Journal of Diabetes and Its Complications 30 (2016) 1017–1024

Contents lists available at ScienceDirect

Journal of Diabetes and Its Complications

journal homepage: WWW.JDCJOURNAL.COM

Metformin attenuates fluctuating glucose-induced endothelial dysfunction through enhancing GTPCH1-mediated eNOS recoupling and inhibiting NADPH oxidase

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ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form 17 April 2016
Accepted 24 April 2016
Available online 27 April 2016

Keywords:
Fluctuating glucose
Guanosine 5′-triphosphate cyclohydrolase 1
Human umbilical vein endothelial cells
Metformin
Type 2 diabetes

ABSTRACT

Aims: The aim of this study was to investigate whether and how metformin ameliorated endothelial dysfunction induced by fluctuating glucose (FG) in human umbilical vein endothelial cells (HUVECs).
Methods: HUVECs, which were exposed to FG to induce endothelial dysfunction, were incubated with nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine-methyl ester (L-NAME), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor apocynin, metformin and/or adenosine monophosphate-activated protein kinase (AMPK) inhibitor compound C. The oxidative stress and endothelial NOS (eNOS) coupling were evaluated.
Results: FG induced endothelial dysfunction as indicated by increased reactive oxygen species (ROS) generation and decreased nitric oxide (NO) production. Although FG increased eNOS phosphorylation, uncoupled eNOS was evidenced by downregulated guanosine 5′-triphosphate cyclohydrolase 1 (GTPCH1) and tetrahydrobiopterin (BH4) levels. FG also upregulated the level of p47-phox, a subunit of NADPH oxidase. Similar to L-NAME and apocynin, metformin ameliorated the FG-induced endothelial dysfunction by decreasing ROS generation. Furthermore, metformin recoupled eNOS through upregulating GTPCH1 and BH4 levels, and attenuated the upregulation of p47-phox in FG-treated HUVECs. Addition of compound C abolished the above effects of metformin.
Conclusion: Metformin improves the FG-induced endothelial dysfunction in HUVECs. The protective effect of metformin may be mediated through activation of GTPCH1-mediated eNOS recoupling and inhibition of NADPH oxidase via an AMPK-dependent pathway.

1. Introduction

Macrovascular disease is a major chronic complication of diabetes mellitus. Endothelial dysfunction, characterized by reduced nitric oxide (NO) bioavailability, is involved in the initiation and progression of diabetic vascular complications (Davignon & Ganz, 2004; Esper et al., 2006). The endothelial nitric oxide synthase (eNOS) is responsible for most of the vascular NO production. eNOS oxidizes substrate L-arginine to L-citrulline and NO (Forstermann & Munzel, 2006). When the expression and activity of eNOS decreases or eNOS becomes uncoupled, reactive oxygen species (ROS) will generate instead of NO, and ultimately lead to endothelial dysfunction (Takimoto et al., 2005). Tetrahydrobiopterin (BH4) is an essential cofactor to sustain the dimer structure and bioactivity of eNOS. BH4 deficiency could induce eNOS uncoupling. Guanosine 5′-triphosphate cyclohydrolase 1 (GTPCH1) is a key rate-limiting enzyme of BH4 synthesis, and plays an important role in maintaining BH4 level and NO production. Inhibition of GTPCH1 results in downregulation of BH4 level and eNOS uncoupling, and thus leads to endothelial dysfunction (Wang et al., 2008).

Postprandial hyperglycemia, as well as hypoglycemic events, is an independent risk factor for cardiovascular complications in diabetic patients (Suh & Kim, 2015). Studies have shown that fluctuating glucose (FG) exhibits a more specific triggering effect on oxidative stress than chronic sustained hyperglycemia in patients with type 2 diabetes (Monnier et al., 2006). Likewise, compared with chronic high glucose, FG can more easily induce apoptosis, upregulation of

Disclosures: No conflicts of interest, financial or otherwise, are declared by the authors.

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http://dx.doi.org/10.1016/j.jdiacomp.2016.04.018

adhesion molecule expression and oxidative stress in cultured human umbilical vein endothelial cells (HUVECs) and in patients with type 2 diabetes (Monnier et al., 2006; Quagliaro et al., 2005; Risso, Mercuri, Quagliaro, Damante, & Ceriello, 2001). However, the underlying mechanisms are still elusive. Whether FG could decrease GTPCH1 expression and BH4 level and accordingly induce eNOS uncoupling is not well described.

Metformin, one of the most widely used anti-diabetic agents, can elicit protective potency in the cardiovascular system independent of glucose-lowering effects (Anfossi, Russo, Bonomo, & Trovati, 2010). Adenosine monophosphate-activated protein kinase (AMPK) signaling pathway plays a pivotal role in the effects of metformin. Notably, a recent study reported that metformin could promote eNOS phosphorylation and increase NO production by activating AMPK, and consequently improve the progression of heart failure in dogs (Sasaki et al., 2009). However, whether metformin could regulate eNOS phosphorylation in vascular endothelial cells, especially in human cells, is not fully known.

In this study, we aimed to investigate the influence of FG on GTPCH1-mediated eNOS coupling in cultured HUVECs, and to investigate whether and how metformin ameliorate oxidative stress in the endothelial cells.

