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Cilostazol promotes mitochondrial biogenesis in human umbilical vein endothelial cells through activating the expression of PGC-1α

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ABSTRACT

Mitochondrial dysfunction is frequently observed in vascular diseases. Cilostazol is a drug approved by the US Food and Drug Administration for the treatment of intermittent claudication. Cilostazol increases intracellular cyclic adenosine monophosphate (cAMP) levels through inhibition of type III phosphodiesterase.

The effects of cilostazol in mitochondrial biogenesis in human umbilical vein endothelial cells (HUVECs) were investigated in this study. Cilostazol treated HUVECs displayed increased levels of ATP, mitochondrial DNA/nuclear DNA ratio, expressions of cytochrome B, and mitochondrial mass, suggesting an enhanced mitochondrial biogenesis induced by cilostazol. The promoted mitochondrial biogenesis could be abolished by Protein kinase A (PKA) specific inhibitor H-89, implying that PKA pathway played a critical role in increased mitochondrial biogenesis after cilostazol treatment. Indeed, expression levels of peroxisome proliferator activator receptor gamma-coactivator 1α (PGC-1α), NRF 1 and mitochondrial transcription factor A (TFAM) were significantly increased in HUVECs after incubation with cilostazol at both mRNA levels and protein levels. Importantly, knockdown of PGC-1α could abolish cilostazol-induced mitochondrial biogenesis. Enhanced expression of p-CREB and PGC-1α induced by cilostazol could be inhibited by H-89. Moreover, the increased expression of PGC-1α induced by cilostazol could be inhibited by downregulation of CREB using CREB siRNA at both mRNA and protein levels. All the results indicated that cilostazol promoted mitochondrial biogenesis through activating the expression of PGC-1α in HUVECs, which was mediated by PKA/CREB pathway.

1. Introduction

Mitochondria are highly dynamic organelles in eukaryotic cells. Endothelial mitochondria have been demonstrated to play a crucial role in vascular pathophysiology [1]. During the life cycle of mitochondria, mitochondrial biogenesis is involved in maintaining mitochondrial homeostasis to meet the physiological needs of eukaryotic cells. Nuclear respiratory factor 1 (NRF 1), which controls the nuclear genes to encode mitochondrial proteins, has been reported to regulate mitochondrial biogenesis in cells and tissues. Another critical factor is named mitochondrial transcription factor A (TFAM), which drives transcription and replication of mitochondrial DNA (mtDNA). The expressions of NRF 1 and TFAM are regulated by peroxisome proliferator activator receptor gamma-coactivator 1α (PGC-1α) [2]. Mitochondria biogenesis is reported to participate in the regulation of endothelial cell metabolism, redox regulation, and signal transduction. Dysregulation of mitochondrial biogenesis is observed in many diseases and is associated with endothelial dysfunction, including cellular energetic imbalance, oxidative stress, and apoptosis [3]. Thus, identification of mechanisms that promote mitochondrial biogenesis in endothelial cells is helpful for the development of improved pharmacological approaches to prevent vascular dysfunction and promote vascular health [4].

Cilostazol is a licensed clinical drug for treating patients suffering from intermittent claudication. It's a phosphodiesterase (PDE) inhibitor, which selectively inhibits PDE3, a cAMP-degrading enzyme, thus elevating intracellular cAMP [5]. Recent studies also showed that cilostazol could prevent coronary artery restenosis post-endovascular treatments. It also displayed the protective effects against transient focal cerebral ischemia [6] and chronic cerebral hypo perfusion injury [7]. In vitro studies found that cilastozol could prevent the lipopolysaccharide (LPS)-induced apoptosis in HUVECs through mitochondrial pathways [8]. But the effects of cilostazol on mitochondria in endothelial cells remain unknown.

To explore whether cilostazol induces mitochondrial biogenesis in endothelial cells, we investigated the effects of cilostazol treatment on mitochondrial mass and the induction of factors

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regulating mitochondrial biogenesis in primary HUVECs. We focused on the mechanistic role of PGC-1α activation and the related pathways in the effects of cilostazol.

