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Combined effect of insulin and high glucose concentration on albumin permeability in cultured rat podocytes

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ABSTRACT

Podocytes play a fundamental role in regulating glomerular permeability to albumin. This mechanism is disrupted in the course of diabetes. Both insulin and high glucose concentrations enhance the permeability of podocytes to albumin by stimulating oxygen free radical production, primarily by NAD(P)H oxidase-4 (NOX4), and by activating protein kinase G, isoform Iα (PKGIα). However, no study has investigated the combined effects of insulin and high glucose concentration. Here, we investigated the effects of applying insulin (INS, 300 nM) and high glucose (HG, 30 mM), both separately and combined, for 5 days, on cultured rat podocyte permeability to albumin. We measured podocyte permeability with a transmembrane albumin flux assay. We measured NOX4 and PKGIα mRNA expression with real-time PCR. We used Western blots to evaluate protein expression levels of NOX4, PKGIα, the myosin-binding subunit of myosin phosphatase 1, and myosin light chain. We found that INS and HG had a synergistic effect on podocyte permeability to albumin, and this synergy was not dependent on NOX4 or PKGIα. These results suggested that the combined action of INS and HG may exacerbate glomerular dysfunction in diabetes.

1. Introduction

Podocytes, with their foot processes, form an important cellular barrier, known as the slit diaphragm, which is involved in regulating glomerular permeability. Disturbance of podocyte function is a central feature in the development of proteinuria in diabetic nephropathy [1,2]. A common feature of proteinuria is retraction of the podocyte foot processes that form the slit diaphragm [3]. At present, the correlation between this retraction and the development of proteinuria is not well understood. A key question is whether podocyte foot processes can regulate slit diaphragm permeability and glomerular ultrafiltration. Studies have shown that the slit diaphragm protein complex is not simply a static molecular sieve; rather, it is highly dynamic [4,5].

Early-stage type 2 diabetes is characterized by hyperglycemia, the generation of excess oxygen free radicals, and insulin resistance, which leads to hyperinsulinemia [6,7]. Podocytes are uniquely sensitive to insulin; they have exhibited insulin-stimulated glucose uptake kinetics and glucose transporter (GLUT) expression, similar to those of skeletal muscle and fat cells [8,9]. Recent studies have shown that insulin could dynamically remodel the actin cytoskeleton of podocytes, and this was critically important in maintaining the integrity of the glomerular filtration barrier. Actin reorganization resulted in changes in the podocyte structure. Indeed, insulin receptor stimulation caused the retraction of podocyte processes. Moreover, in mice, specific deletion of the gene encoding the insulin receptor in podocytes caused loss of podocyte foot processes [10]. Other groups have suggested that insulin played a role in controlling podocyte contractility, which may contribute to glomerular permeability [11,12]. Recently, we demonstrated that insulin increased albumin permeability in both isolated rat glomeruli and podocytes. That study demonstrated that insulin stimulation of reactive oxygen species (ROS) generation in podocytes could result in cell membrane localization and dimerization of the cGMP-dependent protein kinase G, type Iα (PKGIα) [13]. Moreover, high glucose concentrations also induced an increase in podocyte permeability to albumin through a similar

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mechanism, which depended on the expression of two proteins: the NAD(P)H oxidase-4 (NOX4), which regulates ROS generation; and protein kinase G, isoform Iα (PKGIα), which regulates smooth muscle relaxation [14]. Furthermore, increased albumin permeability was observed in glomeruli isolated from Zucker obese rats, a model of hyperinsulinemia/insulin resistance and oxidative stress. In that model, both PKGIα and NOX4 expression were elevated. Together, those studies suggested that insulin may regulate filtration-barrier permeability, and this mechanism may be dysregulated in diabetes [13].

However, no study has investigated whether the combination of insulin and high glucose concentrations might potentiate glomerular barrier dysregulation. In the present study, we investigated the mechanisms underlying the effects of insulin ad high glucose concentrations, both separately and combined, on cultured podocyte permeability to albumin.

