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Nebivolol stimulates mitochondrial biogenesis in 3T3-L1 adipocytes

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ABSTRACT

Nebivolol is a third-generation β-adrenergic receptor (β-AR) blocker with additional beneficial effects, including the improvement of lipid and glucose metabolism in obese individuals. However, the underlying mechanism of nebivolol's role in regulating the lipid profile remains largely unknown. In this study, we investigated the role of nebivolol in mitochondrial biogenesis in 3T3-L1 adipocytes. Exposure of 3T3-L1 cells to nebivolol for 24 h increased mitochondrial DNA copy number, mitochondrial protein levels and the expression of transcription factors involved in mitochondrial biogenesis, including PPAR-γ coactivator-1α (PGC-1α), Sirtuin 3 (Sirt3), mitochondrial transcription factor A (Tfam) and nuclear related factor 1 (Nrf1). These changes were accompanied by an increase in oxygen consumption and in the expression of genes involved in fatty acid oxidation and antioxidant enzymes in 3T3-L1 adipocytes, including nebivolol-induced endothelial nitric oxide synthase (eNOS), as well as an increase in the formation of cyclic guanosine monophosphate (cGMP). Pretreatment with NG-nitro-L-arginine methyl ester (l-NAME) attenuated nebivolol-induced mitochondrial biogenesis, as did the soluble guanylate cyclase inhibitor, ODQ. Treatment with nebivolol and β3-AR blocker SR59230A markedly attenuated PGC-1α, Sirt3 and manganese superoxide dismutase (MnSOD) protein levels in comparison to treatment with nebivolol alone. These data indicate that the mitochondrial synthesis and metabolism in adipocytes that is promoted by nebivolol is primarily mediated through the eNOS/cGMP-dependent pathway and is initiated by the activation of β3-AR receptors.

1. Introduction

β-AR blockers are among the most widely used drugs for the prevention and treatment of cardiovascular disease, but the traditional β-AR blocker has been associated with weight gain and a worsening of insulin resistance, which limited its clinical utilisation [1]. Nebivolol is a third-generation β-AR blocker that has been approved by the U.S. Food and Drug Administration for the treatment of hypertension. The selectivity of nebivolol for the β1-AR is higher than the selectivity of other β-AR blockers, such as bisoprolol, carvedilol or bucindolol. Nebivolol may also have advantages in populations experiencing heart failure with other comorbidities, including diabetes and obesity [2–4]. Therefore, the role of nebivolol in the regulation of insulin resistance and energy metabolism is of increased interest.

White adipose tissue is an important endocrine organ involved in the control of whole-body metabolism and insulin sensitivity. Mitochondria play a central role in the maintenance of energy stores and the regulation of metabolism [5]. Emerging evidence indicates an impairment of mitochondrial glucose and fatty acid metabolism and mitochondrial loss in muscle and adipose tissue in patients with insulin resistance and type 2 diabetes [6,7]. Thus, mitochondrial biogenesis could, in part, underlie the central role of adipose tissue in the control of whole-body metabolism and in the actions of some insulin sensitisers [8].

Our previous study demonstrated that mitochondrial dysfunction appears to be a key contributor to insulin resistance. By identifying natural compounds and nutrients that can target mitochondrial biogenesis and function, we found that mitochondrial nutrients alone or in combination may be effective in regulating fatty acid oxidation either *in vitro* or *in vivo*, and that such regulation may lead to the prevention of insulin resistance [9,10]. Notably, the same effects have been demonstrated by several drugs, including metformin, 5-aminoimidazole-4-carboxamide ribonucleoside [11], the PPARγ agonist pioglitazone/rosiglitazone [8,12], PPARα agonist WY-14, 643 [12–14], and β3-adrenergic receptors agonist CL-316, 243 [15]. Therefore, targeting mitochondria

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through mitochondrial biogenesis stimulation may be an effective strategy for preventing and treating insulin resistance and diabetes [16].

Mitochondrial biogenesis in mammalian tissues is modulated by PGC-1α expression [17]. The upstream signaling involved in the activation of PGC-1α includes calcium/calmodulin-dependent protein kinase IV (CaMKIV), AMP-activated protein kinase (AMPK) and nitric oxide (NO) [18–20]. Increased PGC-1α induces the transcription of the nuclear respiratory factors Nrf1 and Nrf2, leading to the increased expression of Tfam, which translocates into the mitochondrion and stimulates mitochondrial biogenesis, as manifested by the stimulation of mitochondrial DNA replication and mitochondrial gene expression [21,22].

