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(–)-Epicatechin-induced recovery of mitochondria from simulated diabetes: Potential role of endothelial nitric oxide synthase

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Abstract

(–)-Epicatechin increases indicators associated with mitochondrial biogenesis in endothelial cells and myocardium. We investigated endothelial nitric oxide synthase involvement on (–)-epicatechin-induced increases in indicators associated with mitochondrial biogenesis in human coronary artery endothelial cells cultured in normal-glucose and high-glucose media, as well as to restore indicators of cardiac mitochondria from the effects of simulated diabetes. Here, we demonstrate the role of endothelial nitric oxide synthase on (–)-epicatechin-induced increases in mitochondrial proteins, transcription factors and sirtuin 1 under normal-glucose conditions. In simulated diabetes endothelial nitric oxide synthase function, mitochondrial function–associated and biogenesis-associated indicators were adversely impacted by high glucose, effects that were reverted by (–)-epicatechin. As an animal model of type 2 diabetes, 2-month old C57BL/6 mice were fed a high-fat diet for 16 weeks. Fasting and fed blood glucose levels were increased and NO plasma levels decreased. High-fat-diet-fed mice myocardium revealed endothelial nitric oxide synthase dysfunction, reduced mitochondrial activity and markers of mitochondrial biogenesis. The administration of 1 mg/kg (–)-epicatechin for 15 days by oral gavage shifted these endpoints towards control mice values. Results suggest that endothelial nitric oxide synthase mediates (–)-epicatechin-induced increases of indicators associated with mitochondrial biogenesis in endothelial cells. (–)-Epicatechin also counteracts the negative effects that high glucose or simulated type 2 diabetes has on endothelial nitric oxide synthase function.

Keywords

(–)-Epicatechin; endothelial cells; cardiomyocytes; diabetes; endothelial nitric oxide synthase

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Introduction

In the heart, coronary artery endothelial cells are recognized to be intricately involved in local vasomotor control and in crosstalk with cardiomyocytes.^{1,2} Endothelial cells are rich in endothelial nitric oxide synthase (eNOS) leading to the production of nitric oxide (NO), which serves to regulate vascular and myocyte function. Normal NO levels also serve to maintain vascular and heart health in part, by modulating the production of new mitochondria (i.e. mitochondrial biogenesis).³ Studies using eNOS null-mutant (eNOS^{-/-}) mice have evidenced a key role for eNOS in this process.⁴ In other cell types, it has also been demonstrated that NO donors can up-regulate the expression of the ‘master regulator’ of mitochondrial biogenesis, the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) transcription factor, thereby confirming the regulatory role that NO has on mitochondrial biogenesis.⁴ Mitochondrial health is of high relevance since organelle dysfunction is frequently observed with obesity, insulin resistance, pre-diabetes, diabetes and other cardiovascular diseases.⁵ Under these conditions, PGC-1 α levels are significantly reduced, in addition to proteins involved in oxidative phosphorylation, which may be secondary to impaired physiological NO signalling.

It is well known that hyperglycaemia can suppress eNOS activity, which may lead to impaired endothelium-dependent vasodilatation as seen during acute hyperglycaemia in normal subjects^{6,7} or chronically in diabetic patients.^{8,9} Recent studies suggest that hyperglycaemia-induced eNOS dysfunction may be secondary to excess addition O-linked *N*-acetylglucosamine (O-GlcNAc) at key phosphorylation sites leading to diminished NO production as observed in vitro¹⁰ and in vivo.¹¹ Molecules that prevent or limit eNOS dysfunction may thus favourably impact metabolic disorders by positively modulating mitochondrial function.

