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Upregulation of UCP2 by Adiponectin: The Involvement of Mitochondrial Superoxide and hnRNP K

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Abstract

Background: The adipocyte-derived hormone adiponectin elicits protective functions against fatty liver diseases and hepatic injuries at least in part by stimulating the expression of a mitochondrial inner membrane transporter, uncoupling protein 2 (UCP2). The present study was designed to investigate the cellular and molecular mechanisms underlying adiponectin-induced UCP2 expression.

Methodology/Principal Findings: Mice were treated with adiponectin and/or different drug inhibitors. Parenchymal (PCs) and nonparenchymal (NPCs) cells were fractionated from the liver tissues for mitochondria isolation, Western blotting and quantitative PCR analysis. Mitochondrial superoxide production was monitored by MitoSOX staining and flow cytometry analysis. Compared to control mice, the expression of UCP2 was significantly lower in NPCs, but not PCs of adiponectin knockout mice (AKO). Both chronic and acute treatment with adiponectin selectively increased the mRNA and protein abundance of UCP2 in NPCs, especially in the enriched endothelial cell fractions. The transcription inhibitor actinomycin D could not block adiponectin-induced UCP2 expression, whereas the protein synthesis inhibitor cycloheximide inhibited the elevation of UCP2 protein but not its mRNA levels. Mitochondrial content of heterogeneous nuclear ribonucleoprotein K (hnRNP K), a nucleic acid binding protein involved in regulating mRNA transportation and stabilization, was significantly enhanced by adiponectin, which also evoked a transient elevation of mitochondrial superoxide. Rotenone, an inhibitor of mitochondrial respiratory complex I, abolished adiponectin-induced superoxide production, hnRNP K recruitment and UCP2 expression.

Conclusions/Significance: Mitochondrial superoxide production stimulated by adiponectin serves as a trigger to initiate the translocation of hnRNP K, which in turn promotes UCP2 expressions in liver.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the metabolic syndrome components closely associated with obesity, a worldwide pandemic [1]. The presence of steatosis in liver poses significant risks for the development of Type 2 Diabetes, cardiovascular diseases, viral hepatitis, drug-induced hepatotoxicity and alcoholic steatohepatitis [2,3,4]. In western countries, NAFLD is the most frequent hepatic lesion with an estimated prevalence of 10–25% [5]. About 20% to 30% of individuals with NAFLD progress into non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma [6,7].

Adiponectin is an adipocyte-derived hormone possessing a wide range of beneficial functions against obesity-associated medical complications [8,9,10]. The hepatoprotective activities of adiponectin have been demonstrated by evidence derived from clinical, genetic and pharmacological studies [11,12,13,14,15,16,17,18]. Epidemiological investigations suggest that low adiponectin level is an independent risk factor for NAFLD and liver dysfunctions in different ethnic groups [11,12,15,17,18,19,20]. In mice, adiponectin deficiency leads to exacerbated liver injuries induced by chemicals, endotoxins, alcohol consumption and obesity [21,22,23,24], whereas administration of this protein protects against fatty liver diseases, as well as various other forms of hepatic injuries [17,25,26,27,28]. In adiponectin knockout (AKO) mice, there is a pre-existing condition of hepatic steatosis and mitochondria dysfunction, characterized by abnormal ultrastructures and defective mitochondrial respiratory chain (MRC) activity [24]. Adiponectin treatment restores mitochondrial functions, depletes lipid accumulation, and up-regulates the mRNA and protein expression of uncoupling protein 2 (UCP2) in liver tissues of AKO mice. UCP2 is a mitochondrial ion carrier encoded by