Nebivolol exhibits nitric oxide-mediated vasodilating properties resulting from the stimulation of the endothelial β3-AR [23,24]. The stimulation of the β3-AR and the activation of eNOS increase NO release after nebivolol treatment, which causes peripheral vasodilatation and endothelial function improvement [25,26]. Thus, we speculated that the potential beneficial effects of nebivolol on insulin resistance may be mediated through enhanced mitochondrial biogenesis in adipocytes. The present study sought to determine whether the treatment of adipocytes with nebivolol affects mitochondrial mass or the expression of genes and proteins involved in mitochondrial biogenesis.

2. Materials and methods

2.1. Materials

Nebivolol and anti-GAPDH were purchased from Sigma (St. Louis, MO, USA), anti-oxphos complex I and II from Invitrogen (Carlsbad, CA, USA), anti-PGC-1α from Santa Cruz Biotechnology (Delaware Avenue, USA), the anti-p-eNOS and anti-Sirt3 antibodies from Cell Signaling Technology (Beverly, MA, USA), the reverse transcription system kit from Promega (Mannheim, Germany) and HotStarTaq from Takara (Otsu, Shiga, Japan). The Nrf1, Tfam and 18S rRNA primers were synthesised by Bioasia Biotech (Shanghai, China). The peroxidase-conjugated rabbit anti-goat IgG, peroxidase-conjugated rabbit anti-mouse IgG and peroxidase-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, USA). The BCA™ protein assay kit and Pierce ECL western blotting substrate were purchased from Thermo Scientific (Rockford, USA). TRIzol and the other reagents for cell culture were from Invitrogen.

2.2. Methods

2.2.1. Cell culture and adipocyte differentiation

Murine 3T3-L1 pre-adipocytes (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and allowed to reach confluence. Differentiation of pre-adipocytes was initiated with 1 μmol/L insulin, 0.25 μmol/L dexamethasone and 0.5 mmol/L 3-isobutyl-1-methylxanthine in DMEM supplemented with 10% FBS. After 48 h, the culture medium was replaced with DMEM supplemented with 10% FBS and 1.0 μmol/L insulin. The culture medium was changed every other day, using new DMEM containing 10% FBS. Cells were harvested 9–10 days following the induction of differentiation, when at least 90% exhibited the adipocyte phenotype. Cells were made quiescent by incubation in DMEM supplemented with 0.1% BSA for 12 h and were treated with nebivolol for 24 h.

2.2.2. Mitochondrial mass

A fluorescent probe (Mito-Tracker Green FM; Molecular Probes, Eugene, OR, USA) was used to determine the mitochondrial mass of adipocytes. After treatment with nebivolol for 24 h, adipocytes were incubated with 100 nmol/L MitoTracker green for 30 min at 37 °C. Measurements were made using a fluorescence spectrometer with an excitation wavelength of 490 nm and emission wavelength of 516 nm.

2.2.3. Western blot analysis

Cell lysates (10 μg protein per lane) were subjected to SDS–PAGE, then transferred to nitrocellulose membranes and blocked with 5% non-fat milk/Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated with primary antibodies directed against PGC-1α (1:1000), GAPDH (1:5000), OxPhos Complex I (NADH ubiquinol oxidoreductase 39-kDA subunit 1:2000) and OxPhos Complex II (succinate–ubiquinone oxidoreductase 70 kDa subunit, 1:2000), Sirt3 (1:1000), MnSOD (1:2000), Catalase (1:1000) and p-eNOS (1:1000) in 5% milk/TBST at 4 °C overnight. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using ECL (Roche Mannheim, Germany) and quantified by scanning densitometry. Relative protein expression was normalised to GAPDH expression.

2.2.4. DNA isolation and real-time PCR

Total DNA was extracted using a kit (QIAamp DNA Mini kit; Qiagen, Germany), and quantitative PCR was performed using 18S rRNA primers for a nuclear target sequence and primers for a mitochondrial DNA D-loop target. The following primers were used: mitochondrial D-loop forward: 5′-AATCTACCATCCTCCGTG-3′, reverse: 5′-GACTAATGATTCTTCACCGT-3′;

18S rRNA forward: 5′-CATTCGAACGTCTGCCCTATC-3′ and reverse: 5′-CCTGCTGCCTTCCTTGGA-3′. Quantitative PCR was performed in an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA). The ratio of mitochondrial D-loop to 18S was then calculated.

2.2.5. RNA isolation and reverse transcription PCR

Total RNA was extracted using a TRIzol reagent according to the manufacturer's instructions. To synthesise the first strand of cDNA, 1 μg of RNA was reverse-transcribed, and the synthesised cDNA was amplified in triplicate using specific primers. The following primers were used: Nrf1 forward: 5′-GCCGTCGGAGCACTTACT-3′, and reverse: 5′-CTGTTCCAATGTCACCACC-3′; Tfam forward: 5′-CGCAGCACCTTTGGAGAA-3′, and reverse: 5′-CCCGACCTGTGGAATACTT-3′; 18S rRNA forward: 5′-CATTCGAACGTCTGCCCTATC-3′, and reverse: 5′-CCTGCTGCCTTCCTTGGA-3′. Quantitative PCR was performed in the Mx3000P real-time PCR system (Stratagene). The cycling conditions were as follows: 50 °C for 2 min, initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s. The evaluation of relative differences of PCR product amounts among the treatment groups was carried out using the ΔΔCT method. The reciprocal of 2CT for each target gene was normalised to that of the 18S rRNA, followed by a comparison with the relative value for each target gene in control cells.

