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Research Article

High glucose increases glomerular filtration barrier permeability by activating protein kinase G type Iα subunits in a Nox4-dependent manner

Agnieszka Piwkowska[a,*], *Dorota Rogacka*[a], *Irena Audzeyenka*[a], *Stefan Angielski*[a], *Maciej Jankowski*[a,b]

[a]*Mossakowski Medical Research Centre Polish Academy of Sciences, Laboratory of Molecular and Cellular Nephrology Dębinki 7, Gdańsk 80-211, Poland*

[b]*Medical University of Gdańsk, Department of Therapy Monitoring and Pharmacogenetics, Gdańsk, Poland*

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ABSTRACT

Hyperglycemia is a primary factor that disturbs podocyte function in the glomerular filtration process; this disturbance leads to the development of diabetic nephropathy, and ultimately, renal failure. Podocyte function may also be altered by biological agents that modify protein kinase activity, including the cGMP-activated protein kinase type Iα (PKGIα). We hypothesized that hyperglycemia-induced podocyte protein hyperpermeability was dependent on PKGIα activation, and that PKGIα was activated via dimerization induced by reactive oxygen species. This hypothesis was investigated in rat podocytes cultured in high glucose (HG, 30 mM). Protein expression was measured with Western blot and immunofluorescence. Podocyte permeability was measured with a transmembrane albumin flux assay. We found that HG increased podocyte permeability in long-term incubations (1, 3, and 5 days); permeability was increased by 66% on day 5. This effect was abolished with apocynin, a NAD(P)H inhibitor, and Rp-8-Br-cGMPS, a PKG inhibitor. It was also abolished by introducing small interfering RNAs (siRNAs) against Nox4 and PKGIα into cultured podocytes. Furthermore, HG increased PKGIα dimerization by 138% (0.23 ± 0.04 vs. 0.54 ± 0.09; $P < 0.05$); this effect was abolished with a siRNA against Nox4. Our observations suggested that HG could increase albumin permeability across the podocyte filtration barrier via Nox4-dependent PKGIα dimerization.

Introduction

Podocytes are highly specialized cells that encircle the glomerular capillaries and form a key part of the glomerular filtration barrier. They establish the specific size and charge characteristics of solutes allowed to pass the glomerular filtration barrier. The podocyte cell body gives rise to primary processes that branch into foot processes; in turn, the foot processes of neighboring podocytes establish a highly branched, interdigitating pattern, known as the slit diaphragm [1]. This diaphragm is highly permeable to water and small molecules, but not larger proteins, like albumin. Podocyte damage leads to a retraction of the foot processes. Thus, during proteinuric diseases like diabetes mellitus, the foot processes lose their fine structure and collapse into the podocyte cell bodies [2].

*Correspondence author. Fax: +48 58 3492784.
E-mail address: apiwkowska@cmdik.pan.pl (A. Piwkowska).

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Studies in patients with microalbuminuric type 1 diabetes found an increase in podocyte foot process widths compared to podocytes in healthy patients. The foot process width correlated directly with the urinary albumin excretion rate [3]. In addition, the number and density of podocytes have been reported to be markedly reduced in patients with either type 1 or type 2 diabetes [4,5].

The factors involved in the pathogenesis of diabetic nephropathy are multifaceted [6]. One factor is an imbalance between processes that facilitate and inhibit free radical formation, which leads to an excess of free radicals in the kidney [7]. Several investigators have shown that, in the presence of high glucose concentrations, overproduction of reactive oxygen species (ROS) in podocytes induced dysfunctional filtration and increased the excretion of albumin in the urine [8–10]. It was also shown that Nox4, a homolog of NAD(P)H oxidase, is an important source of oxidative stress. In diabetic nephropathy, Nox4 expression is enhanced in the kidney cortex and proximal tubules [11]. We also showed that Nox4 played a major role in disturbing the oxidant-antioxidant balance in podocytes cultured in high glucose conditions. These cells were characterized by enhanced intracellular ROS production and accumulation of hydrogen peroxide (H_2O_2) in the extracellular space [12].

Recently, we showed that the cGMP-activated protein kinase (PKG)-Iα isoform was expressed in cultured rat podocytes. We demonstrated that exposure to exogenous H_2O_2 induced PKGIα to form interprotein disulfide bonds that cross-linked its two subunits [13]. The functional consequence of this PKGIα dimer formation was an increase in podocyte permeability to albumin.

In the present study, we tested the hypothesis that high glucose conditions could cause activation of Nox4, and this could affect both PKGIα activity and podocyte permeability to albumin in cultured rat podocytes. The experimental results suggested a molecular mechanism that could explain podocyte injury and proteinuria in diabetes.