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nuclear genome but functions exclusively in mitochondria [29]. Although the detailed physiological functions of UCP2 remain to be elucidated, it has been suggested that increased expression of UCP2 may help to prevent the development of hepatic steatosis and steatohepatitis [30]. The liver protective functions of adiponectin are significantly attenuated in UCP2 knockout mice [24]. Administration with adiponectin or UCP2 produces similar effects on MRC activity, fatty acyl CoA accumulation, oxidative stress and inflammation in the liver tissues of AKO mice [31]. These information suggest that upregulation of UCP2 plays an essential role in mediating the hepatoprotective functions of adiponectin. On the other hand, the underlying cellular and molecular mechanisms by which adiponectin stimulate UCP2 expression in liver are largely unknown. Results in the present study demonstrate that adiponectin promotes UCP2 expression selectively in nonparenchymal cells, especially in hepatic endothelial cells, by provoking mitochondrial superoxide production, which facilitates the transportation, stabilization and translation of UCP2 mRNA.

Results

Adiponectin treatment enhanced UCP2 expressions in nonparenchymal cells

To determine the effect of adiponectin on UCP2 expression in parenchymal (PCs) and nonparenchymal (NPCs) cells, Western blotting and quantitative RT-PCR (QPCR) were performed on cells isolated from the livers of C57 and AKO mice. The protein and mRNA abundance of UCP2 in PCs isolated from AKO mice was not different from that in C57 mice (Figure 1A). The UCP2 protein abundance was lower in NPCs isolated from AKO mice compared to that in C57 mice (Figure 1A, left and middle panel). The mRNA level of UCP2 in NPCs isolated from AKO mice was ~50% of the C57 mice (Figure 1A, right panel). Administration of adenovirus encoding adiponectin increased the protein and mRNA abundance of UCP2 in NPCs, but not PCs, of AKO mice (Figure 1B). Next, the effect of acute recombinant protein treatment on UCP2 expression was evaluated. Both mitochondrial protein abundance and the mRNA level of UCP2 was significantly up-regulated at 30 minutes following administration of the protein into portal vein of AKO mice liver (Figure 2, A and B). Similarly, the elevated UCP2 expression was mainly found in NPCs (Figure 2C).

UCP2 was induced by adiponectin treatment in hepatic endothelial cells

Liver consists of parenchymal (PC) and nonparenchymal (NPC) cells. Hepatocytes are the components of PCs and other cell types, including Kupffer cells (~30%), endothelial cells (~60%), stellate cells, and leukocytes etc, are grouped in NPCs. To further determine the cellular target of adiponectin, enriched fractions containing Kupffer cells and endothelial cells were used for Western blotting to determine the mitochondrial UCP2 content. The efficiency of cellular enrichment was validated by Western blotting using macrophage marker F4/80 and endothelial cell marker SE-1 (Figure 3A). Compared to that of Kupffer cells, the protein abundance of UCP2 was much lower in endothelial cells. While adiponectin treatment had no significant effect on UCP2 expression in Kupffer cells, it markedly increased the UCP2 content (by approximately 3-fold) in the endothelial fractions.
Figure 2. Acute treatment with adiponectin elevates UCP2 expression in NPCs. PBS or 50 μg of murine adiponectin protein was injected into the portal vein of AKO mice livers. Liver tissues were collected for evaluation of UCP2 expression. The protein abundance in mitochondria (A), mRNA expression in total tissue lysates (B), and the protein content in PC and NPC fractions (C) were analyzed as in Figure 1. *, P<0.05 vs vehicle treated samples, n = 3.
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Figure 3. Adiponectin promotes UCP2 expression in hepatic endothelial cells. AKO mice were treated as in Figure 1B. The NPCs were used for further fractionation to collect those enriched with Kupffer (K)- and sinusoidal endothelial (E) cells. The enrichment of the two cell types were confirmed by Western blotting using macrophage marker F4/80 and sinusoidal endothelial marker SE-1, respectively (A). UCP2 expression was monitored as in Figure 1. After densitometry analysis, the protein ratio of UCP2/β-actin was calculated and presented as fold changes against Luci Kupffer samples (B). UCP2 gene expression was also quantified in four types of cells treated with or without adiponectin (10 μg/ml) (C). *, P<0.05 and **, P<0.01 vs corresponding controls, n = 3.
doi:10.1371/journal.pone.0032349.g003
harvested from AKO mice liver (Figure 3, A and B). To further corroborate the cell type-specific effect of adiponectin, human umbilical vein endothelial cells (HUVEC), hepatoma H4IIE, macrophage RAW 264.7 and stellate LX-2 cell lines were treated with or without adiponectin. QPCR analysis revealed that adiponectin significantly increased UCP2 mRNA level by ~3.5- and ~1.8-fold in HUVEC and LX-2, respectively (Figure 3C). Consistent with the in vivo data, the UCP2 expression in hepatoma cell H4IIE and macrophage cell RAW 264.7 was not affected by adiponectin (Figure 3C).