2.2.6. Mitochondrial respiration

Oxygen consumption by intact cells was measured as has been previously described [12]. Cells from each condition were divided into triplicate aliquots and measured in a BD Oxygen Biosensor System plate (BD Biosciences). Plates were sealed and 'read' on a fluorescence spectrometer (Molecular Probes, Eugene, OR, USA) at 1 min intervals for 60 min at an excitation wavelength of

485 nm and emission wavelength of 630 nm. We have used 2×10^5 cells in the assay. The oxygen consumption rate of cells generally follows Michaelis–Menten kinetics with respect to oxygen concentration. V_{max} is the maximum consumption rate.

3. Statistics

All values are expressed as means ± SE. Statistical significance was determined by using one-way ANOVA with Bonferroni's post hoc tests between the two groups. The criterion for significance was set at $p < 0.05$.

4. Results

4.1. Nebivolol increases mitochondrial biogenesis

MitoTracker green, the dye that accumulates in mitochondria, was used to label and quantify mitochondria in adipocytes. Seven days after the initiation of differentiation, 3T3-L1 adipocytes were exposed to nebivolol for 24 h. Treatment with nebivolol produced a bell-shaped curve in the relative fluorescence intensity in the concentration range of 1–50 μmol/L, with a maximum fluorescence at 10 μmol/L (Fig. 1A). The D-loop is known to be the major site of transcription initiation for both the heavy and light strands of the mtDNA. Nebivolol significantly increased the ratio of mtD-loop/18S rRNA at concentrations of 5–25 μmol/L (Fig. 1B). Nebivolol also showed an increase in mitochondrial electron transport complex I and II protein levels at 5–25 μmol/L (Fig. 1C and D).

4.2. Nebivolol increases mitochondrial function

To determine whether increased mitochondrial biogenesis is accompanied by changes in oxygen consumption, cells were treated with nebivolol at concentrations of 1–50 μmol/L. As shown in Fig. 2A–B, the basal rate of oxygen consumption was increased in adipocytes treated with nebivolol in the concentration range of 10–25 μmol/L, and this increase was statistically significant.

PPAR-α is also known to be an important regulator of mitochondrial biogenesis and β-oxidation in tissues such as the heart and the liver. We have shown here that the relative abundance of mRNA transcripts encoding PPAR-α was up-regulated by nebivolol in 3T3-L1 adipocytes. This upregulation closely correlates with the stimulation of mitochondrial biogenesis and the induction of CPT-1α abundance involved in fatty acid oxidation at 10 μmol/L.

4.3. Expression of mitochondrial biogenesis genes

The treatment of adipocytes with nebivolol produced a bell-shaped effect on PGC-1α expression, with maximum protein expression at concentrations of 10–25 μmol/L nebivolol (Fig. 3A). The transcription factors Nrf1 and Tfam are involved in regulating the expression of major mitochondrial proteins and in mtDNA transcription and replication. Tfam has been identified as a D-loop–DNA-binding protein. Treatment with nebivolol at concentrations of 1–50 μmol/L resulted in a trend toward a dose-dependent increase in the expression of Nrf1 and Tfam mRNAs, with a significant increase at concentrations of 10–25 μmol/L nebivolol (Fig. 3B). Sirt3 functions as a downstream target gene of PGC-1α

