Hypoglycemic effect of catalpol on high-fat diet/streptozotocin-induced diabetic mice by increasing skeletal muscle mitochondrial biogenesis

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Catalpol, an iridoid glycoside, exists in the root of Radix Rehmanniae. Some studies have shown that catalpol has a remarkable hypoglycemic effect in the streptozotocin-induced diabetic model, but the underlying mechanism for this effect has not been fully elucidated. Because mitochondrial dysfunction plays a vital role in the pathology of diabetes and because improving mitochondrial function may offer a new approach for the treatment of diabetes, this study was designed. Catalpol was orally administered together with metformin to high-fat diet/streptozotocin (HFD/STZ)-induced diabetic mice daily for 4 weeks. Body weight (BW), fasting blood glucose (FBG) level, and glucose disposal (IPGTT) were measured during or after the treatment. The results showed a dose-dependent reduction of FBG level with no apparent changes in BW through four successive weeks of catalpol administration. Catalpol treatment substantially reduced serum total cholesterol and triglyceride levels in the diabetic mice. In addition, catalpol efficiently increased mitochondrial ATP production and reversed the decrease of mitochondrial membrane potential and mtDNA copy number in skeletal muscle tissue. Furthermore, catalpol (200 mg/kg) rescued mitochondrial ultrastructure in skeletal muscle, as detected with transmission electron microscopy. The relative mRNA level of peroxisome proliferator-activated receptor gamma co-activator 1 (PGC1)α was significantly decreased in muscle tissue of diabetic mice, while this effect was reversed by catalpol, resulting in a dose-dependent up-regulation. Taken together, we found that catalpol was capable of lowering FBG level via improving mitochondrial function in skeletal muscle of HFD/STZ-induced diabetic mice.

Keywords catalpol; hypoglycemia; muscle; mitochondrial biogenesis

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Introduction

Currently, there is an increasing number of people suffering from diabetic diseases resulting from a sedentary lifestyle, the consumption of a high-caloric diet, obesity, a longer life span, and others [1]. Diverse oral hypoglycemic drugs used for the clinical treatment of diabetes have characteristic side-effect profiles. However, the drugs used for the treatment of diabetes have a number of limitations, such as adverse effects and high rates of secondary failure. The Diabetes Control and Complications Trial demonstrated that even the optimal control of blood glucose could not improve these complications, suggesting that alternative treatment strategies are needed [2].

Mitochondria play a vital role in many cellular functions, including ATP production, the biosynthesis of amino acids and lipids, cytosolic calcium transport [3], and the amplification of apoptotic stimuli [4]. Many diabetic patients have metabolic disorders, such as lower glucose utilization, hyperlipidemia, and metabolic inflexibility which are related to mitochondrial dysfunction. Meanwhile, mitochondrial dysfunctions, including mitochondrial loss and over-production of oxidants, have been suggested to be involved in the development of insulin resistance [5,6]. Therefore, the role of mitochondria in diabetes is of some concern. Biopsies of skeletal muscle from subjects with type 2 diabetes (T2D) showed that...
citrate synthase and NADH-O₂ oxidoreductase activities were decreased compared with those in lean controls [7]. Reduction in subsarcolemmal mitochondria was confirmed with transmission electron microscopy (TEM), which may contribute to the pathogenesis of muscle insulin resistance in T2D [8]. Patients with insulin resistance or T2D also manifest decreased mitochondrial density, oxidative activity, and mitochondrial ATP synthesis [9, 10]. A study on STZ-diabetic rats showed a persistent increase in reactive oxygen and nitrogen species (ROS and RNS, respectively) production and a decrease in the activities of the mitochondrial respiratory enzymes including ubiquinol-cytochrome c oxidoreductase (complex III) and cytochrome c oxidase (complex IV) [11]. Thus, increasing mitochondrial mass and oxidative activity is viewed as a potential therapeutic approach for the treatment of insulin resistance and diabetes, and minimizes the complications of diabetes or both [12].

