Salvianolic acid A promotes mitochondrial biogenesis and function via regulating the AMPK/PGC-1α signaling pathway in HUVECs

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Abstract. Mitochondrial dysregulation is an important pathology that leads to endothelial dysfunction, and the occurrence and development of cardiovascular diseases. Salvianolic acid A (SAA) has been demonstrated to be effective in the treatment of vascular complications of type 2 diabetes mellitus. Limited information has been reported on the effects of SAA on mitochondrial function in endothelial cells. In the present study, the effects of SAA on mitochondrial biogenesis and the related underlying mechanisms were investigated in human umbilical vein endothelial cells (HUVECs). Mitotracker red staining and transmission electron microscopy were used to evaluate the effect of SAA on mitochondrial quality. The effect of SAA treatment on mitochondrial DNA/nuclear DNA ratio of HUVECs was detected by real-time quantitative PCR. Western blot was used to determine the protein expression levels of complex III and Complex IV of mitochondrial oxidative phosphorylation subunit, and ATP production was determined by ATP test kit. Real-time quantitative PCR and Western blot were used to determine the effects of SAA on the expression of peroxisome proliferator-activated receptor γ coactivator (PGC-1α) and its target genes nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM) proteins and genes. Finally, in the presence of 5'AMP-activated protein kinase (AMPK) specific inhibitors, the expression of PGC-1α, NRF1 and TFAM proteins and the phosphorylation levels of AMPK and Acetyl CoA Carboxylase (ACC) were detected by Western blot or real-time quantitative PCR. The results showed that SAA treatment significantly promoted mitochondrial biogenesis and enhanced mitochondrial function of HUVECs. SAA significantly increased the expression levels of PGC-1α and its target genes NRF1 and (TFAM), a key regulator of mitochondrial biogenesis in HUVECs. These enhancements were accompanied by significantly increased phosphorylation of AMPK and ACC, and were significantly inhibited by specific AMPK inhibitors. These results suggest that SAA may promote mitochondrial biogenesis in endothelial cells by activating the AMPK-mediated PGC-1α/TFAM signaling pathway. These data provide new insights into the mechanism of action of SAA in treating diabetic vascular complications.

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Introduction

In recent years, the incidence of type 2 diabetes mellitus (T2DM) has gradually increased (1). T2DM-associated disability and fatality rates are increasing and are mainly caused due to its complications, including diabetic eye disease, diabetic nephropathy and diabetic peripheral nephropathy (2,3). These issues are considered to be vascular complications of T2DM. At present, the main clinical treatment strategies for diabetic vascular complications include the use of antihypertensive and hypoglycemic drugs to prevent and delay their development (4). For example, the aldose reductase inhibitor epalrestat is used to control blood glucose levels (5). Renin-angiotensin system inhibitors, including angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, are commonly used first-line therapeutic agents for the treatment of diabetic nephropathy (6). Losartan and enalapril are used to reduce the progression of diabetic retinopathy (7).

Endothelial dysfunction serves an important role in diabetic vascular complications and mitochondrial dysfunction is the main cause of endothelial dysfunction in patients with diabetes (8,9). Therefore, previous study have addressed the treatment of T2DM and its complications by improving endothelial function via the enhancement of mitochondrial biosynthesis, maintenance of mitochondrial function and the mitigation of mitochondrial dysfunction (10).

Mitochondrial biogenesis serves a crucial role in cellular mitochondrial function (11). Mitochondrial biogenesis is caused by the initiation of mitochondrial DNA (mtDNA) replication, and the expression of mitochondrial proteins encoded

by the nuclear and mitochondrial genomes (12). Peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1)α/β are considered to be the central regulators of mitochondrial biogenesis (13). PGC-1α transactivates nuclear respiratory factor 1 (NRF1), which activates mitochondrial transcription factor A (TFAM). Subsequently, TFAM regulates the transcription and replication of mtDNA. The 5'AMP-activated protein kinase (AMPK)/PGC-1α signaling pathway is closely associated with mitochondrial biogenesis (14).

Salvianolic acid A (SAA) is one of the main active water-soluble constituents of the plant, *Salvia miltiorrhiza* (15). SAA has been demonstrated to serve an important role in the treatment of vascular complications of T2DM (16). It has previously been reported that SAA may improve endothelial vascular function (17); however, the mechanism by which SAA ameliorates endothelial dysfunction remains unclear. Qiang *et al* (17) demonstrated that SAA can improve mitochondrial function of liver cells and skeletal muscle cells in T2DM mice via the activation of AMPK. Therefore, the aim of the present study was to investigate whether SAA could promote the mitochondrial biosynthesis of human umbilical vein endothelial cells (HUVECs) and improve mitochondrial function via activation of the AMPK/PGC-1α signaling pathway, thereby improving endothelial vascular function and ultimately treating diabetic complications.