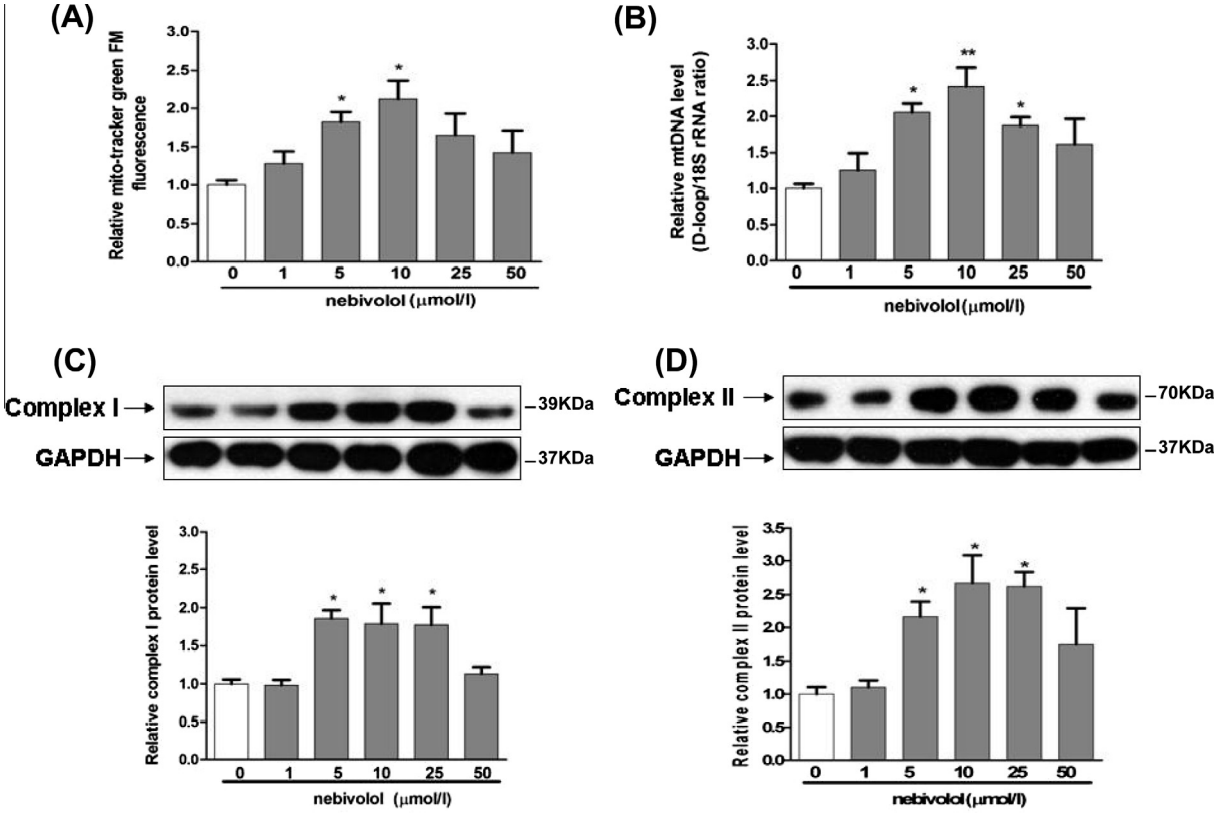

Fig. 1. Nebivolol increases mitochondrial biogenesis in 3T3-L1 adipocytes. (A) Mitochondrial mass. Adipocytes were treated with nebivolol for 24 h, and then incubated with MitoTracker Green FM. Measurements were made with a fluorescence spectrometer. (B) Mitochondrial DNA. The DNA content of mtDNA and the 18S rRNA gene (18S rDNA) were calculated, and the relative ratios of mtDNA content to 18S rRNA gene levels were determined. (C) Expression of mitochondrial complexes I and II (D) Upper: representative western blot image; lower: quantification of protein expression of complexes I and II. Results are presented as the relative fold increase over the control. Data are mean ± SEM of four independent experiments. $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control.