Peroxisome proliferator-activated receptor gamma co-activator 1 (PGC1α) is expressed in several tissues, especially in those with a high oxidative metabolism, such as heart, skeletal muscle, kidney, brown fat, brain, and liver [13]. Importantly, PGC1α regulates mitochondrial biogenesis and functions through interacting with co-activating transcription factors, such as nuclear respiratory factors, PPARs, thyroid hormone, glucocorticoid, and estrogen-related ERRα and γ receptors [14]. In patients with T2D, muscle-biopsy studies showed that the expression of PGC1α is reduced along with the occurrence of mitochondrial dysfunction. Similarly, PGC1α-knockdown mice were found to have defective contractility of skeletal muscle [15]. All these suggested that PGC1α-regulated mitochondrial biogenesis may be a viable target for therapeutic intervention by preventing against mitochondrial dysfunction in diabetic patients.

Catalpol (Fig. 1) is an iridoid glycoside produced in the root of Radix Rehmanniae. Some studies have shown a substantial hypoglycemic effect of catalpol in different diabetic models [16, 17]. Oral administration of catalpol resulted in an increased expression of glucose transporter subtype 4 (GLUT4) in skeletal muscle of STZ-induced diabetic rats [16–18]. Catalpol can also ameliorate diabetes encephalopathy in rats [19]. In addition, catalpol has other pharmacological effects, such as neuron protection [20, 21], against H₂O₂-induced apoptosis [22], and anti-inflammatory properties [23, 24]. Notably, intraperitoneal injection of catalpol significantly reduced mitochondrial dysfunction, enhancing complex I activity and mitochondrial membrane potential, and decreased ROS generation in rotenone-induced mitochondrial damage and brain injury animal model [19, 25, 26]. In this study, catalpol was orally administered to high-fat diet/streptozotocin (HFD/STZ)-induced diabetic mice, and it was found that catalpol can reverse and increase muscle mitochondrial function and biogenesis partly by up-regulating mRNA level of PGC1α. This study is the first report on the protective effect of catalpol against HFD/STZ-induced muscle mitochondrial dysfunction through the up-regulation of PGC1α in skeletal muscle.

Materials and Methods

Chemicals and reagents

STZ was purchased from Sangon Biotech Co., Ltd (Shanghai, China); metformin (>99.8% purity) was made in Sino-American Shanghai Squibb Co., Ltd (Shanghai, China); glucose (Glu), total cholesterol (TC), and triglyceride (TG) test kits were obtained from Whitman Biological Technology Co., Ltd (Nanjing, China); Enliten™ ATP assay kit for ATP concentration measurement was obtained from Promega (Madison, USA); tissue mitochondria isolation kit, mitochondrial membrane potential (ΔΨ_m) detection kit (JC-1 method), and BCA protein assay kit were obtained from the Beyotime Institute of Biotechnology (Haimen, China); catalpol (>95% purity) was a gift from Qinghai Yangzong Pharmaceutical Co., Ltd (Qinghai, China).

Experimental animals

C57BL/6J male mice (18 ± 4 g, 5–6 weeks old) were obtained from the Medical Laboratory Animal Center of Peking University (Beijing, China). Mice were housed five per cage in environmentally controlled conditions maintained at a temperature of 22 ± 2°C and a relative humidity of 55% ± 5% with a 12 h light:12 h dark cycle (07:00 h on, 19:00 h off). Food and water were provided ad libitum, except when noted otherwise before the experiments. This study was approved by the Ethical Committee of China Pharmaceutical University, and Laboratory Animal Management Committee of Jiangsu Province (approval No. 2110827). After having been adjusted to the laboratory environment for 7 days prior to the experiments, mice were randomly divided into six groups: Group I, control (n = 12); Group II, HFD/STZ-model (diabetic) control (n = 12); Group III, HFD/STZ-Met. 200 (n = 12); Group IV, HFD/
STZ-Cat. 50 (n = 12); Group V, HFD/STZ-Cat. 100 (n = 12); Group VI, HFD/STZ-Cat. 200 (n = 12). All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in China.

