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Pycnogenol modulates apoptosis by suppressing oxidative stress and inflammation in high glucose-treated renal tubular cells

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ABSTRACT

Compelling evidence indicates that polyphenolic antioxidants protect against diabetic nephropathy. Pycnogenol is made up of flavonoids, mainly procyanidins and phenolic compounds, and is a known powerful antioxidant. Hyperglycemia is characteristic of diabetic nephropathy and induces renal tubular cell apoptosis. Thus, in this study, we used high glucose-treated renal tubular cells to investigate the protective action of pycnogenol against high glucose-induced apoptosis and diabetic nephropathy. We also sought to further delineate the underlying mechanisms elicited by oxidative stress and inflammation and suppressed by pycnogenol. Results show that pycnogenol significantly suppressed the high glucose-induced morphological changes and the reduction in cell viability associated with cytotoxicity. Bcl2/Bax protein levels indicated pycnogenol's anti-apoptotic effect against high glucose-induced apoptotic cell death. In addition, several key markers of oxidative stress and inflammation were measured for pycnogenol's beneficial effects. Results indicate pycnogenol's anti-oxidative and anti-inflammatory efficacy in suppressing lipid peroxidation, total reactive species (RS), superoxide ($^{\cdot}O_2$), nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^-$), pro-inflammatory inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and nuclear factor-kappa B (NF-κB) nuclear translocation. Based on these results, we conclude that pycnogenol's anti-oxidative and anti-inflammatory properties underlie its anti-apoptotic effects, suggesting further investigation of pycnogenol as a promising treatment against diabetic nephropathy.

1. Introduction

Diabetic nephropathy is a major complication of diabetes, with about 30–40% of patients with type I and 15% with type II diabetes developing end stage renal disease (Francia et al., 2009). However, the cellular and molecular mechanisms underlying the development of diabetic nephropathy are not fully explored.

Renal tubular cells comprise the bulk of the renal cortex, and their role in the tubulointerstitial injury from diabetic nephropathy has been investigated extensively (Huang et al., 2007; Lorz et al., 2009; Stitt-Cavanagh et al., 2009). Because renal tubular cells give us an ideal model from which to explore the cellular mechanisms of tubulointerstitial changes in diabetic nephropathy and decreased renal function, many investigators (Huang et al., 2007; Lorz et al., 2009; Stitt-Cavanagh et al., 2009) have studied the exposure of these cells to high glucose.

Hyperglycemia is characteristic of diabetes and results in several abnormalities in the cellular pathways that make up the critical microenvironments promote apoptosis in various cells. Included are renal tubular epithelial cells, which are known to be a significant factor causing renal functional and pathological changes from diabetic nephropathy (Huang et al., 2007; Lorz et al., 2009).

Apoptosis is a process of normal cell death that maintains tissue homeostasis, but excessive apoptosis or its dysregulation can lead to various pathological processes, such as with diabetes (Francia et al., 2009; Ziyadeh and Wolf, 2008). Apoptosis might contribute to the gradual loss of the renal mass associated with diabetic nephropathy and has been found in renal tubular cells with diabetic nephropathy (Huang et al., 2007).

High glucose is reported to induce oxidative insult and apoptosis in renal tubular cells (Stitt-Cavanagh et al., 2009), and apoptosis

Abbreviations: COX-2, cyclooxygenase-2; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DHR 123, dihydrorhodamine 123; DMEM/F-12, Dulbecco's modified Eagle medium/nutrient mixture F-12; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NBT, nitro blue tetrazolium; NF-κB, nuclear factor-kappa B; PBS, phosphate buffered saline; RS, reactive species; TBARS, thiobarbituric acid reactive substances.

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is associated with the inflammatory state in diabetic nephropathy (Kim et al., 2006). Oxidative stress in the cell occurs from the enhanced generation of reactive species (RS), such as reactive oxygen species and reactive nitrogen species (Manna et al., 2009), and enhanced lipid peroxidation and advanced glycation end products are shown in diabetic nephropathy (Huang et al., 2007). High glucose generates RS that often play a significant role in glucose-induced cellular dysfunction in diabetes (Stitt-Cavanagh et al., 2009; Rohdewald, 2002).

