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EPO Relies upon Novel Signaling of Wnt1 that Requires Akt1, FoxO3a, GSK-3β, and β-Catenin to Foster Vascular Integrity during Experimental Diabetes

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Abstract

Multiple complications can ensue in the cardiovascular, renal, and nervous systems during diabetes mellitus (DM). Given that endothelial cells (ECs) are susceptible targets to elevated serum D-glucose, identification of novel cellular mechanisms that can protect ECs may foster the development of unique strategies for the prevention and treatment of DM complications. Erythropoietin (EPO) represents one of these novel strategies but the dependence of EPO upon Wnt1 and its downstream signaling in a clinically relevant model of DM with elevated D-glucose has not been elucidated. Here we show that EPO can not only maintain the integrity of EC membranes, but also prevent apoptotic nuclear DNA degradation and the externalization of membrane phosphatidylserine (PS) residues during elevated D-glucose over a 48-hour period. EPO modulates the expression of Wnt1 and utilizes Wnt1 to confer EC protection during elevated D-glucose exposure, since application of a Wnt1 neutralizing antibody, treatment with the Wnt1 antagonist DKK-1, or gene silencing of Wnt1 with Wnt1 siRNA transfection abrogates the protective capability of EPO. EPO through a novel Wnt1 dependent mechanism controls the post-translational phosphorylation of the "pro-apoptotic" forkhead member FoxO3a and blocks the trafficking of FoxO3a to the cell nucleus to prevent apoptotic demise. EPO also employs the activation of protein kinase B (Akt1) to foster phosphorylation of GSK-3β that appears required for EPO vascular protection. Through this inhibition of GSK-3β, EPO maintains β-catenin activity, allows the translocation of β-catenin from the EC cytoplasm to the nucleus through a Wnt1 pathway, and requires β-catenin for protection against elevated D-glucose since gene silencing of β-catenin eliminates the ability of EPO as well as Wnt1 to increase EC survival. Subsequently, we show that EPO requires modulation of both Wnt1 and FoxO3a to oversee mitochondrial membrane depolarization, cytochrome c release, and caspase activation during elevated D-glucose. Our studies identify critical elements of the protective cascade for EPO that rely upon modulation of Wnt1, Akt1, FoxO3a, GSK-3β, β-catenin, and mitochondrial apoptotic pathways for the development of new strategies against DM vascular complications.

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Keywords

Akt1; apoptosis; β-catenin; cytokines; diabetes; endothelial; erythropoietin; growth factors; forkhead; FoxO3a; GSK-3β; oxidative stress; phosphatidylserine; protein kinase B; Wnt

INTRODUCTION

Diabetes mellitus (DM) present important health concerns and involves complications throughout the body including cardiovascular, renal, and nervous systems [1-8]. In the nervous system, long-term uncontrolled serum glucose can result in significant functional impairment, dementia [9-11], and generalized neuropathies, especially in conjunction with vascular disease and renal impairment [12, 13], leading to severe disability [14, 15]. Increased risk for vascular dementia also has been found in elders who have DM [11, 16]. Injury of cerebral microvascular endothelial cells (ECs) appears to critically contribute to the complications in the central nervous system (CNS). Recently, cerebral microvascular ECs have been demonstrated to be more susceptible to advanced glycation end products that can occur during DM [17]. In addition, elevated glucose levels in a rat model of DM results in cerebral microvascular EC dysfunction and subsequent disruption of blood-brain barrier permeability [18]. Consequently, for one to alleviate complications in the nervous system that are linked to vascular injury, one needs to focus on the protection of ECs [19, 20].

One novel pathway to consider involves Wnt1 and its signaling pathways that can alter cellular survival [21-25]. The secreted cysteine-rich glycosylated Wnt proteins play a role in a variety of cellular functions that involve gene expression, gene replication, cell differentiation, and cell apoptosis [26-28]. High glucose can down-regulate the expression of some of Wnt proteins, such as Wnt4 and Wnt5. Sustaining Wnt4 and Wnt5 signaling is beneficial for cell survival during elevated glucose exposure [29]. Variations in genes in the Wnt signaling pathway, such as transcription factor 7-like 2 gene, may impart increased risk for type 2 DM in some populations [30]. In addition, experimental work has suggested that some Wnt family members may affect glucose tolerance and insulin sensitivity [31]. The expression of Wnt5b in adipose tissue, the pancreas, and the liver in diabetic patients promotes adipogenesis and contributes to the susceptibility to type 2 DM [32]. Of the Wnt family members, Wnt1 has been closely linked to the control of cell growth and proliferation. Wnt1 can promote cell survival during insults such as serum deprivation [33], amyloid toxicity [34], or oxidative stress [27, 35], while loss of Wnt1 expression leads to apoptosis [27, 34-38]. Interestingly, Wnt1 is expressed in endothelial cells. Wnt1 signaling promotes endothelial proliferation and vessel growth [24, 39] and prevents apoptosis in ECs exposed to elevated glucose [36].

Wnt1 can prevent apoptotic cell injury during DM via inhibition of glycogen synthase kinase (GSK-3β). Active GSK-3β phosphorylates its downstream target β-catenin resulting in its subsequent degradation. Wnt1 can inhibit GSK-3β to prevent the phosphorylation and degradation of β-catenin to allow β-catenin to translocate to the nucleus resulting in the transcription of anti-apoptotic genes [34, 40, 41]. As a result, GSK-3β and β-catenin have been considered as therapeutic targets for a number of disorders [34, 42-44]. In DM, inactivation of GSK-3β prevents high glucose cell toxicity of pancreatic beta cells [45] and has been linked to cardioprotection during experimental DM [46]. In addition, physical exercise, one of the important lifestyle interventions for DM to promote glycemic control in clinical studies [47], has been reported to lead to phosphorylation and inhibition of GSK-3β [48].

Wnt1 also has been shown to be tied to the cytokine and growth factor erythropoietin (EPO) [36, 49, 50]. EPO offers protection in a variety of disorders that involve the cardiovascular system [51-55], renal system [56-60], perinatal disease [61], sepsis [62], cellular ischemia [63-69], and the visual system [70]. In clinical studies with DM, plasma EPO is often low in diabetic patients with [71] or without anemia [72]. Yet, studies with EPO administration in patients with congestive heart failure or DM combined with congestive heart failure also demonstrate an improved cardiac output and a decrease in medical resource utilization [73-75]. In other clinical work, EPO serum levels are significantly associated with the number and function of circulating endothelial progenitor cells and EPO can stimulate postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow [76], suggesting that EPO may be protective for ECs during disease states.

In our present work, we show that EPO employs novel pathways of Wnt1 signaling to protect cerebral ECs against elevated D-glucose exposure. Wnt1 is necessary for EPO cellular protection as illustrated through antibody blockade, pharmacological inhibition, and gene silencing of Wnt1. Wnt1 also modulates posttranslational phosphorylation and cellular trafficking of FoxO3a and β-catenin. Through gene silencing studies, these pathways of FoxO3a and β-catenin appear to be significant determinants of EPO cytoprotection and are integrated with Wnt1 oversight of Akt1 and GSK-3β. Subsequently, EPO relies upon Wnt1 to prevent mitochondrial depolarization, cytochrome c release, and caspase 3 activation.

MATERIALS AND METHODS

Cerebral Microvascular Endothelial Cell Culture

Vascular ECs were isolated from Sprague-Dawley adult rat brain cerebra by using a modified collagenase/dispase-based digestion protocol [19, 64, 77]. Briefly, rat brains were removed and cerebella were cut off aseptically. The cerebral cortices cleaned of white matter were trimmed into blocks (approximately 1-2 mm^3). The blocks were then incubated in dissociation medium containing 1 mg/ml collagenase/dispase (Roche, Mannheim, Germany) in M199E with 0.5% antibiotic-antimycotic solution at 37°C for 2 hours. The cell slurry formed was homogenized and was then re-suspended with 15% dextran (Sigma, Louis, MO, USA). Following centrifugation at 4,000 × g for 20 min (4°C), the pellet was re-suspended in a minimal volume of HEPES buffer and layered onto a pre-prepared colloidal silica gradient solution of 45% Percoll (Sigma, Louis, MO, USA) in Dulbecco′s PBS. The upper band (ECs) was collected following centrifugation at 20,000 × g for 20 min (10°C). Cells were re-suspended in growth medium containing 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 90 μg/ml heparin, 20 μg/ml endothelial cell growth supplement (ICN Biomedicals, Aurora, OH, USA), and 0.5% antibiotic-antimycotic solution in M199E. The cells were plated on gelatin-coated dishes and continuously incubated in a humidified atmosphere of 5% CO_2 and 95% room air. All experiments were performed using the third passage cells. Endothelial cells were identified by positive direct immunocytochemistry for factor VIII-related antigen [19, 64, 77] and by characteristic spindle-shaped morphology with antigenic properties shown to resemble brain endothelium *in vivo* [78].

Experimental Treatments

Elevated D-glucose concentrations in ECs was performed by replacing the media with serum-free M199E media with 2 mmol/l L-glutamine and 90 μg/ml heparin containing 25 mM D-glucose and then incubated at 37°C for 48 hours. In this injury paradigm for elevated D-glucose, hyperosmolarity did not play a significant role in cell toxicity. A mannitol concentration of 25 mM resulted in similar and not significantly different survival rates than untreated control ECs with survival equal to approximately 90%, suggesting that hyperosmolarity was not a significant factor in cell injury. Furthermore, we performed

additional studies with the biologically inactive agent L-glucose plus 5.6 mM D-glucose and have observed that L-glucose at concentrations of 25 mM did not significantly alter cell survival [79, 80].