Induction of diabetes in mice and drug administration
Diabetes was induced in mice by a single intraperitoneal injection of STZ at 85 mg/kg body weight (BW) dissolved in freshly prepared 100 mM citrate buffer (pH 4.5). The STZ injection was administered after 2 weeks of HFD (30% fat + 70% standard chow; Nanjing Jiangning Qinglongshan Animal Farms, Nanjing, China) feeding and immediately after a 16 h fasting period. The normal controls were injected with citrate buffer alone. During the trial, the HFD/STZ-injected mice were orally given 5% Glu (2 ml/kg BW) 24 h after the injection to prevent initial STZ-induced hypoglycemic mortality, and were then tested for fasting blood glucose (FBG) levels 48 h later, and those with FBG levels ranging from 12 to 18 mM were considered as HFD/STZ-diabetic mice.

The HFD/STZ-diabetic mice were orally gavaged each day with 50, 100, or 200 mg/kg BW catalpol or 200 mg/kg BW metformin (the doses were decided according to body surface area method [27] compared with the doses in rats), and both normal and diabetic control mice received no treatment. All the control and experimental groups were then gavaged with distilled water and provided with food and water for 4 weeks. During the trial, individual BW and FBG level were measured weekly.

At the termination of the study, mice were fasted and then sacrificed. Blood sample was collected in a dry test tube and allowed to coagulate at room temperature for 30 min. The blood samples were separated by centrifugation at 2000 g for 10 min and then used for the measurement of serum TG and TC levels. After the mice were sacrificed, muscle tissues were dissected, washed in ice-cold saline, patted dry, and weighed. Muscle samples (100 mg) were removed immediately from the same site of each mouse for the isolation of the mitochondria. ATP content was then detected, and mitochondrial membrane potential (ΔΨm) was measured. The same region of the gastrocnemius muscle samples from normal control, diabetic control, and experimental (200 mg/kg BW catalpol) groups were trimmed into ~1 mm³ strips for mitochondrial ultrastructure examination using TEM. The rest of the muscle tissue was harvested, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

Intraperitoneal glucose tolerance test
After the last week of treatment, 0 min blood sample was immediately taken from normal control, diabetic control, and experimental mice after an overnight fasting. Without delay, a glucose solution (2.5 g/kg BW) was administered intraperitoneally. Blood samples were withdrawn from each mouse at 30, 60, and 120 min interval. The blood samples were used to estimate the level of glucose based on the manufacturer’s instructions. The area under the curve (AUC) was calculated according to the measurement results.

Skeletal muscle mitochondrial preparation
All skeletal muscle mitochondrial isolations were performed at 4°C. Approximately 100 mg of muscle tissue was dissected from the animal and inserted in a tube placed on ice. Then, the muscle tissue was cut into small pieces and homogenized. Six different mitochondrial isolation preparations were made for each experiment using the tissue mitochondria isolation kit. Muscle mitochondrial protein concentrations were determined using the BCA protein assay kit.

Measurement of skeletal muscle mitochondrial ATP content
The Enliten™ ATP assay kit for ATP measurement was used to measure muscle ATP content. The assay uses recombinant luciferase to catalyze the conversion of D-luciferin in the presence of ATP and O₂ to oxyluciferin, PPI, AMP, CO₂, and light at the wavelength of 560 nm. As described above, the isolated mitochondria were suspended in ATP-free water and then vortexed freely. Then, ATP was measured according to the manufacturer’s instruction. The samples were immediately neutralized to pH 7.75 followed by a 2 s delay after a 100 μl Enliten™ rL/L reagent injection and a 10 s RLU signal integration time. Light photons were measured by a luminometer and compared with an ATP standard curve to calculate ATP content. ATP content is expressed as mol/mg protein for muscle samples. Muscle protein concentration was determined using the BCA protein assay kit.