We have previously evidenced the capacity of the flavanol (–)-epicatechin (Epi) to stimulate NO production via eNOS activation through distinct pathways that are dependent and independent of calcium (Ca²⁺).^{12–14} Under normal Ca²⁺ conditions, eNOS activation involves the participation of the phosphoinositide 3-kinase (PI3K) pathway, and in Ca²⁺-depleted cells, Epi activates eNOS through an active complex between eNOS, AKT and HSP90 (heat shock protein 90). We have also provided evidence that Epi can stimulate mitochondrial biogenesis in vitro¹⁵ and in vivo,¹⁶ effects that appear to be NO-dependent. Likewise, we also demonstrated that Epirich cocoa or pure Epi were able to restore mitochondrial structure in type 2 diabetes mellitus (T2DM) subjects¹⁷ and in high-fat diet (HFD)-fed rats,¹⁸ whereby treatment recovered protein levels of PGC-1 α , mitofilin, TFAM (mitochondrial transcription factor A) and other mitochondrial-related proteins towards normal. We, therefore, propose that Epi can reverse hyperglycaemia-induced eNOS dysfunction in human coronary artery endothelial cells (HCAECs) and in diabetic hearts from HFD-fed mice and, thus, protect mitochondria.

The aims of this study were to investigate whether (1) Epi is able to increase indicators associated with mitochondrial biogenesis in HCAECs through eNOS activation, (2) Epi

restores eNOS function and mitochondria markers in high-glucose (HG)-exposed cells, and (3) Epi rescues heart eNOS activity and mitochondria in HFD-fed mice.

Methods

Reagents

HCAEC growth media was from Cell Applications, Inc. Normal-glucose (NG, 5 mM D-glucose) Dulbecco's Modified Eagle Medium (DMEM), HG (25 mM D-glucose) DMEM, trypsin and antibiotics were from GIBCO-BRL Invitrogen. Fetal bovine serum (FBS) was from Omega Scientific, and bovine serum albumin (BSA), Na₃VO₄, NaF, Tween-20 and D-glucose were from Sigma-Aldrich. Polyvinylidene difluoride (PVDF) membranes were from Immobilon and Bradford assay reagent was from Bio-Rad. Epi, protease and phosphatase inhibitor cocktail (P8340, P0044 and P2850), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), D-glucose and anti-TFAM were obtained from Sigma-Aldrich. *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenylcarbamate (PugNac) was purchased from Toronto Research Chemicals. Protein-G-sepharose was from Santa Cruz Biotechnologies.

Cell culture and treatment

HCAECs were obtained from Cell Applications, Inc. Cells were maintained in a humidified atmosphere at 37°C with 5% CO₂ in HCAEC growth medium as previously described.¹² Cells were treated using 100 nM of Epi (diluted in water) or vehicle for 10 min or 48 h as previously described.¹² To evaluate the effects of HG on HCAECs, cells at 75% confluence were incubated for 48 h with NG DMEM or HG DMEM (media was changed every 12 h with fresh Epi).

NO measurements

NO levels were evaluated using a nitrate/nitrite fluorometric assay kit (Cayman Chemical) according to manufacturer's instructions using a fluorometer (FLx800, BioTek Instruments Inc.) at excitation and emission wavelengths of 360 nm and 430 nm, respectively. In cells, NO levels were measured in growth media and normalized to protein content using the Bradford method. In mice, nitrate/nitrite concentration was measured in plasma.

Immunoprecipitation

Immunoprecipitation assays were performed as previously described.¹³ In brief, cells/heart tissues were lysed with 50–100 µl of non-denaturing extraction buffer [0.5%, Triton X-100, 50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, and 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA)] and supplemented with protease and phosphatase inhibitor cocktails, and 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 2 mmol/L Na₃VO₄ and 1 mmol/L NaF and 20 µM of the O-GlcNAcase inhibitor PugNac. Homogenates were incubated on ice with shaking for 15 min and centrifuged (15 min) at 12,000g at 4°C. A total of 0.5 mg protein was pre-cleared by adding 1 µg of normal rabbit IgG control and 20 µL prot-G-agarose with mixing for 30 min (4°C) and subsequent centrifugation at 12,000g for 10 min at 4°C. The supernatants were recovered and incubated at 4°C under mild agitation with 3 µg of immunoprecipitating anti p-eNOS (Ser1177) antibody. A quantity of 20 µL of protein G-

sepharose was added, and the mixture was incubated at 4°C for 3 h with shaking. The immunoprecipitation mixture was centrifuged at 12,000g for 15 min at 4°C, and the supernatant recovered and stored at 4°C. The pellet was washed three times with extraction buffer and centrifuged at 12,000g for 15 min at 4°C. The immunoprecipitated proteins in the pellet and those remaining in the supernatant were applied to a 4%–15% gradient Mini-PROTEAN® TGX™ precast protein gels (Bio-Rad) for immunoblotting.