2. Materials and methods

2.1. Cell culture, treatment and transfection

HUVECs were from Lonza (Walkersville, USA). Cells were cultured in EBM-2 media with supplemental growth factors according to manufacturer's instructions [9]. Cells were treated by 10 μM cilostazol with or without 10 μM PKA inhibitor H89 (Sigma, USA). The PGC-1α, CREB and negative control small interfering RNAs (siRNAs) oligo were from Sigma, USA. siRNAs were transfected into HUVECs using Lipofectamine RNAiMAX (Invitrogen, USA).

2.2. Western blot analysis

HUVECs were lysed in RIPA buffer (Tris–HCl, pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with the complete protease inhibitor and phosphatase inhibitor cocktail (Roche, USA). The extracted protein was then subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). After blocked with 5% non-fat milk, membranes were sequentially incubated with primary antibodies for 3 h and horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature (RT). Finally, blots were developed using Beyo ECL Plus reagent (enhanced chemiluminescence) and exposed to film according to the manufacturer's protocol [10]. The following antibodies were used in this study: rabbit monoclonal antibody for PGC-1α (Calbiochem, USA); rabbit monoclonal antibodies for NRF 1, TFAM, and cytochrome B, p-CREB, CREB and β-actin were from Cell Signaling, USA.

2.3. Real time polymerase chain reaction (PCR)

Total RNA from HUVECs was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA was used as templates for reverse transcription PCR to synthesize cDNA. The synthesized cDNA was then used in quantitative real-time PCR analysis using the step one plus real-time PCR system (Applied Biosystems, USA) with the SYBR Green detection method. The following primers were used in this study: PGC-1α (forward, 5′-CAATGAATGCAGCGGTCTTA-3′; reverse, 5′-ACGTCTTTGTGGCTTTTGCT-3′); NRF 1 (forward, 5′-CTACT CGTG TGGGACAGCAA-3′; reverse, 5′-AATTCCGTCGATGGTGAGAG-3′); TFAM (forward, 5′-GGCACAGGAAACCAGTTAGG-3′; reverse, 5′-CAGAACACCGTGGCTTCTAC-3′); GAPDH (forward, 5′-CCACA TCGCTCAGACACCAT-3′; reverse 5′-CCAGGCGCCCAATACG-3′).

2.4. Determination of mitochondrial DNA (mtDNA) copy number

mtDNA copy number was measured by real-time PCR method. Total intracellular DNA in HUVECs was extracted using QIAamp DNA mini kit (QIAGEN, Germantown, MD, USA) following the manufacturer's instruction. The primers for one subunit of human electron transport chain used for mtDNA amplification were: ND1: forward, 5′-ATGGCCAACCTCCTACTCCT-3′; reverse: 5′-GCGGTGATG TAGAGGGTGAT-3′; Primers for Human nuclear 18S, used for internal control, were: forward, 5′-ACGGACCAGAGCGAAAGCA-3′and reverse, 5′-GACATCTAAGGGCATCACAGAC-3′. Relative amounts of mtDNA and nuclear DNA (nDNA) copy numbers were compared. The ddCt (mtDNA to 18S) represents the mtDNA copy number in a cell.

2.5. Mitochondria staining with MitoTracker red

To stain the mitochondria, HUVECs were plated on cover-slips. After indicated transfection and incubation, cells were washed with HBSS and stained with 20 nM MitoTracker red (Invitrogen) as described previously [11]. Cell nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI). Cells were observed at 100× oil immersion using Zeiss fluorescence microscope. Fifty individual cells were randomly selected in each group. The intracellular integrated optical density (IOD) of red fluorescence was analyzed with Image-Pro Plus software (Version 5.0). The average IOD was used to index mitochondrial mass.

2.6. Measurement of intracellular ATP

ATP levels in HUVECs were determined using a bioluminescence somatic cell assay kit (Invitrogen, USA) according to the manufacturer's instructions. 2×10^4 cells were used for each sample. Chemiluminescence signals were acquired with a Victor X3 Multilabel Plate Readers (PerkinElmer, USA).