Measurement of skeletal muscle mitochondrial membrane potential (ΔΨm)
JC-1 is an ideal fluorescent and cationic dye, and the changes in JC-1 fluorescence is widely applied to analyze ΔΨm. According to the manufacturer’s manual, 100 μl of purified mitochondria containing 100 μg of protein was added in 96-well plates and then incubated with 900 μl of JC-1 staining solution (5 μg/ml) at room temperature and finally rinsed twice with JC-1 staining buffer. Changes in JC-1 fluorescence intensity and mitochondrial JC-1 monomers (λex 514 nm, λem 529 nm) and aggregates (λex 585 nm, λem 590 nm) were determined by an F-4500 fluorescence spectrophotometer. Skeletal muscle mitochondrial ΔΨm is proportional to the fluorescence ratio of red (i.e. aggregates) to green (i.e. monomers) and is expressed as OD590/OD529.
Determination of skeletal muscle mitochondrial DNA copy number

A QIAamp DNA mini kit was used to extract DNA from total muscle tissues. To quantify the amount of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), the real-time quantitative polymerase chain reaction (PCR) was used with EvaGreen fluorescent dye (Bio-Rad, Hercules, USA). ND1 and β-actin were used to represent mtDNA and nDNA, respectively. The following primers were used in this study: ND1 sense primer 5’-CAGCCTGACCCTAGCCATAATA T-3’; antisense primer 5’-TGATTCTCTTCTGTCAAGTGC GAA-3’; nuclear β-actin sense primer, 5’-GAGTGGTGC TCTCTGAAATGGTT-3’, antisense primer, 5’-GAAAGAG GCCAGTCCCATAGTACA-3’ (Sunshine Biotechnology, Nanjing, China). The results are expressed as the mtDNA/nDNA ratio. The PCR was carried out in an iQ5 Optical System (Bio-Rad, Barcelona, Spain) based on the ΔCt method. DNA (10 ng) and 1 x SsoFast EvaGreen Supermix (10 µl) were mixed in a total volume of 20 µl and placed in 96-well plates. The PCR cycle included a hold for the initial 30 s at 95°C followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. The threshold cycle number values of mitochondrial and nuclear products were performed separately. The relative mtDNA content (mtDNA/nDNA) was calculated with the following equation: relative copy number (Re) = 2^{ΔCt}, where ΔCt = C_{β-actin} - C_{mtDNA} [28]. Each melting temperature analysis was performed in triplicate for each DNA sample.

Real-time PCR analysis of mRNA for PGC1α gene in skeletal muscle

Total RNA was prepared from muscle tissues using Trizol reagent (Invitrogen, Carlsbad, USA). One microgram of total RNA from each sample was reverse transcribed to cDNA using PrimeScript™ Reverse Transcriptase (Takara, Osaka, Japan). The real-time PCR reaction mixture contained 10 µl of 1 x SsoFast EvaGreen Supermix, 1 µl of cDNA, 7 µl of RNase/DNase-free water, and 500 nM of each primer in a total volume of 20 µl. The thermal cycler conditions were the same as those used in the previous section. The relative mRNA levels of the target genes were normalized to the level of the housekeeping gene, GAPDH, and the iQ5 Optical System based on the ΔCt method. Primers used for PGC1α and GAPDH are as follows: PGC1α sense primer, 5’-TCTCGGGTGGATTGAGTG GTG-3’; PGC1α antisense primer, 5’-TGTCAGTGCAAA ATGAGGCGC-3’; GAPDH sense primer, 5’-GCACGCGAT CTAACGTGCC-3’; and GAPDH antisense primer, 5’-GAT GCTGCTTACACCCACGCA-3’ (Sunshine Biotechnology). The PCR results were confirmed at least three times for each experiment.

Muscle mitochondrial ultrastructural analysis with TEM

Muscle biopsies were trimmed into ~1 mm² strips and then immediately fixed in 2.5% glutaraldehyde for at least 2 h, post-fixed with 1% osmium tetroxide, dehydrated in a series of graded alcohols, embedded in epoxy resin, and finally polymerized with a Leica automatic microwave electron microscopy tissue processor at 60°C for 24 h. Ultrathin sections (70–80 nm thick) were made on an RMC PowerTome XL ultramicrotome (Butterfield Drive Tucson, Arizona, USA), picked up by copper grids, stained with uranyl acetate and lead citrate, and examined by a JEM-1010 transmission electron microscopy (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. Ten digital photomicrographs were taken randomly from each sample at ×30,000 and ×80,000 magnifications.

Statistical analysis

Data are presented as the mean ± SEM for each group. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. Differences were considered significant when P < 0.05.