For treatments, erythropoietin [EPO) (10 ng/ ml, Sigma, St Louis, MO)), human recombinant Wnt1 protein (100 ng/ml, R&D Systems, Minneapolis, MN)), a mouse monoclenal antibody against Wnt1 (1 μg/ml, R&D Systems, Minneapolis, MN)), and a glycogen synthase kinase (GSK)-3β inhibitors SB216763 [3-(2,4-Dichlorophenyl)-4-(1-methyl-*1*H-indol-3-yl)-*1*H-pyrrole-2,5-dione] (SB21, 5 μM) (Tocris, Ellisville, MO), and Wnt antagonist dickkopf related protein 1 (DKK-1, 0.5 μg/ml, R&D Systems, Minneapolis, MN) were applied 1 hour prior to elevated D-glucose and the treatment were continuous.

Cell Survival and Injury

EC injury was determined by bright field microscopy using a 0.4% trypan blue dye exclusion method following elevated D-glucose treatment per our previous protocols [79, 80]. The mean survival was determined by counting eight randomly selected non-overlapping fields with each containing approximately 10-30 cells (viable + non-viable) in each 24 well plate or 35 mm dish.

DNA Fragmentation

Genomic DNA fragmentation was determined by the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay [81, 82). Briefly, ECs were fixed in 4% paraformaldehyde/0.2% picric acid/0.05% glutaraldehyde and the 3'-hydroxy ends of cut DNA were labeled with biotinylated dUTP using the enzyme terminal deoxytransferase (Promega, Madison, WI) followed by streptavidinperoxidase and visualized with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA).

Assessment of Membrane Phosphatidylserine (PS) Residue Externalization

Externalization of membrane PS residues was determined by using Annexin V labeling per our prior studies [81-84]. A 30 μg/ml stock solution of Annexin V conjugated to phycoerythrin (PE) (R&D Systems, Minneapolis, MN) was diluted to 3 μg/ml in warmed calcium containing binding buffer (10 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$). Plates were incubated with 500 μl of diluted Annexin V for 10 minutes. Images were acquired with "blinded" assessment with a Leitz DMIRB microscope (Leica, McHenry, IL) and a Fuji/Nikon Super CCD (6.1 megapixels) using transmitted light and fluorescent single excitation light at 490 nm and detected emission at 585 nm.

Gene Silencing with siRNA Transfection

ECs were plated into 35 mm dishes. To silence Wnt1 gene expression, commercial reagents using the SMART pool Wnt1 siRNA kit (Upstate, Lake Placid, NY) were used. Transfection of siRNA duplexes was performed with Lipofectamine 2000 reagent according to manufacturer guidelines (Invitrogen, Carlsbad, CA). To silence FoxO3a and β-catenin gene expression, the following sequences were synthesized (Applied Biosystems, Foster City, CA): the FoxO3a, the siRNA sense strand 5'-AGGAACGUGAUGCUUCGCAAtt -3', and the antisense strand 5'-UUGCGAAGCAUCACACGUUCCgg -3'; β-catenin, the sense strand 5'-CUGUUGGAUUGAUUCGAAtt-3', and antisense strand 5'-UUUCGAAUCCAACAGtt-3'. Transfection of siRNA duplexes was performed with lipofectamine 2000 according to manufacturer guidelines (Invitrogen, Carlsbad, CA). Experimental assays were performed 72 hours post-transfection. For each siRNA assay, negative controls contain multiple siRNAs including the target siRNA and positive controls

are absent of the target siRNA. For each siRNA assay, negative controls contain multiple siRNAs including the target siRNA and positive controls are absent of the target siRNA, which results in the absence and presence of Wnt1 expression respectively (data not shown).

Western Blots

Cells were homogenized and each sample (50 μg/lane) was subjected to 12.5 % (Wnt1 and caspase 3) or 7.5% (β-catenin, FoxO3a, and Akt1) SDS-polyacrylamide gel electrophoresis. The membranes were incubated with a mouse monoclonal antibody against Wnt1 (1: 1000, R&D Systems, Minneapolis, MN), a rabbit polyclonal antibody against total FoxO3a (1: 1000), phosphorylated FoxO3a (p-FoxO3a, Ser^{253}, 1: 1000), a rabbit monoclonal antibody against phosphorylated-Akt1 (p-Akt1, Ser^{473}, 1:1000) and total Akt1 (1:1000) (Cell Signaling, Beverly, MA), a rabbit monoclonal antibody against active caspase 3 (1: 1000), a rabbit polyclonal antibody against cytochrome c (Cell Signaling, Beverly, MA), a rabbit polyclonal antibody against phospho-β-catenin (Ser^{33}, 1:200), and β-catenin (1:200, Santa Crutz, Santa Crutz, CA). After washing, the membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (goat anti-mouse IgG for Wnt1, 1:1000, or goat anti-rabbit IgG for FoxO3a, p-FoxO3a, p-β-catenin, β-catenin, and caspase 3, 1: 2000, Invitrogen, Carlsbad, CA). The antibody-reactive bands were revealed by chemiluminescence (Amersham Pharmacia Biotech, NJ) and band density was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at: http://rsb.info.nih.gov/nih-image/).

Preparation of Mitochondria and Cytosol for the Analysis of Cytochrome C Release—Briefly, cells were harvested, homogenized, and the harvested supernatants were centrifuged at 10,000 g for 15 min at 4 °C. The resulting pellet was re-suspended in isolation buffer and used as the mitochondrial fraction. The supernatant was subjected to further ultra-centrifugation at 50,000 g for 1 h, with the resultant supernatant being used as the cytosolic fraction.

Western Blot Analysis for FoxO3a and β-Catenin in the Cytoplasm and the Nucleus—Cells were homogenized, the cytoplasmic and nuclear proteins were prepared by using NE-PER nuclear and cytoplasmic extraction reagents according to manufacture's instruction (Pierce, Rockford, IL). The expression of FoxO3a and β-catenin in nucleus and cytoplasm was determined by Western blot. Each sample (50 μg/lane) was subjected to 7.5% SDS-polyacrylamide gel electrophoresis and Western blot was performed as description as above.

Immunocytochemistry and Double Staining for β-Catenin or FoxO3a, and TUNEL

Cells were fixed for either single or double staining with 50% Methanol, 50% Acetone, blocked with 1.5% Normal Horse Serum, and labeled with antibody (Vector laboratories, Burlingame, CA). For β-catenin and FoxO3a translocation, ECs were incubated with a rabbit antibody against β-catenin (1:100, Santa Crutz, Santa Crutz, CA), FoxO3a (1: 100, Cell Signaling, Beverly, MA) or active caspase 3 (Cell Signaling, Beverly, MA) over night at 4°C. Then β-catenin or FoxO3a was visualized by Texas-red labeled anti-rabbit secondary antibody (1:100, Vector laboratories, Burlingame, CA). Cells were washed in PBS, then stained with DAPI (Sigma, St. Louis, MO) for nuclear identification. For specific double staining of FoxO3a and TUNEL, the 3′-hydroxy ends of cut DNA are labeled with biotinylated dUTP using an enzyme terminal deoxytransferase followed by Fluorescence Avidin (1:50, Vector laboratories, Burlingame, CA)). Cells are then incubated with rabbit anti-FoxO3a (1:100, Cell Signaling, Beverly, MA), then with biotinylated anti-rabbit IgG (1:50) followed by Texas Red streptavidin (1:50). Fluorescence imaging used the wavelengths of 565 nm (red) and 400 nm (DAPI).

Assessment of Mitochondrial Membrane Potential

The fluorescent probe JC-1 (Invitrogen, Carlsbad, CA) was used to assess the mitochondrial membrane potential. EC monolayers in 35 mm plates were washed with M199E growth medium containing 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 90 μg/ml heparin, and 0.5% antibiotic-antimycin. Subsequently, 1.5 ml fresh warmed EC growth medium was applied to each plate. The stock solution of JC-1 was directly administrated to each plate to yield a final concentration of 2 μg/ml. The plates were incubated in a humidified atmosphere of 5% CO_2 and 95% room air at 37 °C for 30 minutes. The cultures were washed three times using fresh M199E growth medium. ECs were analyzed immediately under a Leitz DMIRB microscope (Leica, McHenry, IL, USA) with a dual emission fluorescence filter with 515-545 nm for green fluorescence and emission at 585-615 nm for red fluorescence.

Statistical Analysis

For each experiment, the mean and standard error were determined. Statistical differences between groups were assessed by means of analysis of variance (ANOVA) from 6 replicate experiments with the post-hoc Student's t-test. Statistical significance was considered at $p<0.05$.

RESULTS

Erythropoietin (EPO) Protects Cerebral ECs During Elevated D-Glucose Against Apoptotic DNA and PS Externalization Injury

D-glucose was applied to primary cultures of microvascular ECs at the concentrations of 10, 25, 50, 75, and 100 mM, cell survival was determined 48 hours following elevated D-glucose by using trypan blue exclusion method. As shown in Fig. (**1A**), EC survival was significantly reduced over progressive times following D-glucose administration to 50 ± 4% (25 mM); 42 ± 5% (50 mM), 36 ± 3% (75 mM), and 26 ± 4% (100 mM) when compared with untreated control (91 ± 3%, $P< 0.01$).

To investigate the effects of EPO on the cell survival during HG, EPO (10 ng/ml) was applied to EC cultures 1 hour prior to the administration of D-glucose (25 mM) and cell survival was determined at 6, 24, and 48 hours following HG. The representative picture of trypan blue staining (Fig. **1B**) demonstrated that EPO 1 hour pretreatment significantly reduced the staining 48 hours following elevated D-glucose (25 mM). The quantitative results, as shown in Fig. (**1C**), indicated that administration of D-glucose (25 mM) induced progressively decrease in cell survival in ECs, which was elevated by pretreatment of EPO (10 ng/ml) 1 hour prior to D-glucose application.

