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Protective effects of pioglitazone on vascular endothelial cell dysfunction induced by high glucose via an inhibition of IKK α / β -NF κ B signaling mediated by PPAR γ *in vitro*

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ABSTRACT

Pioglitazone (PIO), a synthetic ligand for peroxisome proliferator activated receptor γ (PPAR γ), is clinically used to treat type 2 diabetes mellitus. However, little is known on its endothelial protective effects and the underlying mechanisms. In this study, we sought to investigate PIO's endothelial protective effects and its likely mechanisms. 95% confluent high glucose concentration (HG, 33 mM)-injured WT-HUVECs and PPAR γ^{Low} -HUVECs were first pretreated with 10 μM of GW9662 for 30 minutes, and then treated with various concentrations of PIO (5, 10, 20 μM) for 24 hours. Finally, the levels of NO, endothelin 1(ET1), tumor necrosis factor alpha (TNF α) and interleukin 6 (IL6) were detected. The cells were utilized to determine the cell viability, caspase3 activity, the levels of inhibitory κB kinase α/β (IKK α/β) mRNA, IKK α/β , and nuclear factor κB (NF κB) p65. Severe dysfunction and activation of IKK α/β -NF κB signaling occurred after exposure of HUVECs to HG. Conversely, treatment with PIO significantly attenuated the dysfunction and the activation of IKK α/β -NF κB signaling induced by HG in a dose-dependent manner. Moreover, the protective effects of PIO were completely abrogated by GW9662 or down-regulation of PPAR γ . Taken together, PIO protects HUVECs against the HG-induced dysfunction through the inhibition of IKK α/β -NF κB signaling mediated by PPAR γ .

Key Words: pioglitazone; high glucose; nitric oxide; endothelial cell; PPAR γ

Abbreviations

DMEM, Dulbecco's modified Eagle's medium

DMSO, dimethyl sulfoxide

ET1, endothelin 1

FBS, fetal bovine serum

HG, high glucose concentration

HUVECs, human umbilical vascular endothelial cells

I κ B α , inhibitory κ B alpha

IKK α/β , inhibitory κ B kinase alpha/beta

IL6, interleukin 6

NF κ B, nuclear factor kappa B

NO, nitric oxide

NRE, NF κ B responsive elements

PIO, pioglitazone

PPAR γ , peroxisome proliferator-activated receptor gamma

PPRE, PPAR responsive elements

RXR, retinoid X receptors

TBP, TATA binding protein

T2DM, type 2 diabetes mellitus

TZDs, thiazolidinediones

TNF α , tumor necrosis factor alpha

VECs, vascular endothelial cells

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia. Chronic hyperglycemia results in target organ impairments by oxidative stress etc., including angiopathy, retinopathy, renopathy and neuropathy so on (Nathan 2014). Among these, macroangiopathy has been considered as a dominant factor of morbidity and mortality in DM (Ghosh et al. 2015). Therefore, control of hyperglycemia and protection of vascular endothelial cells (VECs) are therapeutic strategies for diabetic angiopathy. Clinically, oral hypoglycemic agents, insulin, and insulin sensitizers etc. are the common used drugs for type 2 diabetes mellitus (T2DM) (Fox et al. 2015).

Pioglitazone (PIO), one of thiazolidinediones (TZDs), serves as an insulin sensitizer and is often used for therapy of T2DM (Seong et al. 2015). As for its action mechanism, it is commonly recognized that PIO ameliorates insulin sensitivity via a peroxisome proliferator activated receptor γ (PPAR γ)-dependent insulin signaling trans-activation mechanism (Huang et al. 2012). PPAR γ , a ligand-dependent nuclear transcription factor, belongs to a super-family member of nuclear transcription factors which regulate gene expression in response to the specific ligands including endogenous and exogenous ligands (McCarthy et al. 2013). Once PPAR γ binds with PIO, the heterodimer of PPAR γ -RXR (retinoid X receptors) dissociates first with co-repressors of PPAR γ including NCoR, and Tab2 etc. and associate subsequently with its co-activators such as PGC1 etc. to form a complex of PPAR γ -RXR-PGC1. Ultimately, the complex combines to the PPAR responsive elements (PPRE) of target

genes and launches the transcription of target genes (Yu et al. 2015). The aforementioned process is called as PPRE-dependent trans-activation mechanism. It is through PPRE-dependent trans-activation mechanism that PIO plays the crucial roles in the gluco-lipid metabolism and adipogenesis (Wang et al. 2015).