Pycnogenol, containing procyanidins, phenolic acids, bioflavonoids and catechins, has been reported to have beneficial pharmacological properties. These properties include anti-oxidative, anti-inflammatory actions (Bartlett and Eperjesi, 2008), anti-diabetic retinopathy protection (Zibadi et al., 2008), and the ability to reduce the cardiovascular risk factors associated with type 2 diabetes (Zibadi et al., 2008). However, the protective effect of pycnogenol against diabetic nephropathy is not well delineated.

Here, we propose that pycnogenol may play a protective role against diabetic nephropathy by preventing apoptosis in high glucose-induced renal tubular cells. We examined cell viability, morphological changes, and the anti-apoptotic effect of pycnogenol by measuring pro-apoptotic Bax, and anti-apoptotic Bcl2 protein levels in high glucose-treated renal tubular cells.

To verify the underlying actions of pycnogenol's anti-apoptotic effect, we measured major oxidative stress and inflammatory markers, such as lipid peroxidation, total reactive species (RS), superoxide ($\cdot O_2$), nitric oxide ($NO\cdot$), peroxynitrite ($ONOO^-$), pro-inflammatory inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), and nuclear factor-kappa B (NF-κB) nuclear translocation.

2. Materials and methods

2.1. Reagents

Pycnogenol® was purchased from the Horphag Research Ltd. (Guernsey, UK). Pycnogenol® is the standardized extract of the bark of the French maritime pine (*Pinus pinaster*), and consists of the phenolic constituents, taxifolin and catechin, and oligomers of the latter up to dodecamers. In addition, Pycnogenol® contains phenolic acids: p-hydroxybenzoic, protocatechuic, gallic, vanillic, p-coumaric, caffeic and ferulic acid. Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) was obtained from GIBCO™ (Grand Island, NY, USA), and fetal calf serum (FCS) was purchased from Life Technologies Inc. (Grand Island, NY, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 4′,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), dihydrorhodamine 123 (DHR 123) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Monoclonal anti-COX-2, monoclonal anti-NOS antibody, anti-Bcl2 and polyclonal anti-NF-κB, anti-Bcl2, anti-Bax and peroxidase-labeled secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were of the highest commercial grade available.

2.2. Preparation of pycnogenol

Detailed information regarding pycnogenol's preparation procedure has been reported (Kim et al., 2008). In brief, 1 ml of a solution of Pycnogenol® purchased from the Horphag Research Ltd. (Guernsey, UK) in 60% EtOH (25.0 mg/ml) was mixed with 4.0 ml of 5% mercaptoethanol in 60% EtOH containing concentrated HCl (0.3/100 ml) and heated at 70 °C for 7 h. After cooling, the mixture was analyzed by HPLC. The concentration of catechin, epicatechin, and epicatechin-4-(2-hydroxyethyl)thio ether in the reaction mixture were evaluated based on calibration curves obtained by using standard samples. The mean oligomerization degree of the pycnogenol (1 + molar ratio of extension units/terminal units) was estimated to be 4.87 ± 0.12.

2.3. Cell culture

Renal tubular cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM/F-12 medium supplemented with 5% FCS in humidified atmosphere containing 5% CO_2 in air at 37 °C. All subsequent procedures were performed under these conditions, and all the experiments were determined in triplicate and repeated three times to ensure reproducibility.

2.4. Measurement of cell viability and morphology

The cell viability assay was determined as described by Carmichael et al. (1987) using the MTT method. In short, renal tubular cells were seeded at 2×10^4/ml in 96-well plates and preincubated for 24 h. The cells were pretreated with 5 mM glucose for 24 h before treatment with 30 mM glucose and pycnogenol at the same time. A 50 μl aliquot of MTT solution (1 mg/ml) was added to each well of a 96-well culture plates ($n = 6$/group), incubated for 4 h at 37 °C, and then the medium containing MTT was eliminated. The incorporated formazan crystals in the viable cells were solubilized with 100 μl dimethyl sulfoxide and the absorbance at 540 nm of each well was estimated using a microplate reader (Model 3550-UV, Bio-Rad, Tokyo, Japan). In performing the morphology test, cells were grown on six-well plates, treated with glucose and pycnogenol for 24 h, and photographed.