In order to further investigate the efficacy of EPO during elevated D-glucose (HG) in ECs, EPO at a series of concentrations (0.01~1000 ng/ml) was applied to EC cultures 1 hour prior to the administration of D-glucose (25 mM) and cell survival was determined 48 hours later by using trypan blue exclusion method. The quantitative results indicated that EPO at the concentration from 1 ng/ml to 100 ng/ml significantly increased percent cell survival with maximal efficacy at the concentration of 10 ng/ml 48 hours following HG (Fig. **1D**).

Apoptotic DNA fragmentation and phosphatidylserine (PS) exposure were determined 48 hours following the elevated D-glucose treatment by TUNEL assay and Annexin V labeling method respectively. As shown in Fig. (**1E**), representative pictures demonstrated that elevated D-glucose treatment resulted in apoptosis with chromatin condensation, nuclear fragmentation, and PS exposure with green fluorescence staining of cell membrane in ECs. In contrast, EPO (10 ng/ml) 1 hour pretreatment reduced the staining of TUNEL and PS

residues during elevated D-glucose. In Fig. (**1F**), the quantitative results indicated that percent DNA fragmentation was significantly increased from 9 ± 2% of control cells to 57± 4%, and percent PS exposure from 7 ± 2% to 58 ± 4% 48 hours following administration of D-glucose, but EPO (10 ng/ml) pretreatment significantly reduced percent DNA fragmentation and PS exposure to 27 ± 3% and 30 ± 3% respectively.

EPO Maintains Wnt1 Expression During Elevated D-Glucose in Cerebral ECs

To investigate the effects of EPO on Wnt1 expression, Western blot for Wnt1 in ECs 6, 24, and 48 hours following administration of D-glucose (HG, 25 mM) was performed. A representative result of Western blot in Fig. (**2A**) indicated that Wnt1 expression significantly increased 6 hours after HG, but the expression of Wnt1 was diminished after 24 hours of exposure to elevated D-glucose and approached control level 48 hours following high D-glucose exposure (Fig. **2A**). Application of EPO (10 ng/ml) in EC cultures for 48 hours significantly increased the expression of Wnt1 compared with untreated control cultures (Fig. **2B**).

To further investigate the effects of EPO on the Wnt1 expression during HG, EPO (10 ng/ml) was applied to EC cultures 1 hour prior to the administration of D-glucose (25 mM) and Wnt1 expression was illustrated 6, 24, and 48 hours following HG. As shown in Fig. (**2C**), the expression of Wnt1 was significantly increased to the same level by EPO at all time points observed following HG, suggesting that EPO can prevent the degradation of Wnt1 during elevated D-glucose exposure.

Wnt1 is Necessary for Protection Against Elevated D-Glucose in Cerebral ECs

Application of recombinant human Wnt1 protein (100 ng/ml) in EC cultures 1 hour prior to administration of D-glucose at the concentration of 25 mM, significantly reduced trypan blue uptake and apoptotic chromatin condensation and nuclear fragmentation determined in ECs 48 hours after administration of D-glucose by using trypan blue exclusion method and TUNEL assay respectively (Fig. **2D**). Significant decrease in trypan blue staining from 54 ± 4% of high D-glucose (HG) treated alone to 31 ± 5% and percent DNA fragmentation from 57 ± 5% to 30 ± 3% in ECs with application of Wnt1 100 ng/ml 1 hour prior to elevated D-glucose were observed 48 hours following HG (Fig. **2E**).

Application of Wnt1 antibody (Wnt1 Ab, 1 μg/ml) alone did not significantly change cell survival and DNA integrity when compared to untreated control cultures (data not shown). During exposure to elevated glucose (D-glucose, HG, 25 mM), application of Wnt1 Ab (1 μg/ml) did not significantly change the percentage of cell survival and DNA fragmentation (Fig. **2E**). Yet, when Wnt1 Ab (1 μg/ml) was given 30 min prior to Wnt1 application (100 ng/ml) and D-glucose (25 mM) was administered 1 hour later, Wnt1 Ab could neutralize the protective capacity of Wnt1.

Inhibition of Wnt1 Attenuates the Ability of EPO to Protect ECs Against elevated D-Glucose in ECs

To investigate the requirement of Wnt1 for EPO to protect ECs during elevated D-glucose, EPO (10 ng/ml) was applied to EC cultures 1 hour prior to administration of D-glucose at the concentration of 25 mM and cell survival was determined by trypan blue exclusion method 48 hours after administration of D-glucose (HG). The results indicated that EPO significantly reduced trypan blue uptake in ECs during elevated D-glucose (Fig. **2F**). Interestingly, Wnt1 antibody (Wnt1 Ab, 1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) given 30 min prior to EPO application attenuated the efficacy of EPO resulting in a significant increase in trypan blue staining in ECs. As shown in Fig. (**2G**), the percent cell survival was significantly increased from 46 ± 4% of elevated D-glucose alone to 71 ± 4%

in ECs with EPO 1 hour pretreatment determined 48 hours following administration of D-glucose. Yet, co-application of Wnt1 Ab (1 μg/ml) or DKK-1 (0.5 μg/ml) with EPO attenuated the efficacy of EPO and reduced cell survival to 53 ± 4% and 48 ± 5% respectively. Although slightly decreased, the cell survival was not significantly altered by Wnt1 Ab (1 μg/ml) or DKK-1 (0.5 μg/ml) treated alone during elevated D-glucose in ECs (Fig. **2G**).

In addition, application of EPO (10 ng/ml) to EC cultures 1 hour prior to administration of D-glucose at the concentration of 25 mM, significantly reduced apoptotic chromatin condensation and nuclear fragmentation and PS exposure (Fig. **3A**) determined 48 hours later by TUNEL assay and Annexin V labeling methods respectively. The quantitative results demonstrated that elevated D-glucose induced significant increase in percent DNA fragmentation and percent PS exposure. EPO application significantly decreased percent DNA fragmentation from 54 ±5% in ECs exposed elevated D-glucose alone to 26± 3% and reduced percent PS exposure from 58 ± 5% to 30 ± 5% (Fig. **3B**). Yet, application of Wnt1 Ab (1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) given 30 min prior to EPO application abolished the efficacy of EPO and resulted in a significant increase in percent DNA fragmentation and percent PS exposure, the levels of which are similar to elevated D-glucose alone treated ECs (Fig. **3B**).

Gene Silencing of Wnt1 Blocks EPO Protection in ECs During Elevated D-Glucose Exposure

ECs were transfected with Wnt1 siRNA for 72 hours prior to EPO 1 hour pretreatment and D-glucose administration (25 mM), Western blot for Wnt1 was performed in EC protein extracts (50 μg/lane) obtained at 48 hours following administration of D-glucose (HG, 25 mM). As shown in Fig. (**3C**), representative result of Western blot detection for Wnt1 demonstrated that Wnt1 siRNA transfection resulted in a significantly decrease of Wnt1 expression in ECs of normal untreated control or in high D-glucose treated ECs with or without EPO 1 hour pretreatment.

After transfection with Wnt1 siRNA, ECs were exposed to elevated D-glucose by administration of D-glucose (25 mM) and EC survival was evaluated at 48 hours following high D-glucose exposure by trypan blue exclusion method. In Fig. (**3D**), Wnt1 siRNA did not significantly alter cell survival in ECs of untreated control and in ECs exposed to high D-glucose alone. EC survival was significantly increased by EPO (10 ng/ml) applied 1 hour prior to high D-glucose when compared to high D-glucose treated alone, but greatly reduced by pre-transfection with Wnt1 siRNA. As also shown in Fig. (**3D**), the efficacy of EPO on DNA fragmentation and PS exposure assessed 48 hours after D-glucose administration was also abrogated by gene silencing with Wnt1 siRNA, resulting in significant increase in percent DNA fragmentation and percent PS exposure.

EPO Controls Post-Translational Phosphorylation of FoxO3a to Protect ECs Against Elevated D-Glucose

Western blot assay was performed for phosphorylated FoxO3a (p-FoxO3a) as well as for the expression of total FoxO3a at 48 hours following administration of D-glucose (HG, 25 mM). HG significantly decreased the expression of p-FoxO3a over a 48 hour period when compared to control cultures (Fig. **4A**). In contrast, EPO (10 ng/ml) or Wnt1 (100 ng/ml) applied 1 hour prior to HG significantly increased the expression of p-FoxO3a 48 hours later. The capacity of EPO and Wnt1 to phosphorylate FoxO3a was lost in ECs with the lost of FoxO3a by using FoxO3a siRNA transfection.

During transfection of ECs with FoxO3a siRNA, EC survival was increased from 53 ± 3% of HG alone to 65 ± 4% (Fig. **4B**), demonstrating that elimination of FoxO3a can enhance EC survival during elevated D-glucose. Interestingly, EPO (10 ng/ml) or Wnt1 (100 ng/ml) administration with HG in ECs transfected with FoxO3a siRNA yield a similar survival to EPO or Wnt1 treatment with HG in wildtype cells without a synergistic effect, suggesting that EPO relies upon the prevention of FoxO3a activity to exert cytoprotection (Fig. **4B**).

EPO and Wnt1 Sequester FoxO3a in the Cytoplasm of ECs

Immunofluorescent staining for FoxO3a and DAPI nuclear staining were used to follow the subcellular translocation of FoxO3a in ECs during EPO and high D-glucose (HG) exposure (Figs. **4C, 4D**). During HG exposure alone, significant immunofluorescent staining for FoxO3a in the nucleus of ECs with minimal cytoplasmic staining is present. This is evident by the inability to detect significant DAPI nuclear staining in cells during merged HG images since prominent FoxO3a staining is present in the nucleus (Figs. **4C, 4D**). In contrast, administration of EPO (10 ng/ml) with HG exposure, FoxO3a is retained in the cytoplasm with minimal nuclear staining as shown with the notable presence of DAPI nuclear staining in merged images, illustrating that EPO retains FoxO3a in the cytoplasm at levels similar to or below control levels. However, the ability of sequester FoxO3a in the cytoplasm by EPO is lost during Wnt1 antibody (Wnt1 Ab) treatment or Wnt1 siRNA transfection (Figs. **4C, 4D**), supporting the premise that EPO requires Wnt1 to prevent FoxO3a translocation from the cytoplasm to the nucleus during HG.