Adiponectin enhanced the mRNA stability of UCP2 and promoted hnRNP K translocation to mitochondria

To investigate the mechanism underlying adiponectin-induced UCP2 expression, PBS or adiponectin was administered into the portal vein of AKO mice in the presence or absence of transcriptional inhibitor actinomycin D (ActD) or protein synthesis inhibitor cycloheximide (CHX) (Figure 4). ActD treatment did not block the effects of adiponectin on UCP2 expression. In the presence of ActD, adiponectin increased the UCP2 protein and mRNA levels by ~2.3- and ~3.2-fold, respectively (Figure 4, A and B). The elevation of UCP2 protein expression was observed in both PC and NPC fractions. On the other hand, CHX completely suppressed the stimulatory effect of adiponectin on UCP2 protein expression, which was measured in the lysates derived from both the AKO liver tissues and the NPC fractions (Figure 4, A and D). However, CHX did not prevent adiponectin-induced elevation of UCP2 mRNA expression (Figure 4B).

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is involved in regulating UCP2 mRNA stability and protein synthesis [32]. While the total hnRNP K protein expression was not

![Figure 4](https://www.journal.pone.org/figure/f4.png)

**Figure 4. Adiponectin enhances the mRNA stability of UCP2 and promotes its protein synthesis.** Two inhibitors, actinomycin D (ActD, A, B and C) or cycloheximide (CHX, A, B and D), were administered together with or without adiponectin protein into liver tissues of AKO mice livers. The protein (A) and mRNA (B) abundance of UCP2 was monitored by Western blotting and QPCR, respectively. The relative protein expression was also monitored in PCs/NPCs lysates (C and D). *, P<0.05 vs corresponding controls, n = 3.

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significantly different between C57 and AKO mice (data not shown), the abundance of this protein was much lower in the mitochondria fractions of AKO mice liver compared to those of C57 mice (Figure 5A). Adenovirus-mediated chronic over-expression of adiponectin significantly elevated hnRNP K levels in mitochondria isolated from AKO mice (Figure 5B, top panel). Similarly, injection of recombinant adiponectin, via portal vein, also increased the mitochondrial hnRNP K protein content (Figure 5B, bottom panel). UCP2 mRNA is transported to mitochondria-associated polysomes for protein synthesis. A 123 bp specific fragment of UCP2 mRNA was amplified from mitochondrial RNA samples (Figure 5C). The mitochondrial contents of UCP2 mRNA were quantified by QPCR using the mitochondrial gene cytochrome C oxidase subunit II (COX II) as the internal control (Figure 5D). It was found that UCP2 mRNA content in the mitochondria-associated polysome fraction of AKO mice was only \( \frac{29.1}{\%} \) of that in the C57 group. Adiponectin replacement by either adenovirus administration or portal vein injection restored the UCP2 mRNA contents (Figure 5D).