2. Materials and methods

2.1. Reagents

Metformin, D-glucose, mannitol, β-endothelial cell growth factor, compound C (CC), *N*-nitro-L-arginine-methyl ester (L-NAME), apocynin, and ROS detection kit were purchased from Sigma (St. Louis, MO). Medium 199 and fetal bovine serum were from Hyclone (Logan, UT), and other reagents for cell culture were from Invitrogen (Carlsbad, CA). Anti-eNOS monoclonal antibody and anti-phospho-Ser-1177 eNOS antibody were from BD Biosciences (San Jose, CA); anti-AMPK and anti-phospho-Thr-172 AMPK antibodies were from Cell Signaling Technology (Beverly, MA); anti-GTPCH1 antibody was from Abnova (Taibei, China); and anti-p47-phox and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). IRDye 800CW-conjugated goat anti-rabbit and goat anti-mouse IgGs were purchased from LI-COR Biosciences (Lincoln, NE). Other reagents for Western blot were from Bio-Rad Laboratories (Hercules, CA).

2.2. Cell culture

Human umbilical cord samples were collected from healthy donors. Written informed consent was obtained from pregnant women before labor. HUVECs were isolated by collagenase digestion as previously described (Liu et al., 2011) and cultured in the endothelial cell growth medium (M199, 10% fetal bovine serum, 4.16 ng/mL β-endothelial cell growth factor, 8.2 mg/mL heparin, and 1× GlutaMax). The HUVECs of 5 to 6 passage were used in all experiments, which were examined to ensure specific characteristics of endothelial cells by cytochemical staining. The in vitro model of endothelial dysfunction was established by exposure of HUVECs to FG. The cell treatment conditions were divided into four groups: normal glucose control (NC, 5.6 mmol/L glucose), constant high glucose (HG, 30 mmol/L glucose), FG (5.6 and 30 mmol/L glucose, alternated every 8 h), and hyperosmotic control (HC, 5.6 mmol/L glucose + 24.4 mmol/L mannitol). To detect the effects of metformin, HUVECs were treated with 2 mmol/L metformin in most of the conditions. To clarify the involved signaling pathways, the nitric oxide synthase (NOS) inhibitor L-NAME (300 μmol/L), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor apocynin (100 μmol/L), or AMPK inhibitor CC (10 μmol/L) was added. The cells in all these groups were incubated for 72 h. All experiments were performed four times with each group in triplicate.

2.3. Measurement of NO

NO level in cell supernatant was measured by ELISA kits according to the manufacturer's protocols (Jiamay Biotech, Beijing, China). The NO level in the NC group was set up as 100%.

2.4. Measurement of intracellular ROS

Determination of intracellular oxidant production in the HUVECs was based on oxidation of a ROS probe dye 2′7′-dichilroflurescin diacetate (20 μmol/L) by intracellular ROS, resulting in formation of the fluorescent compound 2′7′- dichilroflurescin (DCF). The DCF fluorescence was monitored with a flow cytometer (BD Biosciences) or a laser confocal microscope (LSM 510 META, Carl Zeiss, Jena, Germany). The intracellular ROS level in the NC group was set up as 100%.

2.5. Measurement of intracellular BH4

High performance liquid chromatography (HPLC) was used for the measurement of intracellular BH4. Briefly, cells were lysed in distilled water containing 1 mmol/L dithiothreitol, 50 mmol/L Tris-HCl and 1 mmol/L EDTA. The cell lysate (90 μL) was treated with acid solution (10 μL, 1.5 mmol/L $HClO_4$: 2 mmol/L H_3PO_4 = 1:1), and centrifuged at 13,000 *g* for 10 min. The supernatant (90 μL) was treated with acid iodine solution (10 μL, 1% iodine and 2% KI in 1 mmol/L HCl solution) and incubated for 60 min in the dark, and then fresh ascorbic acid (5 μL, 20 mg/mL in water) was added to reduce excess iodine. After centrifugation, the supernatant (90 μL) was detected. The above process is called acid oxidation, which is to determine the total biopterin [BH4, dihydropterin (BH2), and oxidized biopterin]. The below process is called alkaline oxidation, which is to determine the levels of BH2 and biopterin. The cell lysate (80 μL) was treated with NaOH (10 μL, 1 mmol/L) and alkaline iodine solution (10 μL, 1% iodine and 2% KI in 1 mmol/L NaOH), and incubated for 60 min in the dark. Then, 20 μL H_3PO_4 (1 mmol/L) and 5 μL fresh ascorbic acid were added. After centrifugation, the supernatant (120 μL) was detected by an HPLC system with an auto sampler and a fluorescence detector (Agilent 1100, Agilent Technologies, Santa Clara, CA). A Hypersil C18 column (4.6 mm × 250 mm, 5 μm) was used for separation of biopterin with a mobile phase of ration of methanol to water (5:95, *v*/*v*) running at a flow rate of 1.0 mL/min. The retention time of biopterin was approximately 14 min, and the excitation and emission wavelengths were 350 and 440 nm, respectively. Compounds were quantitated by their peak height in comparison with external standards. BH4 concentrations, expressed as pmol/mg protein, were calculated by subtracting BH2 + biopterin from total biopterin.

2.6. Western blot analysis

HUVECs were lysed with RIPA cell-lysis buffer (Thermo Fisher Scientific, Waltham, MA) containing protease inhibitor cocktail (Roche, Basel, Switzerland). The protein content was assayed by a BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA). Proteins were loaded to SDS-PAGE and transferred to nitrocellulose membranes. After incubation in blocking buffer (5% BSA in TBS-T), the membranes were incubated with primary antibody (all at 1:1,000 dilutions) overnight at 4 °C, followed by incubation with IRDye 800CW-conjugated goat anti-rabbit or anti-mouse IgG. Protein bands were visualized by Odyssey infrared imaging system (LI-COR Biosciences), and the intensity of each band was measured by the accompanying software.