The translocation of FoxO3a was also determined by Western blot of the expression of FoxO3a in the cytoplasm and the nucleus. Equal amounts of cytoplasmic (cytoplasm) or nuclear (nucleus) protein extracts (50 μg/lane) were immunoblotted with anti-total FoxO3a at 48 h following administration of elevated D-glucose (HG, 25 mM). As shown in Fig. (**4E**) and (**4F**), elevated D-glucose leads to the translocation of FoxO3a from the cytoplasm to the nucleus, EPO prevents FoxO3a translocation to the nucleus during high D-glucose exposure. But this ability of EPO is lost during Wnt1 Ab treatment or gene silencing of Wnt1.

EPO Prevents Apoptotic DNA Degradation through the Inhibition of FoxO3a Nuclear Translocation in ECs During Elevated D-Glucose

Phosphorylation of FoxO3a leads to the association of FoxO3a with 14-3-3 protein and retention of FoxO3a in the cytoplasm rendering it ineffective to initiate apoptosis [77, 79, 80, 85]. We therefore assessed whether EPO altered the nuclear translocation of FoxO3a and thereby prevents apoptosis in ECs during elevated D-glucose. To examine the relationship between FoxO3a nuclear translocation and the induction of apoptosis, we assessed co-localization of FoxO3a and TUNEL with immunofluorescent double staining. Following elevated D-glucose exposure by adding 25 mM D-glucose for 48 hours, merged images confirm nuclear staining for both FoxO3a and TUNEL, indicative of active pro-apoptotic transcriptional activity for FoxO3a leading to nuclear DNA degradation (Figs. **4G, 4H**). In contrast, EPO (10 ng/ml) administered 1 hour prior to the administration of D-glucose (25 mM) significantly reduced the number of ECs that stained positive for FoxO3a and TUNEL in the same cells (Figs. **4G, 4H**). Yet, the co-localization of FoxO3a with TUNEL in the same ECs dramatically increased similar to high D-glucose alone during application of the Wnt1 antibody (Wnt1 Ab, 1 μmol/l) or during gene silencing of Wnt1 (Figs. **4G, 4H**), indicating the necessity of Wnt1 to block the pro-apoptotic properties of FoxO3a.

EPO Uses Akt1 to Phosphorylate Glycogen Synthase Kinase-3β (GSK-3β) and Provide EC Protection During Elevated D-Glucose

We show that EPO modulates the activity of GSK-3β through phosphorylation. This occurs at the conserved regulatory residue of Ser^9 (86, 87]. Elevated D-glucose also initially

increased the expression of p-GSK-3β at 6 and 24 hours when compared to EC control cultures. After 24 hours post elevated D-glucose exposure, expression of p-GSK-3β was lost suggesting that GSK-3β activity was no longer inhibited (Fig. **5A**). Yet, application of EPO (10 ng/ml) or Wnt1 (100 ng/ml) 1 hour prior to elevated D-glucose exposure was able to maintain the inhibition of GSK-3β and significantly promote the expression of p-GSK-3β over a 48 hour course (Fig. **5A**).

To further investigate the potential mechanism of EPO to maintain phosphorylation of GSK-3β, Western blot for active Akt1, p-Akt1, was performed as active Akt1 targets ser 9 of GSK-3β for phosphorylation. The result indicated that the expression of p-Akt1 was mildly increased at 48 hours following administration of D-glucose (Fig. **5B**). Pretreatment with EPO (10 ng/ml) or Wnt1 (100 ng/ml) 1 hour prior to elevated D-glucose exposure significantly increased the expression of p-Akt1. Yet, application of Wnt1 Ab (1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) given 30 min prior to EPO application abolished the efficacy of EPO, resulting in the decrease in the expression of p-Akt1 (Fig. **5B**).

Application of the GSK-3β inhibitor (SB216763, SB21, 5 μM) 1 hour prior to elevated D-glucose exposure (25 mM) decreased trypan blue staining and DNA fragmentation 48 hours following exposure to elevated D-glucose (Fig. **5C**). Application of EPO (10 ng/ml) 1 hour prior to elevated D-glucose exposure (25 mM) resulted in the significant decrease in trypan blue staining and DNA fragmentation in ECs. Interestingly, no synergic effects on trypan blue staining and DNA fragmentation was observed when GSK-3β inhibitor (SB21, 5 μM) was co-applied with EPO (10 ng/ml) during elevated D-glucose, supporting the premise that EPO and Wnt1 utilizes inhibition of GSK-3β activity to prevent EC injury and apoptotic demise (Fig. **5C**). Furthermore, the changes in EC Trypan blue staining and DNA fragmentation during inhibition of GSK-3β activity were not altered by co-application of the Wnt1 or Wnt1 Ab, indicating that modulation of the GSK-3β activity to preserve EC survival occurs downstream from the initial activation of Wnt1 (Fig. **5D** and **5E**).

EPO Relies Upon Wnt1 to Maintain β-Catenin Integrity and Translocation During Elevated D-Glucose

Western blot for phospho-β-catenin (inactive form) was performed in ECs during elevated D-glucose and EPO application, since β-catenin has been closely associated with Wnt1 signaling pathways [24]. EC protein extracts (50 μg/lane) were immunoblotted with anti-phosphorylated β-catenin (p-β-catenin) antibody. In Fig. (**6A**), representative images of Western blot for p-β-catenin performed at 6, 24, and 48 hour time intervals following administration of elevated D-glucose (25 mM) (HG) were illustrated. The expression of p-β-catenin expression was not significantly increased until 48 hours following exposure to elevated D-glucose. Application of EPO (10 ng/ml) or Wnt1 (100 ng/ml) 1 hour prior to the administration of elevated D-glucose significantly decreased p-β-catenin expression 48 hours following elevated D-glucose. But the expression of p-β-catenin was significantly increased in ECs pretreated with Wnt1 antibody (Wnt1 Ab) or transfected with Wnt1 siRNA for 3 days prior to EPO or Wnt1 during D-glucose application, suggesting that EPO modulates the p-β-catenin expression during elevated D-glucose through Wnt1 associated pathways.

Un-phosphorylated β-catenin can translocate to the nucleus and promote anti-apoptotic gene transcriptions. We investigated the effects of EPO on the translocation of β-catenin in ECs during elevated D-glucose. EPO (10 ng/ml) was applied to EC cultures 1 hour prior to the administration of D-glucose (25 mM) and immunofluorescent staining for β-catenin (Texas-red) was performed at 48 hours later. Nuclei of ECs were counterstained with DAPI. As shown in Fig. (**6C**) and (**6D**), in merged images, cells with HG alone show EC nuclei with minimal β-catenin staining (blue/white) and EC cytoplasm with significant β-catenin

staining (red) in contrast to cells with combined EPO with HG, demonstrating significant increase in nuclear staining of β-catenin. Wnt1 Ab or Wnt1 siRNA treatment with combined EPO and HG resulted in the lost of nuclear staining of β-catenin. The similar pattern of β-catenin translocation was also demonstrated by Western blot for β-catenin expression in the cytoplasm and the nucleus (Fig. **6E**).

To further demonstrate the effects of active β-catenin on cell survival in ECs during elevated D-glucose, β-catenin siRNA was transfected into ECs prior to EPO (10 ng/ml), Wnt1 (100 ng/ml), and D-glucose (25 mM). Gene silencing of β-catenin abrogated the expression of β-catenin and significantly reduced the expression of p-β-catenin during elevated D-glucose in ECs (Fig. **7A** and **7B**). In addition, β-catenin siRNA transfection significantly attenuated the efficacy of EPO or Wnt1 to protect ECs against elevated D-glucose (Fig. **7C**), suggesting that EPO protects ECs against elevated D-glucose, at least in part, through modulating the function of β-catenin.

EPO Prevents Mitochondrial Permeability, Cytochrome c Release, and Caspase 3 Activation through Wnt1 and FoxO3a During Elevated D-Glucose

Exposure to elevated glucose (D-glucose, 25 mM, HG) produced a significant decrease in the red/green fluorescence intensity ratio using a cationic membrane potential indicator JC-1 within 24 hours when compared with untreated control cultures (Figs. **8A** and **8B**) with increased release of cytochrome c (Fig. **8C** and **8D**) and subsequent caspase 3 activation (Fig. **8E** and **8F**), suggesting that HG results in mitochondrial membrane depolarization, cytochrome c release, and caspase 3 activation. Application of EPO (10 ng/ml) 1 hour prior to HG exposure significantly increased the red/green fluorescence intensity of the ECs, indicating that mitochondrial permeability transition pore (MPTP) membrane potential was restored to baseline (Figs. **8A** and **8B**). Administration of EPO maintained MPTP function and prevented mitochondrial cytochrome c release and caspase 3 activation as demonstrated by Western analysis (Fig. **8E** and **8F**).

Interestingly, treatment with FoxO3a siRNA prior to the administration of D-glucose also prevents mitochondrial depolarization, cytochrome c release, and caspase 3 activation, but did not change the efficacy of EPO during elevated D-glucose. Yet, treatment with Wnt1 siRNA prior to HG decreases the efficacy of EPO, resulting in an increased mitochondrial depolarization, cytochrome c release, and caspase 3 activation, the level of which is compatible to elevated D-glucose treated ECs alone (Fig. **8A-F**).