Nuclear factor κ B (NF κ B), a predominant transcription factor, plays central roles in inducing the expressions of a wide variety of genes such as cytokines (TNF α , IL6, GM-CSF), chemokines (MIP1 α , MCP1) and adhesion molecules (E-selectin, VCAM1, ICAM1). All these molecules are extensively involved in systemic inflammation (Kaplan et al. 2014). Physiologically, NF κ B exists in the cytoplasm in a heterotrimer of NF κ B p65, p50 and inhibitory kappa B (I κ B α). When cells are stimulated by injuries, hypoxia, and microorganisms etc., the I κ B kinase (IKK α/β), an upstream protein of NF κ B signaling, is activated. Activated IKK α/β phosphorylates I κ B α at serine 32 and 36 and subsequently the degradation of I κ B α occurs. The degradation of I κ B α results in the nuclear translocation of NF κ B p65 and the nuclear NF κ B p65 combines to the NF κ B responsive elements (NRE) of inflammatory genes. Finally, the transcription of inflammatory genes is initiated (Chang et al. 2013).

More recently, there are numerous reports that PIO also possesses anti-inflammatory and anti-cancerous effects and is clinically attempted to treat the patients with atherosclerosis and cancer (Zhao et al. 2013; Reka et al. 2011). Regarding its anti-inflammatory mechanisms, there exist two distinct viewpoints. One is so-called PPAR γ -dependent PPRE-independent trans-repression mechanism, and the other is so-called PPAR γ -independent PPRE-independent trans-repression which

is referred as ‘off-target’ effect (Marder et al. 2013). Moreover, for the former, there are also two views. One is a NF κ B-dependent mechanism while the other is an AP1-dependent mechanism (Monsalve et al. 2013).

A variety of studies demonstrated that cross-talk of PPAR γ -mediated insulin signaling and NF κ B-mediated inflammation signaling fine tunes the onset and development of atherosclerosis induced by hyperglycemia (Marcone et al. 2015). As critical endocrine cells and a crucial barrier between blood and vessel stroma, integrity and normal functionality of VECs are required for the maintenance of vascular homeostasis (Jufri et al. 2015). Unfortunately, little is known on PIO’s endothelial protective effects and its underlying mechanisms. Accordingly, in the present study, we proposed and sought to investigate the hypothesis that PIO protects endothelial cell against dysfunction induced by high glucose (HG) through a PPAR γ -dependent NF κ B trans-repression signaling.

2. Materials and methods

2.1 Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (NY, USA). The assay kits of TNF α , IL6 and ET1 were purchased from R&D Systems, Inc. (MN, USA). The assay kit of NO was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, CHN) and the caspase3 activity assay kit was from Beyotime Institute of Biotech (Shanghai, CHN). Antibodies against IKK α/β , NF κ B p65 and β -actin were purchased from Santa Cruz (CA, USA) and antibody against TATA binding protein (TBP) was from Abcam

(Cambridge, UK). Total RNA extraction and reverse transcription PCR kit were from Invitrogen Co. (USA). The primers of PCR were synthesized by Shenggong Bioengineer Co. (Shanghai, CHN). PIO, GW9662 and other reagents were all purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated elsewhere.

2.2 Cell culture

Wild type human umbilical vascular endothelial cells (WT-HUVECs) were obtained from the American Type Culture Collection (ATCC, Catalog No: CRL-1730, US). HUVECs low PPAR γ expressing (PPAR γ^{Low} -HUVECs) were prepared by infecting adenoviruses containing PPAR γ -RNAi (AD-PPAR γ -RNAi, Genechem Tech Inc., Shanghai, CHN). The cell culture was performed as described in our previous report (Huang et al. 2010). Briefly, the cells were cultured in a 6-well plate or a 24-well plate and propagated in DMEM, which was supplemented with 10% FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. The cells were cultured at 37°C in a 95% O₂-5% CO₂ humidified atmosphere. The HUVECs with 95% confluence were used for the following experiments.