2.7. Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test was used to determine the difference in the means of the two groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

Mitochondria are the major source of ATP. HUVECs were treated with 10 μM cilostazol and the levels of ATP were measured using a standard firefly fluorescence assay. Cilostazol treatment resulted in a significant increase in ATP production. However, this effect was completely abolished by 10 μM H-89, a specific PKA inhibitor (Fig. 1A). To directly determine whether mitochondrial biogenesis was affected, we measured the ratio of mitochondrial DNA to nuclear DNA (mtDNA/nDNA) by real time PCR. It was found that the mtDNA/nDNA was significantly increased in HUVECs (Fig. 1B) after cilostazol treatment. But the increased mtDNA/nDNA was significantly abolished by 10 μM H-89. Moreover, increased expression level of mitochondrial protein, cytochrome B, was also found after cilostazol treatment (Fig. 1C). Correspondingly, H-89 could inhibit the increased expression of cytochrome B. We further measured the mitochondria mass in HUVECs after cilostazol treatment by using Mito-Tracker red staining. Fluorescence microscopy revealed that cilostazol treatment led to a significant increase in Mito-Tracker staining. Correspondingly, the increased Mito-Tracker staining induced by cilostazol was prevented by H-89. The negative effects of H-89 on cilostazol-induced mitochondrial biogenesis demonstrated that PKA pathway played a critical role in increased mitochondrial biogenesis after cilostazol treatment.

Mitochondrial biogenesis is regulated by the PGC-1α-NRF 1-TFAM pathway, so in the further study we measured the expression level of PGC-1α in HUVECs after incubation with cilostazol. Real-time PCR studies showed that PGC-1α mRNA levels were significantly increased in HUVECs after cilostazol treatment (Fig. 2A). Immunoblot analysis also revealed significantly increased total protein levels of PGC-1α in HUVECs after incubation with 10 μM cilostazol (Fig. 2B). We further investigated the expressions of NRF 1 and TFAM. The real time PCR result indicated that cilostazol treatment led to a significant increase in the mRNA levels of NRF 1 and TFAM (Fig. 2C). Accordingly, the protein levels of NRF 1 and TFAM were significantly increased in HUVECs after cilostazol treatment (Fig. 2D). These data suggest increased expressions of func-