DISCUSSION

We demonstrate that primarily cultured cerebral ECs are extremely sensitive to elevations in D-glucose that are similar to clinical D-glucose concentrations known to occur in a range from 15 mM - 25 mM (270 mg/dl - 450 mg/dl) [6, 7, 88, 89]. Elevated D-glucose by adding D-glucose in EC cultures at the final concentration of 25 mM leads to a significant loss in cell survival and correspondingly a significant increase in genomic DNA degradation and externalization of membrane PS residues when compared to control ECs over a 48 hour course. Administration of EPO (10 ng/ml) with a 1 hour pre-treatment maintains the integrity of cell membrane and enhances EC survival during elevated D-glucose. The maximal effective concentration of EPO for cytoprotection both in ECs and neurons has been demonstrated in our previous studies to be 10 ng/ml [66, 67, 77, 90, 91], by which also achieved maximal protection in ECs during elevated D-glucose. The concentration is similar to serum levels of EPO in patients with cardiac or renal disease that have been associated with potential EPO cellular protection [92, 93]. Clinical protocols with EPO administration have been shown to significantly increase plasma EPO levels well above the 1.0 ng/ml range similar to *in vitro* work and confer beneficial results [94, 95].

EPO blocks apoptotic DNA degradation and PS exposure in ECs during elevated D-glucose similar to other models of oxidative stress in cardiac and vascular cell models [54, 55, 64, 77]. DNA fragmentation and PS exposure can independently lead to cellular injury [14, 73, 96] and activate inflammatory cells to remove ECs tagged with PS [81, 97-99]. This protection by EPO against apoptotic injury is directly linked to Wnt1. We demonstrate that endogenous Wnt1 alone may not be sufficient for protection against elevated D-glucose exposure, since application of Wnt1 Ab or gene silencing of Wnt1 did not significantly alter injury in ECs. However, application of exogenous Wnt1 protein significantly increased EC survival and prevented apoptotic EC degeneration during elevated D-glucose exposure. More importantly, administration of the Wnt1 Ab could neutralize the protective capacity of Wnt1, suggesting that Wnt1 is an important component in the cytoprotection of ECs during elevated D-glucose exposure. In addition, EPO protection in ECs during elevated D-glucose exposure relies upon Wnt1 as demonstrated by several observations. First, EPO administration maintains the expression of Wnt1 over a 48 hour course during elevated D-glucose exposure and prevents the loss of Wnt1 expression that would occur in the absence of EPO during elevated D-glucose. Second, administration of the Wnt1 Ab or Wnt antagonist DKK-1 results in the loss of EC protection conferred by EPO, leading to a decrease in cell survival and an increase in apoptotic DNA fragmentation and an increase in PS exposure. Third, gene silencing of Wnt1 with Wnt1 siRNA also can abolish the protective effects of EPO during elevated D-glucose. Our results suggest that Wnt1 is a necessary component for EPO to protect against EC injury and apoptosis during elevated D-glucose.

The forkhead transcription factor FoxO3a also plays an important role in the protective capacity of EPO during elevated D-glucose. The FoxO subclass can modulate myogenesis [100], foster high D-glucose mediated neuronal injury [101], promote anti-tumor activity [85, 102, 103], regulate cellular metabolism [104-107], and mediate early and late stages of apoptotic injury [35, 79, 80, 108-111]. In our current work, we demonstrate that EPO can phosphorylate FoxO3a during elevated D-glucose and prevent its translocation to the nucleus in ECs. Modulation of FoxO3a intracellular trafficking to retain FoxO3a in the cytoplasm blocks apoptotic death in ECs, since nuclear translocation of FoxO3a during elevated D-glucose directly correlates with apoptotic nuclear DNA degradation. Gene silencing of FoxO3a alone or the application of EPO with FoxO3a siRNA leads to similar survival levels without a synergistic increase, suggesting that EPO relies upon the prevention of FoxO3a activity to promote protection in ECs during elevated D-glucose. Interestingly, the ability of EPO to retain FoxO3a in the cytoplasm is lost when Wnt1 antibody or Wnt1 siRNA is co-applied to EC cultures, suggesting that EPO functions through Wnt1 associated pathways to modulate FoxO3a intercellular trafficking.

Downstream in the Wnt1 signaling pathway, GSK-3β activity has been shown to be blocked by the application of EPO to maintain cell integrity during oxidative stress [91]. Inhibition of GSK-3β activity also has been associated with improved beta cell survival [45] and with cardioprotection [46] during DM. As a result of this prior work, we examined whether GSK-3β plays a role in EC injury and EPO protection during elevated D-glucose exposure. We show that through an Akt1 dependent mechanism GSK-3β becomes initially phosphorylated over a 24 hour course but subsequent phosphorylation of GSK-3β declines by 48 hours during elevated D-glucose exposure. In contrast, EPO application in the presence of elevated D-glucose significantly maintains the inhibitory phosphorylation of GSK-3β over a 48 hour period during elevated D-glucose exposure. This inhibition of GSK-3β activity is closely linked to EC survival, since inhibition of GSK-3β activity during administration of SB216763 prevents EC injury and apoptotic cell loss during elevated D-glucose. Moreover, increased EC survival during inhibition of GSK-3β activity was not altered by the co-application of Wnt1 Ab, suggesting that prevention of GSK-3β activity by

EPO to preserve EC survival occurs downstream from the initial activation of Wnt1. Finally, inhibition of GSK-3β activity during co-administration of EPO results in similar survival and DNA fragmentation levels without a synergistic change, also indicating that EPO relies upon blockade of GSK-3β activity to offer cytoprotection in ECs during elevated D-glucose exposure. It is important to note that EPO and Wnt1 employ Akt1 to inactivate and phosphorylate GSK-3β, since inhibition of Akt1 activity attenuates the ability of EPO or Wnt1 to phosphorylate GSK-3β.

Our studies with β-catenin contrasted with those of GSK-3β. Within the initial 24 hours following exposure to elevated D-glucose, the expression of phosphorylated β-catenin was not altered significantly from control. However, by 48 hours after elevated D-glucose exposure, the expression of phosphorylated β-catenin was significantly increased. These observations coincide with the loss of Wnt1 expression that would be expected to lead to a decrease in the phosphorylation state of GSK-3β, resulting in the activation of GSK-3β and the subsequent phosphorylation of β-catenin. In the presence of EPO, EPO during elevated D-glucose exposure prevents the phosphorylation of β-catenin and ultimately would prevent degradation of β-catenin. This ability of EPO to control the phosphorylation of β-catenin is dependent upon the presence of Wnt1 since application of Wnt1 Ab or gene silencing of Wnt1 with siRNA leads to the ability of EPO to prevent the phosphorylation of β-catenin during elevated D-glucose. In addition, EPO is able to translocate β-catenin from the EC cytoplasm to the nucleus to allow for anti-apoptotic transcription activity. Yet, with the loss of Wnt1 through application of the Wnt1 Ab or gene silencing of Wnt1, EPO cannot foster translocation of β-catenin from the cytoplasm to the cell nucleus and is therefore dependent upon Wnt1 for trafficking of β-catenin. Finally, β-catenin also is necessary for EPO and Wnt1 protection during elevated D-glucose exposure since gene silencing of β-catenin blocks cytoprotection of EPO and Wnt1.

During elevated D-glucose exposure, mitochondrial membrane permeability directly affects cellular injury [36, 112-114]. In our prior studies [79, 80] as well as in our current work, we show that elevated D-glucose in ECs leads to mitochondrial membrane depolarization, the release of cytochrome c, and caspase 3 activation. EPO during elevated D-glucose exposure prevents mitochondrial membrane depolarization, the release of cytochrome c, and the activa-tion of caspase 3. Gene silencing of FoxO3a in the presence of EPO during elevated D-glucose exposure also blocks mitochondrial membrane depolarization, the release of cytochrome c, and the activation of caspase 3 but did not synergistically improve survival, suggesting that EPO may rely upon FoxO3a to prevent the loss of mitochondrial membrane permeability and subsequent caspase 3 activation. In addition, loss of Wnt1 abrogates the ability of EPO to modulate mitochondrial membrane permeability during elevated D-glucose exposure, also suggesting that Wnt1 may be required for EPO to prevent mitochondrial depolarization.

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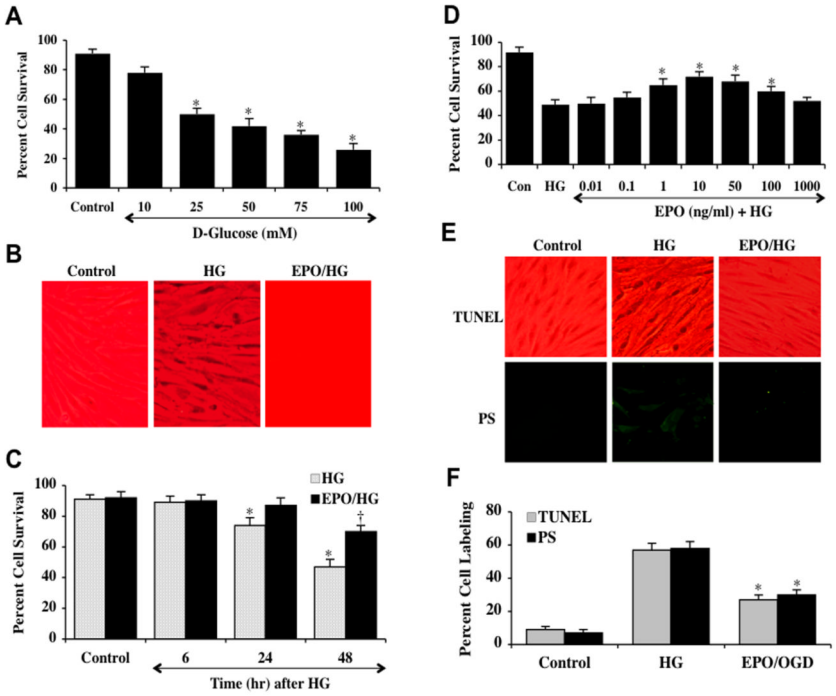

Fig. (1). Erythropoietin (EPO) provides protection against elevated D-glucose exposure in ECs