Treatment with rotenone, a MRC complex I inhibitor, abolished adiponectin-mediated induction of UCP2 and hnRNP K translocation

Western blotting was performed on mitochondria protein lysates of AKO mice treated with recombinant adiponectin in the presence or absence of MRC complexes inhibitors, including rotenone (Rot, complex I inhibitor), antimycin A (AntA, complex III inhibitor) and sodium azide (NaN\(_3\), complex IV inhibitor). The stimulatory effect of adiponectin on mitochondrial UCP2 protein expression was significantly attenuated by rotenone, but not by antimycin A or NaN\(_3\) (Figure 6A). Adiponectin-induced elevation of UCP2 mRNA level was also attenuated by rotenone treatment (Figure 6B). This inhibitor mainly blocked the expression of UCP2 in NPCs (Figure 6C). Rotenone treatment also prevented adiponectin-induced mitochondria translocation of hnRNP K in NPCs (Figure 6D).

MRC is a major source of reactive oxygen species (ROS), which stimulate UCP2 expression [33,34,35]. By applying a fluorescent probe for mitochondrial superoxide, MitoSOX, it was found that
portal vein injection of adiponectin induced a transient superoxide burst (Figure 7A). This was concomitantly associated with the elevation of UCP2 expression and the recruitment of hnRNP K into mitochondrial fractions as shown in Figure 2 and 5, respectively. Note that the positive MitoSOX staining signal was found to be either co-localized with or close to that of von Willebrand factor (VWF), a marker for endothelial cells (Figure 7B). Flow cytometric analysis further confirmed that adiponectin could induce a short burst of mitochondrial superoxide production in HUVEC, which was detected at 30 minutes of treatment. The elevation of ROS was no longer detectable after 1.5 hours (Figure 7C). Adiponectin could not promote the transient induction of mitochondrial superoxide in H4IIE hepatoma cells (data not shown). Treatment with rotenone, but not antimycin A and NaN3, blocked adiponectin-induced mitochondrial superoxide production in HUVEC (Figure 7D). Inhibition of the NADPH oxidase by diphenylene iodonium (0.5 μM) or apocynin (1 mM) had no significant effect on adiponectin-evoked ROS generation and UCP2 expression (data not shown).

Discussion

Uncoupling protein 2 (UCP2) is a mitochondrial inner membrane carrier protein encoded by nuclear genome [30]. The first member of uncoupling protein family, UCP1, was discovered as a physiologic mediator of proton leak and cold-adapted thermogenesis in brown adipose tissue [36]. UCP2 protein shares 59% amino acid identity to that of UCP1 [37]. However, the biological functions of UCP2 remain poorly defined. Nevertheless, a growing body of evidence suggests that UCP2 plays a beneficial role in various stages of fatty liver diseases and could exert anti-steatotic and anti-inflammatory activities through promoting mitochondrial respiration, attenuating non-esterified mitochondrial fatty acid accumulation and ROS production, inhibiting inflammatory cytokine expression and activating AMP-activated protein kinase [30,31,33,35]. In mice, absence of UCP2 is associated with elevated production of ROS, increased oxidative stress, and delayed liver regeneration [33,34,35,38,39]. On the contrary, there lacks sufficient information on whether replacement with UCP2 could alleviate NAFLD [30]. In fatty liver tissues, the lipid-laden hepatocytes express high levels of UCP2, whereas the expression of UCP2 in Kupffer cells decreases [40]. The present study demonstrates that UCP2 expression is significantly reduced in NPCs, but not PCs fractions of AKO livers. Moreover, administration with adiponectin selectively promotes UCP2 expression in hepatic endothelial cells. These evidence suggest that a cell-specific regulation of UCP2 may be crucial for adiponectin to elicit its hepatoprotective functions.