2.7. Statistical analysis

Data were presented as means ± SD. The differences between groups were analyzed by one-way ANOVA. Thereafter, LSD (least

significant difference) was used to analyze the differences between two groups. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS 16.0 J for Windows (SPSS Japan Inc., Tokyo, Japan).

3. Results

3.1. Effects of FG on ROS generation and NO production in HUVECs

Under a confocal microscope, the signal intensity of intracellular ROS was obviously increased in the HUVECs of HG and FG groups relative to the NC group (Fig. 1A). Compared with the NC group, the level of intracellular ROS was significantly increased in the cells of HG ($P = 0.007$) and FG groups ($P < 0.001$) as detected by a flow cytometer. Notably, the ROS level was even higher in the cells of FG group ($P = 0.01$) than that in the HG group (Fig. 1B, Supplementary Table 1). Conversely, the level of NO was significantly reduced in the cells of HG ($P = 0.001$) and FG ($P < 0.001$) groups compared to the NC group. Similarly, an even lower level of NO was found in the cells exposed to FG ($P = 0.008$) than that resulted from HG exposure (Fig. 1C, Supplementary Table 1). However, there was no significant difference in the levels of ROS ($P = 0.899$) and NO ($P = 0.520$) between the NC and HC groups (Fig. 1B and C, Supplementary Table 1). These results suggested that FG induced endothelial dysfunction characterized by increased ROS generation and decreased NO production.

3.2. Effects of FG on eNOS coupling and NADPH oxidase level in HUVECs

Unexpectedly, the level of phosphorylated eNOS (p-eNOS) was remarkably increased in the HUVECs of HG and FG groups (both $P < 0.001$) compared to the NC group (Fig. 2A, Supplementary Table 2). BH4 is an essential cofactor to sustain the bioactivity of eNOS. Therefore, we measured the level of BH4 in the HUVECs by HPLC. Compared with the NC group, the BH4 level was significantly reduced in the cells of HG ($P < 0.001$) and FG ($P = 0.001$) groups (Fig. 2B, Supplementary Table 2). In line with this result, the level of GTPCH1, a key rate-limiting enzyme of BH4 synthesis, was markedly decreased in the cells of HG and FG groups (both $P < 0.001$) compared to the NC group. Notably, the level of GTPCH1 was even lower in the cells of FG group ($P = 0.018$) than that in the HG group (Fig. 2C, Supplementary Table 2). Nevertheless, there was no significant difference in the levels of p-eNOS ($P = 0.757$), BH4 ($P = 0.697$) and GTPCH1 ($P = 0.614$) between the NC and HC groups (Fig. 2A–C, Supplementary Table 2). These results suggested that FG induced eNOS uncoupling by downregulating the levels of both GTPCH1 and BH4.

NADPH oxidases may also induce ROS generation in the vasculature and result in endothelial dysfunction. Our result showed that the level of p47-phox, a subunit of NADPH oxidase, was significantly augmented in the HUVECs of HG and FG groups (both $P < 0.001$) compared with the NC group (Fig. 2D, Supplementary Table 2). Importantly, addition of either L-NAME (an inhibitor of NOS) or apocynin (an inhibitor of NADPH oxidases) significantly attenuated the FG-induced increase of ROS generation (both $P < 0.001$) in the HUVECs (Fig. 2E, Supplementary Table 2). These results indicated that the increased ROS generation in the FG-treated cells was at least partly due to either eNOS uncoupling or upregulated NADPH oxidase.

3.3. Effects of metformin on ROS generation and NO production in the FG-treated HUVECs

Compared with the NC group, FG exposure significantly decreased the level of phosphorylated AMPK (p-AMPK) in the HUVECs ($P < 0.001$). As expected, metformin (0.2–10 mmol/L) dose-dependently increased the level of p-AMPK ($P < 0.05$) in the FG-treated cells (Fig. 3A, Supplementary Table 3). The metformin-induced (2 mmol/L) upregulation of p-AMPK level was blocked by 10 μmol/L CC, an AMPK inhibitor ($P < 0.001$) in these cells (Fig. 3B, Supplementary Table 3). Notably, metformin (2 mmol/L) inhibited the FG-induced augmentation of ROS generation ($P < 0.001$). This effect of metformin was reversed by adding CC ($P = 0.013$) in the FG-impaired cells (Fig. 3C, Supplementary Table 3). Moreover, metformin (2 mmol/L) attenuated the FG-induced downregulation of NO production ($P < 0.001$), which was also diminished by addition of CC ($P = 0.022$) in the FG-treated cells (Fig. 3D, Supplementary Table 3). These results suggested that metformin might attenuate the FG-induced endothelial dysfunction via activation of AMPK signaling pathway.