(A) D-glucose was applied to primary cultures of microvascular ECs at the concentration of 10, 25, 50, 75, and 100 mM, cell survival was determined 48 hours following the application of D-glucose by using trypan blue exclusion method. Cell survival was progressively decreased after application of D-glucose with increasing concentrations (**P*< 0.01 vs. Control. **(B)** Representative pictures demonstrated the staining of the dye of trypan blue in ECs 48 hours following D-glucose application (25 mM). In contrast, EPO) 10 ng/ml) applied 1 hour prior to administration of elevated D-glucose (HG) reduced trypan blue staining. **(C)** The cell survival, determined by trypan blue exclusion method, was progressively decreased following administration of D-glucose (25 mM) over a 48 hour period. In contrast, EPO (10 ng/ml) application 1 hour prior to D-glucose administration significantly increased EC survival 48 hours following application of D-glucose (**P*<0.01 *vs*. Control; †*P*<0.01 vs. HG at 48 hours). Each data point represents the mean and SEM. **(D)** ECs were pretreated with EPO (0.01 to 1000 ng/ml) 1 hour prior to exposure to D-glucose (25 mM) and cell survival was assessed 48 hours later. Protection of EPO against elevated D-glucose was evident in cultures with 1 to 100 ng/ml EPO when compared with cultures exposed to elevated D-glucose alone (**P*<0.01 *vs*. HG treated alone). **(E)** D-glucose was applied to primary cultures of microvescular ECs at the concentration of 25 mM, DNA fragmentation and membrane phosphatidylserine (PS) exposure were determined 48 hours following the application of D-glucose by using TUNEL assay and Annexin V phycoerythrin labeling method respectively. Increased TUNEL positive cell, and annex V staining with illustration of green fluorescence in the cultures with exposure to HG, which was reduced by EPO pretreatment (10 ng/ml). **(F)** The quantitative results indicated that the percent DNA fragmentation and PS exposure were significantly increased 48 hours following administration of D-glucose (25 mM). EPO (10 ng/ml) applied 1 hour prior to high D-glucose significantly decreased apoptotic DNA fragmentation and PS exposure (**P*< 0.01 *vs*. HG). In A, C, D, and F, each data point represents the mean and SEM. In all cases, control = untreated ECs.

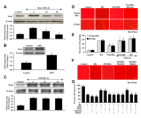

Fig. (2).

Erythropoietin (EPO) modulates the expression of Wnt1 and provides cellular protection in cerebral microvascular ECs through modulation of Wnt1 during elevated D-glucose exposure. (**A**) EC protein extracts (50 μg/lane) obtained at 6, 24, and 48 hours following administration of D-glucose (25 mM) were immunoblotted with anti-Wnt1 antibody. Representative result of Western blot for Wnt1 was illustrated. Wnt1 expression increased at 6 and 24 hours following exposure to elevated D-glucose, but the expression of Wnt1 was lost at 48 hours following high D-glucose treatment (**P*<0.01 *vs*. HG at 6 or 24 hour). (**B**) EPO (10 ng/ml) applied to EC cultures and Western blot for Wnt1 was determined 48 hours later. The expression of Wnt1 was significantly increased after incubation with EPO for 48 hours (**P*< 0.01 *vs*. control). (**C**) Application of EPO (10 ng/ml) 1 hour prior to the administration of D-glucose (25 mM) significantly increased Wnt1 expression 6, 24, 48 hours following high D-glucose treatment (**P*<0.01 *vs*. Control). Control =untreated cultures. (**D**) Recombinant human Wnt1 protein (100 ng/ml) was applied to EC cultures 1 hour prior to administration of D-glucose at the concentration of 25 mM, cell survival and DNA fragmentation were determined 48 hours later by using trypan blue exclusion method and TUNEL assay respectively. The representative pictures indicated that application of Wnt1 reduced the trypan blue staining, chromatin condensation, and nuclear fragmentation during high D-glucose treatment. In contrast, application of Wnt1 antibody (Wnt1Ab, 1 μg/ml) 30 min prior to the administration of Wnt1 protein antagonized the Wnt1 resulting in an increase in trypan blue staining and DNA fragmentation in ECs during high D-glucose treatment. (**E**) A significant decrease in percent trypan blue staining and DNA fragmentation was observed in cultures with Wnt1 (100 ng/ml) 1 hour pretreatment when compared with cultures exposed to elevated D-glucose alone. In contrast, application of Wnt1 antibody (Wnt1Ab, 1 μg/ml) 30 min prior to the administration of Wnt1 protein antagonized the Wnt1 resulting in increase in percent trypan blue staining and DNA fragmentation in ECs during elevated D-glucose treatment (**P*<0.01 *vs*. HG; †*P*<0.01 *vs*. Wnt1/HG). (**F**) EPO (10 ng/ml) was applied to EC cultures 1 hour prior to administration of D-glucose at the concentration of 25 mM, cell survival was determined 48 hours later by using trypan blue exclusion method. The representative pictures indicated that application of EPO reduced the trypan blue staining during high D-glucose treatment. Yet, application of Wnt1 antibody (Wnt1Ab, 1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) 30 min prior to the administration of EPO antagonized the EPO resulting in an increase in trypan blue staining in ECs during high D-glucose treatment. (**G**) A significant increase in percent cell survival was observed in cultures with EPO (10 ng/ml) when compared with cultures exposed to elevated D-glucose alone (**P*< 0.01 *vs*. HG). In contrast, application of Wnt1 antibody (Wnt1Ab, 1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) 30 min prior to the administration of EPO antagonized the EPO resulting in a decrease in percent cell survival of ECs during elevated D-glucose treatment (†*P*<0.01 *vs*. EPO/HG). In all cases, each data point represents the mean and SEM. Control =untreated cultures.

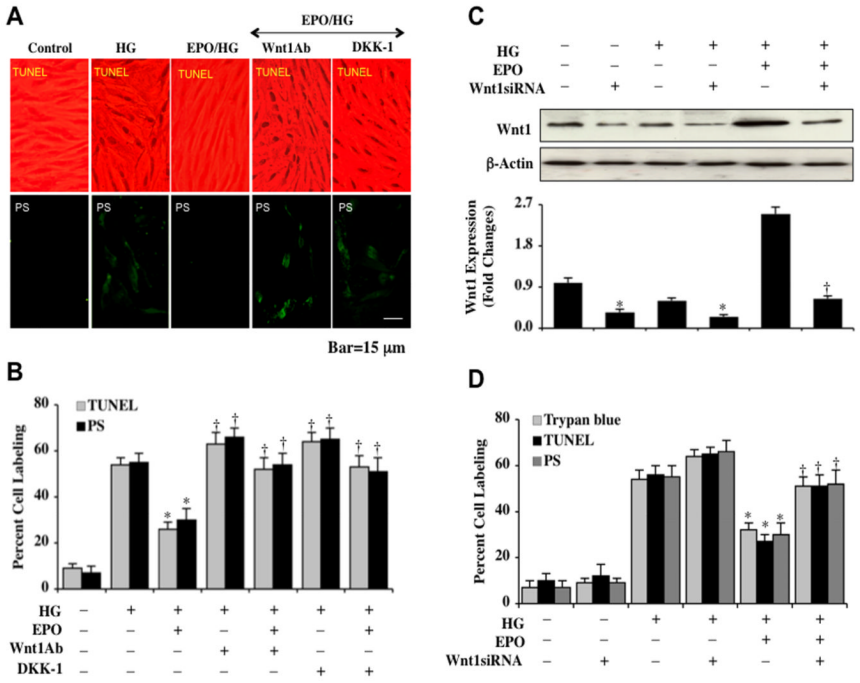

Fig. (3).

Erythropoietin (EPO) requires Wnt1 to prevent apoptotic DNA fragmentation and phosphatidylserine (PS) exposure in cerebral microvascular ECs during elevated D-glucose exposure. (**A**) EPO (10 ng/ml) was applied to EC cultures 1 hour prior to administration of D-glucose at the concentration of 25 mM, DNA fragmentation and PS exposure were determined 48 hours later by TUNEL and Annexin V phycoerythrin labeling methods respectively. Representative pictures indicated that DNA fragmentation was present in high D-glucose treated ECs but greatly reduced in EPO treated ECs (EPO/HG) 48 hours following high D-glucose treatment. Yet, application of Wnt1 antibody (Wnt1Ab, 1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) 30 min prior to the administration of EPO protein antagonized the function of EPO resulting in a increase in cell staining for TUNEL and PS residues during high D-glucose treatment. (**B**) A significant decrease in percent DNA fragmentation and PS exposure was observed in cultures with EPO (10 ng/ml) treatment when compared with cultures exposed to elevated D-glucose alone (**P*<0.01 *vs*. HG). In contrast, application of Wnt1 antibody (Wnt1Ab, 1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) 30 min prior to the administration of EPO resulting in an increase in percent DNA fragmentation and PS exposure of ECs during high D-glucose treatment (†*P*<0.01 *vs*. EPO/HG). (**C**) Gene silencing of Wnt1 was performed prior to EPO pretreatment (10 ng/ml) and D-glucose (25 mM) administration in the cultures of ECs by transfection with Wnt1 siRNA for 3 days and Western blot for Wnt1 was performed 48 hours following elevated D-glucose treatment. Representative result of Western blot for Wnt1 indicated Wnt1 expression was significantly decreased in ECs with or without EPO and high D-glucose treatment (**P*< 0.01 *vs*. Control; †*P*<0.01 *vs*. EPO/HG. (**D**) A significant decrease in percent trypan blue staining, DNA fragmentation (TUNEL), and percent PS exposure was observed in cultures with EPO (10 ng/ml) 1 hour pretreatment when compared with cultures exposed to elevated D-glucose alone (**P*<0.01 *vs*. HG). In contrast, transfection of ECs with Wnt1 siRNA attenuated the efficacy of EPO resulting in an increase in percent staining of Trypan blue, TUNEL, and PS exposure during elevated D-glucose treatment (**P*<0.01 *vs*. HG alone; †*P*<0.01 *vs*. EPO/HG). Each data point represents the mean and SEM. Control =untreated cultures.