Results

Effects of catalpol or metformin on BW

Figure 2 shows that BW was significantly decreased in HFD/STZ-induced diabetic mice compared with normal controls (P < 0.01). During the trial, no apparent changes were observed in BW of the catalpol group compared with diabetic mice (P > 0.05). The administration of metformin to diabetic mice resulted in significant increase in BW compared with diabetic mice 3 weeks after treatment (P < 0.05).

Effects of catalpol or metformin on FBG level

As shown in Table 1, the results demonstrated a dose-dependent reduction of FBG levels during four successive

Figure 2. Effects of catalpol or metformin on BW in HFD/STZ-induced mice Data were presented as mean ± SE (n = 12). One-way ANOVA was followed by Dunnett’s test. **P < 0.01 vs. control group; *P < 0.05 vs. model group. Cat, catalpol; Met, metformin.
weeks of catalpol administration compared with the untreated diabetic mice ($P < 0.05$). Notably, catalpol significantly lowered FBG levels 2 weeks after treatment (Table 1).

**Effects of catalpol or metformin on intraperitoneal glucose tolerance test**

As represented in Fig. 3A, the blood glucose levels in normal mice peaked at 30 and 60 min after the glucose loading, whereas there was a significantly higher glucose disposal rate near baseline levels at 120 min. In the diabetic mice with basal hyperglycemia, the hyperglycemia was exacerbated after the glucose load at 30 and 60 min and did not return to baseline levels after 120 min, indicating glucose intolerance and impaired disposal. However, in the case of diabetic mice treated with 200 mg/kg BW catalpol, there was a significantly higher glucose disposal rate at 30, 60, and 120 min ($P < 0.01$, compared with the diabetic model group at any timepoint); these disposal rates were similar to those of the metformin-treated diabetic mice. The maximum rate of glucose disposal was observed in mice dosed with 200 mg/kg BW catalpol compared with the other two dosages. To evaluate the overall glucose exposure, the AUC for intraperitoneal glucose tolerance test (IPGTT-AUC) was calculated, and a significant improvement in glucose exposure was noted in the diabetic mice treated with 200 mg/kg catalpol compared with that for the other two doses (Fig. 3B).

**Effects of catalpol or metformin on serum TG and TC**

Serum lipids (TG and TC) showed a significant increase in diabetic mice (Fig. 4). The results demonstrated dose-dependent reductions of both TG and TC levels by catalpol treatment. Particularly, both catalpol (200 mg/kg BW) and metformin (200 mg/kg BW) significantly ($P < 0.01$) reduced TG by 30% and 35% (Fig. 4A) and TC by 45% and 36% (Fig. 4B), respectively, compared with the diabetic mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>FBG (mM)</th>
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<tr>
<td></td>
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<td>1 weeks</td>
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<tr>
<td>Control</td>
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<td>3.57 ± 0.28</td>
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<tr>
<td>Model</td>
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<td>15.21 ± 0.29**</td>
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<tr>
<td>Cat</td>
<td>50</td>
<td>15.02 ± 0.41</td>
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<td></td>
<td>100</td>
<td>14.46 ± 0.33</td>
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<td></td>
<td>200</td>
<td>14.37 ± 0.39</td>
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<td>Met</td>
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<td>10.99 ± 0.34##</td>
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Data were presented as mean ± SE ($n = 12$). One-way ANOVA was followed by Dunnett’s test.

**P < 0.01 vs. normal control.

$^bP < 0.05.$

$^mP < 0.01$ vs. diabetic model group.

Cat, catalpol; Met, metformin.

Figure 3. Effects of catalpol or metformin on an IPGTT in HFD/STZ-induced mice (A) FBG levels at any timepoint for the IPGTT; (B) Area under the curve for the IPGTT. Data were presented as mean ± SE ($n = 12$). One-way ANOVA was followed by Dunnett’s test. **P < 0.01 vs. control group; ##P < 0.01 vs. model group. Cat, catalpol; Met, metformin.
ATP content of isolated mitochondria in skeletal muscle

Figure 5 shows that skeletal muscle mitochondrial ATP content was reduced by \( \approx 2.0 \) folds in the diabetic mice compared with the normal control group \((P < 0.01)\), whereas this effect was reversed by the oral administration of catalpol in a dose-dependent manner. Especially, the ATP production was significantly increased by \( \approx 3.0 \) and \( \approx 2.5 \) folds after the administration of 200 and 100 mg/kg BW catalpol \((P < 0.01)\) when compared with the diabetic model mice, respectively. However, the administration of metformin showed no significant changes compared with the diabetic model mice \((P > 0.05)\).