2. Materials and methods

2.1. Preparation and culture of rat podocytes

All experimental procedures were conducted in accordance with Directive 2010/63/EU, and they were approved by the Local Bioethics Commission at the Medical University of Gdansk. Female Wistar rats, which weighed 100–120 g, were anesthetized with thiopental (70 mg/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 and 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 podocyte suspension was seeded in culture flasks and cultivated at 37 °C in an atmosphere of 95% air and 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 [15]. For the different experiments, cells were cultured for five days without or with insulin (300 nM) in normal D-glucose (NG, 5.6 mM) or high D-glucose (HG, 30 mM) concentrations.

2.2. 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. 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. 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 electro-transferred 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. To detect primary antibodies bound to proteins on the immunoblot, the membrane was incubated for 2 h with the appropriate alkaline phosphatase-labeled secondary antibodies. 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.

2.3. RNA interference and cell transfection

We transfected podocytes with small interfering RNAs (siRNA) that targeted PKGIα, NOX4, and a control, non-silencing siRNA (scrambled siRNA, negative control), synthesized by Santa Cruz Biotechnology. Podocytes were cultured in RPMI 1640, supplemented with 10% FBS. One day before transfection, the culture medium was removed, and cells were cultivated in antibiotic-free RPMI 1640, supplemented with 10% FBS. Then, we transfected cells with the siRNA Transfection Reagent (Santa Cruz Biotechnology), according to the manufacturer's instructions. Briefly, the PKGIα siRNA, NOX4 siRNA, or scrambled siRNA were diluted in Transfection Medium (final concentration, 80 nM), mixed with siRNA Transfection Reagent, and incubated for 30 min at room temperature. The transfection mixture was then added to the Transfection Medium, mixed gently, and added to the cells. After 7 h, we added growth medium, which contained 2-fold higher FBS and antibiotics. The cells were incubated for an additional 24 h. After transfection, gene silencing was monitored at the protein level with Western blotting. Podocytes transfected with NOX4 siRNA or PKGIα siRNA showed a significant reduction in NOX4 (37%) or PKGIα (44%) protein expression, respectively, compared to podocytes transfected with scrambled siRNA [14].

2.4. Real-time PCR analysis

Total RNA was isolated from cultured podocytes with TRI Reagent (Sigma) and chloroform/isopropanol extraction. The quantity of isolated RNA was determined by spectrometry (Nanodrop). A sample was considered pure when the A_{260}/A_{280} ratio was 1.8–2.2. Next, the RNA was treated with DNase I (Sigma) to eliminate any contaminating DNA. Then, reverse transcription was performed with 700 ng of RNA (DNAse treated) and 100 U of M-MLV Reverse Transcriptase (Promega) in M-MLV 5× Reaction Buffer, 0.2 mM dNTPs, 10 mM DTT, 0.25 μg Primer $p(dT)_{15}$ (Roche), and 8 U of RNAse Inhibitor (EURx). The mixture was incubated for 1 h at 42 °C, followed by denaturation for 5 min at 95 °C. PKGIα and β-actin mRNAs were amplified with real-time PCR on a Roche Lightcycler® 480 system. The total reaction mix (final volume 20 μl) contained approximately 70 ng of cDNA, 1× LightCycler® FastStart DNA Master SYBR Green I (Roche), 4 mM $MgCl_2$, and 0.5 μM each of sense and antisense intron-spanning primers. The PCR conditions were: pre-incubation 10 min at 95 °C; then, 35 cycles of: denaturation for 10 s at 95 °C, annealing for 5 s at 60 °C, and elongation for 18 s at 72 °C. The primer sequences were: 5′-AAGACGGCAAGCATGAA GCT–3′ (forward), 5′-CCCTTCTGTCCCTGTAAAGGTTT–3′ (reverse) for PKGIα [16]; 5′-CCCTCAGATGTCATGGAACTCCGTA–3′ (forward), 5′-GATTGGATGTCTCTGCAGACCCAGA–3′ (reverse) for Nox4, and 5′TCTATGCCAACACAGTGCTGTCTGG–3 (forward), 5′-GCTTGCTGAT CCACATCTGCTGG–3′ (reverse) for β-actin, which served as a control. The PCR products were verified with a melting curve plot. The products were isolated with electrophoresis in a 2.5% agarose gel, which was stained with ethidium bromide.

