Effects of reduced β2 glycoprotein I on high glucose-induced cell death in HUVECs

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Abstract. Reduced β2 glycoprotein I (β2GPI) has been demonstrated to exhibit a beneficial effect in diabetic atherosclerosis and retinal neovascularization. However, the effect of reduced β2GPI on vascular disorders in diabetic mellitus (DM) remains to be elucidated. The present study established a high glucose-induced injury model using human umbilical cords veins (HUVECs) and evaluated the protective effects of reduced β2GPI against the injury. The data demonstrated that a low concentration of reduced β2GPI (0.5 μM) mitigated high glucose-induced cell loss, decreased nitric oxide (NO) production and resulted in calcium overloading. Mechanically, reduced β2GPI additionally reversed high glucose-induced phosphatase and tensin homolog (PTEN) accumulation, decrease of protein kinase B phosphorylation and nitric oxide synthase activity, and increase of cyclooxygenase-2 activity. It was further confirmed that PTEN inhibitor-bpV (1 μM) exhibited similar effects to those resulting from reduced β2GPI. Overall, the data revealed that reduced β2GPI exerts protective effects from glucose-induced injury in HUVECs, potentially via decreasing PTEN levels. The present study suggests reduced β2GPI may act as a novel therapeutic strategy for the treatment of vascular disorders in DM.

Introduction

Atherosclerosis is one of the severe microvascular complications of diabetes mellitus (DM). Epidemiological studies demonstrate that hyperglycemia is the most consistent symptom that reveals a significant association with the development of vascular disorders in DM (1). However, the mechanisms for the vascular disorders in DM remain to be elucidated. Endothelial cell dysfunction has been demonstrated to occur in diabetes (2-4), and functional recovery of these cells may aid in therapeutically resolving the vascular complications that occur in DM.

β2 glycoprotein I (β2GPI) has been identified as the most prominent antigen in antiphospholipid syndrome (5,6). β2GPI is predominantly synthesized in hepatocytes (7) and consists of five repeating amino acid domains. Domains I-IV include four cysteines each and have the conserved sequences, whereas domain V has an extra 20 amino acid tail with a unique cysteine termination (8). It has previously been demonstrated that the disulfide bond between Cys288 to Cys326 in domain V may be reduced by thioredoxin-1 (TRX-1) or protein disulfide isomerase (PDI) (9), resulting in the reduced state of β2GPI, referred to as reduced β2GPI.

Although β2GPI has been revealed to participate in the autoimmune system, vascular thrombosis, infectious diseases and other systems (10-12), the specific function of β2GPI in DM remains to be elucidated. It has been reported that β2GPI is associated with accelerated atherosclerosis and enhanced oxidative stress (13), whereas reduced β2GPI protects endothelial cells from oxidative stress-induced injury (13). The authors previously demonstrated that reduced β2GPI is beneficial in diabetic atherosclerosis and retinal neovascularization (14). However, no studies have examined the association between reduced β2GPI and microvascular disorders in DM. The present study hypothesizes that reduced β2GPI exhibits beneficial effects in microvascular disorders in DM.

Materials and methods

Cell culture. Briefly, human umbilical cord tissues were harvested from 12 women who had normal delivery (age range 25-35 years, average, 31.4 years) at Tianjin Medical University Metabolic Diseases Hospital (Tianjin, China). Endothelial cells from human umbilical cords veins (HUVECs) were harvested as previously described (15). Written consent was obtained from each patient. All protocols were approved by the Ethical Committee of Tianjin Medical University Metabolic Disease Hospital (Tianjin, China), ethics protocol number: DXBYYhMEC2017-11. Briefly, human umbilical cord tissues

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were digested by 0.05% collagenase for 15 min at 37°C. The cells were collected and grown in M199 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20% fetal calf serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% CO_2. Adherent cells were harvested following trypsin-EDTA treatment (0.05%). Culture medium was replaced every 2 days and adherent cells were identified by their cobblestone appearance at confluence and positive labelling with mouse FITC-conjugated antihuman factor VIII (1:1,000, ab41187; Abcam, Cambridge, UK) detected by flow cytometry as previously described (15).