Mitochondrial membrane potential \((\Delta \Psi_m)\) levels of isolated mitochondria in skeletal muscle

Figure 6 shows that the \( \Delta \Psi_m \) level of diabetic mice in skeletal muscle was reduced by 60% compared with the control group \((P < 0.01)\), whereas this effect was reversed in a dose-dependent manner after catalpol treatment. Especially, the \( \Delta \Psi_m \) level in skeletal muscle of diabetic mice was brought back to near normal upon 200 mg/kg BW catalpol treatment (Fig. 6). However, in the case of metformin administration, there was no apparent change in the level of muscle \( \Delta \Psi_m \) compared with the diabetic model mice \((P > 0.05)\).

mtDNA copy number normalized by nDNA in skeletal muscle

Figure 7 shows the copy number of mtDNA in skeletal muscle of the control, diabetic model, and experimental mice. The mtDNA copy number in the diabetic mice was decreased by 30% compared with the control mice \((P < 0.05)\). There was a significant increase in mtDNA upon catalpol treatment
in a dose-dependent manner compared with the diabetic model group, especially for the 100 and 200 mg/kg BW catalpol groups ($P < 0.05$). In contrast, no change was observed in the 200 mg/kg BW metformin group compared with the diabetic mice ($P > 0.05$).

Mitochondrial ultrastructural analysis in skeletal muscle with TEM
The mitochondrial ultrastructure in skeletal muscle is shown in Fig. 8. In the control mice, the muscle mitochondrial ultrastructure was within the normal range for size and contained well-defined membrane and well-delineated cristae arrangements (Fig. 8A,B). In contrast, the muscle mitochondrial ultrastructure in the diabetic mice showed extensive mitochondrial lesions, including swollen morphology, disrupted cristae arrangement, and the loss of inner- and outer-membranes along with a reduction in mitochondrial density (Fig. 8C,D). The administration of 200 mg/kg BW catalpol rescued muscle mitochondrial injury (Fig. 8E,F); specifically, these cells possessed intact sarcomeres and less swelling as well as an intact mitochondrial membrane and well-organized cristae. The ultrastructural images of the cells from the groups treated with 200 mg/kg BW catalpol were similar to that of the control group (Fig. 8).

Relative mRNA level of PGC1α in skeletal muscle
Figure 9 shows the relative mRNA level of the PGC1α in skeletal muscle from the control and model mice. Real-time PCR results showed that the mRNA level of PGC1α was

Figure 7. mtDNA copy number in skeletal muscle on HFD/STZ-induced mice (normalized to nDNA) Data were presented as mean ± SE ($n = 12$). One-way ANOVA was followed by Dunnett’s test. *$P < 0.05$ vs. control group; **$P < 0.05$ vs. model group.

Figure 8. Mitochondrial ultrastructural analysis of the skeletal muscle in HFD/STZ-induced mice using TEM Representative images at ×30,000 and ×80,000 to compare the differences between the mitochondria of the control group (A,B), model group (C,D), and catalpol-treated (200 mg/kg) group (E,F).
setting this model was time-consuming [29–31]. In addition, STZ could induce hypoinsulinemia, hyperglycemia, and mitochondrial dysfunction, resembling diabetes. Single high-dose injection of STZ (T2DM), to demonstrate the relationships between them and functions largely affect muscle metabolism and have a significant impact on whole-body metabolism [34]. Given their essential function in aerobic metabolism, mitochondria are intuitively of interest with regard to the pathophysiology of diabetes [12]. Our results showed smaller mitochondrial ultrastructures, reduced mitochondrial ultrastructure numbers per unit volume (density), and significant reduction of mitochondrial ATP content and ∆Ψm levels in the skeletal muscle of HFD/STZ-diabetic mice. Currently, T2DM is one of the major diseases all over the world. Although lifestyle modification is suggested as an initial therapy for T2DM, drug treatment is also required in many cases. Metformin that plays a role in glucose-lowering effect as implied by our present study of lowered FBG and the IPGTT, is commonly used in clinic to treat T2DM. There are various mechanisms for metformin that have been demonstrated, including inhibition of complex I of the mitochondrial respiratory chain, decreasing hepatic glucose production, increasing glucose uptake, and stimulation of AMP-dependent protein kinase (AMPK) [36].