2.5. Permeability assay

Transepithelial permeability to albumin was evaluated by measuring the diffusion of FITC-labeled BSA (Sigma) across a podocyte monolayer, as described previously, but with minor modifications [15,17]. Briefly, rat podocytes (1×10^5 cells/cm^2)

were seeded on type IV collagen-coated Cell Culture Inserts (3-μm membrane pore size, 0.32 cm^2 membrane surface area, BD Biosciences). The inserts were placed in 24-well plates, and the cells were allowed to differentiate for one week. Differentiated cells were used for transwell permeability experiments between 7 and 15 days post-seeding. For transwell measurements, podocytes on the inserts were washed twice with PBS and culture medium on both sides, and the inserts were placed into two-compartment chambers with serum-free RPMI 1640 medium (SFM). After 2 h, the medium in the upper compartment was replaced with 0.3 ml fresh SFM, and the medium in the lower compartment was replaced with 1.5 ml SFM containing 1 mg/ml FITC-albumin. After 1 h incubation, 200 μl of the solution in the upper chamber was transferred to a 96-well plate, and the absorbance of the FITC-albumin was determined at 490 nm with a plate spectrophotometer (BioTek EL808).

2.6. Statistical analysis

Statistical analyses were performed with one-way ANOVA, followed by the Student–Newman–Keuls test to determine significance. Values are reported as the mean ± SEM. Significance was set at $P < 0.05$.

3. Results

Cultured rats podocytes were incubated with either INS (300 nM), HG (30 mM), or both for 5 days. Then, we measured albumin permeability and PKGIα and NOX4 protein expression. Fig. 1 shows that either INS or HG alone caused about a 2.5-fold increase in podocyte permeability to albumin. However, the combined effect of INS and HG was more pronounced; the maximum effect was nearly a 5-fold enhancement in permeability to albumin. Downregulation of NOX4 with siRNA reduced the INS effect by about 50% (25.1 ± 4.6 vs. 50.4 ± 6.2 μg/ml, $P < 0.05$, Fig. 1A) and the HG effect by about 35% (36.9 ± 1,2 vs. 56.5 ± 1.9 μg/ml, $P < 0.05$, Fig. 1A). Notwithstanding, the combined effect of INS and HG in the presence of NOX4 downregulation was greater than that of INS or HG alone. Furthermore, downregulation of PKGIα by siRNA reduced the INS effect on permeability to albumin by about 30% (68.4 ± 5.0 vs. 97.4 ± 6.4 μg/ml, $P < 0.05$, Fig. 1B) and reduced the HG effect by about 28% (86.3 ± 4.7 vs. 119.2 ± 5.5 μg/ml, $P < 0.05$, Fig. 1B). Again, the combined effect of INS and HG in the presence of downregulated PKGIα was greater than that induced by these factors alone. Neither siRNA NOX4 nor siRNA PKGIα affected podocyte permeability to albumin in NG conditions (5.6 mM). Thus, these experiments suggested that podocyte permeability to albumin induced by INS and HG was mostly dependent on NOX4 and PKGIα, but also partly dependent on another intracellular signaling pathway.

In contrast to HG, INS affected the expression of PKGIα in cultured rat podocytes (Fig. 2A). INS upregulated the expression of PKGIα protein by 45% (from 0.23 ± 0.03 to 0.33 ± 0.03, $P < 0.05$), without significantly affecting PKGIα mRNA levels (Fig. 3A). Both INS and HG alone increased the expression of NOX4 protein (Fig. 2B); NOX4 protein increased by 41% with INS (from 0.23 ± 0.02 to 0.32 ± 0.01, $P < 0.05$) and by 43% with HG (from 0.23 ± 0.02 to 0.33 ± 0.02, $P < 0.05$). NOX4 mRNA was not significantly affected by HG (Fig. 3B). The combined action of INS and HG had no additional effect on PKGIα and NOX4 protein expression (Fig. 1A, B).