The mitochondrial superoxide anion radicals (O$_2^-$) are generated as the by-products of oxidative phosphorylation, from electrons leakage at complex I and III of the electron transport chain. MitoSOX (Life Technologies) was used to evaluate the mitochondrial superoxide production. Figure 6. Rotenone inhibits adiponectin-stimulated UCP2 expression and hnRNP K translocation to mitochondria. Mitochondrial respiration chain inhibitors, including rotenone (Rot), sodium azide (NaN3) and antimycin A (AntA) were administered into liver tissues of AKO mice as described in Methods. The effects of each inhibitor on adiponectin-induced UCP2 protein expression in mitochondria of liver tissues (A), UCP2 mRNA expression in total tissue lysates (B) were evaluated. The mitochondria protein content of UCP2 (C) and hnRNPK (D) in PCs and NPCs were compared by Western blotting. *, P<0.05 vs corresponding controls, n = 3. doi:10.1371/journal.pone.0032349.g006
Complex I-derived O$_2^•$ not only in cell respiration, but also in cellular/organismal ROS pumping NADH:quinone oxidoreductase) plays an important role in the membrane [46,47]. The high rate of superoxide production by sensitive to the pH gradient across the mitochondrial inner membrane [48]. When tightly controlled, mitochondrial ROS act as signalling messengers for many biological responses, including the antioxidant mechanisms for restoring cellular redox homeostasis [42,43,44]. Here, the results demonstrate that adiponectin treatment induces the generation of mitochondrial superoxide, which can be inhibited by rotenone, the complex I inhibitor but not by antimycin A, the complex III inhibitor. Complex I (H$^+$-pumping NADH:quinone oxidoreductase) plays an important role not only in cell respiration, but also in cellular/organismal ROS homeostasis [45]. Complex I-derived O$_2^•$ production due to reverse electron transport can be inhibited by rotenone and is sensitive to the pH gradient across the mitochondrial inner membrane [46,47]. The high rate of superoxide production by complex I could be abolished by uncoupler [48,49]. However, the mechanisms of superoxide production by complex I are less clear than that of complex III. Nevertheless, the results of the present study suggest that adiponectin preferentially acts through a rotenone-sensitive and complex I-related pathway to induce the superoxide burst and the expressions of UCP2 (Figure 6). Elevated UCP2 subsequently maintains low ROS production in the mitochondrial matrix [39].

Both adenovirus-mediated chronic overexpression of adiponectin and acute injection of the recombinant protein elevate mRNA and protein levels of UCP2 in mitochondria. Transcription inhibitor actinomycin D does not affect the ability of adiponectin to stimulate UCP2 expression. Adiponectin stabilizes and facilitates the transport of UCP2 transcripts to mitochondria-associated polysomes for protein synthesis, which involves hnRNP K, a nucleic acid binding protein. Different hnRNP proteins affect distinct sets of targets. hnRNPK is one of those showing the broadest effects on regulating mRNA transcription, stability and translation of genes involved in the control of apoptosis, oxidative stress, oncogenesis and metabolism [50]. A large number of RNAs associated with hnRNPK are nuclear-encoded but expressed in mitochondria, including UCP2 [51]. Translocation of hnRNPK from nucleus to cytoplasm and the importation of this protein into mitochondria have also been implicated in regulating mitochondrial genome transcription [52]. hnRNP K binds with the 3’-untranslated region and increases the abundance of UCP2 mRNA in mitochondria [32]. Mitochondria content of hnRNPK in AKO livers is much lower than that of C57 mice. Similar to UCP2 elevation, adiponectin-induced mitochondrial translocation of hnRNPK can also be inhibited by rotenone treatment. These evidence indicate that adiponectin-induced hnRNPK translocation to mitochondria could be a downstream response to the transient mitochondria superoxide production (Figure 8). Indeed, previous studies have suggested that a number of redox-linked signal transduction pathways, such as protein kinase C [53], c-Jun N-terminal kinases [54], c-Src [55], and extracellular signal regulated kinases [56], are involved in the activation of hnRNPK.

In summary, the present study demonstrates that adiponectin treatment in liver induces a transient superoxide production from mitochondria, which initiates a signalling event for UCP2 up-regulation through hnRNPK (Figure 8). Based on experiments with pharmacologic inhibitors, complex I inhibition (with rotenone) but not complex III inhibition (with antimycin A) decreases the superoxide generation induced by adiponectin. To characterize the specific sites of superoxide generation and the downstream ROS signals may be attractive targets for the development of adiponectin-based hepatoprotective therapies.