Purification of β2GPI and preparation of reduced β2GPI. β2GPI was purified from normal human plasma extracted from a total of 10 male volunteers (age range 20-40 years, average 35 years) (Tianjin Medical University Metabolic Disease Hospital) as previously described (16). Venous blood (50 ml) was collected and plasma was obtained following centrifugation at 4°C, 987 x g for 10 min. Written informed consent was obtained from each volunteer. Reduced β2GPI was prepared as previously described (17). In brief, purified β2GPI (2 μM) was reduced by TRX-1 (3.5 μM) activated with dithiothreitol (DTT, 70 μM). The thiols of reduced β2GPI were protected by reduced glutathione. The reduced β2GPI was verified using western blotting and liquid chromatography-mass spectrometry analysis as previously described (14).

Cell viability. HUVECs were seeded in a 96-well culture plate and cultured for 5 days at 37°C in a humidified atmosphere containing 5% CO_2. Different concentrations of reduced β2GPI (0.25-1 μM) were incubated with the cells. A total of 24, 48 and 72 h following incubation, the cell viability was detected by an MTT assay. The effect of reduced β2GPI was compared with the non-reduced β2GPI. High glucose (44.4 mM) was applied to model cell injury and the protective effects of reduced β2GPI on cell death compared to vehicle (TRX-1, DTT and glutathione) were analyzed. Following application of the aforementioned treatments, 20 μl MTT (5 mg/ml) was added into the 180 μl culture medium in each well. The medium was removed following 4 h, and 150 μl dimethyl sulfoxide was added into each well to dissolve the precipitation. The absorbance was measured at a wavelength of 490 nm using an automated microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell viability was calculated using the following formula: Cell viability (%) = average absorbance of treated group/average absorbance of control group x 100%.

Detection of intracellular free Ca²⁺. Intracellular free Ca^{2+} levels were detected by a fluo 3/-acetoxymethyl (AM) kit (Beyotime Institute of Biotechnology, Ningbo, China) following the manufacturer's protocol. HUVECs were seeded in a 96-well culture plate and cultured for 5 days. Following various treatments for 48 h, the cells were washed with PBS and incubated in serum-free medium containing 5 μM flura-3/AM for 60 min at 37°C. Following this, the cells were washed with PBS and cultured for a further 25 min to ensure fluro-3/AM conversion into fluro-3 in cells. The excitation and emission wavelengths were set at 488 and 530 nm respectively to measure fluro-3 fluorescence using a microplate reader.

Determination of total nitric oxide (NO), NO synthase (NOS) and cyclooxygenase (COX)-2 activity. HUVECs were seeded in a 12-well culture plate and cultured for 5 days. Following various treatments, the culture medium was collected to measure the NO level using a NO assay kit (A012; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was detected by a spectrophotometer set at a wavelength of 540 nm. Nitric oxide synthase (NOS) activity was detected following the kit manufacturer's protocol (S0025; Beyotime Institute of Biotechnology). Following various treatments, the cells were collected to measure the NOS activity. COX-2 activity was detected following the kit manufacturer's protocol (ab210574; Abcam). Following various treatments, the cells were collected to measure the COX-2 activity. The levels of NO, NOS and COX-2 were calculated as follows: [(Absorbance of treated wells-absorbance of blank wells)/(absorbance of standard wells-absorbance of blank wells)] x standard concentration (100 μM).

