The PKGІα/VASP pathway is involved in insulin- and high glucose-dependent regulation of albumin permeability in cultured rat podocytes

Received April 11, 2020; accepted May 23, 2020; published online June 02, 2020

Patrycja Rachubik 1,*, Maria Szrejder1, Irena Audzeyenka1,2, Dorota Rogacka1,2, Michał Rychłowski3, Stefan Angielski1 and Agnieszka Piwkowska1,2

1Laboratory of Molecular and Cellular Nephrology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Wita Stwosza 63, 80-308 Gdańsk, Poland; 2Faculty of Chemistry, Department of Molecular Biotechnology, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland; and 3Laboratory of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology, University of Gdańsk, Medical University of Gdańsk, Abrahama 58, 80-307 Gdańsk, Poland

*Patrycja Rachubik, Laboratory of Molecular and Cellular Nephrology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Wita Stwosza 63, 80-308 Gdańsk, Poland. Tel.: +48-58-523-54-86, Fax: +48-58-523-54-87, e-mail: prachubik@imdik.pan.pl

Podocytes, the principal component of the glomerular filtration barrier, regulate glomerular permeability to albumin via their contractile properties. Both insulin- and high glucose (HG)-dependent activation of protein kinase G type 1z (PKGІα) cause reorganization of the actin cytoskeleton and podocyte disruption. Vasodilator-stimulated phosphoprotein (VASP) is a substrate for PKGІα and involved in the regulation of actin cytoskeleton dynamics. We investigated the role of the PKGІα/VASP pathway in the regulation of podocyte permeability to albumin. We evaluated changes in high insulin- and/or HG-induced transepithelial albumin flux in cultured rat podocyte monolayers. Expression of PKGІα and downstream proteins was confirmed by western blot and immunofluorescence. We demonstrate that insulin and HG induce changes in the podocyte contractile apparatus via PKGІα-dependent regulation of the VASP phosphorylation state, increase VASP colocalization with PKGІα, and alter the subcellular localization of these proteins in podocytes. Moreover, VASP was implicated in the insulin- and HG-dependent dynamic remodelling of the actin cytoskeleton and, consequently, increased podocyte permeability to albumin under hyperinsulinaemic and hyperglycaemic conditions. These results indicate that insulin- and HG-dependent regulation of albumin permeability is mediated by the PKGІα/VASP pathway in cultured rat podocytes. This molecular mechanism may explain podocytopathy and albuminuria in diabetes.

Keywords: high glucose; insulin; podocytes; protein kinase G type 1z/VASP.

Abbreviations: DN, diabetic nephropathy; FPs, foot processes; HG, high glucose; MLC, myosin light chain; PKGІα, protein kinase G type 1z; pMLC, MLC phosphorylation; pVASP Ser239, VASP phosphorylation at Ser239; SD, slit diaphragm; SFM, serum-free medium; SG, standard glucose; siRNAs, small-interfering RNAs; VSMCs, vascular smooth muscle cells; VASP, vasodilator-stimulated phosphoprotein; VEGF, vascular endothelial growth factor.

Chronic hyperglycaemia and hyperinsulinemia are conducive to the development of diabetic nephropathy (DN), one of the main complications of diabetes mellitus (1). The first clinical sign of DN is albuminuria due to increasing glomerular barrier permeability (2). The main component of the glomerular filtration barrier is podocytes, which are highly specialized, terminally differentiated cells that surround glomerular capillaries (3). Podocytes consist of a cell body, major processes and interdigitating foot processes (FPs) forming the filtration slit. Neighbouring FPs are connected by cell–cell junctions called a slit diaphragm (SD), which is the major glomerular filter sieve impermeable to plasma proteins, such as albumin (2, 4, 5).