Western blotting

HCAECs were homogenized in lysis buffer and proteins isolated as previously described.¹³ For heart tissue, approximately 50 mg were homogenized with a polytron in 500 µL lysis buffer (1% triton X-100, 20 mM Tris·HCl, 140 mM NaCl, 2 mM EDTA and 0.1% sodium dodecyl sulphate) with protease and phosphatase inhibitor cocktails supplemented with 0.15 mM PMSF, 5 mM Na₃VO₄, and 3 mM NaF. Homogenates were sonicated for 30 min at 4°C and centrifuged (12,000g) for 10 min at 4°C. The total protein content of cell of heart homogenates was measured in the supernatant using the Bradford method. A total of 30–40 µg of protein were loaded onto a 4%–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad), electrotransferred to a PVDF using a Trans-Blot® SD Semi-Dry system (16 V, 60 min) for low molecular weight proteins, or using a Mini Trans-Blot® Cell system (70 V, 2 h) for high molecular weight proteins. The membranes were incubated for 1 h in blocking solution [5% nonfat dry milk in Tris-buffered saline (TBS) plus 0.1% Tween 20 (TBS-T)], followed by 1 h to overnight incubation at 4°C with primary antibodies. Primary antibodies were typically diluted 1:1000–2000 in TBS-T plus 5% BSA. Antibodies against total eNOS and phospho Ser 1177 eNOS were from Cell Signaling. To analyze glycosylation of proteins, we use the RL2-(O-GlcNAc) antibody (Abcam). To examine mitochondrial biogenesis-related proteins, we evaluated PGC-1α, sirtuin-1 (SIRT1) (Cell Signaling) and TFAM. To examine mitochondrial structure/function-related proteins, we examined mitofilin porin and oxidative phosphorylation complex I, II, III and V (MitoSciences). S6RP (Cell Signaling) was used as a loading control. Membranes were washed (3× for 5 min) with TBS-T and incubated for 1 h at room temperature with specific horseradish peroxidase (HRP)-conjugated secondary antibodies. The immunoblots were developed using an enhanced chemiluminescence (ECL) detection kit and the band intensities were digitally quantified. The densitometric analysis was performed using the ImageJ software.

Transfection with eNOS small interfering RNA

HCAEC were transfected with small interfering RNA [siRNA; sieNOS (sc-36093) or scrambled RNA duplex as a control (sc-37007) from Santa Cruz Biotechnology] using the transfection reagent (sc-29528, Santa Cruz Biotechnology) according to the manufacturer's instructions. Briefly, 2×10^5 HCAECs were seeded on 6-well plates and grown until 70% confluency. Thereafter, cells were electroporated using the siRNA transfection reagent and incubated for 8 h with 60 nmol siRNA based on the manufacturer's protocol. Experiments were performed for 72 h after siRNA transfection. The protein knockdown efficiency was assessed by the Western blot analysis.

Animal model

As an animal model of T2DM, 2-month-old C57BL/6 mice were fed ad libitum with a HFD for 6 months (rodent chow containing 60% kcal from fat, Research Diets, Inc.). Same age (i.e. 6 months old) C57BL/6 wild-type mice fed with normal chow were used for comparison purposes. Mice were then treated by gavage for 15 days with 1 mg Epi/kg of body weight as described before.¹⁶ Control mice were treated with vehicle (water). After 15 days of treatment, mice were anaesthetized by inhalation of isoflurane (5% in a 100% oxygen mix) and then decapitated. Immediately, heart samples and plasma were collected and stored at -80°C until use. Each of the three groups studied included five animals. All procedures were performed in accordance with the National Institutes for Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the University of California, San Diego (UCSD) Institutional Animal Care and Use Committee.

Morphometrics and glucose levels

Body weight (BW), heart weight (HW), fasting and fed blood glucose were measured at 6 months of age and after 15 days of Epi treatment. Fasting glucose was measured after fasting mice for 8 h. Glucose challenge was given via oral gavage (1.5 mg/g of mouse BW) followed by blood glucose measurements 1 h later.