Immunoblot analysis. Cytoplasmic protein was isolated using the Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The cells were treated with high glucose and/or β2GPI and washed with 1 ml ice-cold PBS, and collected and centrifuged for 5 min at 1,158 x g at 4°C. Protein concentration was detected using the BCA method (Beyotime Institute of Biotechnology), and an equal amount of protein (20 μg) was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The nitrocellulose membranes were blocked with 5% non-fat milk for 2 h at room temperature and subsequently incubated with anti-phosphatase and tensin homolog (PTEN, 1:1,000; no. 9552), anti-protein kinase B (AKT, 1:1,000; no. 9272), anti-phospho (p)-AKT (1:1,000; no. 12694) and anti-actin (1:5,000; no. 12262) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) primary antibodies at 4°C overnight. Following washing, the membranes were incubated with a secondary antibody (1:5,000; ab131368; Abcam) for 2 h at room temperature. The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 kit; GE Healthcare Life Sciences, Chalfont, UK). Grey density was analyzed using Quantity One analysis software v1.4.6 (Bio-Rad Laboratories, Inc. Hercules, CA, USA).

Immunochemical staining. Following treatment with indicated drugs, the cells were fixed in 4% paraformaldehyde at room temperature for 2 h. Non-specific staining was blocked by 0.1 M PBS containing 10% goat serum (Hyclone; GE Healthcare Life Sciences) with 0.4% Triton X-100 (Sigma-Aldrich; Merck KgaA, Darmstadt, Germany) at room temperature for 2 h. The cells mounted on coverslip were incubated with anti-PTEN antibody (1:400, no. 9552; Cell Signaling Technology, Inc.) in 0.1 M PBS containing 5% goat serum and 0.4% Triton X-100 overnight at 4°C. Sections were washed three times (15 min each) in PBS and incubated with Alexa Fluor 594 goat anti-rabbit IgG (1:200, B40944; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. Fluoroshield mounting media
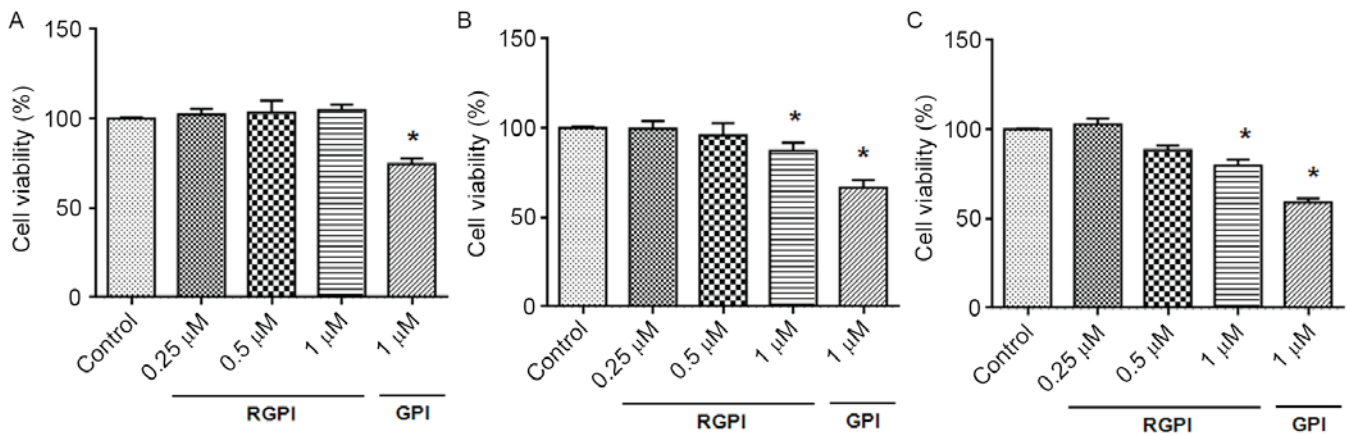
Figure 1. Effects of reduced β2GPI on cell viability in human umbilical cord veins. (A) The cells were incubated with non-reduced or reduced β2GPI at differing concentrations for (A) 24, (B) 48 and (C) 72 h. Data were presented as the mean ± standard error of the mean A total of 6 repeats were carried out in each group. *P<0.05 vs. control. β2GPI, β2 glycoprotein I; RGPI, reduced GPI.

(ab104140; Abcam) was used to preserve the fluorescence of the stained samples. The images were obtained using Olympus Fluorescence Microscopy (Olympus Corporation, Japan).