Materials and methods

Cell culture. Primary HUVECs were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cellular experiments were performed using 1st-5th generation cells. The cells were cultured in RPMI-1640 medium (Procell Life Science & Technology Co., Ltd.) containing 10% FBS (Biological Industries) and 1% penicillin-streptomycin (Procell Life Science & Technology Co., Ltd.). The cells were cultured in an incubator at 37˚C in a 5% CO_2 humidifying atmosphere.

Drug treatment. SAA (purity, 98%) was provided as a lyophilized powder by The Institute of Materia Medica, Chinese Academy of Medical Sciences. The cells were cultured in petri dishes in a medium of 37˚C humidified with 5% CO_2. Following their attachment to the wells, different concentrations of SAA (10^{-3}, 10^{-2} and 10^{-1} μM) were added and incubated at 37˚C for 24 h. To determine the role of SAA in mitochondrial biogenesis, the inhibitor group was treated with 2.5 μM AMPK inhibitor compound C (MedChemExpress) at 37˚C for 30 min prior to treatment with 10^{-2} μM SAA. The AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; 0.5 mM; MedChemExpress) at 37˚C for 30 min as a positive control.

Mitochondrial quantification. Cells were treated at the above dosing concentration at 37˚C for 24 h and were subsequently examined via electron microscopy. Briefly, the cells were fixed in 2.5% glutaraldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 4 h and then rinsed with phosphate buffer and immobilized with 2% osmium tetroxide at 4˚C for 1.5 h. Dehydrated with a series of graded ethanol to 70% ethanol, in a saturated uranium acetate solution prepared with 70% ethanol or acetone, dyed for 2 h or more, cleaned with epoxy propane, embedded in Spurrs low viscosity resin and sectioned. Thin sections with thickness of 70 nm were observed via transmission electron microscopy (Olympus Corporation). Image-Pro Plus (version 5.0; Media Cybernetics, Inc.) software was used to quantify the number of mitochondria in each image and the data were expressed as mitochondrial density (number of mitochondria/cytoplasmic region). At least 20 cells were assessed per treatment.

Adenosine triphosphate (ATP) assay. The ATP levels of cells were determined via a colorimetric method using an ATP test kit (Elabscience Biotechnology, Inc.). Creatine kinase catalyzes the reaction of creatine and ATP to generate creatine phosphate; creatine phosphate content is detected using a colorimetric method to reflect the ATP content (18). The cells were cultured in 6-well plates in 37˚C humidified with 5% CO_2 until adherent. Following adherence, the cells were treated with different concentrations of SAA (10^{-3}, 10^{-2} and 10^{-1} μM) and incubated at 37˚C for 24 h. The collected cells were added with 0.3 ml boiling deionized water for each 10^6 cells, tightly covered and mixed, then inserted into the floating float and placed in the boiling water bath for 10 min, then mixed in vortex for 1 min at room temperature of 1,000 x g and centrifuged for 10 min. The ATP levels of the supernatant were assessed according to the instructions provided by the manufacturer. The optical density was measured at 636 nm using a microplate reader and the ATP levels were determined using the manufacturer's formula.

Western blotting. Following treatment with SAA, the cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology) containing a 1% protease inhibitor cocktail (MedChemExpress) on ice for 15 min. The protein concentration was assessed using a BCA kit (Elabscience Biotechnology, Inc.). Protein samples (20 μg were separated with 10% SDS-PAGE (Epizyme). Following separation, the protein was transferred to a PVDF membrane (Beijing Solarbio Science & Technology Co., Ltd) and blocked with 5% skimmed milk at room temperature for 1.5 h. After blocking, the membrane was incubated with primary antibodies against the following: AMPK (1:1,000; cat. no. 5831; Cell Signaling Technology, Inc.), phosphorylated (p)-AMPK (1:1,000; cat. no. 2535; Cell Signaling Technology, Inc.), acetyl-CoA carboxylase (ACC; 1:1,000; cat. no. 3676; Cell Signaling Technology, Inc.), p-ACC (1:1,000; cat. no. 11818; Cell Signaling Technology, Inc.), PGC-1α (1:1,000; cat. no. 2178; Cell Signaling Technology, Inc.), NRF1 (1:1,000; cat. no. 46743; Cell Signaling Technology, Inc.), TFAM (1:1,000; cat. no. 8076; Cell Signaling Technology, Inc.), complex III (1:1,000; cat. no. ab182330; Abcam), complex IV (1:1,000; cat. no. ab14705; Abcam) and GAPDH (1:2,000; cat. no. E-AB-20059; Elabscience Biotechnology, Inc.) at 4˚C overnight. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (cat. nos. E-AB-1003 and E-AB-1001; 1:10,000; Elabscience Biotechnology, Inc.) for 2 h at room temperature. A ChemiDoc™ Imager (VILBER, Ltd.) was used to detect the chemiluminescence signals by dropping chemiluminescence solution (Elabscience Biotechnology, Inc.) Image-pro Plus (Version 5.0; Media Cybernetics, Inc.) software was used to quantify protein expression in each image.