Citrate synthase activity

Heart tissue samples (25 mg) were homogenized with a polytron in 250 μL of cold extraction buffer (20 mM Tris-HCl, 140 mM NaCl, 2 mM EDTA and 0.1% sodium dodecyl sulphate) with protease inhibitors (P2714, Sigma-Aldrich), 5 mM Na_3VO_4 and 3 mM NaF. Homogenates were centrifuged at 10,000g for 15 min at 4°C . Supernatants were recovered and used to measure CS activity as described previously. The HCAEC extracts (20 μg protein) used for Western blotting were employed to assess citrate synthase (CS). All samples were tested in duplicates and measured at room temperature.

Statistical analysis

For cell culture experiments, at least three independent experiments each in triplicate were performed. For animal studies, five mice per group were included. Results are expressed as mean $\pm$ standard error of the mean (SEM). Data analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A p value of <0.05 was considered to be statistically significant.

Results

eNOS knockdown and L-NAME inhibition blocks Epi-induced increases of indicators associated with mitochondrial biogenesis in HCAEC

To evaluate the participation of NO in Epi-induced mitochondrial biogenesis, we knocked-down or chemically blocked eNOS using siRNA or L-NAME (150 μM), respectively. siRNA reduced eNOS protein levels to $\sim 35\%$ versus control [Figure 1(a)], whereas in scrambled treated cells [(-)siRNA] no significant changes were observed [Figure 1(a)]. Using cell

media we measured NO production 10 min after stimulation with Epi 100 nM, as this concentration and time evoke a significant effect on HCAEC eNOS activity.¹² As expected, eNOS knockdown and L-NAME blocked Epi-induced NO production [Figure 1(b)]. We then examined levels of mitochondrial proteins and transcription factors in controls and eNOS knockdown and L-NAME-treated cells. As shown in Figure 2(a) and (b), mitofilin and complex I, II, III and V were increased with Epi, an effect that was blocked by either eNOS knockdown or L-NAME treatment. Likewise, SIRT1, TFAM and PGC-1 α protein levels were increased with Epi, while siRNA and L-NAME treatment blunted Epi's effects [Figure 2(c) and (d)].

HG decreases NO production and eNOS phosphorylation and increases eNOS O-GlcNAc levels in HCAECs

It has been demonstrated that HG conditions can impair eNOS activity in endothelial cells.¹⁰ We, thus, evaluated NO production, eNOS phosphorylation and eNOS-O-Glc-Nac levels in Epi-treated and untreated cells under HG conditions. As illustrated in Figure 3(a), HG diminished NO production whereas Epi treatment recovered NO levels towards those observed under NG conditions. Similarly, HG decreased eNOS phosphorylation and Epi reverted this reduction towards NG levels [Figure 3(b)]. HG also increased eNOS-O-GlcNAc levels at Ser1177, an effect that was reverted by Epi [Figure 3(c)].

HG decreases levels of indicators associated with mitochondrial biogenesis

As HG impaired eNOS function, we further evaluated the impact of treatment on mitochondria endpoints. As shown in Figure 4(a) and (b), mitofilin, SIRT1, PGC-1 α and TFAM protein levels were reduced by HG media. However, concomitant treatment with Epi blocked the suppressive effect of HG towards levels observed under NG conditions [Figure 4(a) and (b)]. Treatment of cells with Epi cultured in NG media also resulted in an increase in mitochondrial endpoints [Figure 4(a) and (b)]. Mitochondrial function as assessed by CS activity levels was also reduced by HG media, which was reverted by Epi [Figure 4(c)]. In HG conditions, blockade of eNOS by L-NAME blunted the effects of Epi on mitochondrial biogenesis markers [Figure 4(a) and (b)] and function [Figure 4(c)].