Statistical analysis. The data are presented as the mean ± standard error of mean. Statistical analyses of the data were performed using Student's t-test or one-way analysis of variance followed by Newman-Keuls multiple comparisons post-hoc test, using version 17.0 of SPSS (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Reduced β2GPI decreases high glucose-induced cell death in HUVECs. Initially, the present study detected the cell viability following reduced β2GPI treatment. As presented in Fig. 1, the results demonstrated that reduced β2GPI at the concentration range of 0.25 to 1 μM did not affect cell viability following 24 h treatment. Conversely, 1 μM β2GPI treatment for 24 h significantly decreased the cell viability to a 70% level. A total of 48 h following treatment, 1 μM reduced β2GPI slightly decreased the viability and 1 μM β2GPI significantly decreased the cell viability. A total of 72 h following treatment, reduced β2GPI at the concentration of 1 μM significantly decreased the cell viability of HUVECs. A total of 0.5 μM reduced β2GPI did not have cytotoxic action in HUVECs following 24-48 h treatment. However, β2GPI is toxic to HUVECs. In the following experiments, 0.5 μM reduced β2GPI was selected to investigate the potential mechanisms.

HUVECs are sensitive to high glucose. As presented in Fig. 2, following 44.4 mM glucose treatment for 48 h, the cell viability decreased to 50%, whereas reduced β2GPI significantly mitigated high glucose-induced cell loss (cell viability, 85%). Non-reduced β2GPI further decreased the cell viability compared with high glucose group. However, the vehicle (TRX-1, DTT and glutathione) did not have protective action. As the control, reduced β2GPI did not affect cell viability in HUVECs. These results suggested that 0.5 μM reduced
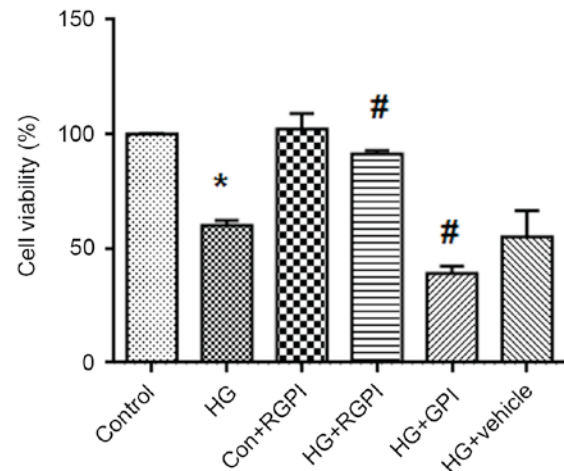

Figure 2. Effects of reduced β2GPI on high glucose induced cell death in human umbilical cord veins. The cells were incubated with reduced β2GPI or vehicle and high glucose for 48 h. Data are presented as the mean ± standard error of the mean. A total of six repeats were carried out in each group. *P<0.05 vs. control. #P<0.05 vs. HG group. β2GPI, β2 glycoprotein I; HG, high glucose; RGPI, reduced GPI; Con, control.

β2GPI was beneficial for cell survival in high glucose treated HUVECs.