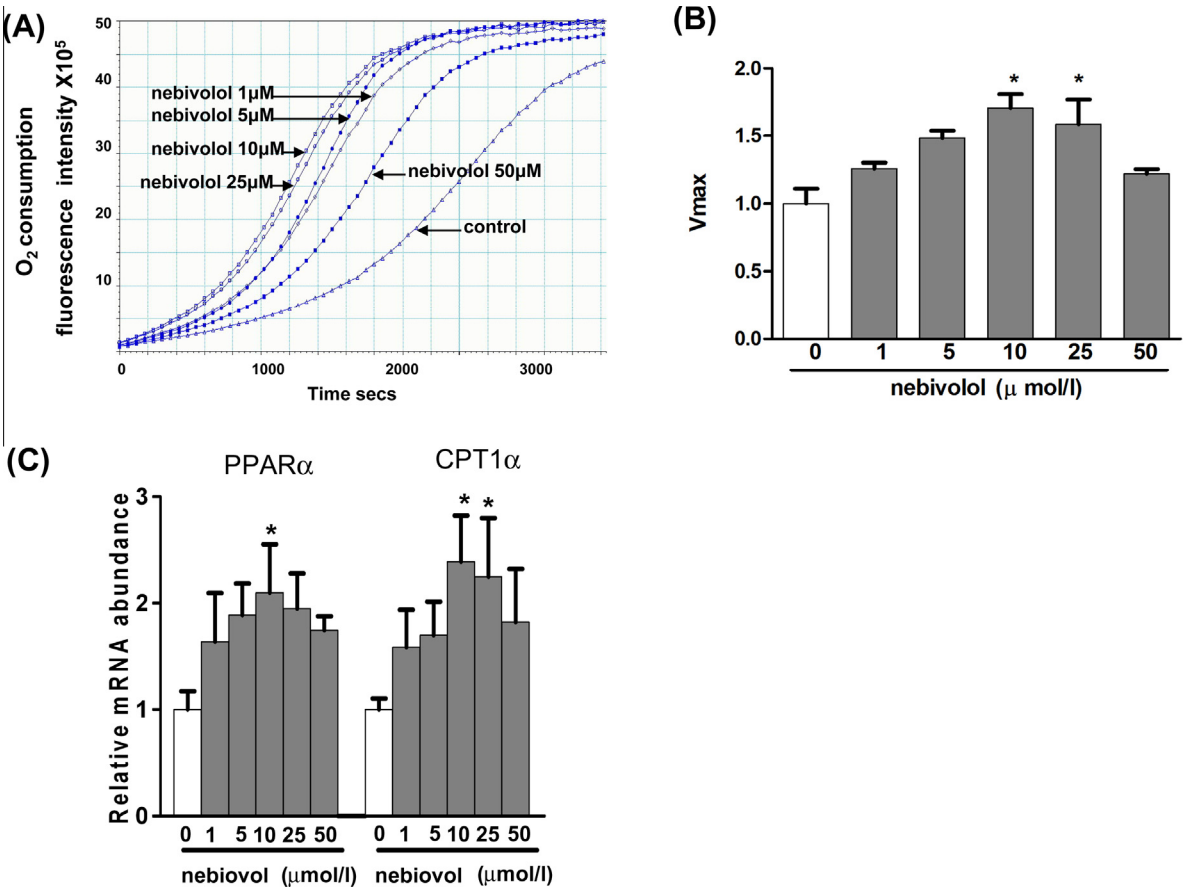

Fig. 2. Nebivolol increases mitochondrial function. (A) Representative oxygen consumption curves. (B) Quantitative changes in the respiratory rate of adipocytes exposed to each condition were calculated by kinetic measurements. V_{max} is the maximum consumption rate.(C) Effect of nebivolol on expression of PPAR-α and CPT-1α mRNA in adipocytes. Results are presented as the relative fold increase over the control. All values are mean ± SEM of four independent experiments. $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control.

and mediates the effects of PGC-1α on cellular ROS production and mitochondrial biogenesis. As shown in Fig. 3C–D, nebivolol produced a bell-shaped distribution of Sirt3, catalase and MnSOD protein levels when added in the range of 1–50 μmol/L, with a maximum at 5–25 μmol/L.

4.4. Nebivolol activates endothelial nitric oxide synthase and increases cGMP levels

To investigate the role of endogenous NO, p-eNOS protein levels were measured in adipocytes incubated with nebivolol. Fig. 4A shows nebivolol induced phosphorylation of eNOS in time-dependent manner. To determine the receptor responsible for the effects of nebivolol on eNOS activation, adipocytes were exposed to pharmacological blockade of β-ARs. Pharmacological blockade of nebivolol by the β3-AR blocker SR59230A (10 μmol/L), β1-A R-blocker metoprolol (10 μmol/L) or β2-AR blocker butoxamine (10 μmol/L) revealed that only SR59230A pretreatment inhibited eNOS activation induced by nebivolol (Fig. 4B), whereas β1, β2AR blocker did not affect it (Fig. 4B).

To investigate whether eNOS/cGMP play a role in the mitochondrial biogenesis that is induced by nebivolol, we tested its effects on cGMP. As shown in Fig. 4C, nebivolol (10 μmol/L) increased cGMP levels in adipocytes after 24 h incubation, and the increase was abolished in the presence of either the eNOS inhibitor l-NAME (100 μmol/L) or SR59230A. Further, pretreatment with l-NAME or ODQ (10 μmol/L) significantly inhibited nebivolol-induced expression of PGC-1α and decreased complex I and II protein levels (Fig. 4D). l-NAME or ODQ treatment blocked the nebivolol-mediated upregulation of Sirt3 expression, as well as MnSOD and catalase protein levels (Fig. 4E).

4.5. Blockage of β3R-attenuated mitochondrial biogenesis caused by nebivolol

Consistent results were obtained by the blockage of these β3 receptors, in that SR59230A completely reversed the increase in PGC-1α and mitochondrial protein levels, while the nebivolol-induced increase in the levels of Sirt3 and antioxidant enzymes was significantly suppressed by the blockage of β3-AR (Fig. 4C, D). These results indicated that nebivolol promotes mitochondrial synthesis initiated by the activation of the β3-AR receptor.