Discussion

Nowadays, there is a multiple of literatures on whether mitochondrial dysfunction could play a relevant role in developing insulin resistance and/or T2D, while the causal relationship between them is still controversial. In this study, it is essential for us to develop a suitable animal model of hyperglycemia, insulin resistance, and mitochondrial dysfunction, resembling the characteristics seen in human type 2 diabetes mellitus (T2DM), to demonstrate the relationships between them and to develop diabetic drugs. Single high-dose injection of STZ could induce hypoinsulinemia, hyperglycemia, and mitochondrial dysfunction, resembling type 1 diabetes in human, but setting this model was time-consuming [29–31]. In addition, obesity, insulin resistance, and diabetes induced by HFD alone did not lead to any impairment of mitochondrial performance [30–33]. In order to shorten the 3-month modeling time, the combination of STZ injection and HFD has been tried to develop the skeletal muscle mitochondrial dysfunction and insulin resistance in diabetic mice, which took only 4 weeks [9,31].

In our study, the results showed that HFD/STZ diabetic mice had a significant reduction in BW, and an obvious increase in blood glucose, serum TG, and serum TC levels. No apparent change was observed in BW upon treatment with catalpol when compared with diabetic mice. The hypoglycemic action of this drug was observed in a dose-dependent manner and was demonstrated by the higher rate of glucose disposal (IPGTT). Using TEM, we observed that in the catalpol-treated group (200 mg/kg BW), the skeletal muscle mitochondrial morphology showed less damage, and the mitochondrial density was increased. This phenomenon was further supported by the observation that the mtDNA copy number was increased compared with the diabetic mice. Furthermore, the mitochondrial ATP content and the ∆Ψm level in the skeletal muscle were also reversed in a dose-dependent manner by the catalpol supplementation. Interestingly, the expression of PGC1α, which plays an essential role in improving muscle mitochondrial biogenesis and function, in skeletal muscle was also significantly up-regulated after four successive weeks of catalpol treatment, which is consistent with the existing reports [14,34]. In single STZ-treated mice, the levels of mitochondrial respiratory chain complexes and mitochondrial function were markedly reduced, resulting from the down-regulation of PGC1α-mediated expression of mitochondrial genes [31]. Therefore, HFD/STZ-induced diabetic model is a useful animal model for screening hypoglycemic drugs and studying the corresponding mechanisms. Interestingly, mitochondrial function in the skeletal muscle was also enhanced in the control mice treated with catalpol (200 mg/kg BW) alone (data not shown). On the basis of the current investigation, it is strongly suggested that the hypoglycemic effect of catalpol is mediated through the amelioration of muscle mitochondrial dysfunction.

Mitochondria, which convert nutrients into energy through cellular respiration, are the principal energy source of the cell [35]. Mitochondrial adaptations (biogenesis and dynamics) and functions largely affect muscle metabolism and have a significant impact on whole-body metabolism [34]. Given their essential function in aerobic metabolism, mitochondria are intuitively of interest with regard to the pathophysiology of diabetes [12].
Particularly, some literatures reported that treatment of T2DM with metformin was associated with increased plasma ghrelin concentrations [37]. Furthermore, in our study, BW gain in the metformin-treated group was observed, which probably was involved in increased plasma ghrelin concentrations, resulting in the appetite stimulation. Thus, therapy targeting mitochondria may provide a new way to treat diabetes. Our results suggested that the treatment of diabetic mice with catalpol significantly increased their aerobic capacity, as evidenced by their increased skeletal muscle ATP content. Surprisingly, catalpol significantly lower FBG levels after 2 weeks. Furthermore, in the HFD/STZ diabetic model, blood glucose is accompanied by an increase in serum TG and TC levels, which were rescued to near normal with the treatment of catalpol. The significant reduction in TC and TG levels was presumably involved in increasing the fatty acid β-oxidation by improved mitochondrial function, decreasing the intracellular fatty acyl CoA and diacylglycerol, and promoting to the uptake of glucose in skeletal muscle [9].