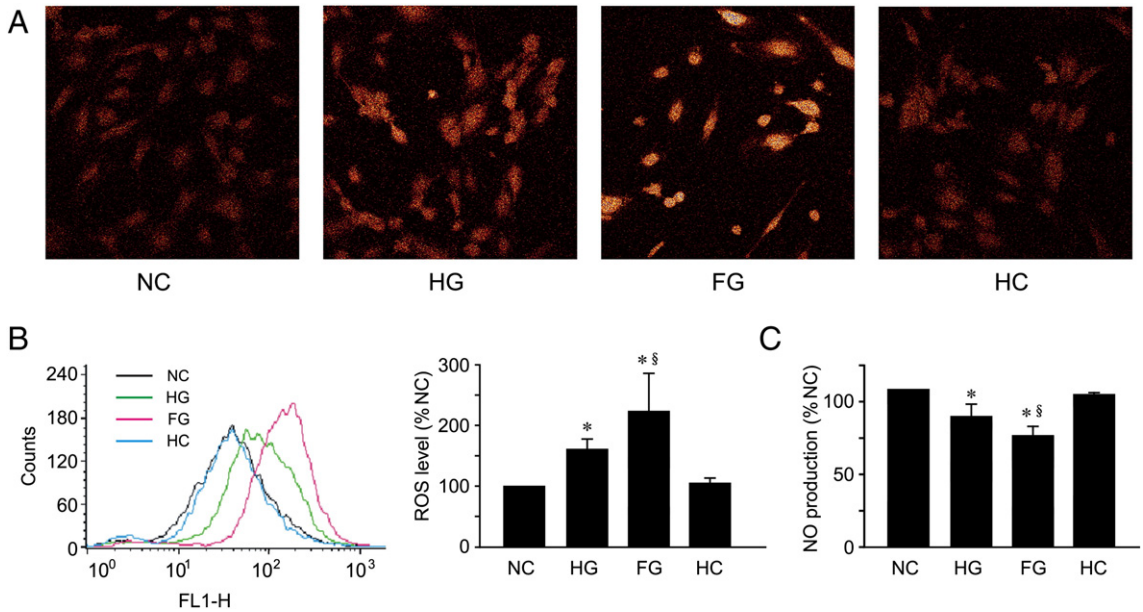

Fig. 1. Effects of FG on ROS generation and NO production in HUVECs. A: Representative photographs of intracellular ROS signals detected by a laser confocal microscope. B: Representative photographs (left panel) and quantification (right panel) of ROS level detected by a flow cytometer. C: Quantification of NO level measured by ELISA. Values are expressed as means ± SD. All experiments were performed four times with each group in triplicate. *$P < 0.05$, vs NC group; §$P < 0.05$, vs HG group. NC, normal glucose control; HG, constant high glucose; FG, fluctuating glucose; HC, hyperosmotic control.

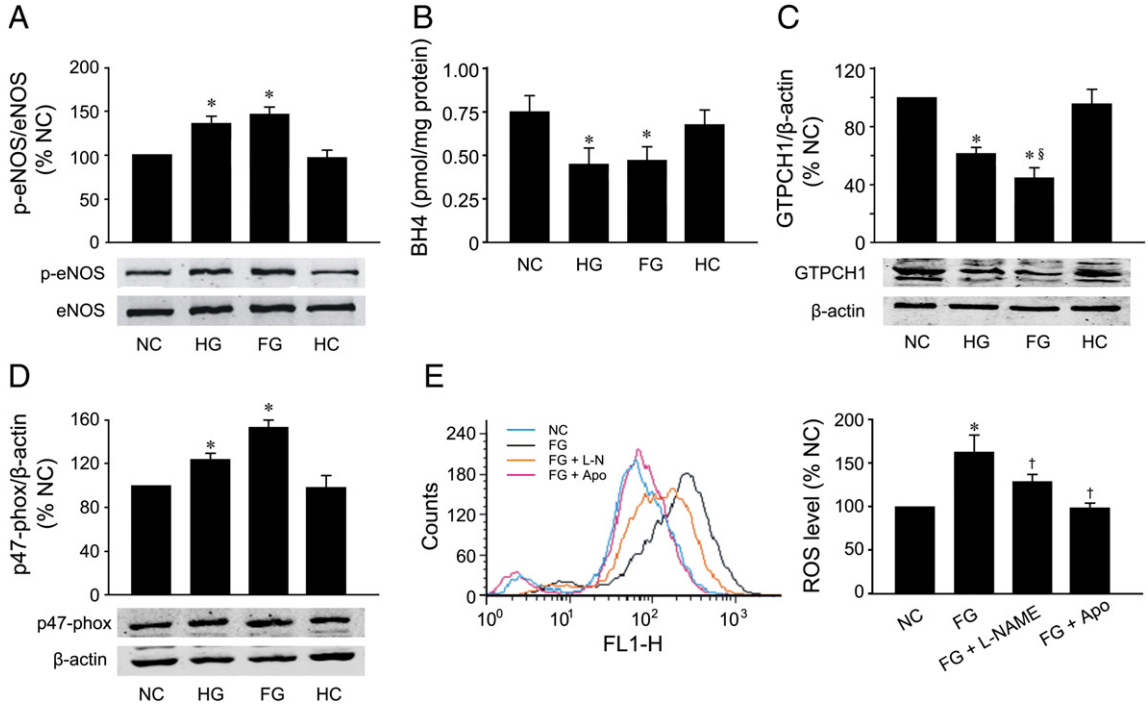

Fig. 2. Effects of FG on eNOS coupling and NADPH oxidase level in HUVECs. A: Representative photographs and quantification of phosphorylated eNOS (p-eNOS) level measured by Western blot. B: Quantification of BH4 level detected by HPLC. C and D: Representative photographs and quantification of GTPCH1 (C) and p47-phox (D) levels measured by Western blot. E: Representative photographs (left panel) and quantification (right panel) of ROS level detected by a flow cytometer. Values are expressed as means ± SD. All experiments were performed four times with each group in triplicate. $^{*}P < 0.05$, vs NC group; $^{\S}P < 0.05$, vs HG group; $^{\dagger}P < 0.05$, vs FG group. NC, normal glucose control; HG, constant high glucose; FG, fluctuating glucose; HC, hyperosmotic control; L-NAME, *N*-nitro-L-arginine-methyl ester, an NOS inhibitor; Apo, apocynin, an inhibitor of NADPH oxidases.