Next, we measured the effects of INS and HG on the expression and phosphorylation of proteins involved in regulating the podocyte contraction apparatus. The basal phosphorylation of the regulatory myosin binding subunit (MYPT1; 0.88 ± 0.06) was increased by 37% with INS and by 33% with HG (Fig. 2C). The basal

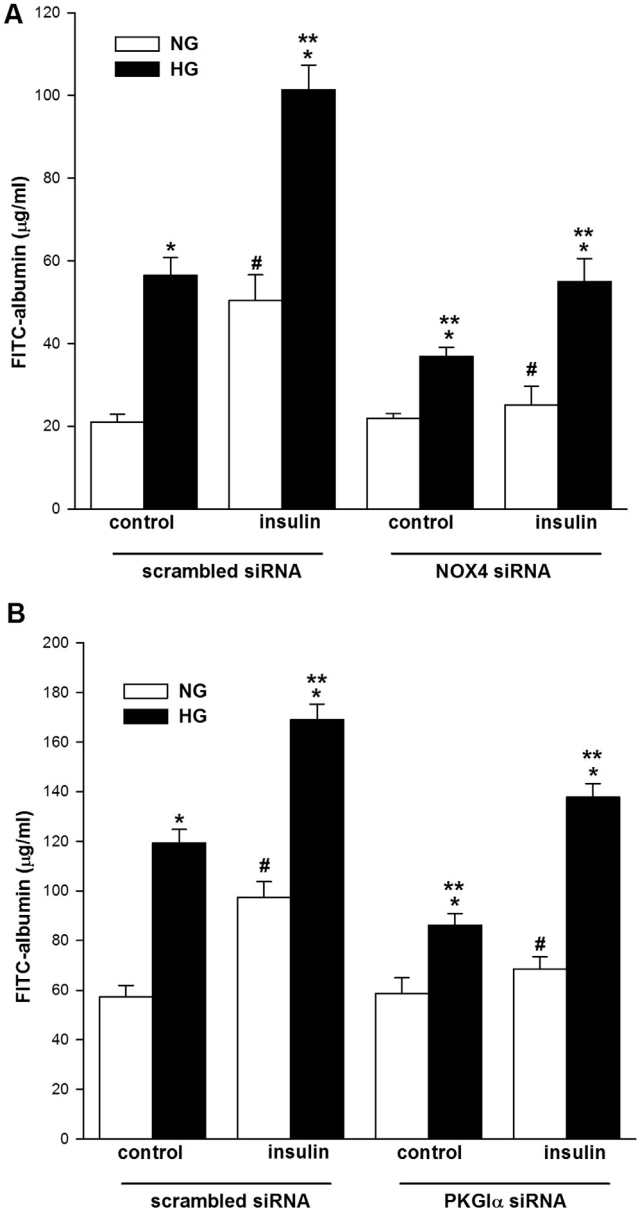

Fig. 1. NOX4 and PKGIα downregulation affected insulin-evoked podocyte albumin permeability. Rat podocyte monolayers were transfected with small-interfering RNAs (siRNAs) that targeted (A) Nox4 or (B) PKGIα. Controls were run with a scrambled siRNA. Monolayer permeability to FITC-tagged albumin was tested under normal (NG, 5.6 mM) and high (HG, 30 mM) glucose conditions. Values represent the means ± SEM of four independent experiments. $^{*}P < 0.05$ compared to appropriate control in NG; $^{**}P < 0.05$ compared to scrambled siRNA in HG; $^{\#}P < 0.05$ compared to scrambled siRNA in NG.

phosphorylation of myosin light chain (MLC; 0.15 ± 0.02) was reduced 51% with INS and 40% with HG (Fig. 2D). The combined action of INS and HG had no additional effect on the phosphorylation of these proteins. Moreover, the expression of MYPT1 and MLC proteins was not affected under these conditions. These results suggested that the combined effects of INS and HG on permeability to albumin probably did not affect the balance between p-MYPT1 and MYPT1 or between p-MLC and MLC, at least at the phosphorylation sites recognized by the antibodies used in these experiments.

The effects of INS and HG on PKGIα disulfide bond formation in non-reducing conditions (100 mM maleimide) is shown in Fig. 4A.