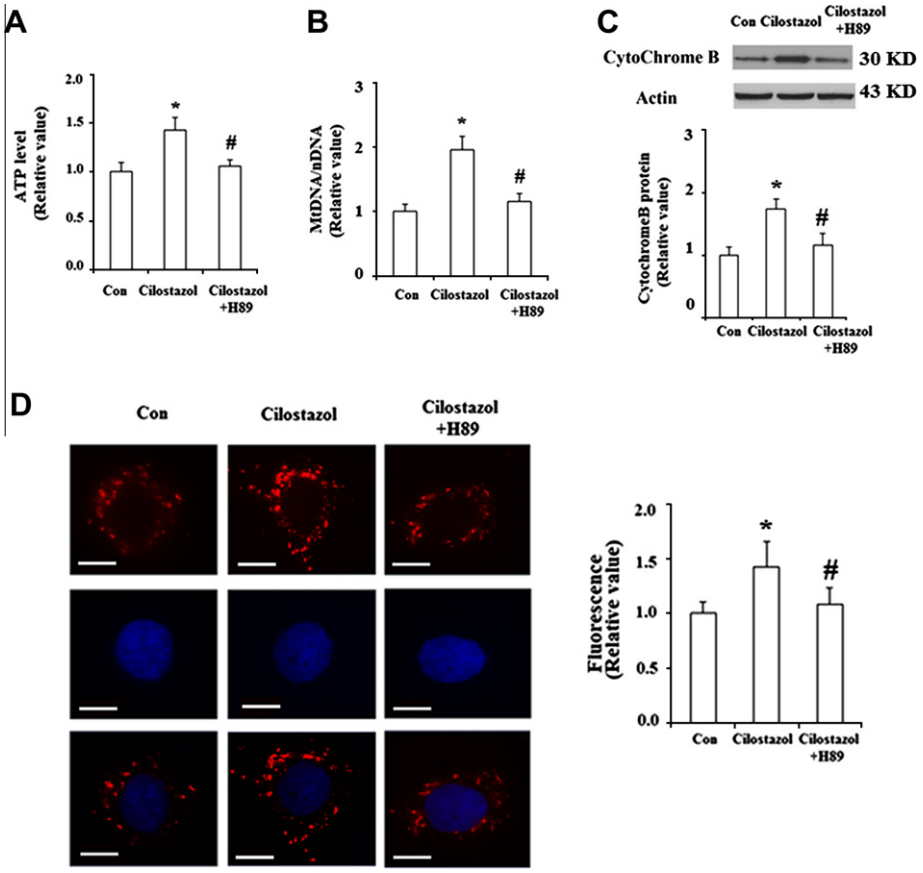

Fig. 1. Cilostazol increased mitochondrial mass in HUVECs, which could be abolished by H-89. HUVECs were plated 8 h prior to treatment with 10 μM cilostazol with or without H-89 for 24 h. Con, control group. (A) Cilostazol increased ATP production. After cell lysis, levels of ATP were determined as described in experimental procedures (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group); (B) mitochondrial-to-nuclear DNA ratios were determined by quantitative real time PCR and were expressed relative to control cells (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group); (C) representative immunoblot and quantification analysis revealed that the level of cytochrome B was significantly increased by cilostazol treatment, but the increased expression of cytochrome B was abolished by H-89 (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group); (D) cells were incubated with 10 μM cilostazol followed by staining with MitoTracker red dye and DAPI as described in methods. The cells were visualized by fluorescence microscopy. Scale bar, 20 μM. The integrated fluorescence intensity was analyzed (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group).

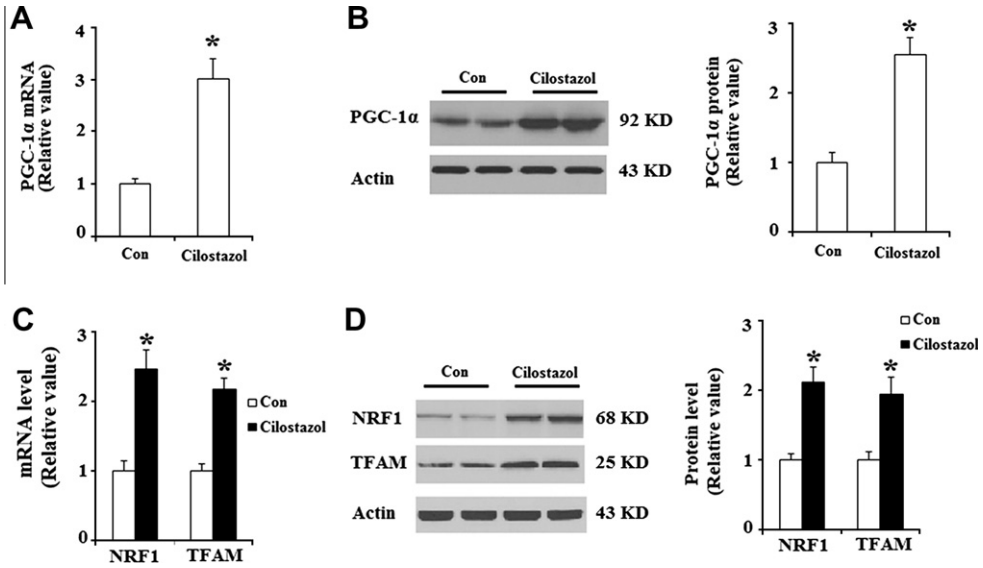

Fig. 2. Cilostazol increased the expressions of mitochondrial biogenesis proteins. Con, control groups. (A) mRNA levels of PGC-1α were significantly increased by incubation with 10 μM cilostazol for 24 h (*$P < 0.01$ vs. control group); (B) immunoblot and quantification analysis revealed that protein levels of PGC-1α were significantly increased after incubation with 10 μM cilostazol for 24 h (*$P < 0.01$ vs. control group); (C) real time PCR results revealed that mRNA levels of NRF 1 and TFAM were significantly increased after incubation with 10 μM cilostazol for 24 h (*$P < 0.01$ vs. control group); (D) immunoblot and quantification analysis revealed that protein levels of NRF 1 and TFAM were significantly increased after incubation with 10 μM cilostazol for 24 h (*$P < 0.01$ vs. control group).