Materials and methods

Materials

Cell culture reagents were from Sigma-Aldrich (St. Louis, MO), with the exception of fetal bovine serum (FBS), which was purchased from Gibco, Invitrogen (Carlsbad, CA). Reagents for SDS-PAGE were purchased from MP Biochemicals, with the exception of the protein standard (Bio-Rad, Hertfordshire, UK) and protease inhibitor cocktail (Sigma-Aldrich). Sources of primary antibodies were as follows: goat polyclonal antibody to PKGIα and mouse monoclonal antibody to actin were from Sigma-Aldrich; rabbit polyclonal antibody to Nox4 was from Abcam (Cambridge, UK). Alkaline phosphatase-conjugated secondary antibodies and donkey anti-goat, goat anti-rabbit, and donkey anti-mouse antibodies were from Santa Cruz Biotechnology. Cy3-conjugated anti-goat secondary antibody was from Rockland (Gilbertsville, PA). All other reagents were purchased from Sigma-Aldrich.

Preparation and culture of rat podocytes

The experimental procedures were in accordance with Directive 2010/63/EU and were also approved Local Bioethical Commission at the Medical University of Gdansk. Female Wistar rats weighing 100–120 g were anesthetized with thiopental (70 mg per kg body weight, i.p.). The kidneys were excised and minced with a scalpel, then pressed through a system of sieves with decreasing pore diameters (160, 106, and 53 μm) to obtain a suspension of glomeruli in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The final suspension of glomeruli was plated in 75 cm^2 type I collagen-coated culture flasks (Becton Dickinson Labware, Beckton, UK) and maintained at 37 °C in an atmosphere of 95% air/5% CO_2 for 5–7 days. The outgrowing podocytes were trypsinized and passed through sieves with 33-mm pores to remove the remaining glomerular cores. The suspension of podocytes was seeded in culture flasks and cultivated at 37 °C in an atmosphere of 95% air/5% CO_2. Experiments were performed with podocytes that had been cultivated for 12–20 days. The phenotype of the podocytes and cell viability were determined as described previously based on immunocytochemical methods. Briefly, podocyte-specific antibodies to Wilm's tumor-1 protein (WT-1; Biotrend Koeln, Germany) and to synaptopodin (Progen, Heidelberg, Germany) were used to determine cell phenotype; lactate dehydrogenase leakage was used to detect viability [14].

For the different experiments, cells were cultured in normal D-glucose (NG, 5.6 mM) or high D-glucose (HG, 30 mM) concentrations or in 5.6 mM D-glucose plus 24.4 L-glucose (LG), as an osmotic control, for the indicated times.

Western blot analysis

Podocytes were treated with lysis buffer (1% Nonidet P-40, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 10% glycerol) in the presence of a protease inhibitor cocktail (Sigma-Aldrich) and homogenized at 4 °C by scraping. The cell homogenates were centrifuged at 9500 × *g* for 20 min at 4 °C. Supernatant proteins (20 μg) were separated on an SDS-polyacrylamide gel (10%) and electrotransferred to a nitrocellulose membrane. The membrane was blocked for 1.5 h with Tris-buffered saline (TBS) (20 mM Tris–HCl, 140 mM NaCl, 0.01% NaN_3) containing 3% non-fat dry milk. After blocking, the membrane was washed with TBS containing 0.1% Tween-20 and 0.1% bovine serum albumin (BSA) and incubated overnight at 4 °C with primary antibody. The following primary antibodies were diluted in TBS containing 0.05% Tween-20 and 1% BSA: anti-PKGIα (1:400, Santa Cruz Biotechnology), anti-Nox4 (1:650, Abcam), and anti-actin (1:3000, Sigma-Aldrich). To obtain non-reducing conditions, we used maleimide (100 mM) in the homogenization and lysis buffers; this alkylated the thiols and prevented thiol disulfide exchanges. To detect primary antibodies bound to the immunoblot, the membrane was incubated for 2 h with the appropriate alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit IgG-AP, goat anti-mouse IgG-AP, or donkey anti-goat IgG-AP; Santa Cruz Biotechnology). The protein bands were detected with the colorimetric 5-bromo-4-chloro-3-indolylphasphate/nitroblue tetrazolium (BCIP/NBT) system. The density of the bands was measured quantitatively with the Quantity One program (Bio-Rad). Protein content was measured with the Lowry method.

RNA interference and cell transfection

A small interfering RNA (siRNA) that targeted PKGIα and a control, nonsilencing siRNA (scrambled siRNA, negative control) were