Reduced β2GPI reverses high glucose-induced increase of PTEN. The present study detected PTEN expression following high glucose treatment for 48 h. As presented in in Fig. 3A, high glucose treatment for 48 h significantly elevated PTEN levels. Reduced β2GPI significantly decreased PTEN expression in high glucose-treated cells, whereas reduced β2GPI did not influence PTEN level in untreated HUVECs. Immunochemical staining verified the results in Fig. 3B. In control, control + reduced β2GPI and high glucose + reduced β2GPI groups, PTEN was weakly expressed. PTEN is a negative regulator of AKT phosphorylation. The present study additionally detected p-AKT and AKT levels. It was demonstrated that high glucose decreased the p-AKT/AKT level, which was reversed by reduced β2GPI treatment (Fig. 3C). In addition, the PTEN inhibitor bpV was applied. As indicated,
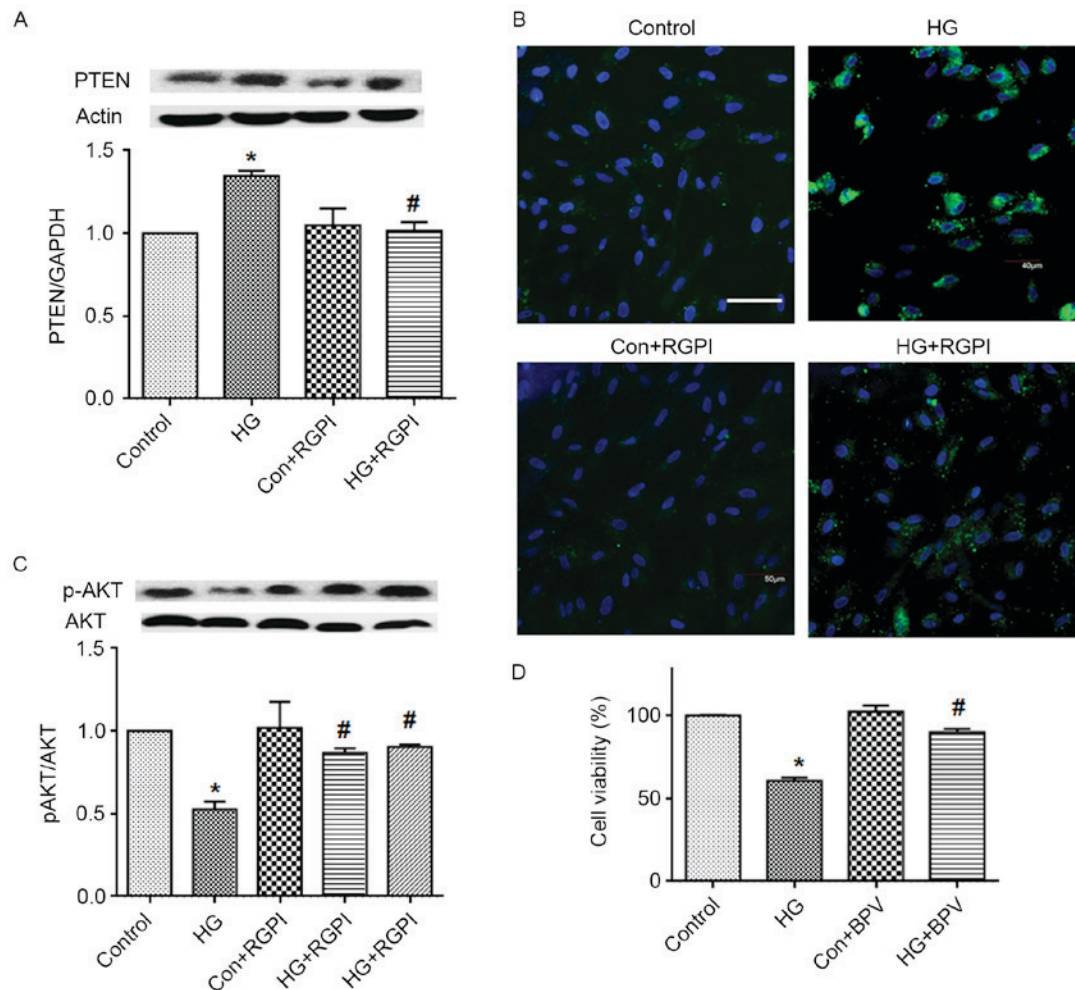
Figure 3. Effects of reduced β2GPI on high glucose-induced PTEN expression in human umbilical cord veins. Cells were incubated with reduced or non-reduced β2GPI for 48 h in absence or presence of high glucose. Representative images of PTEN expression levels detected via (A) immunoblotting and (B) immuno-histochemical staining. Scale bar: 50 μm. (C) Detection of p-AKT expression via immunoblotting. (D) PTEN inhibitor reversed high glucose-induced cell death. Data are presented as the mean ± standard error of the mean. A total of six repeats were carried out in each group. *P<0.05 vs. control. #P<0.05 vs. HG group. β2GPI, β2 glycoprotein I; HG, high glucose; RGPI, reduced GPI; Con, control; PTEN, phosphatase and tensin homolog; BPV, PTEN inhibitor; AKT, protein kinase B; p, phosphorylated.

