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Pin1 inhibitor Juglone prevents diabetic vascular dysfunction

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ABSTRACT

Background: Atherosclerosis is a major cause of mortality in patients with diabetes. However, novel breakthrough therapies have yet to be approved in this setting. Prolyl-isomerase-1 (Pin1) is emerging as a key molecule implicated in vascular oxidative stress and inflammation. In the present study, we investigate whether pharmacological inhibition of Pin1 may protect against diabetes-induced oxidative stress, endothelial dysfunction and vascular inflammation.

Methods and Results: Experiments were performed in human aortic endothelial cells (HAECs) exposed to normal (5 mmol/L) or high glucose (25 mmol/L) concentrations, in the presence of Pin1 inhibitor Juglone (10 μM) or vehicle (<1% ethanol). In parallel, streptozotocin-induced diabetic mice were treated with Juglone i.p. every other day for 30 days (1 mg/Kg). Organ chamber experiments were performed in aortic rings to assess endothelium-dependent relaxations to acetylcholine (Ach 10^{-9} to 10^{-6} mol/L). Mitochondrial oxidative stress, organelle integrity as well as NF-kB-dependent inflammatory signatures were determined both in HAECs and aortas from diabetic mice. In HAECs, ambient hyperglycemia increased mitochondrial superoxide anion generation while treatment with Juglone prevented this phenomenon. Pharmacological inhibition of Pin1 also preserved mitochondrial integrity, nitric oxide availability and endothelial expression of adhesion molecules. Interestingly enough, endothelial dysfunction, oxidative stress and NF-kB-driven inflammation were significantly attenuated in diabetic mice chronically treated with Juglone as compared to vehicle-treated animals.

Conclusion: Pharmacological inhibition of Pin1 by Juglone prevents hyperglycemia-induced vascular dysfunction. Taken together, our findings may set the stage for novel therapeutic approaches to prevent vascular complications in patients with diabetes.

1. Introduction

Prevalence of diabetes mellitus (DM) is alarmingly increasing worldwide and accounts for incident cardiovascular morbidity and mortality [1,2]. Among the different factors involved, atherosclerotic vascular disease carries the largest burden for diabetic patients and explains most of cardiovascular deaths [3,4]. This aspect deserves attention since new breakthrough therapies have yet to be approved in this setting. Hence, deciphering early molecular networks of vascular disease will be instrumental to develop novel therapeutic strategies in people with DM [5].

Prolyl-isomerase 1 (Pin1) is emerging as an important regulator of cellular function *via* conformational changes of proteins [6–8]. Pin1 isomerizes specific phosphorylated Ser/Thr–Pro bonds in many substrate proteins, including glycolytic enzyme, protein kinases, protein phosphatases, transcription activators and regulators [6,9]. Such Pin1-mediated isomerization alters the structure and activity of these proteins, thereby regulating an array of distinct functions such as cell metabolism, cell mobility, tumor development, and oxidative stress [10]. Specifically, Pin1 has the ability to recognize phosphorylated motifs of the pro-oxidant adaptor $p66^{Shc}$, endothelial nitric oxide synthase (eNOS) and nuclear factor kappa-B (NF-kB), thus affecting their functions [11–13]. Indeed, Pin1-dependent conformational changes foster accumulation of free radicals, reduced nitric oxide availability while triggering NF-kB-dependent transcriptional programs. Although previous work has provided mechanistic insights on the role of Pin1, it remains unknown whether its pharmacological inhibition may prevent reactive oxygen species (ROS)-dependent pathways as well as endothelial dysfunction in the diabetic vasculature. The analysis of recent randomized trials has shown that targeting oxidative stress using ROS scavengers is an ineffective and sometimes harmful therapeutic strategy to combat cardiovascular disease [14]. Hence, the characterization of mechanism-based therapeutic approaches remains the most promising strategy to counteract molecular networks of vascular damage. Growing evidence indicates that Juglone, also known as 5-hydroxy-1,4-naphthoquinone, is a reactive molecule produced by walnut trees which irreversibly inhibits the enzymatic activity of Pin1 by forming Michael adducts with Cys113 residue [15]. In the present study, we

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investigate whether pharmacological inhibition of Pin1 by Juglone attenuates features of DM-related vascular disease.

2. Methods

A detailed description of the methods used in this study is provided in Supplementary material.

2.1. Cell culture

Human aortic endothelial cells (HAECs, passages 5 to 7) were exposed for 72 h either to normal glucose (5 mmol/L) or high glucose concentrations (25 mmol/L), in the presence or in the absence of Juglone (5-hydroxy-1,4-naphthoquinone, 10 μM) or vehicle (ethanol, <1% final concentration). Mannitol at the final concentration of 25 mmol/L was used as an osmotic control.

2.2. Real time-PCR

Real time PCR amplification was performed in an MX3000P PCR cycler (Stratagene) using the SYBR Green JumpStart kit (Sigma Aldrich, St. Louis, USA). Primers used are available upon request.

2.3. Measurements of mitochondrial O_2^- and nitric oxide by ESR spectroscopy

Mitochondrial O_2^- generation and nitric oxide (NO) release were assessed by electron spin resonance (ESR) spectroscopy, as previously reported [16,17].

2.4. Assessment of mitochondrial network, swelling and DNA fragmentation

Mitochondrial network extent and organelle swelling were assessed by fluorescence microscopy and optical density, respectively. Mitochondrial DNA damage was assessed by quantitative PCR-based amplification of a large fragment of mitochondrial DNA [18].