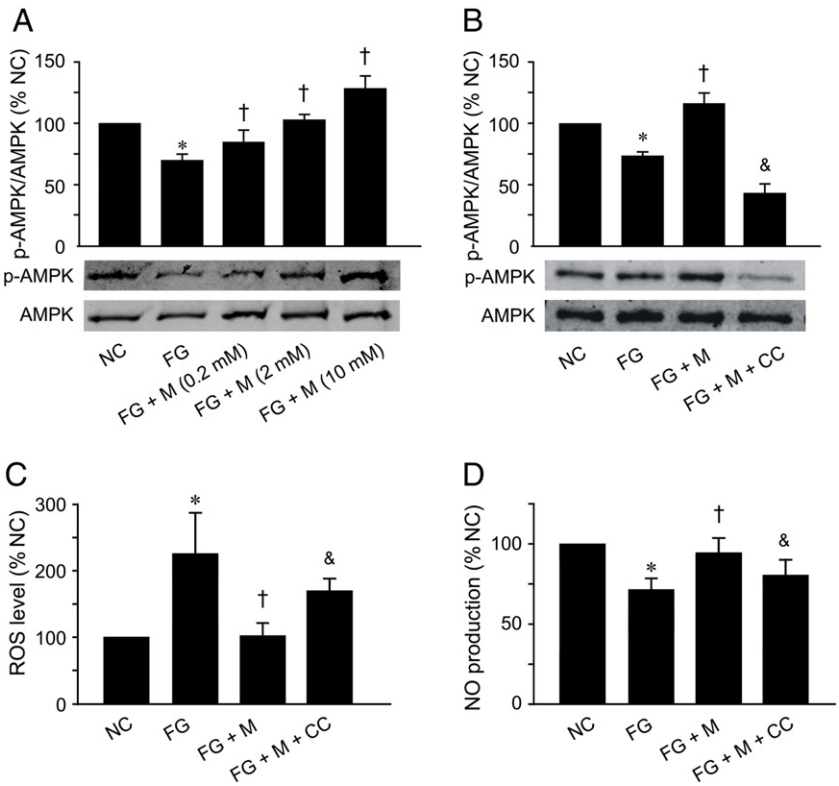

Fig. 3. Effects of metformin on AMPK phosphorylation and the levels of ROS and NO in HUVECs exposed to FG. A and B: Representative photographs and quantification of phosphorylated AMPK (p-AMPK) level measured by Western blot. C: Quantification of ROS generation detected by a flow cytometer. D: Quantification of NO production measured by ELISA. Values are expressed as means ± SD. All experiments were performed four times with each group in triplicate. $^{*}P < 0.05$, vs NC group; $^{\dagger}P < 0.05$, vs FG group; $^{\&}P < 0.05$, vs FG + M group. NC, normal glucose control; FG, fluctuating glucose; M, metformin; CC, compound C.

3.4. Effects of metformin on eNOS coupling and NADPH oxidase level in the FG-treated HUVECs

As shown in Fig. 4A and Supplementary Table 4 (also see Fig. 2A), the level of p-eNOS in the HUVECs was significantly upregulated by FG ($P < 0.001$). Interestingly, metformin (2 mmol/L) could further increase the level of p-eNOS in the FG-treated cells ($P < 0.001$). The AMPK inhibitor CC (10 μmol/L) remarkably enhanced the metformin-induced upregulation of p-eNOS level ($P < 0.001$), instead of inhibiting it, in the FG-impaired cells. We next investigated the effects of metformin on the levels of BH4 and GTPCH1. As shown in Fig. 4B and C and Supplementary Table 4, metformin significantly attenuated the FG-induced reduction of BH4 and GTPCH1 levels (both $P < 0.001$) in the HUVECs. Importantly, addition of CC abolished the above effects of metformin on BH4 and GTPCH1 levels (both $P < 0.001$) in the FG-treated cells. These results indicated that metformin promoted eNOS recoupling through activation of AMPK signaling pathway.

As shown in Fig. 4D and Supplementary Table 4, metformin also significantly inhibited the FG-induced upregulation of p47-phox level in the HUVECs ($P < 0.001$). Likewise, this effect of metformin in the FG-treated cells was blocked by adding CC ($P < 0.001$), suggesting that metformin downregulated the level of this NADPH oxidase subunit via activation of AMPK signaling pathway.

4. Discussion

Our studies showed that FG, similar or even significant to HG, induced oxidative stress as indicated by increased ROS generation and decreased NO production in the HUVECs. The endothelial dysfunction might be mediated via upregulation of the NADPH oxidase subunit p47-phox, as well as induction of eNOS uncoupling through downregulation of GTPCH1 and BH4 levels. Metformin upregulated GTPCH1 and BH4 levels, promoted eNOS recoupling, and subsequently inhibited ROS generation and increased NO production via AMPK signaling pathway. Furthermore, metformin might also function as a protective factor of the endothelial cells against oxidative stress by downregulating the NADPH oxidase.