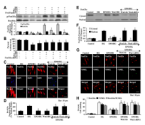

Fig. (4).

Erythropoietin (EPO) and Wnt1 modulates phosphorylation of FoxO3a and intracellular trafficking of FoxO3a through Wnt1 to block nuclear DNA degradation during elevated D-glucose. (**A**) EPO (10 ng/ml) or Wnt1 (100 ng/ml) was applied to EC cultures 1 hour prior to D-glucose (25 mM) administration, EC protein extracts (50 μg/lane) were immunoblotted with anti-phosphorylated-FoxO3a (p-FoxO3a, Ser^{253}) and FoxO3a at 48 hours following high D-glucose (HG) treatment. Elevated D-glucose induced down regulation of p-FoxO3a expression, but EPO or Wnt1 induced a significant increase in the expression of p-FoxO3a 48 hours following high D-glucose treatment. Transfection with FoxO3a siRNA significantly reduced expression of p-FoxO3a and FoxO3a in ECs of treatment either with HG, EPO/HG, or Wnt1/HG (**P*<0.01 *vs*. untreated control; †*P*<0.01 *vs*. EPO/HG or Wnt1/HG). (**B**) Gene silencing with FoxO3a siRNA in normal ECs did not change cell survival, but transfection with FoxO3a siRNA significantly increased EC survival 48 hours following elevated D-glucose. Yet, FoxO3a transfection did not change the cell survival significantly in ECs with EPO (10 ng/ml) or Wnt1 (100 ng/ml) treatment during elevated D-glucose (**P*<0.01 *vs*. untreated control; †*P*<0.01 *vs*. HG alone). (**C**) EPO (10 ng/ml) combined with HG was followed at 48 hours with immunofluorescent staining for FoxO3a (Texas-red). Nuclei of ECs were counterstained with DAPI. In merged images, cells with EPO combined with HG show EC nuclei with minimal FoxO3a staining (blue/white) and EC cytoplasm with significant FoxO3a staining (red) in contrast to cells with HG alone, Wnt1 antibody (Wnt1Ab), or Wnt1siRNA transfection combined with EPO and HG with minimal FoxO3a staining, demonstrating the inability of EPO to sequester FoxO3a in the cytoplasm. (**D**) EPO prevents FoxO3a translocation to the nucleus during high D-glucose (HG), but this ability of EPO is lost during Wnt1Ab treatment or gene silencing of Wnt1 (**P*<0.01 *vs*. HG alone; †*P*<0.01 *vs*. EPO/HG). Intensity of FoxO3a nuclear staining was performed using the public domain NIH Image program and control = untreated ECs. (**E**) Equal amounts of cytoplasmic (cytoplasm) or nuclear (nucleus) protein extracts (50 μg/lane) were immunoblotted with anti-total FoxO3a at 48 h following administration of elevated D-glucose (HG = high D-glucose, 25 mM). Elevated D-glucose leads to the translocation of FoxO3a from the cytoplasm to the nucleus, EPO prevents FoxO3a translocation to the nucleus during high D-glucose (HG) exposure. But this ability of EPO is lost during Wnt1Ab treatment or gene silencing of Wnt1. (**F**) Quantification of the Western band intensity was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image) and demonstrates that significant expression of FoxO3a translocates to the cell nucleus 48 h following elevated D-glucose (HG = high D-glucose, 25 mM), but EPO prevents the translocation of FoxO3a. Wnt1 antibody (Wnt1Ab) or transfection with Wnt1siRNA abolishes the ability of EPO resulting in the translocation of FoxO3a to the EC nucleus during HG (**P* < 0.01 *vs*. HG; †*P* < 0.01 *vs*. EPO/HG,). Each data point represents the mean and SEM from 3 experiments. (**G**) Immunofluorescent double staining for FoxO3a and TUNEL in ECs was performed at 48 hours after administration of D-glucose (25 mM). EPO (10 ng/ml) application 1 hour prior to elevated D-glucose prevents nuclear DNA degradation and FoxO3a nuclear translocation in the same ECs with no overlap of staining in merged images. In contrast, the merged images show both nuclear FoxO3a and TUNEL staining (yellow) in ECs with high D-glucose alone, with combined EPO/HG and Wnt1 Ab (1 μg/ml), or with combined EPO/HG and Wnt1 siRNA gene silencing, illustrating that EPO requires Wint1 to prevent FoxO3a nuclear translocation that leads to apoptotic DNA degradation. (F) EPO (10 ng/ml) during elevated D-glucose significantly prevents FoxO3a and TUNEL nuclear staining in the same ECs, but this ability

of EPO is lost during combined EPO/HG with Win1Ab (1 μg/ml), or with combined EPO/HG and Wnt1 siRNA gene silencing (*P<0.01 *vs*. HG; †P<0.01 *vs*. EPO/HG).

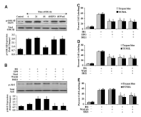

Fig. (5).

Erythropoietin (EPO) protects ECs by inhibiting the activity of glycogen synthase kinase-3β (GSK-3β) through Akt1 during high D-glucose treatment. (**A**) EC protein extracts (50 μg/lane) were immunoblotted with anti-phosphorylated GSK-3β (anti-p-GSK-3β) antibody. Representative images of Western blot for p-GSK-3β performed at 6, 24, and 48 hour time intervals following administration of elevated D-glucose (25 mM) (HG) were illustrated. The expression of p-GSK-3β was increased at 6 and 24 hours following exposure to high D-glucose, but the expression of p-GSK-3β was lost 48 hours following high D-glucose treatment. In contrast, application of EPO (10 ng/ml) or Wnt1 (100 ng/ml) 1 hour prior to HG increased the expression of p-GSK-3β 48 hours following HG (**P* < 0.01 *vs*. 6 hours or 24 hours of HG; $^{\dagger}P$ < 0.01 *vs*. HG 48 hours). (**B**) EC protein extracts (50 μg/lane) were immunoblotted with anti-phosphorylated Akt1 (anti-p-Akt1) antibody. Representative images of Western blot for p-Akt1 performed at 48 hours following administration of elevated D-glucose (25 mM) (HG) were illustrated. The expression of p-Akt1 was mildly increased at 48 hours following exposure to high D-glucose. But, application of EPO (10 ng/ml) or Wnt1 (100 ng/ml) 1 hour prior to HG significantly increased the expression of p-Akt1 48 hours following HG (**P* < 0.01 *vs*. Control). In contrast, application of Wnt1 antibody (Wnt1Ab, 1 μg/ml) or Wnt antagonist DKK-1 (0.5 μg/ml) 30 min prior to the administration of EPO antagonized the EPO resulting in a decrease in p-Akt1 expression during elevated D-glucose treatment (†*P*<0.01 *vs*. EPO/HG). (**C**) EPO (10 ng/ml) and GSK-3β inhibitor SB21 (5 μM) were applied to EC cultures 1 hour prior to the administration of D-glucose (25 mM) (HG), cell survival and DNA fragmentation were determined by Trypan blue and TUNEL labeling respectively 48 hours following HG. EPO and SB21 pretreatment significantly decreased Trypan blue staining and apoptotic DNA fragmentation of ECs 48 hours following elevated D-glucose. EPO (10 ng/ml) combined with SB21 (5 μM) application resulted in a similar percent trypan blue staining and DNA fragmentation to ECs with EPO or SB21 treated alone following HG (**P*< 0.01 *vs*. HG). (**D**) Wnt1 (100 ng/ml) and GSK-3β inhibitor SB21 (5 μM) were applied to EC cultures 1 hour prior to the administration of D-glucose (25 mM) (HG), cell survival and DNA fragmentation were determined by Trypan blue and TUNEL labeling respectively 48 hours following HG. Wnt1 and SB21 pretreatment significantly decreased Trypan blue staining and apoptotic DNA fragmentation of ECs 48 hours following elevated D-glucose. Wnt1 (100 ng/ml) combined with SB21 (5 μM) application resulted in a similar percent trypan blue staining and DNA fragmentation to ECs with Wnt1 or SB21 treated alone following HG (**P*< 0.01 *vs*. HG). (**E**) Wnt1Ab (1 μg/ml) and GSK-3β inhibitor SB21 (5 μM) were applied to EC cultures 1 hour prior to the administration of D-glucose (25 mM) (HG), cell survival and DNA fragmentation were determined by Trypan blue and TUNEL labeling respectively 48 hours following HG. SB21 application significantly decreased trypan blue staining and apoptotic DNA fragmentation of ECs 48 hours following elevated D-glucose. Co-application of Wnt1Ab with SB21 did not change percent trypan blue staining and apoptotic DNA fragmentation of ECs 48 hours following elevated D-glucose compared with group of SB21/HG (**P* < 0.01 *vs*. HG).

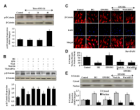

Fig. (6).