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total intracellular RNA was extracted from HUVECs using RNA simple Total RNA kit (Tiangen Biotech Co., Ltd.). A Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.) was used to determine the concentration and quality of the extracted RNA. A total of 1 μg RNA was reverse-transcribed into cDNA using an Evo M-MLV RT Mix kit with gDNA Clean for qPCR (GATC Biotech AG). The cDNA was analyzed via qPCR (QuantStudio3, Thermo Fisher Scientific, Inc.) using SYBR-Green Master Mix (GATC Biotech AG) to determine the mRNA expression levels of the target genes. The thermocycling conditions were as follows: 1 cycle of 95˚C for 10 min; 95˚C for 10 sec; 60˚C for 15 sec; 72˚C for 20 sec (40 cycles), and 72˚C for 10 min. The starting template was quantitated using the CT value estimated by the real-time PCR recorder. β-actin was used as the internal control .The qPCR primers used were as follows: PGC-1α forward (F), 5'-ATCTACTGCCTGGGGACCTT-3' and reverse (R), 5'-ATGTGTCGCCTTCTTGCTCT-3'; NRF1 F, 5'-CGCAGCACCTTTGGAGAA-3' and R, 5'-CCCGACCTG TGGAATACTTG-3'; TFAM F, 5'-GGCACAGGAAACCAG TTAGG-3' and R, 5'-CAGAACACCGTGGCTTCTAC-3'; and β-actin F, 5'-ACGGCCAAGTCATCACTATTG-3' and R, 5'-AGCCACCGATCCACACAGA-3'.

Determination of mtDNA copy number. Total DNA was extracted from cells using TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd.). Using the aforementioned method, qPCR was used to quantify mtDNA by assessing the proportion of the mitochondrial D-loop region and the 18S ribosomal (r)RNA expression levels; 18S rRNA was used to represent nuclear DNA (nDNA). The primers used in the experiment were as follows: Mitochondrial D-loop F, 5'-ATGGCCAAC CTCCTACTCCT-3' and R, 5'-GCGGTGATGTAGAGGGTG AT-3'; and 18S F, 5'-CATTCGAACGTCTGCCCTATC-3' and R, 5'-CCTGCTGCCTTCCTTGGA-3'.

Mitochondrial mass assessment. The cells were incubated in a confocal glass dish in 37˚C humidified with 5% CO_2 until adherent. Following adherence, the cells were treated with different concentrations of SAA (10^{-3}, 10^{-2} and 10^{-1} μM) and incubated at 37˚C for 24 h. After washing with PBS, Mitotracker red (Shanghai Yeasen Biotechnology Co., Ltd.) at a final concentration of 50 nM was added and cells were incubated at 37˚C for 30 min in the dark. Then the nuclei were stained with 10 μg/ml DAPI (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 5 min. Staining was assessed using a confocal microscope (Zeiss AG). The integrated optical density of the stained cells was analyzed using Image-Pro Plus (version 5.0) software to assess the mitochondrial mass.

Statistical analysis. All results are presented as the mean ± SEM of at least three independent experiments. Statistical analysis was performed using GraphPad Prism (version 5.0; GraphPad Software Inc.). One-way ANOVA followed by Tukey's post hoc test was used to assess the statistical significance of the differences between more than two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SAA promotes mitochondrial biogenesis. To assess the effects of SAA on mitochondrial biogenesis, Mitotracker red staining was used to detect the mitochondrial mass of HUVECs. SAA treatment significantly increased the mitochondrial mass in a dose-dependent manner compared with cells treated with vehicle only (Fig. 1A). The number of mitochondria was also observed using electron microscopy. Compared with the vehicle group, the number of mitochondria in the SAA group significantly increased in a dose-dependent manner, with the highest number of mitochondria in cells treated with 10^{-1} μM SAA (Fig. 1B). Increased copy number of mtDNA indicated mitochondrial biogenesis (Fig. 1C). The mtDNA levels were increased by 1.8, 2.4, and 3.2 times with SAA treatment at 10^{-3}, 10^{-2} and 10^{-1} μM, respectively. These results indicated that SAA treatment significantly increased mtDNA levels in HUVECs compared with the vehicle-only group. The results suggested that SAA promotes mitochondrial biogenesis.