2.3 Model establishment of HG-induced endothelial cell dysfunction

The model establishment of HG-induced endothelial cell dysfunction was performed as described in our previous report (Liu et al. 2014). In brief, both WT-HUVECs and PPAR γ^{Low} -HUVECs were seeded in a 6-well plate at 2×10^5 cells per well. When grown to a 95% confluence, the cells were exposed to a complete DMEM containing HG (33 mM) for 48 hours. Cell supernatants were collected to detect the levels of NO and ET1 and the cells were used to detect cell viability and

caspase3 activity.

2.4 Experimental protocols

2.4.1 Effects of various concentrations of PIO on the cell dysfunction induced by HG

WT-HUVECs were seeded in a 6-well plate at 2×10^5 cells per well and cultured in a complete DMEM containing 5.5 mM of glucose. When grown to a 95% confluence, the cells were first exposed to a complete DMEM containing HG for 48 hours, and then treated further with a fresh complete DMEM containing indicated PIO concentrations (5, 10, 20 μ M) or DMSO (a vehicle), respectively, for additional 24 hours, while the cells untreated with anything were considered as a control. Eventually, the cell supernatants were collected to detect all biochemical parameters and the cells were used to measure the cell viability, the caspase3 activity, IKK α / β mRNA and its protein levels and cytoplasmic and nuclear NF κ B p65 levels.

2.4.2 The protective effects of PIO on the cell dysfunction induced by HG were mediated by PPAR γ

The 95% confluent WT-HUVECs were first exposed to HG for 48 hours, and then pretreated with 10 μ M of GW9662 for 30 minutes, and finally treated with 20 μ M of PIO for another 24 hours. Meanwhile, the PPAR γ^{Low} -HUVECs were also carried out the same treatment as the WT-HUVECs. The levels of NO, ET1, TNF α and IL6 were detected.

2.5 MTT assay

The cell viability was determined by MTT assay according to the method described

in our previous report with a minor modification (Liu et al. 2014). Briefly, the WT-HUVECs were seeded in a 96-well plate at a density of 2×10^4 cells per well and cultured in the DMEM. When grown to a 95% confluence, the cells were treated as described in the section 2.4.1. Subsequently, the cells were further incubated with 10 microliters of MTT (5 mg/ml) for 4 hours and treated with 100 microliters of DMSO for 15 minutes. Finally, optical density values (OD) at wavelength of 490 nm were measured.

2.6 Determination of caspase3 activity

The caspase3 activity was determined using a caspase3 activity assay kit which was based on the capability of caspase3 to cleave acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) producing yellow formazan product p-nitroaniline (pNA). The procedure was performed following the manufacturer's instructions. Briefly, cells were lysed with the lysis solution followed by incubation on ice for 15 minutes and centrifugation (16,000g) for 10 minutes at 4°C. The supernatants were collected and added with the reaction buffer and the coupling substrate followed by 37°C water bath for 1 hour. The absorbance values were determined by enzyme-linked assay at 405 nm wavelength. The values were regarded as the relative activity of caspase3.

2.7 Quantitative assays of the levels of ET1, TNF α and IL6 by ELISA

The quantitative assays of the levels of ET1, TNF α and IL6 were performed using the Quantikine ELISA Kits according to the manufacturer's instructions. Briefly, 50 microliters of cell supernatants were first added to a 96-well polystyrene microplate pre-coated with indicated monoclonal antibodies and incubated for 2 hours at room

temperature on the shaker. Next, after five times of gentle washes with PBS, 100 microliters of secondary antibodies conjugated to horseradish peroxidase were added and further incubated for 2 hours. Then, 100 microliters of substrate solutions were added and treated for 30 minutes at room temperature. Ultimately, 100 microliters of stop solutions were added and the OD values were read at 450 nm in a microplate reader (Bio-Rad Laboratories).