5. Discussion

Obesity and metabolic syndrome are associated with mitochondrial dysfunction. Mitochondrial biogenesis and remodelling in white adipocyte tissue enhances fatty acid uptake and oxidation by increasing oxygen consumption [9]. The present study indicates that nebivolol was able to increase mitochondrial DNA copy number, mitochondrial protein levels and the expression of transcription factors involved in mitochondrial biogenesis, including PGC-1α, Sirt3, Tfam and Nrf1. These changes were accompanied by an increase in oxygen consumption and in the genes involved in fatty acid oxidation in 3T3-L1 adipocytes. PPARα level was upregulated by nebivolol treatment in 3T3-L1 adipocytes. The

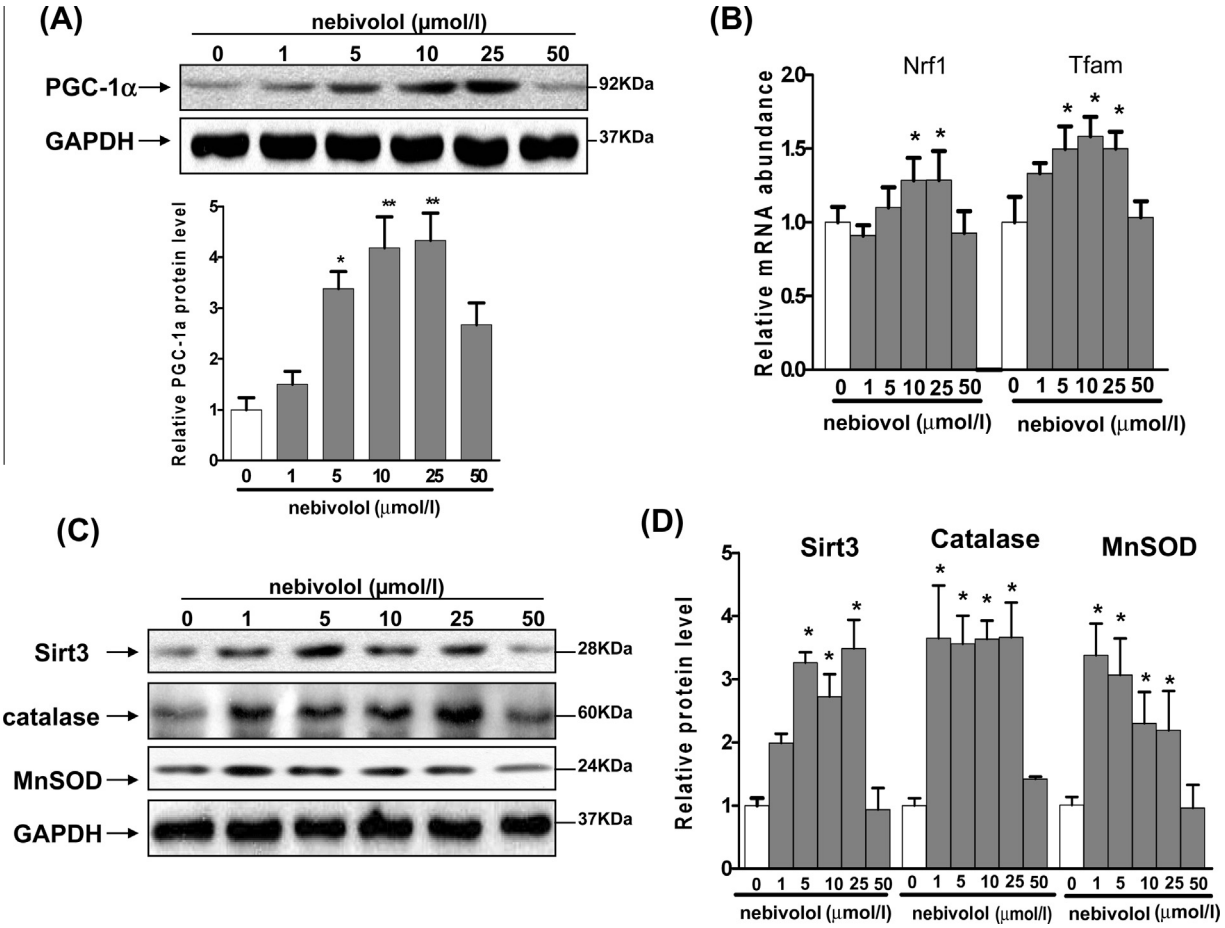

Fig. 3. Effects of nebivolol on mitochondrial biogenesis genes. (A) Protein expression of PGC-1α. Upper: representative western blot image; lower: quantification of PGC-1a protein expression of adipocytes incubated with nebivolol for 24 h. (B) mRNA abundance of Nrf1 and Tfam. Results are presented relative to their values in untreated control cells. (C) Protein expression of Sirt3, MnSOD and Catalase. Right: representative Western blot image; left: quantification of Sirt3, MnSOD and Catalase protein expression in adipocytes incubated with nebivolol for 24 h. Results are presented as the relative fold increase over the control. All values are the mean ± SEM of four independent experiments. $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control.

upregulation closely correlates with the stimulation of mitochondrial biogenesis and the induction of CPT1α involved in fatty acid oxidation. It shows that nebivolol may be a promoter of mitochondrial biogenesis.