SAA enhances mitochondrial function. Mitochondrial biogenesis increases mitochondrial function and intracellular ATP is produced by the mitochondria (19). To assess whether SAA treatment increased mitochondrial function, intracellular ATP levels were quantified in HUVECs and the protein expression levels of the related mitochondrial complex III and IV were determined via western blotting. As presented in Fig. 2A, the protein expression levels of complex III and IV were significant increased following SAA treatment. In addition, compared with the vehicle group, intracellular ATP levels increased significantly after SAA treatment in a dose-dependent manner, which suggested that SAA potentially enhanced mitochondrial function (Fig. 2B).

SAA promotes the expression of PGC-1α, NRF1 and TFAM. It has previously been reported that mitochondrial biogenesis is associated with the PGC-1α/NRF1/TFAM signaling pathway (20). It was therefore hypothesized that the promotion of mitochondrial biogenesis by SAA may also be related to the PGC-1α/NRF1/TFAM signaling pathway. Therefore, the effect of SAA on the mRNA and protein expression levels of PGC-1α, NRF1 and TFAM in HUVECs was investigated. The results indicated that the mRNA and protein expression levels of PGC-1α, NRF1 and TFAM were all significantly increased following the treatment of HUVECs with SAA at concentrations of 10^{-3}, 10^{-2} and 10^{-1} μM compared with the vehicle group (Fig. 3). These results suggested that SAA increased the expression of PGC-1 α, NRF1, TFAM, which are associated with mitochondrial biogenesis.

SAA promotes AMPK signaling pathway-mediated mitochondrial biogenesis. As mentioned above, SAA significantly increased the expression levels of PGC-1α, NRF1 and TFAM, which thereby promoted mitochondrial biogenesis. PGC-1α expression is dependent mainly on upstream AMPK activation (21). Therefore, it was hypothesized that the effects of SAA on mitochondrial biogenesis may be caused via the activation of AMPK. The results demonstrated that the AMPK specific inhibitor compound C significantly reduced the expression levels of PGC-1α, and its downstream target proteins