2.8 Measurement of nitrite levels

Nitrite levels in the supernatants are commonly considered as an indicator of NO production. The levels of nitrite were determined by an assay kit according to the manufacturer's instructions. Briefly, a standard curve was prepared using a series of nitrite concentrations. One hundred microliters of samples were added in a 96-well microplate, and then the Griess reagent $\square$ and $\square$ were sequentially added and mixed. Next, the mixture was incubated at 37°C for 60 minutes. Finally, the OD values were determined at 550 nm with a spectrophotometer (Bio-Rad Laboratories).

2.9 Total RNA extraction and RT-PCR analysis for IKK α / β mRNA

Total RNA was extracted using a TRIzol (Invitrogen, USA) following the manufacturer's instructions. All reagents for complementary DNA (cDNA) synthesis and amplification were obtained from Invitrogen Co. (USA). Two micrograms of total RNA were used for cDNA synthesis. PCR was performed using Taq DNA polymerase. The primers were as follows: IKK α , forward, 5'-GCGTGAAA CTGGAATAAATACTGG-3', and reverse, 5'-GCTGCCCTTTGTCCCTGA-3', 327 bp; IKK β , forward, 5'-AAGCCAGAAAACATCGTC-3', and reverse, 5'-CACCGTT

CCATTCAAGTC-3', 321 bp; β -actin, forward, 5'-CTCCATCCTGGCCTCGCTGT-3' and reverse, 5'-GCTGTCACCTTCACCGTTCC-3', 268 bp. PCR for IKK α/β was carried out according to the following procedures: hot start of 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute; and final extension of 72 °C for 5 minutes. PCR for β -actin according to the following procedures: hot start of 94°C for 3 minutes; 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute; and final extension of 72 °C for 5 minutes. Ten microliters of amplified products were separated on 1.5% agarose gels in Tris–acetate–EDTA buffer (40 mmol/l Tris–acetate, 2 mmol/l Na₂EDTA, pH 8.5) and then visualized under UV light. Finally, the assay of signal intensity was performed by image system (Bio-Rad).

2.10 Extraction of cytoplasmic and nuclear protein and immunoblotting analysis

The whole extraction procedure was performed following the method previously reported by Luo et al. with a minor modification (Luo et al. 2014). In the following procedure, all samples, reagents and tubes were pre-chilled and kept on ice. All centrifugations were performed at 12,000 rpm and 4°C. Briefly, HUVECs were collected and pelleted by centrifugation for 30 seconds in a 1.5 ml micro-centrifuge tube. The supernatants were removed and the cell pellets were re-suspended and washed in 1 ml of ice-cold Dulbecco's modified phosphate buffered saline (DPBS). After another centrifugation, the pellets were re-suspended in a volume of hypo-osmotic lysis buffer (0.3 M sucrose, 2% (v/v) Tween 40, 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF,

2 ng/ml pepstatin A, and 2 ng/ml leupeptin). The cells were homogenized by pipetting 100 times using a micropipette with a 200 μ l pipette tip. Enucleated samples were overlaid on 1 ml of 1.5 M sucrose buffer (1.5 M sucrose, 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/ml pepstatin A, and 2 ng/ml leupeptin) and centrifuged for 10 minutes. Supernatants were collected for cytoplasmic protein immunoblotting analysis and the nuclear pellets were re-suspended in 1 ml of low-salt wash buffer (10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/ml pepstatin A, and 2 ng/ml leupeptin) and pelleted again by centrifugation for 30 seconds. After the supernatants were removed, the washed nuclear pellets were re-suspended in 50 μ l of high-salt extraction buffer (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/ml pepstatin A, and 2 ng/ml leupeptin) and placed on ice for 20 minutes with occasional vortexing. The samples were centrifuged for 20 minutes and the supernatants were used to immunoblotting analysis. Protein concentration was determined with a BCA protein quantification kit. Equal amount of protein (35 μ g) was separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, immunoblotted with specific primary antibodies at a 1:1000 dilution and second antibodies at a 1:2000 dilution and detected by chemiluminescence with the ECL detection reagents (Amersham Biosciences).

2.11 Statistical analysis

All data were expressed as the means $\pm$ S.E.M. Significance was tested with

unpaired t test, one-way ANOVA and homogeneity test of variance. A *P* value <0.05 was considered statistically significant.

