mitochondrial targeting nutrient, is able to promote mitochondrial function by stimulating mitochondrial biogenesis.

Mitochondrial fatty acid oxidation is one of the key processes in ATP production. Mitochondrial biogenesis and remodeling in white adipocyte tissue enhance fatty acid uptake and oxidation, indicated by increased oxygen consumption. The increase in oxygen consumption is accompanied by an increase in expression of *Cpt1* and *Ppara*, suggesting that HT stimulates mitochondrial biogenesis, leading to increased fatty acid oxidation.

We hypothesized that the protective effects of mitochondrial antioxidants and nutrients on mitochondria may include (a) protecting mitochondria from oxidative damage and thus slowing down the loss of mitochondria, (b) stimulating repair of damaged mitochondria, (c) stimulating degradation of damaged mitochondria (by lysosomes) and (d) stimulating de novo mitochondrial biogenesis [21]. α-Lipoic acid and acetyl-L-carnitine are two examples of mitochondrial nutrients. In our previous experiments, we [22] have demonstrated that treatments using a combination of *R*-α-lipoic acid and acetyl-L-carnitine at concentrations of 0.1, 1 and 10 μM for 24 h significantly increased mitochondrial mass, expression of mtDNA, mitochondrial complexes, oxygen consumption and fatty acid oxidation in 3T3-L1 adipocytes. These changes were accompanied by an increase in the mRNA expression of *Cpt1a* and expression of several transcription factors involved in mitochondrial biogenesis, including *Ppargc1α*, *Tfam*, *Nrf1* and *Nrf2*. We concluded that the combination of *R*-α-lipoic acid and acetyl-L-carnitine may act as *Pparg* and *Ppara* dual ligands to complementarily promote mitochondrial synthesis and adipocyte metabolism.

HT has long been considered as a potent antioxidant polyphenol [14,15,17]. However, its effect on mitochondrial biogenesis has never been studied. Therefore, this is the first study to show that HT is able to act as a mitochondrial targeting nutrient and provides a new mechanism of the efficacy of the Mediterranean diet on lowering the risk of various diseases, including cardiovascular disease, cancer, diabetes and obesity. As we know, cardiovascular disease is the most common and most serious complication of diabetes and obesity. Because mitochondrial respiration plays a critical role in glucose metabolism, mitochondrial dysfunction has been shown to be associated with diabetes and obesity. The Mediterranean diet, including a high intake of HT, may stimulate mitochondrial biogenesis and function (enhancement of fatty acid oxidation) and, thus, reduce the risk of obesity and diabetes, leading to a lowered risk of cardiovascular disease. It seems that mitochondrial biogenesis and the phase II antioxidant system are closely related or coupled because the transcriptional coactivator PPARGC1α was shown to suppress ROS and neurodegeneration [40]. Therefore, it is possible that HT, a potent antioxidant and Phase II enzyme inducer, may enhance mitochondrial biogenesis and improve mitochondrial function by suppressing ROS and stimulating the Phase II antioxidant system to strengthen the cell's antioxidant defenses, in addition to its direct effect on mitochondrial assembly as demonstrated here.

Mitochondrial biogenesis is a complicated process. The activation of PPARGC1α is associated with a number of signaling pathways involving the activation of AMPK [34], intracellular calcium and the

subsequent activation of calcium-sensitive signaling of calcium/calmodulin-dependent protein kinase [41] and nitric oxide [42] and cAMP-responsive element binding protein [29–31]. We investigated the possible involvement of the AMPK signaling pathway by detecting the phosphorylation of AMPK and ACC (Fig. 10). Whether HT also affects other signaling pathways of PPARGC1α activation needs to be studied further.

In addition to stimulating mitochondrial biogenesis, AMPK was also shown to increase muscle fatty acid oxidation and insulin sensitivity. The antidiabetic drug metformin activates AMPK [43]. gACRP30 or globular adiponectin, the globular subunit of ADIPOQ, improves insulin sensitivity and increases fatty acid oxidation. The mechanism by which gACRP30 exerts these effects is possibly due to activation of AMPK and inactivation of ACC [44]. The potent effect of HT on phosphorylation of AMPK and ACC is consistent with the decrease in FFA and the increases in Cpt1 and Ppara as indexes of increased fatty acid oxidation. The potent effect of HT on phosphorylation of AMPK and ACC in our 3T3-L1 adipocytes suggests that HT might be potentially effective in increasing fatty acid oxidation and improving insulin sensitivity in diabetes and obesity.