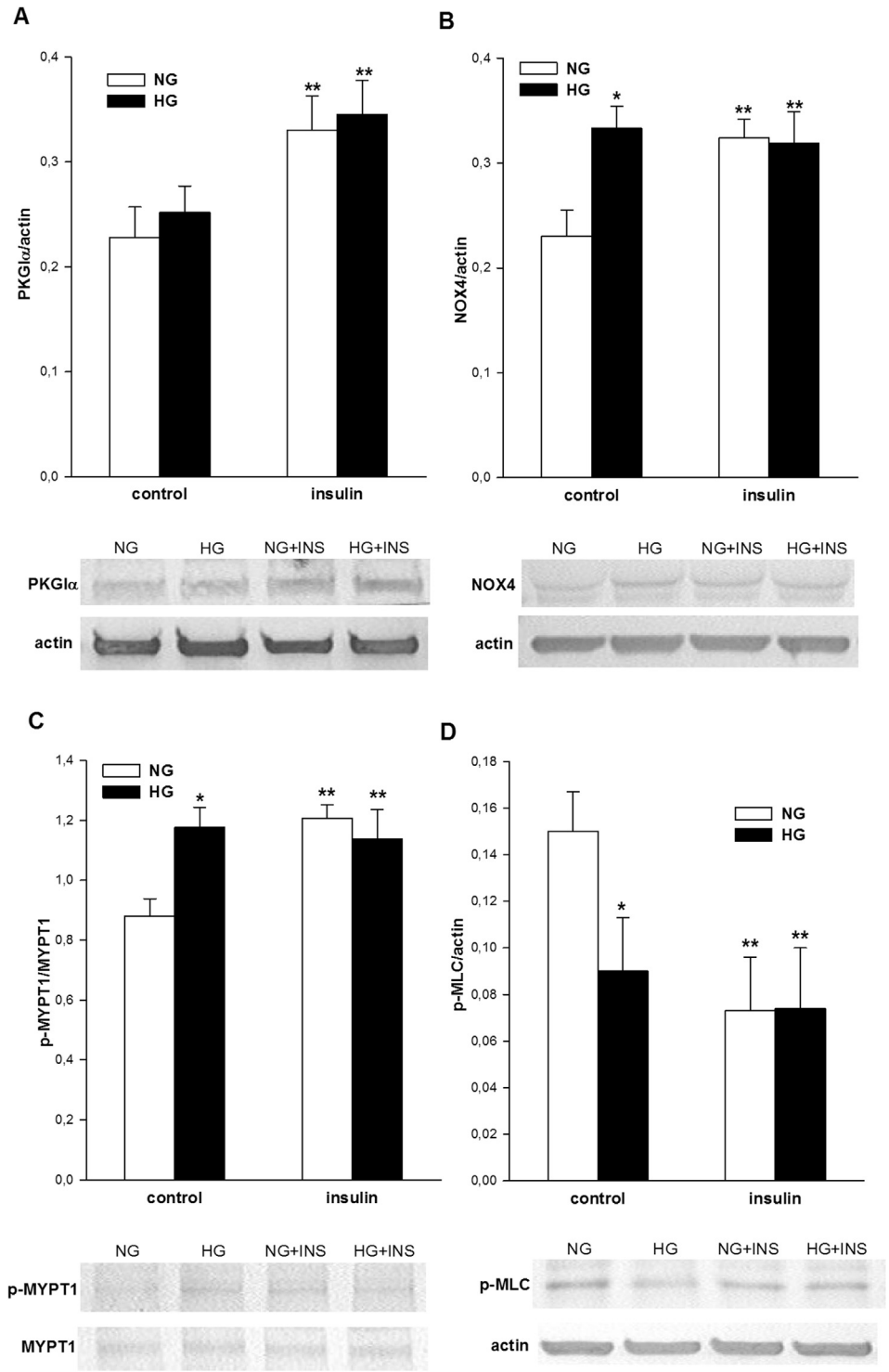

Fig. 2. Insulin-induced changes in protein expression and phosphorylation. Cultured rat podocytes were incubated in normal (NG 5.6 mM) and high (HG, 30 mM) glucose concentrations for five days, with or without insulin. Proteins (20 μg) were separated by SDS-PAGE, immunoblotted with anti-PKGIα, anti-NOX4, anti-MYPT1, anti-phospho-MYPT1 (Ser695), anti-phospho-MLC (Ser19), and anti-β-actin antibodies, and visualized with an alkaline phosphatase-reaction. Representative immunoblots show that insulin induced increases in (A) PKGIα and (B) NOX4 protein expression levels, and insulin increased (C) MYPT1 phosphorylation and (D) MLC dephosphorylation. Densitometric quantifications are reported as the ratios of band intensities, as follows: (A) PKGIα to β-actin, (B) NOX4 to β-actin, (C) p-MYPT1 to MYPT1, and (D) p-MLC to β-actin. Values represent means ± SEM of 4–6 independent experiments. $^{*}P < 0.05$ compared to control in NG, $^{**}P < 0.05$ compared to the appropriate control.

In non-stimulated podocytes, PKGIα was mainly (87%) present in the monomeric form (75 kDa); this finding was not affected by downregulating NOX4 expression with siRNA. INS and HG increased the percentage of dimerized PKGIα (150 kDa) by 176% and 171%, respectively. Because INS also increases the expression of the NOX4 in podocytes, we examined the role of NOX4 on PKGIα dimerization and expression. NOX4 siRNA abolished the INS- and HG-induced dimerization of PKGIα; therefore, this process was NOX4-dependent. The combined action of INS and HG had no additional effect on percentage of dimerized PKGIα.