The upregulation of eNOS in endothelial cells by nebivolol treatment has been previously reported [27]. We suggest that NO possesses an autocrine function in mediating the effects of nebivolol in adipocytes. Accordingly, treatment with nebivolol was found to mimic the effects of NO, resulting in increased mtDNA content, mitochondrial ETC. complex expression levels, PGC-1α protein levels, and Nrf1 and Tfam mRNA levels, with concomitant increases in oxygen consumption. Similarly, we found that l-NAME prevented nebivolol-induced mitochondrial biogenesis and the upregulation of mitochondrial biogenesis factors in differentiated 3T3-L1 adipocytes. In our study, nebivolol was found to increase cGMP levels in adipocytes. Therefore, it seems that NO-mediated mitochondrial biogenesis is dependent on cGMP. This assumption was examined by studying the effects of the selective guanylate cyclase inhibitor ODQ. Pretreatment with the selective guanylate cyclase inhibitor ODQ significantly inhibited nebivolol-induced increases in mtDNA content and lowered levels of the PGC-1α protein and Nrf1 and Tfam mRNA. Thus, the eNOS–cGMP pathway may play an important role in regulating nebivolol-induced mitochondrial biogenesis in adipocytes.

Sirt3 is a member of the sirtuin family of NAD(+)-dependent deacetylases that is localised to and functions within the mitochondria. It is possible that Sirt3 plays an important role in regulating global mitochondrial protein acetylation levels [28], improves antioxidant defenses by deacetylating MnSOD and catalase[29,30], and appears to be important for the maintenance of mitochondrial integrity and metabolism. Our study found that nebivolol could increase Sirt3 expression, and this increase could be inhibited by pretreatment with l-NAME or ODQ. This result is consistent with a previous report that Sirt3 functions as a downstream target of PGC-1α [31]. Thus, the stimulation of Sirt3 by nebivolol may depend on the eNOS–cGMP-PGC-1α pathway.

β-AR is known to play an important role both in proliferation and in mobilization of fat stores. Monjo et al. [32] analyzed the relative expression of the different β-AR subtypes in preadipocytes and adipocytes and found that β-ARs were upregulated with fold changes of 1.9, 3.7, and 578 for β1-, β2-, and β3-AR in adipocytes, respectively. It means that β3-AR is selectively expressed in mature adipocytes. We found that the increase in the mitochondrial content induced by treatment with nebivolol for 24 h could be prevented by the pharmacological blockage of β3-AR. Consistent with this finding; nebivolol increased the expression levels of PGC1α and Tfam, which positively regulate mitochondrial biogenesis, in a β3-AR-dependent manner. The changes in adipocyte mitochondrial content mirrored the increased mitochondrial protein levels of complex I and complex II. Nebivolol also possesses endothelium-dependent arterial and venous vasodilation properties that are largely attributed to its stimulation of nitric oxide production [33,34]. The nebivolol-induced activation of eNOS was prevented by the pharmacological blockade of β3-AR, but not by that of β1