Macroangiopathy is one of the main chronic complications in type 2 diabetes, which significantly increases mortality risk. Hyperglycemia, which induces vascular endothelium damage by oxidative stress, non-enzymatic glycosylation and other mechanisms, is an important initial factor of diabetic angiopathy and atherosclerosis (Brownlee, 2001). In recent years, a series of studies have shown that diabetic vascular complications not only are associated with mean blood glucose level, but also have a close relationship with glucose variability (Monnier & Colette, 2008). Endothelial cell damage caused by FG may be more severe than that resulted from HG (Monnier et al., 2006; Quagliaro et al., 2005; Risso et al., 2001). In line with those observations, our study showed that higher ROS generation and lower NO production were found in the HUVECs of FG group than that in HG group, suggesting that FG could induce more severe endothelial dysfunction than HG.

Endothelial dysfunction, a common pathophysiological basis of diabetic angiopathy, is characterized by decreased NO bioavailability (Davignon & Ganz, 2004; Esper et al., 2006). NO is derived from

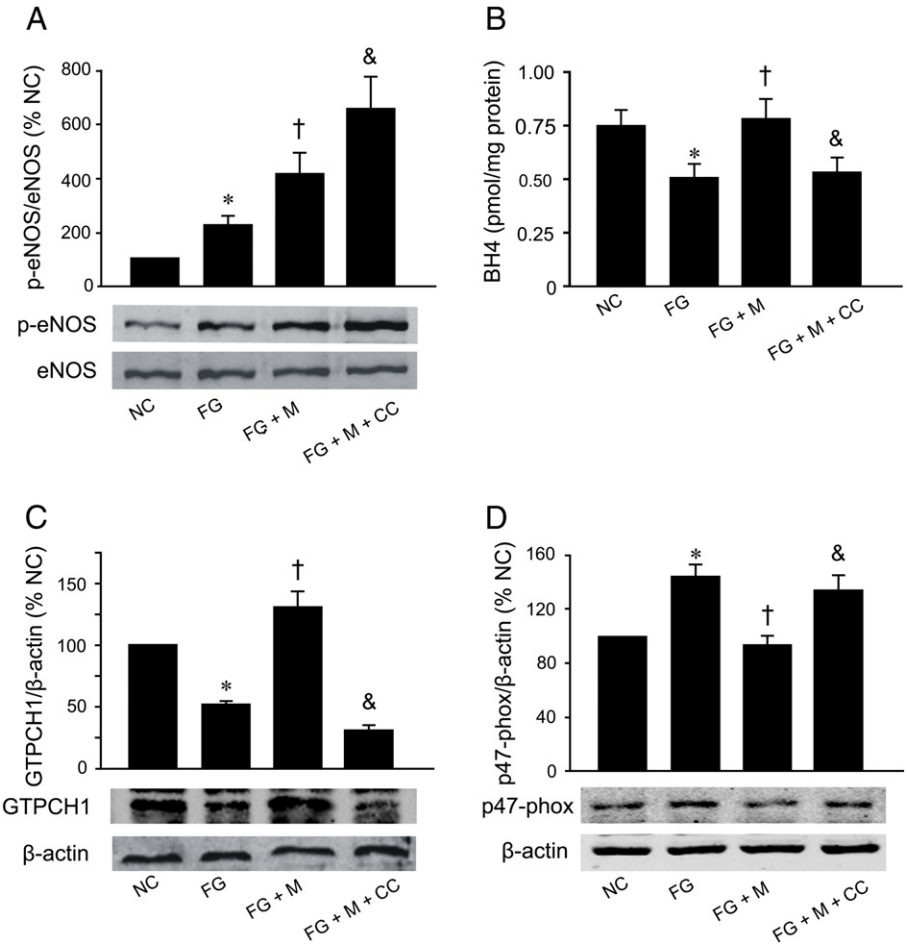

Fig. 4. Effects of metformin on eNOS coupling and NADPH oxidase level in HUVECs exposed to FG. A: Representative photographs and quantification of p-eNOS level measured by Western blot. B: Quantification of BH4 level detected by HPLC. C and D: Representative photographs and quantification of GTPCH1 (C) and p47-phox (D) levels measured by Western blot. Values are expressed as means ± SD. All experiments were performed four times with each group in triplicate. $^{*}P < 0.05$, vs NC group; $^{\dagger}P < 0.05$, vs FG group; $^{\&}P < 0.05$, vs FG + M group. NC, normal glucose control; FG, fluctuating glucose; M, metformin; CC: compound C.

L-arginine in the catalysis of eNOS. Under the normal physiological conditions, as an important regulator in vascular homeostasis, NO can regulate vascular tone and blood pressure, and inhibit platelet aggregation and adhesion (Napoli et al., 2006). However, under the pathophysiological conditions such as diabetes, the bioavailability of NO is significantly decreased. The declined NO bioavailability is mainly due to decreased production of NO and increased inactivation of NO, the latter of which is mainly caused by the ultra oxygen anion. A number of studies have demonstrated that decreased NO production is not entirely caused by decreased eNOS expression. Surprisingly, overexpression of eNOS accelerates atherosclerosis in ApoE-deficient mice (Ozaki et al., 2002). A likely explanation is that eNOS overexpression in the ApoE-deficient mice leads to eNOS uncoupling. Evidence for eNOS uncoupling has been also apparent in patients with endothelial dysfunction resulting from diabetes or hypercholesterolemia (Li, Horke, & Forstermann, 2014). When eNOS uncoupled, superoxide is generated at the expense of NO production. The uncoupled eNOS becomes the new sources of ROS and mediates the damage on vascular endothelium (Li & Forstermann, 2013). Our results showed that both HG and FG upregulated eNOS phosphorylation. Since this alteration was in parallel to the upregulation of ROS generation and downregulation of NO production, we concluded that the FG-induced upregulation of phosphorylated eNOS might be uncoupled. Under this condition, eNOS was converted from an NO-producing enzyme to a molecule that generated superoxide. It is worth noting that eNOS uncoupling is not an "all or none" phenomenon. Uncoupled and coupled eNOS can exist at the same time (Ponnuswamy et al., 2012).