Erythropoietin (EPO) promotes the activity of β-catenin during elevated D-glucose through modulation of Wnt1 in cerebral microvascular ECs. (**A**) EC protein extracts (50 μg/lane) were immunoblotted with anti-phosphorylated β-catenin (p-β-catenin) and total β-catenin antibodies. Representative images of Western blot for p-β-catenin performed at 6, 24, and 48 hours following administration of elevated D-glucose (25 mM) (HG) were illustrated. The expression of p-β-catenin was increased at 48 hours following exposure to elevated D-glucose (**P*< 0.01 *vs*. untreated control). (**B**) Application of EPO (10 ng/ml) or Wnt1 (100 ng/ml) 1 hour prior to the administration of D-glucose (25 mM) significantly decreased p-β-catenin expression 48 hours following elevated D-glucose treatment. The expression of p-β-catenin was significantly increased in ECs pretreated with Wnt1 antibody (Wnt1Ab) or transfected with Wnt1siRNA for 3 d prior to EPO or Wnt1 combined with D-glucose application (**P*<0.01 *vs*. untreated control; †*P*< 0.01 *vs*. HG treated alone). (**C**) Administration of EPO (10 ng/ml) combined with D-glucose (HG, 25 mM) was followed at 48 hours by immunofluorescent staining for β-catenin (Texas-red). Nuclei of ECs were counterstained with DAPI. In merged images, cells with HG alone show EC nuclei with minimal β-catenin staining (blue/white) and EC cytoplasm with significant β-catenin staining (red) in contrast to cells with combined EPO with HG, demonstrating significant increase in nuclear staining of β-catenin. Wnt1Ab or Wnt1siRNA treatment with combined EPO and HG resulted in the lost of nuclear staining of β-catenin. (**D**) EPO prevents HG induced β-catenin translocation to the cytoplasm during HG, but this ability of EPO is lost during Wnt1Ab treatment or gene silencing of Wnt1 (**P*<0.01 *vs*. Control; †*P*< 0.01 *vs*. HG). Intensity of β-catenin nuclear staining was performed using the public domain NIH Image program and control = untreated ECs. (**E**) Equal amounts of cytoplasmic (cytoplasm) or nuclear (nucleus) protein extracts (50 μg/lane) were immunoblotted with anti-total β-catenin at 48 h following administration of elevated D-glucose (HG = high D-glucose, 25 mM). EPO (10 ng/ml) 1 h pretreatment prevents HG induced β-catenin translocation to the cytoplasm during HG, but this ability of EPO is lost during Wnt1Ab treatment or gene silencing of Wnt1 (**P*<0.01 *vs*. Control; †*P*< 0.01 *vs*. HG). Density of β-catenin was determined using the public domain NIH Image program and control = untreated ECs.

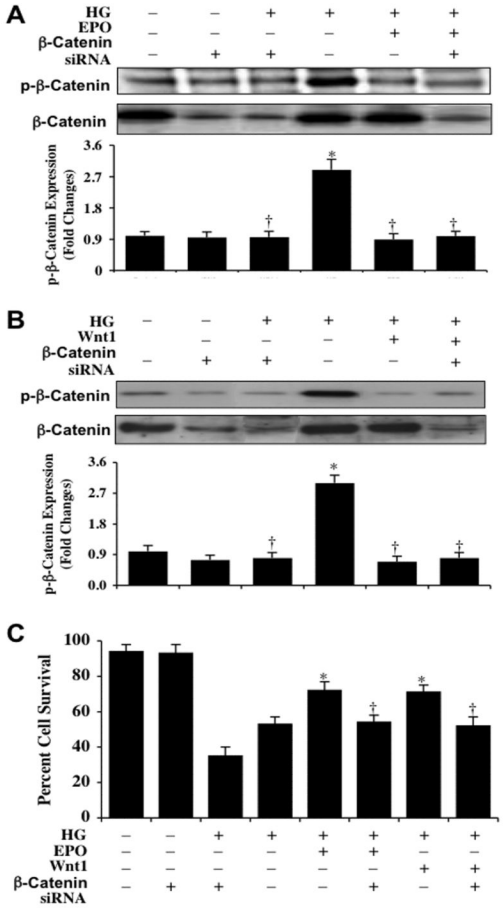

Fig. (7).

Gene silencing of β-catenin attenuates protective effects of erythropoietin (EPO) during elevated D-glucose in ECs. (**A**) ECs were transfected with β-catenin siRNA for 72 hours prior to EPO (10 ng/ml) 1 hour pretreatment and D-glucose administration (HG, 25 mM). EC protein extracts (50 μg/lane) were immunoblotted with anti-phosphorylated β-catenin (p-β-catenin) and antiβ-catenin antibodies. Representative images of Western blot performed at 48 hours following HG were illustrated. The expression of p-β-catenin was increased at 48 hours following exposure to elevated D-glucose. Application of EPO (10 ng/ml) 1 hour or β-catenin siRNA transfection prior to the administration of elevated D-glucose significantly decreased p-β-catenin expression 48 hours following elevated D-glucose treatment (**P*< 0.01 *vs*. untreated control; †*P*< 0.01 *vs*. HG treated alone). (**B**) The expression of p-β-catenin was significantly decreased in ECs transfected with β-catenin siRNA for 3 d or ECs pretreated with Wnt1 (100 ng/ml) 1 hour prior to D-glucose application (**P*<0.01 *vs*. untreated control; †*P*< 0.01 *vs*. HG treated alone). (**C**) Gene silencing with β-catenin siRNA significantly attenuates the ability of EPO (10 ng/ml) or Wnt1 (100 ng/ml) to increase cell survival during elevated glucose (**P*<0.01 *vs*. HG; †*P*<0.01 *vs*. EPO/ or Wnt1/HG).

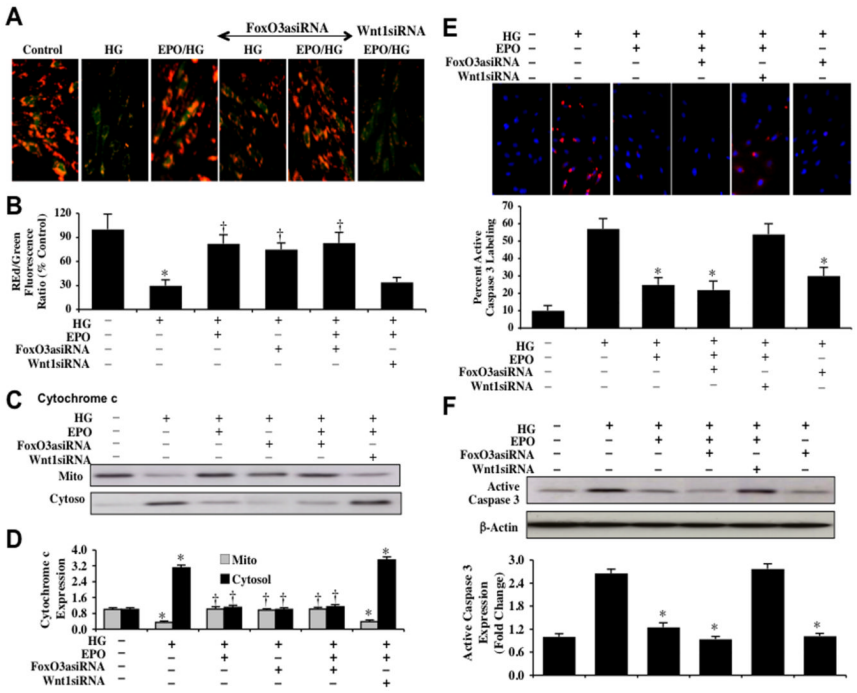

Fig. (8).

EPO prevents mitochondrial membrane depolarization, cytochrome c release, and caspase activation during elevated D-glucose. (**A**) EPO (10 ng/ml) was applied directly to EC cultures 1 hour prior to the administration of D-glucose (25 mM) (HG) and mitochondrial staining with a membrane potential indicator (JC-1 2 μg/ml, 30 minutes) was performed 24 hr later. Images were acquired using a dual emission fluorescence filter (515-545 nm for green fluorescein and emission at 585-615 nm for red fluorescein). Representative pictures are illustrated. Compared to untreated control cultures (**A** and **B**), HG exposure resulted in mitochondrial membrane depolarization as shown by the significant decrease in red/green fluorescent intensity ratio. In contrast, application of EPO (10 ng/ml) or with FoxO3a siRNA transfection prevented mitochondrial depolarization (**A** and **B**). Transfection with Wnt1 siRNA prior to administration of D-glucose attenuated the efficacy of EPO resulting in the mitochondrial depolarization. (**B**) The relative ratio of red/green fluorescent intensity of mitochondrial staining was measured in 3 independent experiments per the experimental paradigms in (A). Data was digitalized using NIH computer imaging software. Twenty-four hours post exposure to HG resulted in a significant decrease in the red/green fluorescence ratio. EPO (10 ng/ml) applied 1 hour prior to HG exposure, significantly prevented the decrease in fluorescence ratio (**P*<0.01 for Control; †*P*<0.01 *vs*. HG treated alone). No significant difference was observed in the fluorescence ratio in FoxO3a siRNA transfected cells between EPO treated and the untreated cultures. In contrast, Wnt1 siRNA transfection significantly decreased red/green ratio in EPO treated cells during HG. (**C**) and (**D**) A representative Western blot result for cytochrome c in mitochondria and cytosol is illustrated. Cytochrome c expression in mitochondria was significantly reduced and cytochrome c in cytosol was significantly increased 24 hours following exposure to elevated D-glucose. Application of EPO (10 ng/ml) significantly prevented mitochondrial cytochrome c release during elevated D-glucose. FoxO3a siRNA treatment prevented cytochrome c release from mitochondria and, in contrast, Wnt1 siRNA increased the release of cytochrome c during elevated D-glucose (**P*<0.01 for control; †*P*<0.01 *vs*. HG treated alone). (**E**) Administration of EPO (10 ng/ml) combined with D-glucose (HG, 25 mM) was followed at 24 hours with immunofluorescent staining for active caspase 3 (Texas-red).

Nuclei of ECs were counterstained with DAPI. In merged images, cells with HG alone show EC with significant caspase 3 staining (red) in contrast to cells with combined EPO with HG. FoxO3a siRNA alone or combined with EPO treatment resulted decreased staining of active caspase 3. In contrast, Wnt1siRNA treatment with combined EPO and HG resulted in an increase in the staining of active caspase 3 (**P*<0.01 vs. HG alone). (**F**) A representative image of western blot for active caspase 3 and quantitative results indicated that HG induced an increased expression of active caspase 3, which was inhibited by pre-treatment with EPO or FoxO3a siRNA. Wnt1siRNA treatment attenuated the efficacy of EPO, resulting in an increase in the expression of active caspase 3 (**P*<0.01 *vs*. HG alone).