HFD-fed mice have altered myocardial eNOS function and reduced levels of indicators associated with mitochondrial biogenesis

HFD-fed mice have been linked to altered balance in glucose homeostasis.¹⁹ We therefore, used this model to explore the in vivo effects of simulated T2DM on eNOS function. As expected, fasting blood glucose (Figure 5(a)) and fed blood glucose (Figure 5(b)) were higher in HFD-fed mice versus those fed a normal chow. Oral treatment with Epi at 1 mg/kg for 15 days partially reverted these effects [Figure 5(a) and (b)]. HFD-fed mice also evidenced decreased NO levels [Figure 5(c)] that were accompanied by decreased heart eNOS phosphorylation levels versus normals [Figure 5(d)]. Epi treatment reverted the effects of HFD on NO and eNOS phosphorylation levels towards those observed in normals [Figure 5(c) and (d)]. eNOS-O-GlcNAc levels were higher in HFD-fed mice versus mice fed normal chow, an effect that was reversed by Epi treatment [Figure 5(e)].

Structural mitochondrial proteins and transcription factor levels were decreased in HFD-fed mice versus normals [Figure 6(a) and (b)]. Epi treatment was able to fully recover SIRT1, PGC-1 α , mitofilin and porin protein levels [Figure 6(a) and (b)], while partially restoring TFAM and complex V [Figure 6(a) and (b)]. Mitochondrial function was also reduced in HFD-fed mice compared to normal mice, which was reverted by Epi treatment [Figure 6(c)].

Discussion

In this study, by using siRNA and a chemical blocker of eNOS, we evidence a key role for the enzyme in mediating Epi-induced increases of indicators associated with mitochondrial biogenesis in HCAEC. We demonstrate that HG impairs eNOS activity as shown by reduced NO levels, phosphorylation and increased O-GlcNAc residues on Ser1177. These effects are reverted by Epi treatment. HG also led to decreased mitochondrial function and protein levels of the key regulator PGC-1 α that was also restored by Epi. L-NAME treatment blocked the effects of Epi on mitochondrial markers and function under HG conditions. Additionally, using a mouse model of T2DM we demonstrate that eNOS function is impaired and accompanied by reduced myocardial mitochondrial function and related proteins in mouse hearts. Fifteen days of Epi treatment was capable of preventing these alterations. Results suggest that Epi protects eNOS function from the insult that diabetes-like conditions evoke on its structure, thereby positively impacting endothelial and cardiac mitochondria structure and function.

NO produced from physiological activation of eNOS has multiple effects on the cardiovascular system. NO can modulate cardiac function through both vascular-dependent and vascular-independent effects.²⁰ In coronary arteries, NO is linked to the regulation of coronary vessel tone, which can indirectly affect cardiac function. In cardiomyocytes, NO also has a direct effect on contractility and mitochondrial respiration.²⁰ Hence, molecules that target the eNOS function offer the possibility to favourably impact the cardiovascular function.

Our study¹² and that by Brossette et al.²¹ have evidenced the capacity of the flavanol Epi to stimulate the production of NO in endothelial cells via eNOS phosphorylation, which can activate mitochondrial biogenesis.¹⁵ By using siRNA and L-NAME, we provide further evidence for the participation of NO in mediating the effects of Epi (at relevant physiological concentrations)²² on indicators associated with mitochondrial biogenesis in HCAEC. Similarly, Csiszar et al.²³ using HCAEC demonstrated that the inhibition of eNOS using L-NAME (300 μ M) blunted the effects of 10 μ M resveratrol on indicators of mitochondrial biogenesis.

In the setting of T2DM, there is a high risk of developing cardiovascular disease, which is largely attributed to the adverse effects of hyperglycaemia on the vascular system.²⁴ Endothelium-dependent vasodilatation, which involves NO production, is known to be impaired during acute hyperglycaemia in normal subjects^{6,7} and in diabetic patients,^{8,9} suggesting that HG levels alter eNOS activity. Hence, it is reasonable to expect that HG can negatively affect endothelial mitochondrial structure/function. To assess this scenario, we investigated the effects of simulated diabetes on eNOS function and mitochondrial function

and biogenesis in HCAEC. We evidence a decrease in eNOS activity followed by reduced mitochondrial function and protein levels of structural proteins and transcription factors involved in the modulation of mitochondrial biogenesis, effects that were prevented by Epi. These protective effects of Epi were blunted by L-NAME, which suggests that eNOS mediates Epi protection on mitochondria under simulated diabetes. Interestingly, Valle et al. demonstrated that overexpression of PGC-1 α prevented HG-induced mitochondrial damage in endothelial cells.²⁵ Here, we demonstrate the capacity of Epi to increase PGC-1 α protein levels under basal and HG conditions, which, in part, can contribute to its restorative effects on endothelial mitochondria exposed to simulated diabetic conditions.