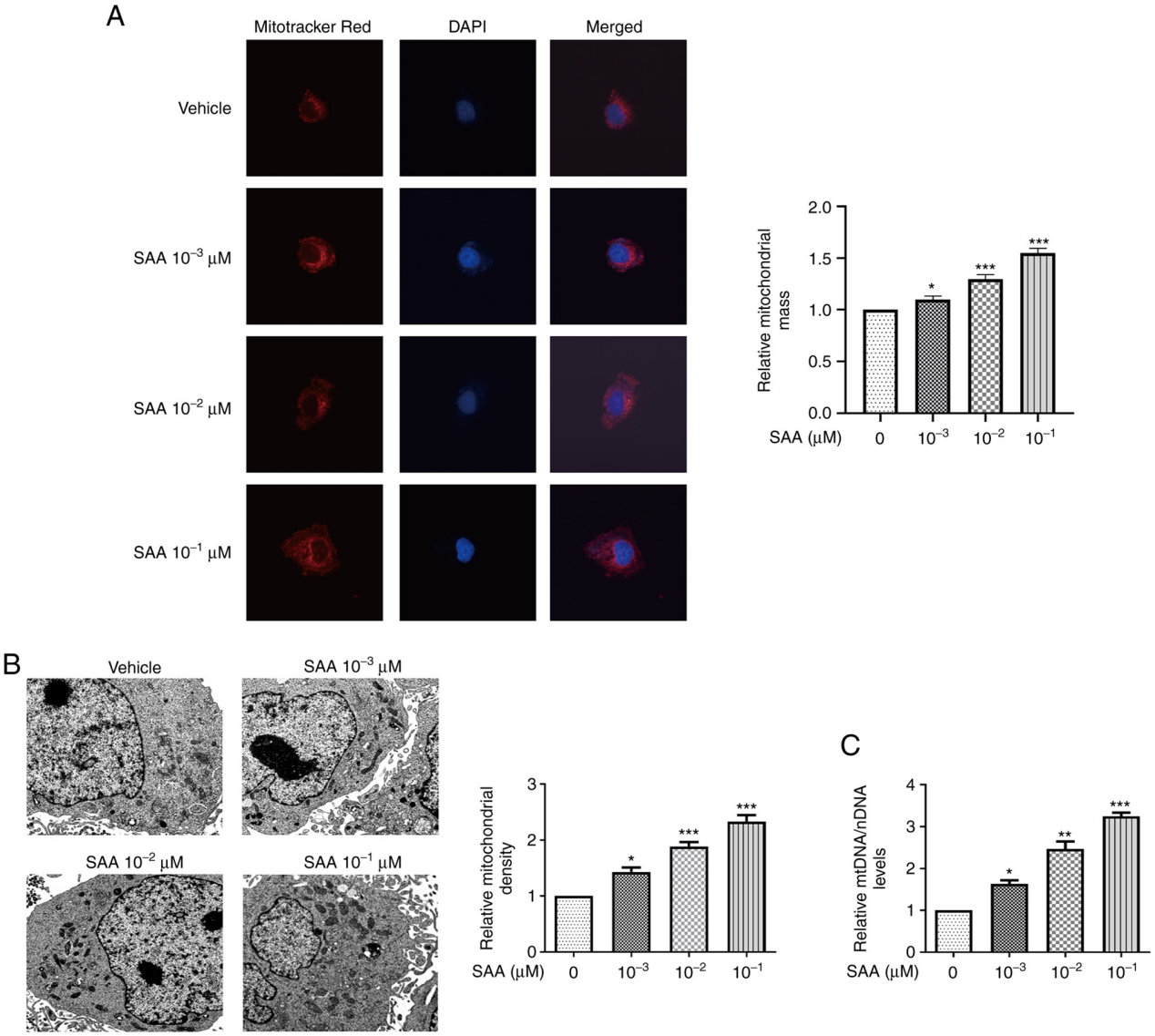

Figure 1. SAA promotes mitochondrial biogenesis in HUVECs. HUVECs were treated for 24 h with SAA at concentrations of 10^{-3}, 10^{-2} and 10^{-1} μM. (A) Mitochondria were stained with Mitotracker red dye. The integrated fluorescence intensity was analyzed to assess the mitochondrial mass relative to the vehicle group. (B) Mitochondrial density was assessed using transmission electron microscopy (magnification, x6,000). (C) mtDNA/nDNA was determined using reverse transcription-quantitative PCR and is presented relative to the control cells. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle. SAA, salvianolic acid A; mtDNA/nDNA, mitochondrial/nuclear DNA.

NRF1 and TFAM compared with cells only treated with SAA (10^{-2} μM) (Fig. 4A and B). Moreover, the SAA-mediated enhancement of mtDNA levels was also significantly reduced following treatment with compound C (2.5 μM) compared with cells treated with SAA (10^{-2} μM; Fig. 4C). AICAR, which is an AMPK activator, was used as a positive control. The results showed that SAA significantly increased phosphorylation levels of AMPK and ACC compared with SAA untreated group. However, compound C significantly inhibited the phosphorylation of AMPK and ACC by SAA (Fig. 4D).

Discussion

Vascular complications of diabetes are the most common, and the leading cause of death and disability (19,22). Endothelial vascular dysfunction is the main pathological basis of diabetic vascular complications (23). Mitochondria are the most abundant organelles in cells, and their reduced number and dysfunction serve an important role in diabetes-related endothelial cell damage, as well as in the occurrence and development of diabetic vascular complications (24). Therefore, the improvement of mitochondrial function in endothelial cells and the induction of mitochondrial biogenesis may be an important strategy to prevent diabetic vascular complications (25).

In the present study, SAA treatment significantly increased mtDNA levels and mitochondrial mass in HUVECs compared with the control group, which indicated that SAA may have promoted mitochondrial biogenesis. Furthermore, the protein expression levels of the mitochondrial respiratory chain complex III and IV, as well as mitochondrial ATP production, were also significantly increased by SAA treatment. These results indicated that SAA may promote mitochondrial biogenesis and improve mitochondrial function in HUVECs.