Overall, the possible mechanism for the relationship between the glucose-lowering action and the anti-triglyceridemic effect of catalpol could be correlated to ameliorating mitochondrial dysfunction and fatty acid β-oxidation in the skeletal muscle of diabetic mice.

PGC1α is a factor for most nuclear receptors and several other transcription factors. Notably, PGC1α induces and coordinates gene expression; and it stimulates the thermogenic program in brown fat, fiber-type switching in skeletal muscle, and metabolic pathways linked to the fasting response in the liver [38]. Notably, the increased PGC1α expression in skeletal muscle results in the induction of a wide array of genes involved in mitochondrial biogenesis and function [39–41]. Furthermore, the muscle-specific disruption of the PGC1α gene in mice results in the decreased expression of mitochondrial genes, resulting in a switch from oxidative fibers to more glycolytic fibers, and impairing their endurance capacity [15]. So, catalpol may affect mitochondrial function through this transcriptional co-activator.

PGC1α is highly regulated at both the transcriptional and post-translational levels, primarily through histone acetyltransferase GCN5-regulated acetylation [42] and NAD+-dependent deacetylase SIRT1-regulated acetylation [43]. The acetylation/deacetylation regulatory mechanism affects PGC1α activity, leading to increased mitochondrial biogenesis. When the balance between acetylation and deacetylation is altered from damage due to diabetes, PGC1α acetylation, regulated by SIRT1, is at high levels, which is consistent with its activity and mRNA levels at a low level, resulting in mitochondrial dysfunction. This reversal of balance could be significant for the treatment or prevention of diabetes. In addition, it has been recently shown that AMPK activation results in a net increase of NAD\(^+\) levels with a consequent induction of SIRT1 activity or PGC1α phosphorylation [44]. We hypothesize that the hypoglycemic effect of catalpol was most likely due to the involvement of the AMPK-SIRT1-PGC1α-regulated mitochondrial biogenesis signaling pathway in skeletal muscle. Meanwhile, chronic metformin treatment for 14 days increased the β-hydroxyacyl-CoA dehydrogenase activity, cytochrome c protein content, and the PGC1α content in the soleus muscle, which suggest that metformin enhances the PGC1α expression and mitochondrial biogenesis in the skeletal muscle [45]. Therefore, we think that metformin may be a right positive drug in our experiment. Furthermore, it has been proposed that AMPK activation by metformin could enhance mitochondrial biogenesis proteins including PGC1α in cardiac ischemic cells [46,47]. However, no change was observed on the PGC1α mRNA expression levels of metformin, so we suggest that PGC1α is presumably regulated at the post-translational level primarily through AMPK phosphorylation. Studies in our laboratory showed that the effect of metformin on HFD/STZ-induced diabetic mice in the skeletal muscle mitochondrial function was not so significant; however, this probably is an advantage of catalpol to play the role in hypoglycemic effect.

Catalpol possesses a wide range of biological and pharmacological activity as described above. Whether oral administration of catalpol has mitochondrial protective activity in skeletal muscle to treat diabetes is still not clear. Our present study showed that catalpol could ameliorate mitochondrial dysfunction in the skeletal muscle of diabetic mice. The increase of aerobic capacity and the decrease in blood glucose levels might be related to the increase in the mRNA expression of PGC1α in skeletal muscle after catalpol treatment.

In summary, it has been indicated that stimulating PGC1α-regulated mitochondrial biogenesis can improve mitochondrial function, decrease oxidative stress, and then ameliorate insulin resistance [5,6,14,48]. Thus, catalpol, targeting mitochondria, provides hope for treating insulin resistance and/or T2D.

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