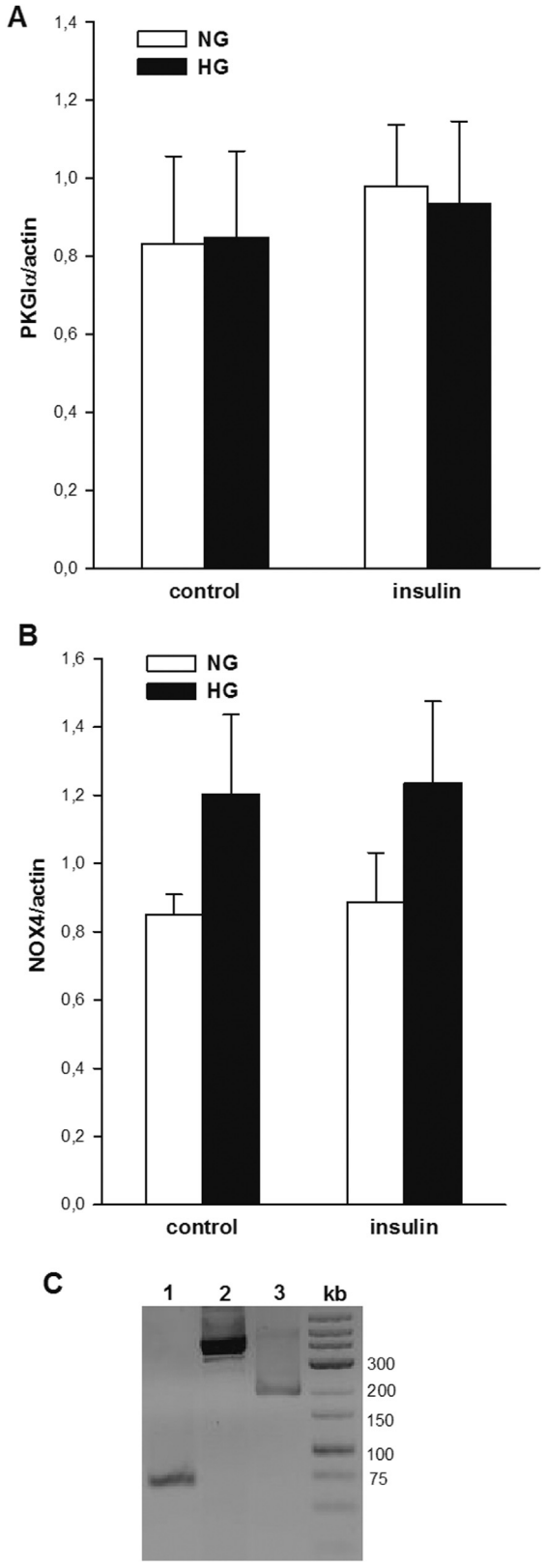

Fig. 3. Insulin influenced PKGIα and NOX4 expression. Rat podocytes were cultured in normal (NG, 5.6 mM) and high (HG, 30 mM) glucose concentrations. (A) PKGIα, (B) NOX4, and β–actin gene expression profiles were analyzed with real-time PCR. The PCR products are shown in the 2.5% agarose gel: lanes (1) PKGIα (72 kb) and (2) NOX4 (450 kb); β-actin (201 kb) (C). Values are the means ± SEM (n = 4–6).

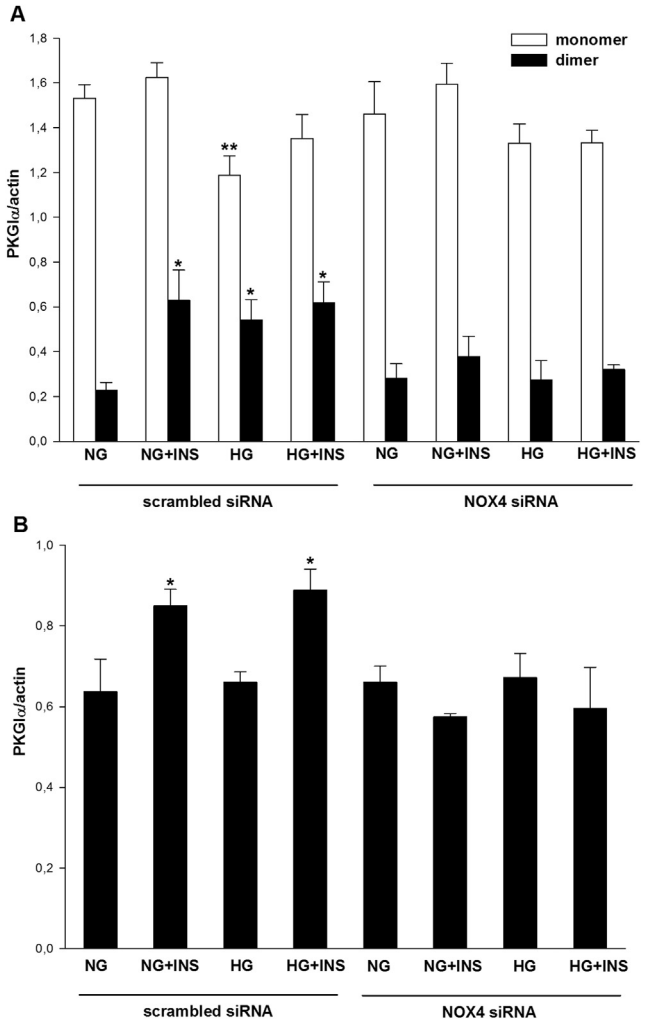

Fig. 4. The effect of NOX4 gene silencing on PKGIα dimerization and protein expression. Podocytes were incubated with normal (NG, 5.6 mM) and high (HG, 30 mM) glucose concentrations for five days with or without insulin. NOX4 expression was silenced with small, interfering RNAs (siRNAs). Western blot analyses were performed to evaluate (A) PKGIα dimerization and (B) PKGIα protein expression. Densitometry of the PKGIα band was normalized to the β-actin band. The values represent the means ± SEM of four independent experiments. $^{*}P < 0.05$ compared to appropriate control, $^{**}P < 0.05$ compared to scrambled siRNA of monomer form in NG.

4. Discussion

In this study, we revealed a mechanism for INS regulation of the glomerular filtration barrier by measuring albumin permeability in podocytes cultured in HG conditions. Previous studies showed that both INS and HG alone could increase podocyte permeability to albumin. Both effects depended on NOX4 and PKGIα, which regulated the phosphorylation state of the cytoskeletal contraction apparatus. However, in the present study, a new, important finding was that INS and HG had a synergistic effect on podocyte permeability to albumin through a mechanism that was probably not dependent on NOX4 and PKGIα.

