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Insulin increases glomerular filtration barrier permeability through PKGIα-dependent mobilization of BK_{Ca} channels in cultured rat podocytes

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ABSTRACT

Podocytes are highly specialized cells that wrap around glomerular capillaries and comprise a key component of the glomerular filtration barrier. They are uniquely sensitive to insulin; like skeletal muscle and fat cells, they exhibit insulin-stimulated glucose uptake and express glucose transporters. Podocyte insulin signaling is mediated by protein kinase G type I (PKGI), and it leads to changes in glomerular permeability to albumin. Here, we investigated whether large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) were involved in insulin-mediated, PKGIα-dependent filtration barrier permeability.

Insulin-induced glomerular permeability was measured in glomeruli isolated from Wistar rats. Transepithelial albumin flux was measured in cultured rat podocyte monolayers. Expression of BK_{Ca} subunits was detected by RT-PCR. BK_{Ca}, PKGIα, and upstream protein expression were examined in podocytes with Western blotting and immunofluorescence. The BK_{Ca}–PKGIα interaction was assessed with co-immunoprecipitation.

RT-PCR showed that primary cultured rat podocytes expressed mRNAs that encoded the pore-forming α subunit and four accessory β subunits of BK_{Ca}. The BK_{Ca} inhibitor, iberiotoxin (ibTX), abolished insulin-dependent glomerular albumin permeability and PKGI-dependent transepithelial albumin flux. Insulin-evoked albumin permeability across podocyte monolayers was also blocked with BK_{Ca} siRNA. Moreover, ibTX blocked insulin-induced disruption of the actin cytoskeleton and changes in the phosphorylation of PKG target proteins, MYPT1 and RhoA. These results indicated that insulin increased filtration barrier permeability through mobilization of BK_{Ca} channels *via* PKGI in cultured rat podocytes. This molecular mechanism may explain podocyte injury and proteinuria in diabetes.

1. Introduction

Podocytes are highly specialized cells that wrap around glomerular capillaries, and they comprise a key component of the glomerular filtration barrier. Podocyte damage leads to a retraction of the foot processes, and proteinuria ensues [1].

Podocytes consist of three morphologically and functionally different segments: a cell body, major processes, and foot processes. 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]. The slit diaphragm represents a signaling platform that regulates podocyte function and includes many proteins, including nephrin, podocin, Neph1, CD2AP, TRPC6, BK_{Ca}, and actin [2–5].

Podocytes express proteins that are characteristic of the smooth muscle cell contractile system [6,7]. The presence of F-actin, myosin, and α-actinin in foot processes was proposed to facilitate glomerulus adaptation to changes in pressure gradients; rearrangements of these proteins can modify the surface area for filtration. Podocytes also express receptors for factors that regulate contraction and relaxation; this suggested that podocyte function may be regulated by vasoactive hormones and autocrine/paracrine factors [8,9]. Moreover, mechanical stress induces F-actin reorganization. Podocyte processes become thin and elongated in response to mechanical stress. The podocyte cell body size is smaller in stressed compared to unstressed conditions. Podocytes cultured *in vitro* exhibit a unique ability to reorganize the actin cytoskeleton, which depends on Ca^{2+} influx and Rho kinase.

Abbreviations: BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; PKGIα, protein kinase G type Iα; MLC, myosin light chain; MLCP, myosin light chain phosphatase; MLCK, myosin light chain kinase; MYPT1, myosin phosphatase target subunit 1

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This reorganization results in the formation of radial stress fibers connected to an actin-rich center. Thus, podocytes can be regarded as intrinsically mechanosensitive cells [8]. In addition, hormonal regulation of podocytes affects the size-selectivity of the filtration barrier. This regulation may be similar to that of smooth muscle cells. The size-selective properties of podocytes are also regulated by cGMP-dependent changes in proteins, like protein kinase G type I alpha (PKGIα), which may regulate the cytoskeleton and slit diaphragm.

The PKGIα isoform stimulates myosin light-chain phosphatase (MLCP) activity by phosphorylating its regulatory subunit, MYPT1, at Ser 695 and Ser 852 [10,11]. Increased MLCP activity reduces the level of MLC phosphorylation and causes relaxation [12]. Moreover, PKGIα opposes the inhibitory effect of RhoA/ROCK on MLCP activity. PKGIα directly inhibits RhoA by phosphorylation at Ser188 [13], and it directly phosphorylates large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) [14].

BK_{Ca} channels are a unique class of ion channel; they couple intracellular chemical and electrical signals in podocytes [4,15]. These channels interact with podocyte proteins that are functionally connected to the foot process cytoskeleton, including nephrin [15], the nephrin-like protein, Neph1 [16], synaptopodin [17], and the transient receptor potential cation channel, TRPC6 [18]. The binding of mechanosensitive TRPC6 to BK_{Ca} channels may allow the activation of BK_{Ca} channels by Ca^{2+} influx during podocytes stretch [18]. Moreover, an intact actin cytoskeleton is required for normal expression of BK_{Ca} channels on the podocyte cell surface [19].