bpV (1 μM) additionally upregulated the p-AKT/AKT level compared with the high glucose treated group. If reduced β2GPI prevents high glucose-induced cell loss via upregulation of PTEN, application of a PTEN inhibitor should exhibit a similar effect on high glucose-induced cell loss. As presented in Fig. 3D, 1 μM bpV mitigated high glucose-induced cell loss, however did not affect cell viability in normal HUVECs.

Reduced β2GPI reverses high glucose-induced intracellular free Ca²⁺ level. The intracellular free Ca^{2+} level was addition-ally detected. As presented in Fig. 4, high glucose incubation increased the intracellular calcium level, which was subse-quently reversed by reduced β2GPI treatment. Conversely, reduced β2GPI did not influence the calcium influx in normal HUVECs compared with the control.

Reduced β2GPI reverses high glucose-induced decrease of NO production and NOS activity. The present study detected NO production and NOS activity. As demonstrated in Fig. 5, high glucose significantly decreased NO production following 48 h treatment. The treatment with reduced β2GPI and bpV
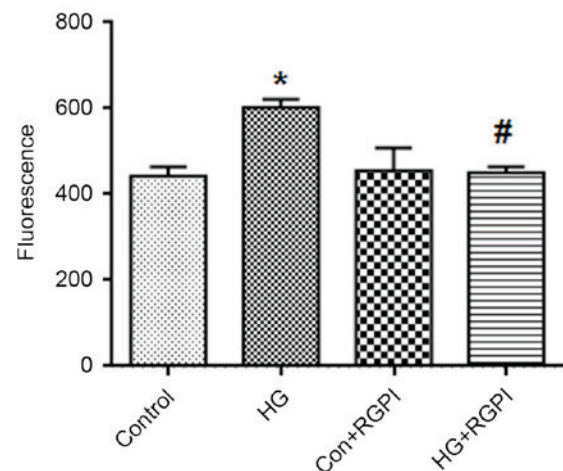

Figure 4. Effects of reduced β2GPI on intracellular free calcium levels in high glucose-induced human umbilical cord veins. The cells were incubated with reduced or non-reduced β2GPI for 48 h in the presence or absence of high glucose. Data are presented as the mean ± standard error of the mean. A total of six repeats were carried out in each group. *P<0.05 vs. control. #P<0.05 vs. HG group. β2GPI, β2 glycoprotein I; HG, high glucose; RGPI, reduced GPI; Con, control.