The precise mechanism through which hyperglycaemia impairs eNOS activity is not well understood. However, post-translational modifications of eNOS (i.e. O-GlcNAc) via the hexosamine pathway (which is activated by hyperglycaemia) has been linked to impaired eNOS function.¹⁰ Here, we demonstrate increased eNOS-O-GlcNAc levels in HG conditions that are reverted by Epi. We, therefore, suggest that HG causes eNOS O-GlcNAc modification, which in consequence can lead to impaired mitochondrial biogenesis.

In the heart, NO can modulate the cardiac function in a paracrine/autocrine manner and proper NO levels are necessary for normal function.²⁰ Several studies have provided evidence that also supports a protective role for eNOS-derived NO in isolated cardiomyocytes.²⁶ eNOS-deficient mice develop progressive cardiac hypertrophy, which suggests a role for NO also mediating cardiac structure.²⁷ Our results demonstrate that eNOS activity is impaired in the hearts of HFD-fed mice. Interestingly, the anti-diabetic drug, metformin, has also been shown to positively affect heart structure/function via eNOS activation in diabetic (db/db) mice.²⁸ Likewise, the *thiazolidinedione* pioglitazone evokes positive effects on rabbit infarcted hearts through the activation of eNOS.²⁹ We also evidence decreased levels of mitochondrial proteins and regulators of mitochondrial biogenesis. We therefore, suggest that impaired mitochondrial biogenesis may be secondary to decreased eNOS activity and demonstrate that Epi is capable of preventing these alterations as elicited by simulated diabetes. Interestingly, Lagouge et al.³⁰ showed a lack of effects of resveratrol on the heart using HFD-fed mice (C57Bl/6J) as assessed by changes in gene expression of PGC-1 α and related mitochondrial biogenesis genes after 15 weeks of treatment with resveratrol at 400 mg/kg/day. Several reports suggest that resveratrol evokes its effects on mitochondria through the activation of SIRT1. However, in the above study, resveratrol was not able to affect the mRNA levels of SIRT1 from HFD mice's heart, while, in this study, Epi (even at lower doses) restored diminished SIRT1 protein levels, as well as other mitochondrial biogenesis-related proteins.

In summary, using in vitro and in vivo models, we provide evidence indicating that Epi is capable of augmenting the levels of indicators associated with mitochondrial biogenesis through eNOS activation in normal and simulated diabetes conditions. The capacity of Epi to favourably affect eNOS function may explain its capacity to restore mitochondria from hyperglycaemia insults where eNOS function is compromised by its O-GlcNAc modification. This scenario suggests that Epi or Epirich foods may be considered as a supplement in the treatment of T2DM patients. However, additional work is necessary to explore this possibility in humans using well-designed clinical trials.

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Figure 1. eNOS knockdown and its inhibition abrogates Epi-induced nitric oxide (NO) production. Knockdown of eNOS was achieved by treating cells with 60 nM small interfering RNA (siRNA) against eNOS or scrambled control siRNA (controls) for 72 h. Cells were lysed and eNOS protein content was evaluated by Western blot analysis. (a) eNOS and GAPDH (loading control) protein levels. Data were normalized versus non-treated cell values (100%). (b) Cells either treated with siRNA or L-NAME (150 μ M, 2 h) were stimulated with Epi (100 nM, 10 min) and NO levels determined. NO levels were normalized by protein content. Results are expressed as mean $\pm$ SEM ($n = 3$) and analyzed by one-way ANOVA followed by Tukey's post hoc test.

* $p < 0.05$ versus control, ^ $p < 0.05$ versus Epi.