Materials and Methods

**Materials**

Sodium azide (NaN$_3$), rotenone (Rot), antimycin A (AntA), cycloheximide (CHX) and actinomycin D (ActD) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) and mito-hydroethidine [MitoSox, 3,8-dihydro-6-phenyl-5,6-phenanthridine diamine, 5-6-triphenyl phosphonium hexyl]-5,6 dihydro-6-phenyl] were purchased from Molecular Probes (Invitrogen, Carlsbad, CA). Bicinchoninic acid (BCA) protein quantitation kit was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Polyvinylidene fluoride (PVDF) membrane and chemiluminescence solution were obtained from GE Healthcare (Piscataway, NJ, USA). Unless otherwise stated, all chemicals were purchased from USB Corporation (Cleveland, Ohio, USA). Rat hepatoma cell H4IIE, human umbilical vein endothelial cell...
translocation hnRNP K, which subsequently promotes the stabilization of UCP2 mRNA and its protein synthesis in mitochondria of streptomycin at 37°C (40M)

Murine adiponectin recombinant protein was purified as described in the NIH publication 86-23 revised 1985. Animal studies

C57BL/6j mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). AKO mice in C57BL/6 background was a kind gift from Prof. Lawrence Chan (Baylor College of Medicine, Houston, TX, USA) [24]. All animals were kept under 12-hour light-dark cycles at 22–24°C in a humidified atmosphere of 5% CO2. Mice were sacrificed under deep anaesthesia. Liver perfusion was performed as described [58]. Adiponectin and Liver

Isolation of PCs and NPCs from liver tissues

Mice were fed with standard chow containing 20% protein, 10% fat and 70% carbohydrates (LabDiet 5053, Purina mills. Richmond, IN). Male mice at the age of 6–8 weeks were used for this study. Adenovirus expressing luciferase or adiponectin was administered into mice by tail vein injection at the optimal dosage of 1×109 pfu per mouse [24,31]. Isolation of PCs and NPCs from liver tissues

After centrifuging at 800 g for 15 minutes at 4°C, endothelial cell-enriched fractions were collected from the intermediate zone between the two Percoll layers. Fractions containing Kupffer cells were obtained from the 50% Percoll layer. Mitochondria were isolated from total liver lysates, PCs and NPCs by following the protocol described previously [24].

In vivo and in vitro detection of mitochondrial reactive oxygen species (ROS)

Mitochondrial superoxide was detected by MitoSOX REDTM, a fluorescent probe targeted to mitochondria. MitoSOX exhibits fluorescence after oxidation by superoxide (excitation 510 nm, emission 580 nm). For in vivo evaluation, MitoSOX with or without adiponectin (50 µg per mouse) in a total volume of 100 µl was administered into portal vein. The liver tissues were collected at different time points for preparing the 5 μm cryo-sections. After fixation in ice-cold acetone and counter-stained with DAPI, images were captured with Olympus BX41 microscope (Olympus, Tokyo, Japan). von Willebrand factor (VWF) was used as an endothelial cell marker for immuno-staining in the liver tissue sections. For determination of mitochondrial superoxide by flow cytometry, HUVEC were preloaded with 5 μM MitoSOX for 30 min in medium without phenol red. After washing, adiponectin with or without various inhibitors were added into the cells for different periods prior to flow cytometric measurement [59]. Real time quantitative polymerase chain reaction (QPCR)