The eNOS uncoupling can be induced by many risk factors, such as diabetes and hypertension. Several mechanisms for eNOS uncoupling are implicated, such as deficiency and/or oxidative degradation of the eNOS cofactor BH4, depletion of the substrate L-arginine, and accumulation of the endogenous eNOS inhibitor asymmetrical dimethylarginine, among which BH4 deficiency is the pivotal factor (Li & Forstermann, 2013). In the present study, both FG and HG reduced BH4 level in the HUVECs. Addition of the NOS inhibitor L-NAME partially attenuated the FG-induced augmentation of ROS generation in the HUVECs. These results indicate that FG may cause endothelial dysfunction by inducing eNOS uncoupling due to decreased BH4 level.

GTPCH1 is a key rate-limiting enzyme for de novo synthesis of BH4, and thereby affects eNOS coupling state. In cultured aortic endothelial cells, inhibition of GTPCH1 by either drugs or RNA interference results in eNOS uncoupling and endothelial dysfunction (Wang et al., 2008). Thus, the inhibited expression and activity of GTPCH1 may be one of the mechanisms leading to endothelial dysfunction in diabetics. In this study, significant decrease of GTPCH1 level in parallel to reduction of BH4 level was found in the HUVECs of HG and FG groups, which led to eNOS uncoupling, and thus resulted in endothelial dysfunction.

NADPH oxidases, the multi-subunit enzyme complexes, are major sources of ROS in the vasculature. p47-phox, a regulatory subunit of NADPH oxidase, is required for activation of Nox catalytic subunit in vascular cells (Li et al., 2014). A recent study reported that HG induced p47-phox translocation and phosphorylation in cultured human aortic endothelial cells (Batchuluun et al., 2014). In this study, we found that FG markedly upregulated p47-phox level and thus augmented NADPH oxidase activity, which might also contribute to the increased ROS generation in the FG-treated HUVECs. Intriguingly, when oxidative stress was enhanced, superoxide modestly and peroxynitrite strongly oxidize BH4 to BH2, leading to BH4 deficiency (Li & Forstermann, 2013). Therefore, the enhanced BH4 oxidation due to oxidative stress, together with the reduced de novo BH4 synthesis due to downregulation of GTPCH1, significantly decreased BH4 level and thus led to eNOS uncoupling. The uncoupled eNOS generated more superoxide and accelerated oxidative stress. As a result, a vicious circle sets up (Laursen et al., 2001). Since oxidative stress is involved in endothelial dysfunction and atherosclerosis (Li et al., 2014), it is conceivable that antioxidants including carotenoids may be beneficial to cardiovascular system (Ciccone et al., 2013).

Metformin, a biguanide, is the most widely used anti-diabetic agent. A series of animal and clinical studies have demonstrated its beneficial and protective effects on cardiovascular system. The United Kingdom prospective diabetes study (UKPDS) showed that metformin could reduce risk of cardiovascular death and myocardial infarction in obese or overweight patients with type 2 diabetes (UKPDS-Group, 1998). The 10 years follow-up study after UKPDS further indicated that the beneficial effects of metformin in the intensive treatment group could be sustained (Holman, Paul, Bethel, Matthews, & Neil, 2008). Other studies reported that metformin significantly improved vascular endothelial function as assessed by flow-mediated dilation, and resulted in the regression of carotid intima-media thickness in patients with type 2 diabetes and those with metabolic syndrome (Meaney et al., 2008). One explanation of these benefits may be that metformin has direct vascular endothelial protective effects independent of its glucose-lowering effect (Anfossi et al., 2010). A previous report showed that oral administration of metformin in diabetic mice improved the damaged endothelium-dependent vasodilation function by suppressing 26S proteasome-mediated GTPCH1 degradation (Wang, Xu, Song, Viollet, & Zou, 2009). One recent study demonstrated that metformin inhibited p47-phox translocation and NADPH oxidase activation, and prevented the HG-induced oxidative stress in cultured human aortic endothelial cells (Batchuluun et al., 2014). Another study reported that metformin increased eNOS phosphorylation at serine 1179 (Ser1179), increased its combination with heat shock protein 90 by activating AMPK signaling pathway, and thereby activated eNOS, which increased NO production and thus improved endothelial function (Davis, Xie, Viollet, & Zou, 2006). Likewise, our results showed that metformin reversed the FG-induced reduction of GTPCH1 and BH4 levels, upregulation of p47-phox level, increase of ROS generation, and reduction of NO production in the HUVECs. Taken together, metformin does have a direct protective effect on the endothelial cells.