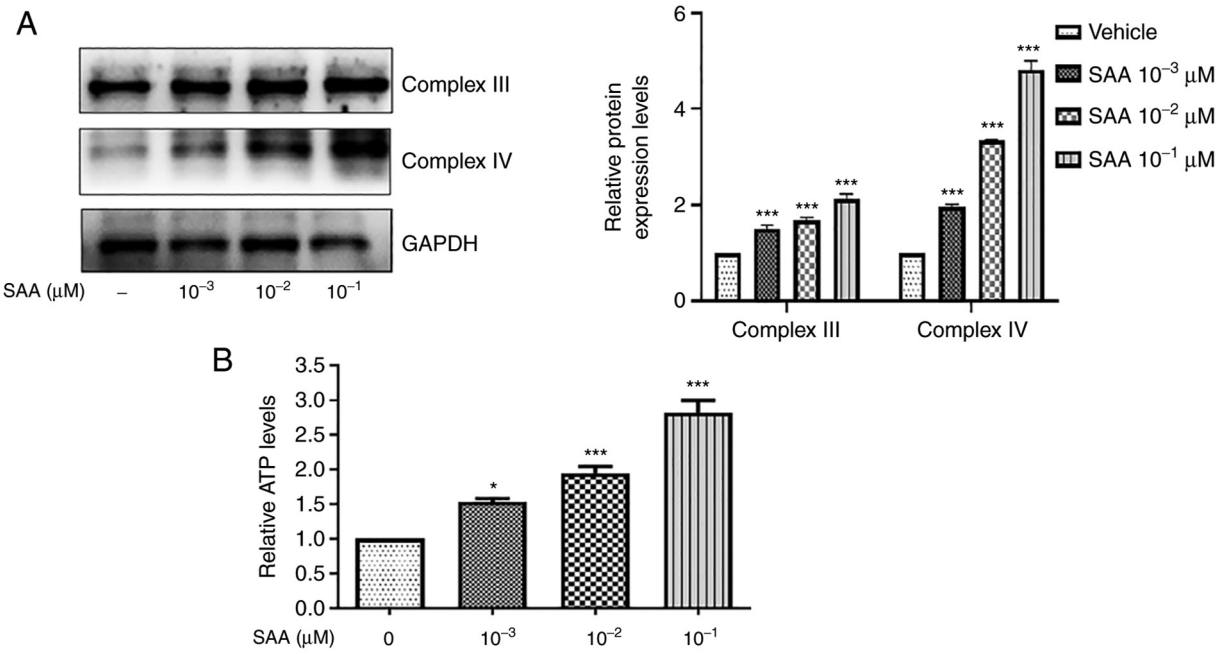

Figure 2. SAA enhances mitochondrial function. (A) Western blotting of complex III and complex IV protein expression levels following SAA treatment of the cells. (B) Intracellular ATP levels were assessed using colorimetry. *P<0.05, ***P<0.001 vs. vehicle. SAA, salvianolic acid A.

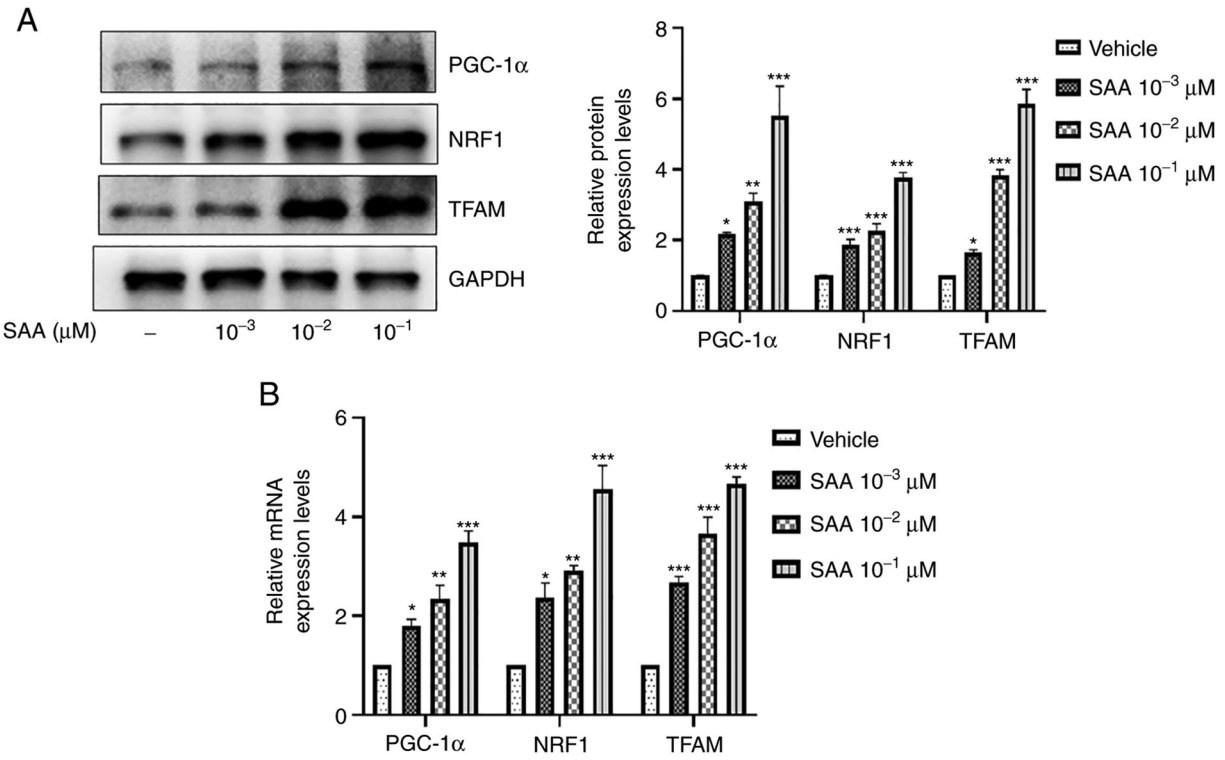

Figure 3. SAA promotes the expression of PGC-1α, NRF1 and TFAM. (A) Following SAA treatment, western blotting was performed to assess PGC-1α, NRF1 and TFAM protein expression levels. (B) mRNA expression levels of PGC-1α, NRF1, and TFAM were determined using reverse transcription-quantitative PCR. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle. SAA, salvianolic acid A; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A.