Figure 2.

eNOS knockdown and its inhibition abrogates Epi-induced increases of indicators associated with mitochondrial biogenesis. eNOS function was decreased by either small interfering RNA (siRNA) or chemical inhibition using L-NAME (150 μ M for 48 h). Cells were stimulated for 48 h with 100 nM Epi and then lysed for Western blot analysis. (a) Representative blots and (b) quantification of mitochondrial proteins. (c) Quantification and (d) representative blots from transcription factors involved in mitochondrial biogenesis. S6RP was used as a loading control. Control values were arbitrarily set to 100%. Results are expressed as mean $\pm$ SEM ($n = 3$) and analyzed by one-way ANOVA followed by Tukey's post hoc test.

* $p < 0.05$ versus control, ^ $p < 0.05$ versus Epi.

Figure 3.

Epi reverts high-glucose (HG)-induced changes in eNOS activity and O-glycosylation (O-GlcNAc). Cells were incubated in either HG (25 mM glucose) or normal glucose (NG, 5 mM) for 48 h with or without 100 nM Epi. eNOS function was examined by measuring (a) nitric oxide (NO) production and (c) eNOS phosphorylation (at Ser1177). (b) Cells were immunoprecipitated using phospho (p)-eNOS antibody and Western blot analysis was performed for the detection of eNOS-O-GlcNAc levels. One representative blot out of three is shown. p-eNOS and p-eNOS-O-GlcNAc protein levels were normalized using total eNOS and S6RP protein levels, respectively. NG levels were arbitrarily set to 100%. Results are expressed as mean $\pm$ SEM ($n = 3$) and analyzed by one-way ANOVA followed by Tukey's post hoc test.

* $p < 0.05$ versus NG, ^ $p < 0.05$ versus Epi.

Figure 4.

Epi restores high-glucose (HG)-induced reductions in indicators associated with mitochondrial biogenesis. Cells were incubated in either HG (25 mM glucose) or normal glucose (NG, 5 mM glucose) for 48 h with or without 100 nM Epi. Cells were lysed and subjected to Western blot analysis. (a) Representative blots of the mitochondrial proteins analyzed. (b) Quantification of mitochondrial proteins. S6RP was used as a loading control. (c) Citrate synthase activity levels, L-NAME was used at 150 μM 1 h before Epi treatment and for 48 h. NG levels were arbitrarily set to 100%. Results are expressed as mean ± SEM (n = 3) and analyzed by one-way ANOVA followed by Tukey's post hoc test.

* $p < 0.05$ versus NG, ^ $p < 0.05$ versus Epi.

Figure 5.

Epi reverts high-fat diet (HFD)-induced changes in myocardial eNOS activity and O-glycosylation (O-GlcNAc) in mice. Two-month-old C57BL/6 mice were fed either a HFD or normal chow for 6 months. HFD-fed mice were treated with vehicle or Epi 1 mg/kg for 2 weeks and plasma and heart muscle endpoints examined. (a) Fasting and (b) fed blood glucose levels were evaluated. (c) Nitric oxide production was measured in plasma. (d) Myocardial eNOS activity and (e) O-GlcNAc levels were examined in the heart by Western blot analysis. One representative blot out of three is shown. p-eNOS and p-eNOS-O-GlcNAc protein levels were normalized using eNOS and S6RP protein levels, respectively. Values from mice-fed normal chow (Ctrl) were arbitrarily set to 100%. Results are expressed as mean $\pm$ SEM ($n = 5$) and analyzed by one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, NS: non-significant.

Figure 6.

Epi restores high-fat diet (HFD)-impaired levels of indicators associated with mitochondrial biogenesis. Two-month-old C57BL/6 mice were fed with either HFD or normal chow for 6 months. HFD-fed mice were treated with vehicle or Epi 1 mg/kg for 2 weeks and myocardial mitochondrial endpoints examined by Western blot analysis. (a) Representative blots from the mitochondrial proteins. (b) Quantification of the densitometry values from the mitochondrial proteins. S6RP was used as a loading control. (c) Citrate synthase activity levels. Values from mice-fed normal chow (Ctrl) were arbitrarily set to 100%. Results are expressed as mean $\pm$ SEM ($n = 5$) and analyzed by one-way ANOVA followed by Tukey's post hoc test.

* $p < 0.05$ versus control, ^ $p < 0.05$ versus HFD.