Figure 6. Effects of reduced β2GPI on COX-2 activity in high glucose-induced human umbilical cord veins. The cells were incubated with reduced β2GPI or BVP with high glucose for 48 h. Data are presented as the mean ± standard error of the mean. A total of six repeats were carried out in each group. *P<0.05 vs. control. #P<0.05 vs. HG group. β2GPI, β2 glycoprotein I; HG, high glucose; RGPI, reduced GPI; Con, control; NO, nitric oxide; NOS, nitric oxide synthase; BVP, phosphatase and tensin homolog inhibitor; COX-2, cyclooxygenase-2.
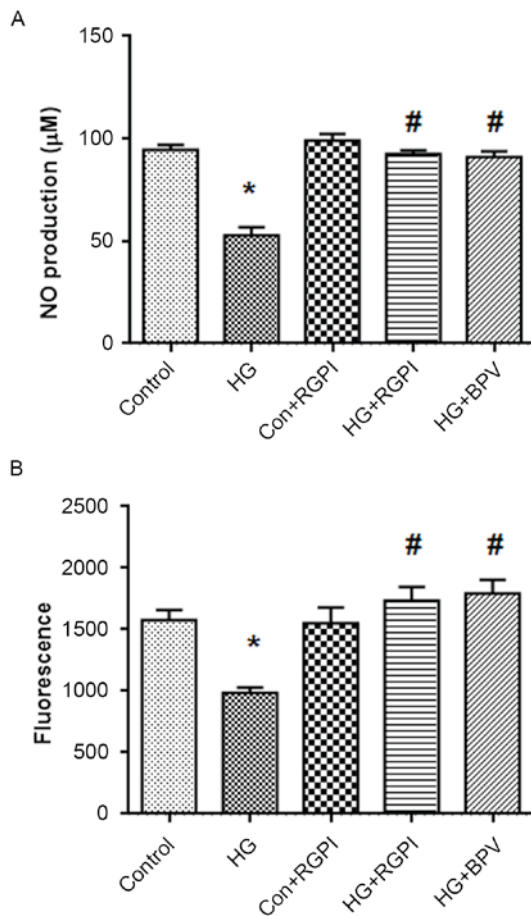
Figure 5. Effects of reduced β2GPI on NO production in high glucose-induced human umbilical cord veins. The cells were incubated with reduced β2GPI or BVP with high glucose for 48 h and (A) NO production and (B) NOS activity were detected. Data are presented as the mean ± standard error of the mean. A total of six repeats were carried out in each group. *P<0.05 vs. control. #P<0.05 vs. HG group. β2GPI, β2 glycoprotein I; HG, high glucose; RGPI, reduced GPI; Con, control; NO, nitric oxide; NOS, nitric oxide synthase; BVP, phosphatase and tensin homolog inhibitor.

eliminated the effects of high glucose on NO production. NOS is a primary enzyme responsible for NO synthesis, therefore its activity was detected. It was demonstrated that high glucose treatment decreased NOS activity. Reduced β2GPI and bpV significantly increased the NOS activities.

Reduced β2GPI reverses high glucose-induced increase of COX-2 activity. The present study detected COX-2 activity following reduced β2GPI treatment. As presented in Fig. 6, high glucose treatment for 48 h significantly elevated COX-2 activity. Reduced β2GPI, in addition to bpV, significantly decreased the COX-2 activity.

Discussion

The present study demonstrated the novel effect of RGPI on high glucose-induced injury in HUVECs. Reduced β2GPI reversed high glucose-induced cell injury, however, these effects were not observed with non-reduced GPI. High glucose resulted in accumulation of PTEN in HUVECs, which inhibited AKT phosphorylation, whereas RGPI reversed that signaling pathway.
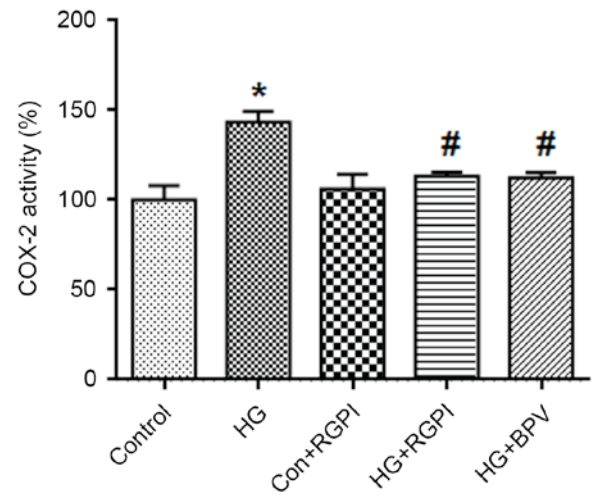
It has previously been reported that the proportion of reduced β2GPI is decreased in antiphospholipid syndrome (18,19). This suggests that reduced β2GPI may exhibit a protective role in the development of the disease. Reduced β2GPI, however not non-reduced β2GPI, has been revealed to protect EAhy926 from oxidative stress induced endothelial cell damage (20). The present study demonstrated that a low concentration of reduced β2GPI protected against high glucose-induced cell death. Furthermore, reduced β2GPI did not affect the cell viability at a concentration of 0.25-1 μM, following 24 h incubation. It was observed that 1 μM reduced β2GPI was toxic to HUVECs following 48 or 72 h incubation. The toxicity may have resulted from the re-oxidation of β2GPI, as the non-reduced β2GPI was additionally detrimental to HUVECs. In contrast to previous studies which revealed that β2GPI and reduced β2GPI improve kidney fibrosis (21), the present study demonstrated that only the reduced form of β2GPI exerted protective effects in high glucose-induced cell injury in HUVECs. Regarding the function of reduced β2GPI, potential re-oxidation may restrict its application. Therefore, the maintenance of the reduced state of β2GPI is critical for its protective function.