The cDNA reversely transcribed from total RNA was used to analyze gene expression levels. Fluorescent SYBR Green (Qiagen, Hilden, Germany) was used to quantify the PCR product on an Applied Biosystems Prism 7000 sequence detection system. The primer sequences were: Forward 5′-GGGATCTGCTAAAGTCCCGTGT-3′ and Reverse 5′-CCATTAGGAGGCTGCTG-3′ for UCP2 (NM_003355.2); Forward 5′-TAGTGGCAGACGCGCACGG-3′ and Reverse 5′-AGCTCATCTGCCGGTCCG-3′ for mouse Ucp2 (NM_011671.4); Forward 5′-GAGATTCAAGGGCTAGCGC-3′ and Reverse 5′-GCTTGAGAGGTCTCTTGTTA-3′ for rat Ucp2 (NM_001130033.1); Forward 5′-GAGATTCAAGGGCTAGCGC-3′ and Reverse 5′-GCTTGAGAGGTCTCTTGTTA-3′ for rat Ucp2 (NM_001130033.1); Forward 5′-GGGATCTGCTAAAGTCCCGTGT-3′ and Reverse 5′-CCATTAGGAGGCTGCTG-3′ for UCP2 (NM_003355.2); Forward 5′-TAGTGGCAGACGCGCACGG-3′ and Reverse 5′-AGCTCATCTGCCGGTCCG-3′ for mouse Ucp2 (NM_011671.4); Forward 5′-GAGATTCAAGGGCTAGCGC-3′ and Reverse 5′-GCTTGAGAGGTCTCTTGTTA-3′ for rat Ucp2 (NM_001130033.1); Forward 5′-GAGATTCAAGGGCTAGCGC-3′ and Reverse 5′-GCTTGAGAGGTCTCTTGTTA-3′ for rat Ucp2 (NM_001130033.1);
TGACCCGATCATTTTGGAGA-3' and Reverse 5'-AGTC-CATCAGGATGCGAGT-3' for human ACTB (NM_001101.3); Forward 5'-AGTTGGACGGTGATGCGAGT-3' and Reverse 5'-CCACGGATGACCCAGAAG-3' for mouse - Actb (NM_002933); Forward 5'-CAGGCCCCGAGTACCCGCT-3' and Reverse 5'-GCCATACCCACCATCACACC-3' for rat Actb (NM_0311432); Forward 5'-AGCAATACCAACCCGGTA-3' and Reverse 5'-GGCAGAAGCTGCGGTATGTC-3' for mouse MT-Co2 (NC_005089.1); β-actin gene (ACTb or actb) was used as the QPCR internal control for cell and tissue RNA samples. Cytochrome C oxidase II (COXII) gene (ACTB, NM_001101.3); Forward 5'-ACGAAATCAACCCGGTA-3' and Reverse 5'-AGTC-3' was used as the internal control for mitochondrial polyrnome-associated RNA samples. The amplified products were checked by separation on a 2% agarose gel.

Western blotting
Tissues were homogenized in lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Nonidet P 40, 0.1% (v/v) SDS, 1% (v/v) sodium deoxycholate, 1 mM NaF, 1 mM Na3VO4, 1% (v/v) protease inhibitor cocktails (Roche, Mannheim, Germany)]. Protein lysates were heated at 95°C for 5 min, separated by SDS-PAGE, and transferred to PVDF membrane for immunoblotting with the specific antibodies (Roche, Mannheim, Germany). Protein relationships were visualized by using enhanced chemiluminescence. 

Statistical analysis
All results were derived from at least three sets of experiments. The density of protein bands in Western blotting results were quantitatively analyzed by ImageJ software for calculating the expression ratios against loading control. Representative Western blotting images were shown. Values are expressed as mean ± SEM. The statistical calculations were performed with the Statistical Package for the Social Sciences version 11.5 software package (SPSS, Inc., Chicago, Ill.). Differences between groups were determined by Student’s t-test. Comparisons with P<0.05 were considered as statistically significant.

Author Contributions
Conceived and designed the experiments: AX PT KL YW. Performed the experiments: MZ BH YL. Analyzed the data: YW MZ AX PT KL YW. DW YY. Wrote the paper: MZ AX PT KL YW.