In addition to the effects on increasing the mRNA expression of Ppara and Cpt1a for stimulation of fatty acid oxidation, HT also induced gene mRNA expression, but not transactivation, of Pparg. Oil Red O staining indicates smaller oil droplets in adipocytes. At the same time, the cellular triglyceride level did not increase (Fig. 9), and the FFA levels in the cell culture supernatant were reduced, suggesting that HT promoted fat burning. This reasoning is in accordance with Wilson-Fritch et al. [23], who describe that PPARγ plays an important role not only in adipogenesis but also in regulating lipid metabolism in mature adipocytes. PPARγ activity can be modulated by direct binding of low-molecular-weight ligands. For example, the clinically effective antidiabetic drugs such as TZD are high-affinity agonist ligands for PPARγ, leading to a net flux of fatty acids from the circulation and other tissues into adipocytes [8]. Interestingly, HT did not increase the levels of cellular triglycerides and FFAs but rather led to decreased level of FFAs in the cell culture supernatant and to smaller lipid droplets in adipocytes. Since HT only up-regulates PPARα/PPARγ expression but has no PPAR agonistic activity, these findings could be interpreted by the presence of sufficient PPAR agonistic molecules (e.g., fatty acids) in the cellular system, to favor adipocyte differentiation.

Our findings indicate that HT in adipocytes may support adipocyte differentiation but does not result in increased fat storage. This is in accordance with observations that a 2-week supplementation of HT did not cause any change in body weight in normal rats [Albino: HanWistar (SPF), doses up to 450 mg/kg]. Also, HT did not influence body weight or body composition in mice [C57BL/6NCrl: (SPF), doses up to 300 mg/kg]. The net effect of HT on adipogenesis can be compared with the action of the novel non-TZD selective PPARγ modulator (nTZDpa). Berger et al. [45] found that, in cell-based assays for transcriptional activation, nTZDpa served as a selective, potent PPARγ partial agonist and was able to antagonize the activity of PPARg full agonists; nTZDpa also displayed partial agonist effects when its ability to promote adipogenesis in 3T3-L1 cells was evaluated. Interestingly, nTZDpa, unlike the TZD, caused reductions in weight gain and adipose depot size when it was administered to fat-fed C57BL/6J mice.

In conclusion, we showed that HT is a nutrient that effectively stimulates mitochondrial biogenesis and function. This mitochondrial targeting property may provide a possible mechanism for the efficacy of the Mediterranean diet for lowering the risk of cardiovascular disease and also suggests that HT may be used as a therapeutic intervention for preventing and treating type 2 diabetes and obesity.

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## References

[1] Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 2005;39:359–407.
[2] Choo HJ, Kim JH, Kwon OB, Lee CS, Mun JY, Han SS, et al. Mitochondria are impaired in the adipocytes of type 2 diabetic mice. Diabetologia 2006;49:784–91.
[3] Hammarstedt A, Jansson PA, Wesslau C, Yang X, Smith U. Reduced expression of PGC-1 and insulin-signaling molecules in adipose tissue is associated with insulin resistance. Biochem Biophys Res Commun 2003;301:578–82.
[4] Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nature Genetics 2003;34:267–73.
[5] Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, et al. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A 2003;100:8466–71.
[6] Semple RK, Crowley VC, Sewter CP, Laudes M, Christodoulides C, Considine RV, et al. Expression of the thermogenic nuclear hormone receptor coactivator PGC-1alpha is reduced in the adipose tissue of morbidly obese subjects. Int J Obes Relat Metab Disord 2004;28:176–9.
[7] Bogacka I, Xie H, Bray GA, Smith SR. Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue in vivo. Diabetes 2005;54:1392–9.
[8] Lehrke M, Lazar MA. The many faces of PPARgamma. Cell 2005;123:993–9.
[9] Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. Science 2005;307:384–7.
[10] McCarty MF. Up-regulation of PPARgamma coactivator-1alpha as a strategy for preventing and reversing insulin resistance and obesity. Med Hypotheses 2005;64:399–407.
[11] Fernandez E, Gallus S, La Vecchia C. Nutrition and cancer risk: an overview. The J Br Menopause Soc 2006;12:139–42.
[12] Ordovas JM, Kaput J, Corella D. Nutrition in the genomics era: cardiovascular disease risk and the Mediterranean diet. Mol Nutr Food Res 2007;51:1293–9.
[13] Ortega RM. Importance of functional foods in the Mediterranean diet. Public Health Nutr 2006;9:1136–40.
[14] Bendini A, Cerretani L, Carrasco-Pancorbo A, Gomez-Caravaca AM, Segura-Carretero A, Fernandez-Gutierrez A, et al. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. Molecules (Basel, Switzerland) 2007;12:1679–719.
[15] Bertelli AA. Wine, research and cardiovascular disease: instructions for use. Atherosclerosis 2007;195:242–7.
[16] Fito M, de la Torre R, Covas MI. Olive oil and oxidative stress. Mol Nutr Food Res 2007;51:1215–24.
[17] Wahle KW, Caruso D, Ochoa JJ, Quiles JL. Olive oil and modulation of cell signaling in disease prevention. Lipids 2004;39:1223–31.
[18] Casalino E, Calzaretti G, Sblano C, Landriscina V, Felice Tecce M, Landriscina C. Antioxidant effect of hydroxytyrosol (DPE) and $Mn^{2+}$ in liver of cadmium-intoxicated rats. Comp Biochem Physiol C Toxicol Pharmacol 2002;133:625–32.
[19] Gordon MH, Paiva-Martins F, Almeida M. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. J Agric Food Chem 2001;49:2480–5.
[20] Liu Z, Sun L, Zhu L, Jia X, Li X, Jia H, et al. Hydroxytyrosol protects retinal pigment epithelial cells from acrolein-induced oxidative stress and mitochondrial dysfunction. J Neurochem 2007;103:2690–700.
[21] Liu J, Ames BN. Reducing mitochondrial decay with mitochondrial nutrients to delay and treat cognitive dysfunction, Alzheimer's disease, and Parkinson's disease. Nutr Neurosci 2005;8:67–89.
[22] Shen W, Liu K, Tian C, Yang L, Li X, Ren J, et al. *R*-alpha-Lipoic acid and acetyl-L-carnitine complementarily promote mitochondrial biogenesis in murine 3T3-L1 adipocytes. Diabetologia 2008;51:165–74.
[23] Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S, et al. Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. Mol Cell Biol 2003;23:1085–94.
[24] Boudina S, Sena S, O'Neill BT, Tathireddy P, Young ME, Abel ED. Reduced mitochondrial oxidative capacity and increased mitochondrial uncoupling impair myocardial energetics in obesity. Circulation 2005;112:2686–95.
[25] Wilson-Fritch L, Nicoloro S, Chouinard M, Lazar MA, Chui PC, Leszyk J, et al. Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone. J Clin Invest 2004;114:1281–9.
[26] Hayakawa T, Noda M, Yasuda K, Yorifuji H, Taniguchi S, Miwa I, et al. Ethidium bromide-induced inhibition of mitochondrial gene transcription suppresses glucose-stimulated insulin release in the mouse pancreatic beta-cell line betaHC9. J Biol Chem 1998;273:20300–7.