The present study identified the mechanisms involved in the protection of reduced β2GPI in high glucose induced cell injury. Consistent with a previous study (15), it was demonstrated that high glucose elicited cell death of HUVECs. Apoptosis of HUVECs occurs following high glucose treatment (22,23). Kinase signaling pathways are important in the regulation of apoptosis. The AKT signaling pathway is an important cell survival signaling pathway (24,25). The present study demonstrated that following high glucose treatment, p-AKT levels were decreased, which was consistent with the results of a previous study (26). In the reduced β2GPI treated group, the p-AKT level was increased compared with the model group. These data suggested that reduced β2GPI

activated the survival signaling pathway. PTEN is a negative regulator of AKT phosphorylation (27). The accumulation of PTEN attenuates AKT phosphorylation. In accordance with a previous study (28), it was observed that the PTEN level was increased following high glucose treatment. Notably, the PTEN level decreased following reduced β2GPI treatment. The increased PTEN is critical for high glucose-induced cell death of HUVECs, as a PTEN inhibitor reversed high glucose-induced cell injury.

Calcium overloading is an important factor in cell death (29). The present study detected intracellular free calcium levels. High glucose elicited calcium overloading in HUVECs, which was eliminated by reduced β2GPI. Calcium activates calpain to elicit cell death. m-calpain requires a high concentration of calcium to exert its function to specifically degrade PTEN (30,31). Therefore, the accumulation of PTEN was contrary to the increased calcium level following high glucose treatment. As PTEN may additionally be degraded by the ubiquitin proteasome system (UPS) (32), the accumulation of PTEN may result from damage of the UPS system under high glucose conditions. However, these interactions require further investigation.

NO is an important neurotransmitter secreted by vascular endothelial cells and has numerous functions, including inhibiting monocyte-macrophage and platelet adhesion, decreasing the monolayer permeability of vascular endothelial cells and reducing vascular endothelial cell and smooth muscle cell proliferation (33). A total of 30% NO in the blood is derived from endothelial nitric oxide synthase genes expressed by vascular endothelial cells (34). The present study detected NO level and NOS activity under high glucose conditions. Results demonstrated that high glucose decreased NO level and NOS activity and the abnormalities of NO genesis were prevented by reduced β2GPI application. Notably, impaired NO production and the NOS activity were reversed following PTEN inhibitor application.

Prostaglandins COX may additionally contribute to hyperglycemia-induced endothelial dysfunction. The present study demonstrated that high glucose treatment induced upregulation of COX-2, which was consistent with previous studies (35,36). As suggested, increased function of COX-2 may result in the reduced NO availability and altered prostanoid profile in human endothelial cells (35). Additionally, PTEN is involved in the regulation of COX-2 activity and application of the PTEN inhibitor reversed the high glucose-induced upregulation of COX-2 activity.

In conclusion, the present study provides novel data suggesting the protective effects of reduced β2GPI in high glucose induced cell injury in HUVECs. High glucose-induced accumulation of PTEN may be important in the increase of cell death, decrease in NO production and COX-2 activation. Further studies are required in order to maintain the reduced state of β2GPI, however the findings identify the therapeutic potential of reduced β2GPI in the treatment of vascular disorders in DM.

Acknowledgements

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