3. Results

3.1 Effects of PIO on cell viability, caspase3 activity and cell function in HUVECs

In this study, we evaluated the cell injury degree, apoptosis and function by detecting the cell viability, caspase3 activity and the levels of NO and ET1, respectively. As shown in Fig.1, Compared with the control group, exposure of HUVECs to HG for 48 hours notably decreased the cell viability and the levels of NO, while significantly elevated the caspase3 activity and the levels of ET1. In the contrary, treatment of PIO for 24 hours significantly antagonized the decreases in the cell viability and the NO levels and the elevations in the caspase3 activity and ET1 levels induced by HG, indicating PIO protects the cells against the injury, apoptosis and dysfunction induced by HG. Moreover, the protective effects manifest a certain degree of dose-dependent manner.

3.2 PIO attenuated the elevations of the IKK α / β expression levels induced by HG

Since IKK α / β -NF κ B signaling is a typical inflammation pathway and diabetic vascular complication such as atherosclerosis is also a chronic inflammation process, we next sought to investigate whether treatment with PIO affects the IKK α / β expression. As anticipated, exposure of HUVECs to HG for 48 hours up-regulated considerably the levels of IKK α / β mRNA and its protein. However, treatment with PIO for 24 hours markedly attenuated the elevations of the IKK α / β expression levels

induced by HG in a dose dependent manner (Fig.2).

3.3 PIO inhibited nuclear translocation of NFκB p65 induced by HG

Nuclear translocation of NFκB p65 from cytoplasm to nucleus, an important subunit of NFκB, plays a critical role in the secretion of inflammatory cytokines including IL-6 and TNFα etc.. Therefore, we subsequently evaluated the effect of treatment with PIO on the nuclear translocation of NFκB p65 under the condition of HG. As shown in Fig.3, exposure of HUVECs to HG considerably induced the nuclear translocation of NFκB p65, manifesting a decrease in the cytoplasmic NFκB p65 levels while an increase in the nuclear NFκB p65 levels. Conversely, treatment with PIO for 24 hours markedly inhibited the nuclear translocation of NFκB p65 induced by HG in a dose-dependent manner.

3.4 PIO alleviated the elevations of inflammatory cytokines induced by HG

Inflammatory cytokines including IL-6 and TNFα etc. are the terminal factors of IKKα/β-NFκB signaling, which play predominant roles in inflammation. Hence, we ultimately observed the changes in these cytokines. As expected, exposure of HUVECs to HG considerably elevated the levels of IL-6 and TNFα. Instead, treatment with PIO for 24 hours markedly alleviated the elevations of inflammatory cytokines induced by HG in a dose-dependent manner (Fig.4).

3.5 Inhibitory effects of PIO on inflammatory cytokines are mediated by PPARγ

Finally, we wondered whether the inhibitory effects of PIO on inflammatory cytokines are mediated by PPARγ. Two approaches were used to address this question. One is to employ an antagonist of PPARγ and the other is to utilize the

RNAi technique to specifically silence *ppary* gene. As displayed in Fig.5, in WT-HUVECs, HG resulted in the release of a greater amount of IL-6 and TNF α than that of control; nonetheless, treatment with 20 μ M of PIO for 24 hours markedly alleviated the elevations of inflammatory cytokines induced by HG; however, pretreatment with GW9662, an antagonist of PPAR γ , completely abolished the PIO's effect. In PPAR γ^{Low} -HUVECs, HG also resulted in the release of a greater amount of IL-6 and TNF α ; however, treatment with either PIO20 alone or PIO20 combined with GW9662, failed to alleviate the elevations of inflammatory cytokines induced by HG. Taken together, these data demonstrate that inhibitory effects of PIO on inflammatory cytokines are mediated by PPAR γ .