AMPK, a heterotrimetric protein composed of α, β and γ subunits, has been implicated in regulating both energy metabolism and vascular homeostasis. Activation of AMPK requires AMPK phosphorylation at Thr 172, and the activated AMPK exerts various beneficial effects on glucose homeostasis and vascular system (Eriksson & Nystrom, 2014; Ewart & Kennedy, 2011). AMPK suppresses 26S proteasome-mediated GTPCH1 degradation and thus increases GTPCH1 level (Wang et al., 2009). Moreover, AMPK is a physiological suppressor of NADPH oxidase in multiple cardiovascular cell systems (Song & Zou, 2012). In the present study, the protective effects of metformin on the FG-impaired endothelial cells were diminished by addition of the AMPK inhibitor CC, indicating that metformin functions via activation of AMPK signaling pathway. However, the precise mechanism of this process needs to be further investigated.

In conclusion, FG induces eNOS uncoupling, enhances oxidative stress and causes endothelial cell injury in the HUVECs. Metformin may attenuate the FG-induced oxidative endothelial cell damage through upregulating GTPCH1 and BH4 levels, and thereby promoting eNOS recoupling. Moreover, metformin downregulates the level of an NADPH oxidase subunit p47-phox, thus decreasing ROS generation and BH4 oxidation. These protective effects of metformin are at least partially mediated via activation of AMPK signaling pathway. The discoveries in this paper are summarized in Fig. 5.

Acknowledgments

This work was supported by research grants from the Chinese National 973 Program (2012CB517502), the National Natural Sciences Foundation of China (81570692, 81400798, 81400767, 81401142 and 81270858), and the Beijing Natural Sciences Foundation of China (7163233).

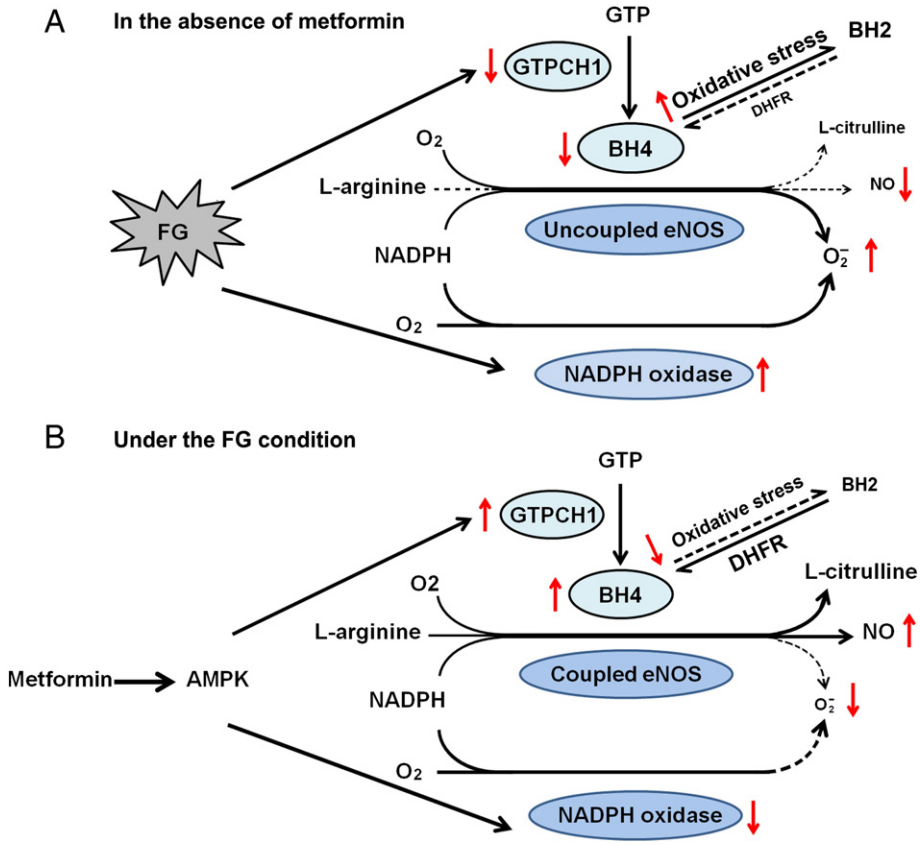

Fig. 5. Proposed scheme of the findings in this paper. A: FG induces oxidative stress in cultured HUVECs. FG decreases the levels of GTPCH1 and BH4, thus inducing eNOS uncoupling. The uncoupled eNOS produces ROS at the expense of NO. Moreover, FG increases the level of a NADPH oxidase subunit p47-phox and accordingly produces more ROS. These processes potentiate oxidative stress in the cells. On the other hand, the oxidative stress decreases BH4 level by oxidizing BH4 to BH2, which further aggravates eNOS uncoupling and oxidative stress. The vicious cycle finally leads to endothelial cell injury. B: Metformin attenuates the FG-induced oxidative stress in the HUVECs. Metformin ameliorates the FG-induced endothelial cell damage probably through upregulating GTPCH1 and BH4 levels, and thereby promoting eNOS recoupling. The recoupled eNOS produces less ROS and more NO. Metformin also decreases ROS generation derived from the NADPH oxidase. The diminished oxidative stress leads to less oxidation of BH4 to BH2; instead, BH2 is reduced to BH4 by dihydrofolate reductase (DHFR). The vicious cycle of oxidative stress is blocked by metformin. Notably, the protective effects of metformin on the endothelial cells are at least partially mediated *via* AMPK-dependent pathway.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jdiacomp.2016.04.018.

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