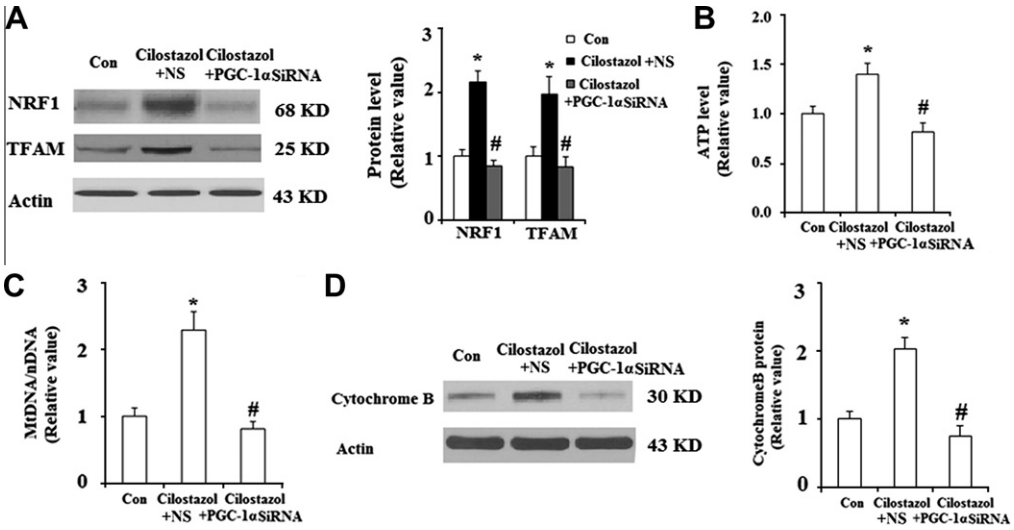

Fig. 3. Inhibition of PGC-1α could abolish the promoted mitochondrial biogenesis signal induced by cilostazol. HUVECs were transiently transfected with PGC-1α siRNA 24 h prior to treatment with 10 μM cilostazol. Con, control group; NS, non-specific siRNA. (A) Representative immunoblot and quantification analysis revealed that the increased levels of NRF 1 and TFAM induced by cilostazol were significantly abolished by inhibition of PGC-1α (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group); (B) firefly luciferase assay showed that the cilostazol-induced ATP production were abolished by transient transfection with PGC-1α siRNA (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group); (C) the induced levels of mitochondrial-to-nuclear DNA ratios were inhibited by transient transfection with PGC-1α siRNA (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group); (D) representative immunoblotting and quantification analysis revealed that cytochrome B was significantly reduced after inhibition of PGC-1α (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group).

tional factors should be responsible for the promoted mitochondrial biogenesis in cilostazol treated HUVECs.

To assess whether the increased expression of PGC-1α played a causal role in the increased mitochondrial biogenesis and function in HUVECs, we investigated the effects of knockdown of PGC-1α in the promoted mitochondrial biogenesis induced by cilostazol. HUVECs were transiently transfected with PGC-1α siRNA. Forty eight hours after transfection, expression of mitochondrial biogenesis proteins and mitochondrial parameters were determined. Indeed, the increased expressions of the downstream proteins including NRF 1 and TFAM induced by cilostazol were significantly inhibited by transfection with PGC-1α siRNA (Fig. 3A). Most importantly, the inhibition of PGC-1α abolished the increased ATP level (Fig. 3B) along with mtDNA/nDNA (Fig. 3C) and Cytochorome B (Fig. 3D) levels induced by cilostazol.