Mitochondrial biogenesis is a complex process. PGC-1α is the main activator that controls the mitochondrial biogenesis signaling pathway via activation of NRF1 and TFAM. This signaling pathway serves an important role in mitochondrial biogenesis (26). The PGC-1α/NRF1/TFAM signaling pathway has previously been demonstrated to be involved in mitochondrial biogenesis in numerous diseases (27,28). Jiang *et al* (29) demonstrated that perampanel could improve mitochondrial biogenesis in neuronal cells via activation of the PGC-1α/NRF1 signaling pathway, which thereby exerts an anti-epileptic effect.

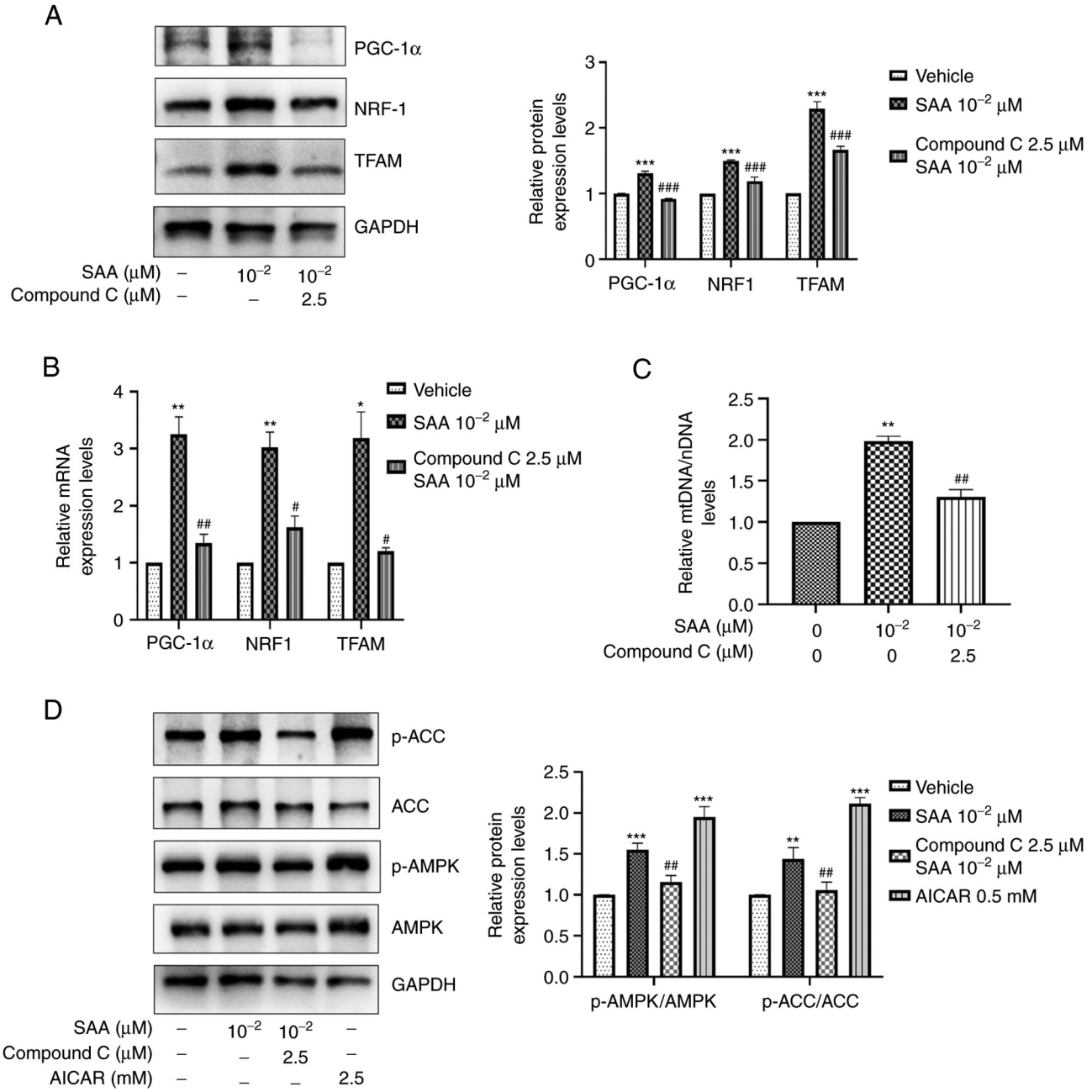

Figure 4. SAA promotes mitochondrial biogenesis via the AMPK signaling pathway. (A) Following SAA and compound C stimulation, western blotting was performed to assess PGC-1α, NRF1 and TFAM protein expression levels. (B) mRNA expression levels of PGC-1α, NRF1 and TFAM were determined using RT-qPCR. (C) mtDNA/nDNA was determined using RT-qPCR and is presented relative to vehicle. (D) Following treatment of the cells with SAA, compound C and AICAR stimulation, western blotting was performed for the detection of p-AMPK and p-ACC expression levels. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. SAA treatment group. SAA, salvianolic acid A; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, 5'AMP-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; RT-qPCR, reverse transcription-quantitative PCR; mtDNA/nDNA, mitochondrial/nuclear DNA, p, phosphorylated; ACC, acetyl-CoA carboxylase.

Sun *et al* (30) demonstrated that activation of the PGC-1α signaling pathway was required to protect mitochondria and promote mitochondrial biosynthesis in electroacupuncture preconditioning-induced ischemia tolerance. Li *et al* (31) reported that the preservation or promotion of mitochondrial function via activation of the PGC-1α/NRF1/TFAM signaling pathway could lead to mitochondrial DNA and protein synthesis. The production of new mitochondria is a potential therapeutic target for the treatment of neurodegenerative diseases (32). Chandrasekaran *et al* (33) demonstrated that LY379268, a selective metabolomic glutamate receptor 2/3 agonist, also upregulated the PGC-1α/TFAM signaling pathway and prevented diabetic peripheral neuropathy via glutamate recycling in Schwann/satellite glial cells and via the improvement of dorsal root ganglion neuronal mitochondrial function. In the present study, SAA significantly increased the mRNA and protein expression levels of PGC-1α, and its downstream targets NRF1 and TFAM, which suggested that the PGC-1α/NRF1/TFAM signaling pathway is important for SAA-induced mitochondrial biogenesis.