3.6 Protective effects of PIO on the cell dysfunction induced by HG are mediated by PPAR γ

Exposure of WT-HUVECs to HG resulted in severe dysfunction, reflecting a reduction of the NO levels and an elevation of the ET1 levels. However, treatment with PIO for 24 hours markedly alleviated the dysfunction induced by HG. Moreover, the protective effects of PIO on the dysfunction were completely abrogated by GW9662. Likewise, after silencing the *ppary* gene, treatment with either PIO20 alone or PIO20 combined with GW9662, failed to alleviate the dysfunction induced by HG, indicating that the protective effects of PIO on the dysfunction induced by HG are mediated by PPAR γ (Fig.6).

4. Discussion

T2DM remarkably increases the risk of cardiovascular diseases which result in a

high incidence of morbidity and mortality (Favero et al. 2014). Impairment of VECs plays a critical role in the development of cardiovascular diseases resulted from chronic hyperglycemia (Onat et al. 2011). Due to prolonged hyperglycemia in patients with T2DM, VECs suffer from the injury and dysfunction through several mechanisms including oxidative stress, advanced glycosylation endproducts and inflammation so on (Kvietys and Granger 2013; Pollreisz et al. 2010). VECs function as a barrier and the endocrine cells. As predominant endocrine cells, the VECs maintain vascular homeostasis via the release of multiple vaso-active factors which exert central roles in the maintenance of vessel wall tone and blood fluidity. It is well known that the most important vaso-active factors are the NO and ET1, which function oppositely and harmoniously (Flammer et al. 2012). Indeed, our present results showed that exposure of HUVECs to HG for 48 hours resulted in the endothelial cell injury, apoptosis and dysfunction, exhibiting the decreases in the cell viability and the levels of NO as well as the increases of the apoptosis and the levels of ET1, consistent with others' results.

How does HG result in the injury of VECs? A wide variety of studies have reported that hyperglycemia or HG leads to the injury or dysfunction of VECs via a canonical NF κ B-dependent signaling cascade (Kolluru et al. 2012). It is well-known that T2DM and atherosclerosis belong to chronic inflammation diseases. Moreover, all members of the NF κ B-dependent signaling cascade are activated in these patients. To our knowledge, NF κ B is a super-family member of nuclear transcriptional factors and is involved in numerous physiological and patho-physiological processes (Vancurova

and Vancura 2012). Under the physiological conditions, NF κ B exists in cytoplasm in an inactive heterotrimer former of P50-P65-I κ B. However, under the pathological conditions such as hyperglycemia, hyperlipidemia or hypertension, etc., IKK α/β , a serine or threonine upstream kinase of NF κ B, is activated. I κ B α is phosphorylated at serine 32 and serine 36 and subsequently degraded. After that, the degradation of I κ B α results in the translocation of NF κ B-p65 from cytoplasm to nucleus and the nuclear NF κ B p65 combines to the NRE of inflammatory genes. Finally, the transcription of inflammatory genes is launched (Basu 2012). In the present study, we observed that the expression levels of IKK α/β substantially increased both at the transcriptional and translational levels under the HG-stimulation. Furthermore, the elevations in the nuclear translocation of NF κ B p65 and inflammatory cytokines including TNF α and IL6 were also observed.

Another question how HG activates the IKK α/β was to be raised. Our previous results demonstrated that HG increased markedly the levels of malondialdehyde and reactive oxygen species (ROS), while notably inhibited the activities of superoxide dismutase and glutathione peroxidase in mitochondria, accompanied by the activation of the IKK α/β (Liu et al. 2014). These results suggest that HG induces severe oxidative stress and the activation of the IKK α/β in mitochondria. Then, we wondered whether the relation of the oxidative stress and the IKK α/β activation induced by HG is accompanying or causal. To address this question, vitamin E, an anti-oxidant, and myxothiazole, an inhibitor of ROS production, were used. Our results revealed that treatment with vitamin E or myxothiazole dramatically counteracted the activity

elevation of IKK α/β induced by HG. As a result, we drew a conclusion that HG activates the IKK α/β through ROS-dependent pathway (data not shown).