[27] Sun L, Luo C, Long J, Wei D, Liu J. Acrolein is a mitochondrial toxin: effects on respiratory function and enzyme activities in isolated rat liver mitochondria. Mitochondrion 2006;6:136–42.
[28] Koo SH, Satoh H, Herzig S, Lee CH, Hedrick S, Kulkarni R, et al. PGC-1 promotes insulin resistance in liver through PPARα-dependent induction of TRB-3. Nat Med 2004;10:530–4.
[29] Hood DA, Irrcher I, Ljubicic V, Joseph AM. Coordination of metabolic plasticity in skeletal muscle. J Exp Biol 2006;209:2265–75.
[30] Liu J. The effects and mechanisms of mitochondrial nutrient alpha-lipoic acid on improving age-associated mitochondrial and cognitive dysfunction: an overview. Neurochem Res 2008;33:194–203.
[31] Reznick RM, Shulman GI. The role of AMP-activated protein kinase in mitochondrial biogenesis. J Physiol 2006;574:33–9.
[32] Choi YS, Kim S, Pak YK. Mitochondrial transcription factor A (mtTFA) and diabetes. Diabetes Res Clin Pract 2001;54(Suppl 2):S3–9.
[33] Nguyen MT, Satoh H, Favelyukis S, Babendure JL, Imamura T, Sbodio JI, et al. JNK and tumor necrosis factor-alpha mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. J Biol Chem 2005;280:35361–71.
[34] Hardie DG. AMP-activated protein kinase: a key system mediating metabolic responses to exercise. Med Sci Sports Exerc 2004;36:28–34.
[35] Park SH, Gammon SR, Knippers JD, Paulsen SR, Rubink DS, Winder WW. Phosphorylation–activity relationships of AMPK and acetyl-CoA carboxylase in muscle. J Appl Physiol 2002;92:2475–82.
[36] Favero CB, Mandell JW. A pharmacological activator of AMP-activated protein kinase (AMPK) induces astrocyte stellation. Brain Res 2007;1168:1–10.
[37] Armstrong JS. Mitochondrial medicine: pharmacological targeting of mitochondria in disease. Br J Pharmacol 2007;151:1154–65.
[38] Fosslien E. Mitochondrial medicine—molecular pathology of defective oxidative phosphorylation. Ann Clin Lab Sci 2001;31:25–67.
[39] Larsson NG, Luft R. Revolution in mitochondrial medicine. FEBS Letters 1999;455: 199–202.
[40] St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, et al. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 2006;127:397–408.
[41] Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, et al. Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science 2002;296:349–52.
[42] Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, et al. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science 2003;299:896–9.
[43] Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles WGT, Schlattner U, et al. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species. J Biol Chem 2004;279:43940–51.
[44] Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang Cc C, Itani SI, et al. Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. Proc Natl Acad Sci U S A 2002;99:16309–13.
[45] Berger JP, Petro AE, Macnaul KL, Kelly LJ, Zhang BB, Richards K, et al. Distinct properties and advantages of a novel peroxisome proliferator-activated protein [gamma] selective modulator. Mol Endocrinol 2003;17:662–76.