The principal action of cilostazol is to elevate intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) levels [12]. The expression of PGC-1α is regulated by transcription factor CREB [13]. cAMP, a PKA activator, could increase phosphorylated CREB and activity through PKA pathway. Thus, we hypothesized that cilostazol increased the expression of PGC-1α and mitochondrial biogenesis through PKA-CREB pathway. The results in Fig. 1 showing that PKA inhibitor H-89 abolished the promoted mitochondrial biogenesis induced by cilostazol supported this

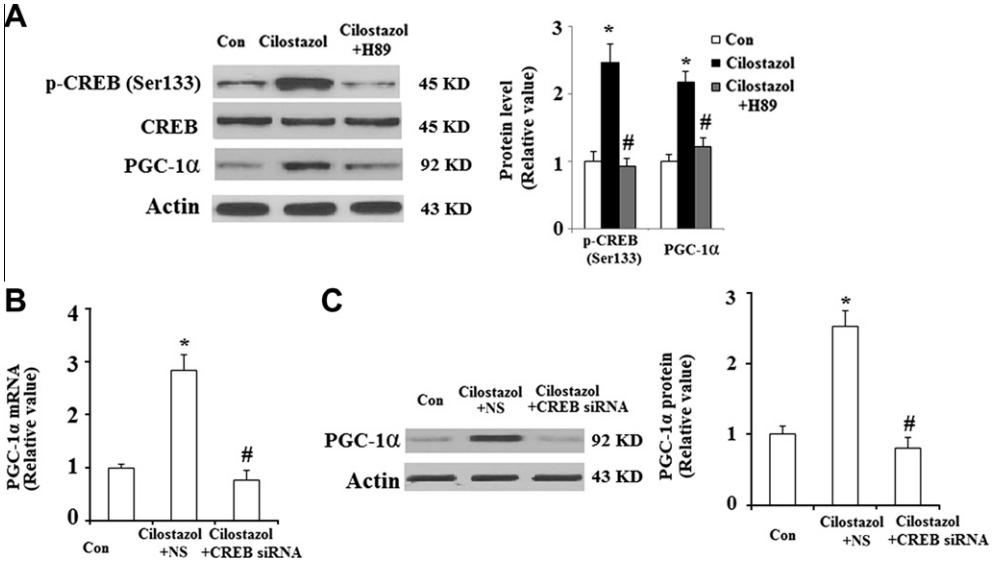

Fig. 4. PKA/CREB pathway played a critical role in the increased expression of PGC-1α induced by cilostazol. Con, control group; NS, non-specific siRNA. (A) Immunoblotting and quantification analysis revealed that the increased levels of p-CREB and PGC-1α induced by cilostazol were significantly inhibited by PKA inhibitor H89 (10 μM); (B) HUVECs were transiently transfected with CREB siRNA 24 h prior to treatment with 10 μM cilostazol. Real time PCR results revealed that cilostazol-induced expression of PGC-1α could be abolished by knockdown of CREB (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group); (C) representative immunoblotting and quantification analysis revealed that cilostazol-induced expression of PGC-1α could be abolished by knockdown of CREB (*$P < 0.01$ vs. control group; #$P < 0.01$ vs. cilostazol treated group).

hypothesis. To explore the mechanisms underlying cilostazol-induced expressions of PGC-1α, we investigated the effect of H-89 on the phosphorylation of CREB (Ser133) and expression of PGC-1α in HUVECs after cilostazol treatment. Indeed, cilostazol-induced phosphorylation of CREB and increased expression of PGC-1α were completely abolished by 10 μM H-89 (Fig. 4A), suggesting that PKA played a critical role in the increased expression of p-CREB and PGC-1α in cilostazol treated HUVECs. To further examine whether CREB mediated cilostazol-induced expression of PGC-1α, the effect of cilostazol on PGC-1α expression was investigated in HUVECs following siRNA mediated downregulation of CREB. Indeed, the increased expression of PGC-1α induced by cilostazol could be abolished by downregulation of CREB at both the mRNA (Fig. 4B) and protein levels (Fig. 4C).