First, we demonstrated that INS and HG, separately or combined, could significantly increase podocyte permeability. The increase in permeability with insulin and/or HG was reduced with the downregulation of NOX4 and with the downregulation of PKGIα. Our data suggested that oxidative modulation of PKGIα in podocytes played an important role in the regulation of filtration barrier permeability. We investigated the roles of NOX4, a primary source

of ROS in podocytes, and PKGIα in regulating podocyte permeability to albumin in response to insulin and HG.

Recently, it was shown that PKGIα is a redox-sensitive molecule. In the presence of H_2O_2, interprotein disulfide bonds formed between adjacent Cys42 residues in the PKGIα homodimer complex. This bonding stimulated catalytic activity independent of cGMP. In contrast, disulfide oxidation inhibited PKGIα activity; it had little effect on the V_{max} of PKGIα, but decreased the K_m by seven-fold [18]. In the present study, we examined the effect of INS and HG on PKGIα disulfide bond formation. We showed that the percentage of dimerized PKGIα increased by about 176% in the presence of INS and by 171% in the presence of HG. According to previous observations, PKGIα dimerization induced by both INS and HG was NOX4-dependent. Importantly, the combined action of INS and HG did not affect the maximal percentage of dimerized PKGIα. This suggested that the synergistic effect that we observed with these factors on podocyte albumin permeability was not dependent on activating PKGIα by dimerization. On the other hand, insulin may stimulate PKGIα activity by a mechanism involving increased cGMP production and a change in the NADH/NAD + redox state. This mechanism was shown in vascular smooth muscle cells, where insulin increased the availability of iNOS-derived NO and stimulated cGMP production [19].

PKGI mediates vasorelaxation; this activity may lead to an increase in glomerular albumin permeability, through both calcium-dependent and calcium-independent pathways. PKGI regulates intracellular calcium concentrations by modifying the activity of many proteins, including large conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}), IRAG, RGS-2, phospholipase-Cβ3, and TRPC6. Phosphorylation activates BK_{Ca}, which mediates K^+ efflux to extracellular spaces, causes cellular hyperpolarization, and increases Ca^{2+} intake through calcium L type channels [20]. PKGI mediates relaxation in smooth muscle cells by regulating contraction-associated proteins, like myosin light chain kinase (MLCK). Activation of MLC by phosphorylation at Ser-19 leads to microfilament contraction. The opposite effect is observed with activation of the myosin phosphatase complex (MLCP). PKGIα binds directly to the regulatory subunit (MYPT1) of MLCP through a leucine zipper interaction. This binding targets PKGIα to the stress fibers, where it mediates smooth muscle cell relaxation and vasodilation in response to rises in intracellular cGMP [21]. Moreover, PKGIα mediates activation of MLCP by phosphorylating Ser-695 on the MYPT1 subunit [22].

Recently, we showed that activation of the NOX4 subunit of NAD(P)H oxidase induced changes in MYPT1 and MLC phosphorylation by activating PKGIα [13]. When we used changes in MLC phosphorylation to indicate PKG activity, we confirmed that INS activated PKGIα. In a previous study, we also showed that a non-metabolized analog of cGMP, 8-Br-cGMP, induced dephosphorylation of MLC without the formation of a disulfide bridge between PKGIα molecules [15]. We also observed a decrease in podocyte MLC phosphorylation after a 5-day incubation in HG. These results suggested that INS and HG promoted relaxation of the podocyte contraction apparatus. Importantly, synergistic effects of these factors suggested the possibility that another mechanism was involved in mediating cell relaxation. One possible mechanism was the phosphorylation of a serine/threonine kinase and the downstream effector of PI3-kinase. A previous study reported the role of Akt phosphorylation in insulin-induced relaxation of rat vascular smooth muscle cells [23]. Future studies should focus on the role of Akt in INS- and HG-induced changes in glomerular filter permeability.

In summary, results from the present study suggested that INS- and HG-dependent changes in PKGI activity may be responsible, at least in part, for increased urinary albumin excretion in patients with elevated levels of insulin or glucose.

Conflict of interest

None.

Acknowledgments

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Transparency document

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