Podocytes play a key role in regulating glomerular filtration barrier permeability through actin filament reorganization in the FPs. The FPs are actin-rich structures in which longitudinally orientated thick actin bundles are surrounded by a sub-plasmalemmal network of cortical branched actin filaments that link the SD domain, the basal domain of FPs, and the apical domain of FPs to the actin bundle (6). The FP actin cytoskeleton is a complex contractile apparatus that enables podocytes to be dynamic and rearrange themselves rapidly depending on the needed changes in filtration; therefore, podocyte morphology and function are strictly associated with the actin cytoskeleton (7). Podocyte injury is characterized by alterations in SD function, leading to the effacement of FPs, increased podocyte motility and increasing proteinuria (8, 9). In patients suffering from diabetes-associated nephropathy, podocyte structure and function are altered and correlate with the urinary albumin excretion rate (10, 11).
Insulin is a hormone that directly influences podocyte actin rearrangement and is extremely important in maintaining glomerular filtration barrier integrity. Stimulation of the insulin receptor causes retraction of actin-rich FP s, increasing cell motility and migration, whereas knocking down insulin receptor in mice podocytes leads to FP decline, resulting in albuminuria (12). In vitro studies have confirmed a stimulating effect of insulin on podocyte albumin permeability (13, 14). The evidence indicates a significant relationship between insulin signalling, actin cytoskeleton reorganization and glomerular permeability.

Protein kinase G type 1z (PKG1z) is involved in insulin-dependent regulation of the glomerular filtration barrier (14–16). The PKG1z isoform present in cultured rat podocytes is activated by insulin through the formation of interprotein disulphide bonds between its two subunits (17). Piwkowska et al. (14) demonstrated that insulin influences actin rearrangement and increases podocyte permeability to albumin in a PKG1z-dependent manner. Moreover, a high glucose (HG) concentration also augments podocyte permeability to albumin in a PKG1z-dependent manner, suggesting a crucial role of PKG1z in the development of DN (18, 19).

Contractile system of cells depends on myosin light chain (MLC) phosphorylation at Ser19 (20). The phosphorylation state of MLC may serve as a biological marker of PKG1z activation in cultured rat podocytes. Insulin- and HG-induced activation of PKG1z decreases the level of MLC phosphorylation (pMLC; 18, 21). This mechanism leads to relaxation of the podocytes and increases their permeability to albumin (14, 17).

PKG1z activation, actin reorganization, and alterations in protein permeability in a podocyte filtration monolayer are closely related. Unfortunately, the indirect link between PKG1z activation and actin filament reorganization in FPs is still not known, and further studies are needed to explain the effect of their mutual interaction on glomerular barrier permeability.

Vasodilator-stimulated phosphoprotein (VASP) belongs to the group of proteins that regulate the organization of the actin cytoskeleton. VASP belongs to the family of Ena/VASP actin-associated proteins (22) and localizes to the actin microfilaments, focal adhesions, stress fibres, cell–cell junctions and dynamic membrane regions (23–26). Functionally, VASP participates in actin filament elongation and cell migration, whereas phosphorylation of VASP influences its subcellular localization, affects the regulation of actin polymerization and attenuates VASP affinity for actin by 40-fold (27–29). PKG phosphorylates VASP at Ser239 (30), the functional consequence of which is inhibition of actin filament assembly (27, 31). In vascular smooth muscle cells (VSMCs), effective vascular contractility depends on the elongation of actin filaments, which is mediated by VASP (29). Activation of the PKG/VASP signalling pathway, including VASP phosphorylation at Ser239 (pVASP Ser239), results in vascular relaxation in VSMCs (32). Unfortunately, little is known about the role of VASP and its phosphorylated form in podocyte function. Recently, inhibition of PAR1 phosphatase was demonstrated to result in augmented podocyte motility, dependent on pVASP Ser239 (33). These data suggest that the properties of VASP render it a suitable receptor-mediated transmitter of molecular signals to the podocyte actin cytoskeleton, and that it may be involved in podocyte pathophysiology.