4. Discussion

Cilostazol is a drug widely used in the treatment of peripheral vascular diseases in humans [14]. Although cilostazol is effective in relieving the symptoms of peripheral vascular disease, neither the mechanism of action of the drug nor its role in the treatment of the vascular disease are fully understood. Here, we report for the first time, to our knowledge, that cilostazol could promote mitochondrial biogenesis and increase mitochondrial contents. In this study, we demonstrated that the ATP levels, mtDNA/nDNA ratio, and mitochondrial contents were significantly increased in HUVECs after cilostazol stimulation. Consistent with this notion, we also found that cilostazol treatment led to the induction of PGC-1α, a key factor in the biogenesis of the mitochondria, followed by significant alterations in the expression patterns of molecules involved in mitochondrial biogenesis, including NRF 1 and TFAM. These data suggested that cilostazol could promote mitochondrial biogenesis in HUVECs. Importantly, inhibition of PGC-1α almost completely abolished the stimulated mitochondrial biogenesis induced by cilostazol in HUVECs. Moreover, we further demonstrated that the promoted mitochondrial biogenesis and induction of PGC-1α could be prevented by PKA inhibitor H-89. We also revealed that the increased expression of PGC-1α induced by cilostazol could be abolished by knockdown of CREB. Based on these observations, we concluded that PKA/CREB pathway played an important role in cilostazol-induced expression of PGC-1α and mitochondrial biogenesis.

Increasing evidence indicates that mitochondrial damage and dysfunction occur in different kinds of diseases, including vascular diseases, such as atherosclerosis, and may contribute to the multiple pathological processes underlying the disease [15]. A novelty previous study demonstrated that reduced expressions of PGC-1α, and its downstream targets NRF 1, NRF 2, and TFAM were associated with impaired mitochondrial biogenesis pathways in Alzheimer's disease (AD). Importantly, the study reported that reduced expression of p-CREB and PGC-1α in AD cell models could be rescued by cAMP in a dose-dependent manner [16]. Although these findings were presented in central neural systems, they were still in consistence with our results and supported our hypothesis. In agreement with our findings, another study demonstrated that PKA mediated activation of CREB enhanced Hepatocyte nuclear factor-4α (HNF-4α) transcriptional activity via induction of the PGC-1α gene in HepG2 cells [17], which suggested that the activated PGC-1α might play a critical role in a variety of physiological activities. Mitochondrial DNA abnormalities can be used as a key marker for diseases differentiation and effectiveness of the treatment. Oxidative stress induced mitochondrial DNA deletion has been considered as a hallmark for the drug development in the context of the cerebrovascular diseases [18]. Mitochondria are the dominant source of ATP, continuous quality control is mandatory to ensure their ongoing optimal function. As a PDE3 inhibitor, cilostazol could increase intracellular cAMP levels, it would be expected that it could increase ATP release in response to activation of heterotrimeric G protein Gi. Indeed, a previous study demonstrated that when type 2 diabetes (DM2) erythrocytes were exposed to cilostazol (10 μM), ATP release from DM2 erythrocytes was potentiated [19], which is in agreement with our study. Mitochondria are also a potent source of free radicals and pro-apoptotic factors. As such, maintaining mitochondrial homeostasis is essential to cell survival. Cilostazol was reported to protect HUVECs against LPS-induced apoptosis by suppressing mitochondria-dependent apoptotic signaling. Cilostazol could destroy the mitochondrial permeability transition, cytosolic release of cytochrome C, and subsequent activation of caspases, stimulating extracellular signal-regulated kinase (ERK1/2) and p38 MAPK signaling, and increasing Bcl-2 expression, while suppressing Bax expression [8]. Consistently, another study demonstrated that cilostazol and its analogs exerted a strong protection against apoptotic cell death in HUVECs by scavenging hydroxyl radicals and intracellular ROS with reduction in TNF-α formation [20]. Mitochondria represent approximately one-third of the mass of the heart and play a critical role in maintaining cellular function [21]. Mitochondrial dysfunction has been suggested as a potential cause for heart failure. Downregulated mitochondrial coactivator PGC-1α along with reduced oxidative capacity has been found in failing hearts [22]. Increased mitochondrial biogenesis through PGC-1α activation is suggested to be a potential therapeutic approach for mitochondrial disorders in cell and animal models of mitochondrial diseases [23,24]. Our data suggested that cilostazol might have a potential effect in therapeutic mitochondrial disorders through stimulating the expression of PGC-1α and mitochondrial biogenesis in vascular diseases.

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