2.5. Assay of lipid peroxidation by thiobarbituric acid reactive substances (TBARS)

The concentration of TBARS was estimated according to Buege and Aust (1978) with slight modification. Cells were plated on 96-well plates and pretreated with 5 mM glucose for 24 h, and then they were treated with 30 mM glucose with and without pycnogenol for 24 h. Then, the media was collected, treated with 0.67% TBA and 20% trichloroacetic acid, and then boiled at 100 °C for 45 min. The mixture was cooled with ice and extracted with *n*-BuOH. The fluorescence of the *n*-BuOH layer was determined using a fluorescence spectrophotometer (model RF-5300PC; Shimadzu, Kyoto, Japan). The maximum absorption of this complex was measured at a wavelength of 532 nm.

2.6. Determination of total RS generation

Total RS production was estimated in culture supernatant as described by Wang and Joseph (1999). Cells were plated on 96-well plates and pretreated with 5 mM glucose for 24 h, and then they were treated with 30 mM glucose with and without pycnogenol for 24 h. Afterward, the cells were washed with calcium and magnesium-free phosphate buffered saline (PBS) and 25 mM DCFH-DA was added to incubation medium. After 15 min, the medium was removed, and the cells were incubated with fresh medium for 1 h. Changes in fluorescence were determined using a fluorescence plate reader (Tecan, Switzerland; excitation was read at 486 nm and emission was read at 530 nm for 30 min).

2.7. Measurement of $\cdot O_2$

The $\cdot O_2$-scavenging activity was determined by estimating the reduction in the ratio of the decrease of nitro blue tetrazolium (NBT), as described by Ewing and Janero (1995). Cells were plated on 96-well plates and pretreated with 5 mM glucose for 24 h, and then they were treated with 30 mM glucose with and without pycnogenol for 24 h. The culture supernatant was added to the reaction buffer [50 mM PBS (pH 7.4) with 125 μM EDTA, 62 μM NBT and 98 μM NADH] containing 33 μM 5-methylphenazium methyl sulfate. The absorbance at 540 nm, as an index of NBT reduction, was read after 5 min using a microplate reader (Model 3550-UV, Bio-Rad, Tokyo, Japan).

2.8. Measurement of $NO\cdot$

$NO\cdot$ production was assessed by evaluating the accumulation of nitrite in the conditioned medium by the Griess assay. In short, 100 μl of culture supernatant was allowed to react with 100 μl of Griess reagent (Green et al., 1982) and then incubated at room temperature for 5 min. The optical density at 540 nm of the samples was measured using a microplate reader.

2.9. Assay of $ONOO^-$ level

$ONOO^-$-dependent oxidation of DHR 123 to rhodamine 123 was assayed, according to the method described by Kooy et al. (1994). Samples were added to the rhodamine buffer (pH 7.4) containing 6.25 μM DHR 123 and 125 μM diethylenetriamine pentaacetic acid and incubated 5 min at 37 °C. The absorbance was determined at 500 nm, the absorbance of rhodamine 123.

2.10. Measurement of NF-κB nuclear translocation

After incubation and treatment, renal tubular cells were fixed with 3.7% paraformaldehyde for 30 min at 4 °C, washed with PBS, and permeabilized with 0.2% Triton X-100 for 30 min at 4 °C. The cells were rinsed with PBS, blocked with 2% bovine serum albumin for 1 h and treated with anti-NF-κB antibody for 2 h at 4 °C. The anti-NF-κB stained cells were washed with PBS, incubated with FITC-conjugated anti-rabbit IgG for 1 h at 4 °C, washed with PBS and then stained with DAPI for 5 min at room temperature. The cells were washed twice more with PBS and analysis was performed using a fluorescent microscope.