2.5. Immunocytochemistry

HAECs were washed with PBS, fixed in 4% PFA for 20 min and blocked with 1% BSA for 1 h. Cells were incubated for 1 h at room temperature with anti-cytochrome *c* antibodies (1:400, Santa Cruz Biotechnology, Nunningen, Switzerland). Fluorescence-labeled secondary antibody (mouse IgG 488, Molecular Probes, USA) was applied for 1 h. Successively, slides were incubated with 4,6 diamidino-2-phenylindole hydrochloride (DAPI) solution (Vector Laboratories, CA, USA) for 10 min and analyzed by fluorescence microscopy (Olympus).

2.6. NF-κB p65 binding activity

The DNA binding reaction was carried out with 5 μg of nuclear protein in a 96-well plate coated with consensus sequences for NF-κB (GGGACTTTCC) for 1 h at room temperature. After washing, NF-κB p65 antibody (Active Motif, Rixensart, Belgium) was added and incubated for 1 h, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Finally, NF-κB p65 DNA binding was assessed spectrophotometrically at 450 nm.

2.7. Streptozotocin-induced diabetic mice

Four- to six-month-old male C57BL/6 mice were administered with a single high dose of streptozotocin (STZ, 180 mg/Kg, *via* intraperitoneal injection) dissolved in sterile 0.025 M citrate buffer (pH 4.5) and injected within 10 min. Control animals received an equal volume of citrate buffer. Hyperglycemia was defined as 3 random blood glucose levels >13.9 mmol/L following STZ injection [17]. Mice were housed in temperature-controlled cages (20–22 °C), fed *ad libitum*, and maintained on a 12:12-h light/dark cycle. All animal studies were conducted in accordance with the guidelines approved by the Institutional Animal Care Committee of the University of Zürich (Kommission für Tierversuche des Kantons Zürich, Switzerland). Mice were divided into four experimental groups: 1) controls; 2) diabetics; 3) diabetic mice treated with Pin1 inhibitor Juglone or 4) vehicle. Juglone was dissolved in 100% ethanol at a concentration of 14 mM (2.44 mg/mL). For injection between 100 and 150 μL of this solution (depending on the animal weight) Juglone was diluted in 5 mL saline and injected i.p. (1 mg/Kg) within 30 min, every other day for 1 month. Control animals received weight appropriate volume of 100% ethanol in 5 mL saline [19].

2.8. Organ chamber experiments

Mice were euthanized by intraperitoneal administration of 50 mg/Kg sodium pentobarbital. The entire aorta from the heart to the iliac bifurcation was excised and placed immediately in cold modified Krebs-Ringer bicarbonate solution (pH 7.4, 37 °C, 95% O_2; 5% CO_2) of the following composition (mmol/L): NaCl (118.6), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25.1), glucose (11.1), and calcium EDTA (0.026). The aorta was cleaned from adhering connective tissue under a dissection microscope, and either snap-frozen in liquid nitrogen and stored at −80 °C or used immediately for organ chamber experiments. For isometric tension studies, aorta was cut into 2 mm rings. Each ring was then connected to an isometric force transducer (Multi-Myograph 610M, Danish Myo Technology), suspended in an organ chamber filled with 6 mL Krebs-Ringer bicarbonate solution (37 °C, pH 7.4), and bubbled with 95% O_2, and 5% CO_2. After a 30 min equilibration period, rings were gradually stretched to the optimal point of their length–tension curve, as determined by the contraction in response to potassium chloride (80 mmol/L). Several rings from the same aorta were studied in parallel. Responses to Ach (10^{-9} to 10^{-6} mol/L) in the presence or absence of PEG–SOD (150 U/mL) were recorded during submaximal contractions to NE (10^{-7} mol/L). The cyclooxygenase inhibitor indomethacin was used at the concentration of 10^{-5} mol/L. The NO donor SNP (10^{-10} to 10^{-5} mol/L) was added to test endothelium-independent responses. Relaxations were expressed as a percentage of the precontracted tension.

2.9. Statistical analysis

All data are presented as mean ± SEM. Statistical comparison were made by using the Student *t* test for unpaired data and 1-way ANOVA, followed by Bonferroni's post-hoc test, when appropriate. Probability values less than 0.05 were considered statistically significant. All analyses were performed with GraphPad Prism (version 5.0) and SPSS (version 20) software.

3. Results

3.1. Pin1 inhibition by Juglone prevents glucose-induced oxidative stress and mitochondrial disruption in human endothelial cells

In human aortic endothelial cells (HAECs), exposure to high glucose (HG) significantly increased mitochondrial generation of superoxide anion (O_2^-) as compared to normal glucose (NG) (Fig. 1A). By contrast, mannitol, used as osmotic control, did not affect ROS production (data not shown). Hyperglycemia-induced mitochondrial oxidative stress was associated with a marked loss of mitochondrial networks characterized by small punctuate mitochondria, as shown by fluorescence microscopy (Fig. 1B). Glucose-induced mitochondrial damage was confirmed by increased DNA fragmentation (Fig. 1C). To further explore defects of mitochondrial functionality, the rate of mitochondrial swelling was determined by light scattering in isolated organelles challenged with calcium overload. Organelles from HAECs exposed to NG showed