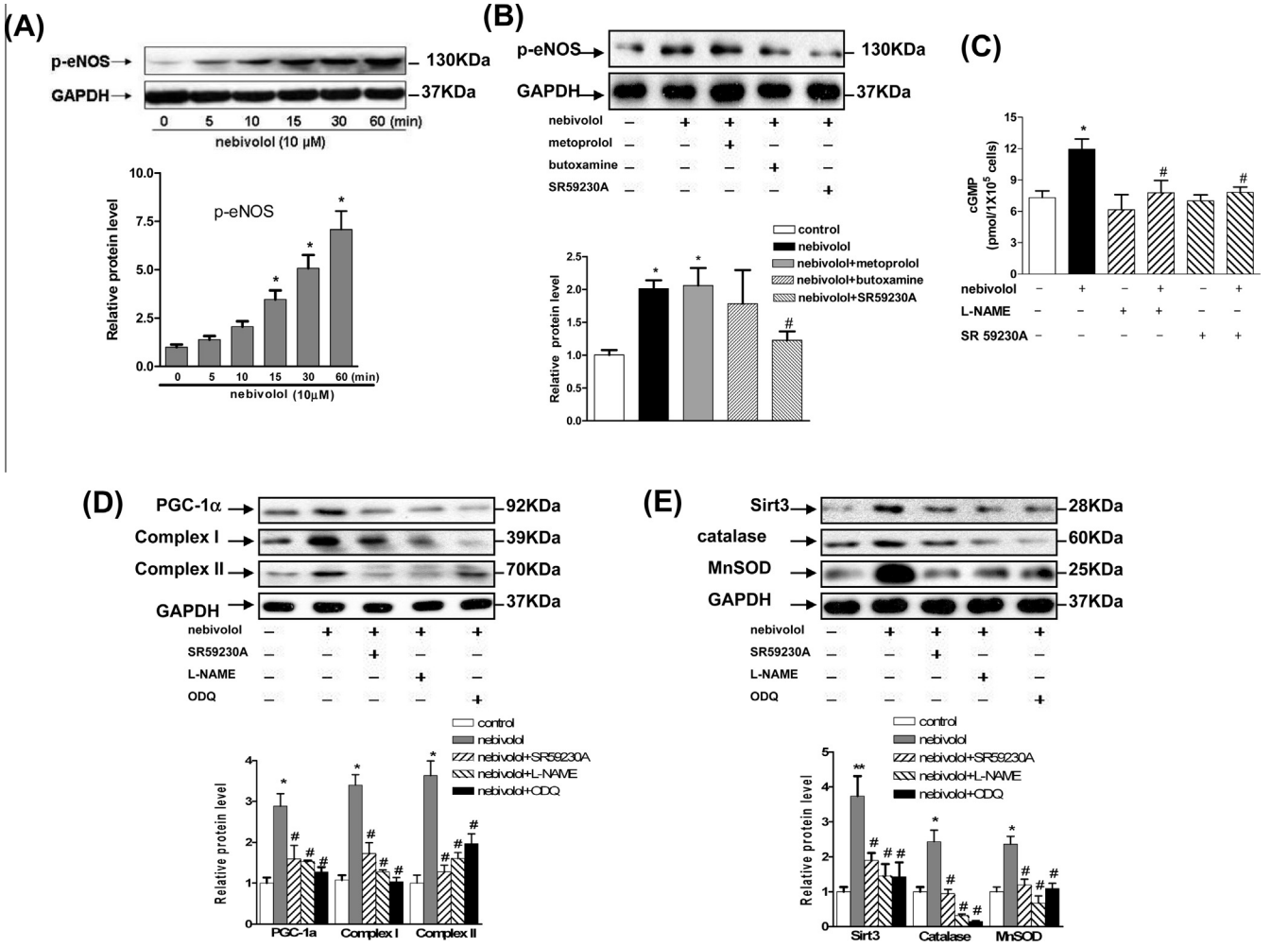

Fig. 4. Nebivolol induces endothelial nitric oxide synthase activation and increases cGMP levels. (A) Effect of treatment with nebivolol on p-eNOS expression. 3T3-L1 adipocytes incubated with nebivolol (10 μmol/L) at different time points. (B) Adipocytes were preincubated with β1-AR blocker metoprolol, β2-AR blocker butoxamine or β3-AR blocker SR59230A for 1 h prior to a 15 min treatment with nebivolol. (C) Effect of treatment with nebivolol on cGMP levels. Adipocytes were preincubated with l-NAME or the β3-AR blocker SR59230A for 1 h prior to a 24 h treatment with nebivolol. (D) Effect of treatment with nebivolol on PGC-1α, complex I and complex II protein levels. Adipocytes were preincubated with l-NAME, ODQ or the β3-AR blocker SR59230A for 1 h prior to a 24 h treatment with nebivolol. (E) Effect of treatment with nebivolol on Sirt3, MnSOD and Catalase protein levels. Representative western blot image (upper) and quantification (lower) analysis are shown. Results are presented as the relative fold increase over the control. The data are the mean ± SEM of four independent experiments. $^{*}p < 0.05$ vs. control, $^{**}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. nebivolol.

or β2-AR blocker *in vitro*. Similar reports have indicated that nebivolol is unable to induce nitric oxide production in the heart in the presence of a β3-AR antagonist. The actions of nebivolol on cardiac tissue were mediated via the stimulation of β3-adrenenergic receptors to release nitric oxide and promote neoangiogenesis [24,35]. Indeed, recent studies have demonstrated that nebivolol, as well as two other specific β3-adrenegic agonists, protects against myocardial ischemia reperfusion injury via rapid activation of endothelial and neuronal nitric oxide synthase and increases nitric oxide bioavailability [36].

In conclusion, we determined that nebivolol was effective in stimulating mitochondrial biogenesis in adipocytes and that the stimulation of mitochondrial biogenesis by nebivolol depended on the eNOS–cGMP pathway. Due to the lack of pure β3-AR agonists available for clinical use at present time, nebivolol acts as a partial agonist of β3-AR and thus can be used to induce mitochondrial biogenesis in adipocytes.

6. Funding

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