Since the vascular complications induced by T2DM belong to the inflammation diseases, the NF κ B-dependent signaling has become therapeutic targets for atherosclerosis and T2DM (Flach et al. 2015). TZDs are insulin-sensitizing drugs acting through PPAR γ , and applied to treat the patients with T2DM (Gupta et al. 2010). PPAR γ belongs to a super-family member of nuclear transcription factors and plays critical roles in gluco-lipid metabolism, adipogenesis etc.. A large number of researches have reported that PPAR γ plays actions in two manners. One is a PPRE-dependent trans-activation pathway, while the other is a PPRE-independent trans-repression pathway (Maniati et al. 2011; Zhang et al.2015). Both experimental and clinical data have clearly shown that TZDs improve the insulin sensitivity and slow down the atherogenic processes, etc. (DeFronzo 2010; Wyatt et al. 2010). PIO, one of the potent synthetic PPAR γ agonists, has been used in the treatment of T2DM. Numerous studies also documented that TZDs decrease the circulating triglycerides as well as fatty acids while increase HDL-cholesterol (Karak et al. 2013; Hulsmans et al. 2013). Despite of these, all these actions of TZDs are through a PPAR γ -dependent insulin pathway trans-activation mechanism, that is, liganded PPAR γ trans-activates its target genes including those involving in the insulin pathway and the metabolism of glucose as well as lipid, etc. (Ahmadian et al. 2013).

Besides, it was recently reported that PIO also exhibits other effects such as anti-inflammatory and anti-atherogenic effects, which contribute to reverse the

development of late diabetic micro-vascular and macro-vascular complications (Zheng et al. 2014). Unfortunately, little is known about its exact mechanisms underpinning anti-inflammation and anti-atherosclerosis. In this study, we indeed observed that PIO has the protective effects against the cell injury and dysfunction induced by HG, presenting that it reversed the reductions in the cell viability and NO levels and the elevations in caspase3 and the ET1 levels induced by HG. Moreover, we further found that treatment with PIO significantly antagonized the increases in the levels of IKK α/β mRNA and its protein, TNF α and IL6 induced by HG. In addition, PIO markedly inhibited the nuclear translocation of NF κ B p65. Taken together, these data demonstrate PIO plays protective effects via inhibiting the NF κ B signaling.

Finally, we wondered whether the inhibitory effects of PIO on NF κ B signaling were through a mediation by PPAR γ . To address this issue, two approaches were used. One is to employ an antagonist of PPAR γ and the other is to utilize the RNAi technique to specifically silence ppar γ gene.. As expected, pretreatment of GW9662, an antagonist of PPAR γ , abrogated the protective effects of PIO on the cell dysfunction and reversed the inhibitory effects of PIO on NF κ B signaling. Moreover, the down-regulation of PPAR γ also achieved similar results. All these findings indicate that the protective effects of PIO on the cell dysfunction and the inhibitory effects on NF κ B signaling are mediated by PPAR γ .

Of course, the protective effects of PPAR γ agonists on endothelial cell injury were identified by numerous other reports. It was shown that rosiglitazone, a PPAR γ ligand, counteracts vascular dysfunction correlated with increased release of proinflammatory

substance elicited by micro-particles, a kind of plasma membrane vesicles containing pro-coagulant and pro-inflammatory properties. In addition, the underlying mechanisms were accounted for that activation of PPAR γ by rosiglitazone attenuated the micro-particle-mediated increases in both interleukin IL6 and IL8 through the up-regulation and activation of NF κ B (Ren et al. 2011). It is noteworthy that oxidative stress is tightly relevant with inflammation, and ROS triggers inflammatory response mainly through IKK-NF κ B signaling pathways. Thus, as the major target of inflammation, the pro-inflammatory transcription factor NF κ B plays a pivotal role in the process of inflammatory responses (Harmon et al. 2011). The present study is only an investigation *in vitro*, and more researches *in vivo* need to be further performed.

5. Conclusions

In sum, the present study observed that PIO has a capability to protect HUVECs against the HG-induced injury and dysfunction in a dose-dependent manner and further confirmed that the underlying mechanism is a non-canonical PPAR γ -mediated IKK α/β -NF κ B inhibitory pathway. These findings highlight an insight into the possible intracellular signaling regarding hyperglycemia-induced endothelial cell injury and dysfunction and provide a novel potential for PIO to prevent and treat the vascular complications resulted from T2DM.