AMPK is an important upstream regulatory factor of PGC-1α and a master regulator of mitochondrial biogenesis (34). AMPK has also been demonstrated to promote mitochondrial biogenesis and to improve endothelial dysfunction (35,36). Previous studies have reported that the activation of AMPK via phosphorylation promotes the expression of PGC-1α, which thereby enhances mitochondrial biogenesis (37). For example, ginger (*Zingiber officinale*) extract has been shown to promote mitochondrial biogenesis and improve mitochondrial function via activation of the AMPK/PGC-1α signaling pathway (38). Similarly, naringenin can promote mitochondrial biogenesis via activation of the AMPK/PGC-1α signaling pathway (39). In the present study, to explore the potential regulatory mechanism of SAA, the phosphorylation of AMPK and downstream ACC were investigated following treatment of HUVECs with SAA. The specific inducer, AICAR, and inhibitor, compound C, of AMPK were used to assess this. The results indicated that SAA significantly increased the phosphorylation levels of AMPK and ACC in HUVECs, which was similar to the results obtained using AICAR. Moreover, compound C significantly inhibited the phosphorylation of AMPK and ACC following SAA treatment of the cells. Compound C also significantly reduced the protein expression levels of PGC-1α, and its downstream targets NRF-1 and TFAM following SAA treatment of the cells. These results suggested that AMPK may be a potential target of SAA, by which the induction of PGC-1α expression and the activation of mitochondrial biogenesis were achieved.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that SAA promoted mitochondrial biogenesis in HUVECs via the AMPK/PGC-1α/NRF1/TFAM signaling pathway. These data provided an important theoretical basis for the use of SAA in the treatment of diabetic complications via improving endothelial vascular dysfunction. However, there are other pathways involved in mitochondrial biogenesis. For example, mitochondrial biogenesis can be promoted by upregulation of AKT/STAT3 signal to inhibit apoptosis and mitochondrial biogenesis of adipocytes can be promoted by activation of p38/CREB pathway (40,41). Whether SAA promotes mitochondrial biogenesis and enhances mitochondrial function is involved in other pathways remains to be studied.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS and ZH designed the study. XW was responsible for the data collection and manuscript writing. All authors participated in the experiments. MiZ and MeZ were responsible for data acquisition and analysis. YH was responsible for statistical analysis. XC and WZ were responsible for literature searches. JS and ZH reviewed and revised the manuscript. XW and MiZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Qingdao University (Qingdao, China; approval no. QYFYWZLL27047).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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