Growing evidence has suggested that insulin plays important roles in podocyte metabolism and function [20]. We previously demonstrated that insulin increased the activation of PKGIα subunits, which led to podocyte dysfunction [21]. We found relationships between PKGIα activation, oxidative stress, actin reorganization, and changes in the permeability of the filtration layer to albumin [22]. Other studies showed that insulin stimulated the surface expression of BK_{Ca} channel pore-forming subunits (Slo1 proteins) in mouse podocytes. This expression was accompanied by an increase in BK_{Ca} channel activity [19]. In the present study, we investigated whether BK_{Ca} was involved in insulin-mediated, PKGIα-dependent regulation of filtration barrier permeability.

2. Materials and methods

2.1. Isolation of renal glomeruli

Rat kidneys were removed and placed in ice-cold PBS, pH 7.4, supplemented with 5.6 mM glucose. Glomeruli were isolated with a gradual sieving technique [23]. Briefly, the renal capsule was removed, and the cortex was minced with a razor blade to a paste-like consistency. This was strained through a steel sieve with a pore size of 250 μm. The material that passed through this sieve was suspended in ice-cold PBS and then passed through two consecutive steel sieves (120 and 70 μm pores). The glomeruli retained on top of the 70 μm sieve were washed off with ice-cold PBS and resuspended in ice-cold PBS buffer. The final suspension consisted of decapsulated glomeruli devoid of afferent and efferent arterioles. The tubular contamination was less than 5%, assessed under the light microscope. The entire procedure was carried out in an ice bath and it was completed in no more than 1 h.

2.2. Preparation and culture of rat podocytes

All experiments were approved by the local ethics committee (No. 11/2007). 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). The final material comprised glomeruli suspended in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The glomeruli were 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% CO2 for 5–7 days. The outgrowing podocytes were trypsinized and passed through sieves with 33-mm pores to remove the remaining glomerular cores. The suspended podocytes were seeded in culture flasks and cultivated at 37 °C in an atmosphere of 95% air and 5% CO2. Experiments were performed with podocytes that were cultivated for 12–20 days. The podocyte phenotype and cell viability were determined as described previously with immunocytochemical methods. Cell phenotype was determined with podocyte-specific antibodies against Wilm's tumor-1 protein, WT-1 (Biotrend Koeln, Germany) and synaptopodin (Progen, Heidelberg, Germany). Cell viability was determined by detecting lactate dehydrogenase leakage.

2.3. Western blot analysis

Podocytes were suspended in 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% NaN3) containing 3% non-fat dry milk. Then, membranes were washed with TBS containing 0.1% Tween-20 and 0.1% bovine serum albumin (BSA). Next, membranes were incubated overnight at 4 °C with primary antibodies diluted in TBS containing 0.05% Tween-20 and 1% BSA. The following primary antibodies were used: anti-p-RhoA (Ser188) (1:400, Sigma-Aldrich), anti-RhoA (1:400, Santa Cruz Biotechnology), anti-PKGIα (1:400, Santa Cruz Biotechnology), anti-MYPT1 (1:400, Santa Cruz Biotechnology), anti-p-MYPT1 (Ser-695) (1:400, Santa Cruz Biotechnology), anti-Slo1 (1:1000, Abcam), anti-MaxiKβ (1:400, Santa Cruz Biotechnology), and anti-actin (1:3000, Sigma-Aldrich). To detect primary antibodies bound to proteins on the immunoblot, the membrane was incubated for 2 h with the appropriate alkaline phosphatase (AP)-conjugated secondary antibodies (goat anti-rabbit IgG-AP, goat anti-mouse IgG-AP, or goat anti-rabbit IgG-AP, Santa Cruz Biotechnology). The protein bands were detected with the colorimetric 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) system. The band density was measured quantitatively with the Quantity One program (Bio-Rad). Protein content was measured with the Lowry method.

2.4. Immunofluorescence

Podocytes were seeded on coverslips coated with type-I collagen (Becton Dickinson Labware, Beckton, UK) and cultured in RPMI 1640 supplemented with 10% FBS. Then, cells were fixed in PBS containing 2% formaldehyde for 10 min at room temperature. Next, the coverslips were placed on ice, and the cells were permeabilized by adding 0.3% Triton-X 100 for 3–4 min, and then, cells were blocked with a PBSB solution (PBS containing 2% FBS, 2% BSA, and 0.2% fish gelatin) for 60 min. After blocking, cells were incubated with anti-PKGIα and anti-Slo1 antibodies in PBSB (1:100) at 4 °C for 1 h. Non-specific staining was evaluated by substituting the primary antibodies with PBSB. Next, cells were washed three times with cold PBS and incubated for 45 min with secondary anti-mouse antibodies conjugated with Alexa Fluor 488 (1:100) or anti-goat antibodies (1:100) conjugated with Cy3. After three 5-min washes, the coverslips were attached to slides with Mowiol 4-88 diluted in glycerol-PBS (1:3 v:v), and the cells were viewed with a confocal laser scanning microscope (Olympus FluoView FV10i) or with a fluorescence microscope (Olympus IX51).

F-actin network was labeled and visualized by fluorescence microscopy as described by Pubill et al. with minor modifications [24].