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Figure captions**Fig.1 Effects of PIO on cell viability, caspase3 activity and cell function in HUVECs**

95% confluent HUVECs were first exposed to a complete DMEM containing HG for 48 hours, and then treated further with a fresh complete DMEM containing indicated PIO concentrations (5, 10, 20 μ M) or DMSO (a vehicle), respectively, for additional 24 hours; while the cells untreated with anything were considered as a control. Eventually, the cells were used to measure the cell viability (A) and the caspase3 activity (B), and the cell supernatants were collected to detect the levels of NO (C) and ET1 (D). All data were expressed as the means $\pm$ S.E.M of 3 independent experiments. $**P < 0.01$, vs. Control; $\# P < 0.05$, $##P < 0.01$, vs. HG.

Fig.2 PIO attenuated the elevations of the IKK α / β expression levels induced by HG

The treatment procedure of HUVECs was performed as described in Fig.1. Eventually, the cells were collected to measure the IKK α / β mRNA levels by RT-PCR (A) and its protein levels by immunoblotting analysis(B). All data were expressed as the means $\pm$ S.E.M of 3 independent experiments. $**P < 0.01$, vs. Control; $\# P < 0.05$, $##P < 0.01$, vs. HG.

Fig.3 PIO inhibited nuclear translocation of NF κ B p65 induced by HG

The treatment procedure of HUVECs was performed as described in Fig.1. The cytoplasmic proteins and nuclear proteins in HUVECs were extracted respectively to detect cytoplasmic (A) and nuclear (B) NF κ B p65 levels with a monoclonal antibody

against p65 by immunoblotting analysis. β -actin and TBP were regarded as the cytoplasmic and nuclear internal control proteins, respectively. All data were expressed as the means $\pm$ S.E.M of 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, vs. Control; $\# P < 0.05$, $##P < 0.01$, $###P < 0.01$, vs. HG.

Fig.4 PIO alleviated the elevations of inflammatory cytokines induced by HG

The treatment procedure of HUVECs was performed as described in Fig.1. The cell supernatants were collected to detect the levels of IL6 (A) and TNF α (B). All data were expressed as the means $\pm$ S.E.M of 3 independent experiments. $**P < 0.01$, vs. Control; $\# P < 0.05$, $##P < 0.01$, vs. HG.

Fig.5 Inhibitory effects of PIO on inflammatory cytokines are mediated by PPAR γ

The 95% confluent WT-HUVECs were first exposed to HG for 48 hours, and then pretreated with 10 μ M of GW9662 for 30 minutes, and finally treated with 20 μ M of PIO for another 24 hours. Meanwhile, the whole treated procedure of the PPAR γ^{Low} -HUVECs was the same as that of the WT-HUVECs. The levels of TNF α (A) and IL6 (B) were detected. All data were expressed as the means $\pm$ S.E.M of 3 independent experiments. $*P < 0.05$, $**P < 0.01$, vs. Control; $\# P < 0.05$, $##P < 0.01$, vs. HG; $\& P < 0.05$, $\&\& P < 0.01$, vs. HG+PIO20; $\$ \$ P < 0.01$, vs. HG+PIO20 in WT-HUVECs.

Fig.6 Protective effects of PIO on the cell dysfunction induced by HG are mediated by PPAR γ

The treatment procedure and grouping of both WT-HUVECs and

PPAR γ^{Low} -HUVECs were the same as described in Fig.5. Finally, the cell supernatants were used to detect the levels of NO (A) and ET1 (B). All data were expressed as the means $\pm$ S.E.M of 3 independent experiments. $**P < 0.01$, vs. Control; $##P < 0.01$, vs. HG; $\&\& P < 0.01$, vs. HG+PIO20; $$$ P < 0.01$, vs. HG+PIO20 in WT-HUVECs.

Draft

Fig.1

214x182mm (300 x 300 DPI)

Fig.2

150x81mm (300 x 300 DPI)

Fig.3

139x64mm (300 x 300 DPI)

Fig.4

118x53mm (300 x 300 DPI)

Fig.5

161x82mm (300 x 300 DPI)

Fig.6

160x80mm (300 x 300 DPI)