In this study, we investigated whether VASP is involved in regulating the podocyte actin cytoskeleton and permeability through insulin- and/or HG-dependent activation of PKG1z. This research will provide new insight into the role of VASP in podocyte function under physiological and pathophysiological conditions. Moreover, our research suggests VASP phosphorylation state as a potential biomarker of DN.

Materials and Methods

Preparation and culture of rat podocytes

All experiments were performed in accordance with directive 2010/63/EU for animal experiments and the protocol approved by the local ethics committee of UTP University of Science and Technology, Bydgoszcz, Poland.

Female Wistar rats weighing 120–140 g were used for primary podocyte culture as described previously (14). All experiments were performed using podocytes cultured for 12–20 days. Cell phenotypes were confirmed by podocyte-specific antibodies against Wilms tumor-1 protein (Biotrend Koeln, Germany) and synaptopodin (Progen, Heidelberg, Germany). Previous studies had shown that cells incubated for 5 days in HG become insulin resistant (34). Therefore, before different experiments, podocytes were incubated for 5 days in SG medium (RPMI-1640 medium, 10% fetal bovine serum (FBS), 11.1 mM D-glucose) or HG medium (RPMI-1640 medium, 10% FBS, 30 mM D-glucose) and in the presence or absence of PKG1z modulators. Medium with appropriate modulator was changed two times. L-glucose served as an osmotic control for HG.

Western blotting

To obtain podocyte lysates, the cells were treated with lysin buffer (1% Nonidet P-40, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 10% glycerol) in the presence of protease (Sigma-Aldrich) and phosphatase (Roche) inhibitor cocktails and homogenized at 4°C by scraping. Proteins in the supernatant (15 μg) were separated on a 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes. The following primary antibodies were used for western blotting: anti-VASP (1:28000, Sigma-Aldrich), anti-p-VASP (Ser239 (1:667, Abcam), anti-cGKI (1:400, Santa Cruz Biotechnology), anti-p-MLC2 (Ser19) (1:667, Cell Signaling Technology) and anti-actin (1:16000, Sigma-Aldrich). To detect the primary antibodies, the membranes were incubated with the appropriate alkaline phosphatase-labelled secondary antibodies (Sigma-Aldrich). The protein bands were visualized using the colorimetric 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium system. Densitometric quantification of bands was performed using Quantity One software (Bio-Rad).

Small-interfering RNA transfection

Podocytes were transfected with small-interfering RNAs (siRNAs) targeting VASP, PKG1z or non-silencing siRNA (scrambled siRNA, negative control; OriGene). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS. One day before the experiment, the culture medium was changed to antibiotic-free RPMI-1640 supplemented with 10% FBS. The cells were transfected with siRNAs using siRNA Transfection Reagent (OriGene) according to the manufacturer’s instructions. Brieﬂy, the targeted siRNA or scrambled siRNA was diluted in Transfection Medium (ﬁnal concentration, 80 nM), mixed with siRNA Transfection Reagent, and incubated for 30 min at room temperature. Next, the Transfection Mixutre was added to the transfection medium gently, and added to the podocytes. After 7 h, growth medium supplemented with 2× higher concentrations of FBS and antibiotics was added to the cells. Afterwards, the podocytes were incubated for an
additional 24 h. After transfection, gene silencing was checked at the protein level by western blotting.

The siRNA targeting VASP was synthesized by OriGene (cat. no. SR515513). A set of VASP Rat siRNA Oligo Duplex consists of three unique 27mer siRNA duplexes. The experiments with all these three siRNA Duplexes (A–C) were conducted. The siRNA targeting PKGγ was synthesized by Santa Cruz Biotechnology (cat. no. sc-270330) and consisted of pools of three to five target-specific 19 × 25 nucleotide siRNAs.

**Immunofluorescence**

Podocytes were seeded on coverslips coated with Type 1 collagen (Becton Dickinson Labware, Becton, UK) and cultured in RPMI-1640 medium supplemented with 10% FBS. Cells were fixed in PBS plus 4% formaldehyde for 20 min at room temperature. Fixed podocytes were permeabilized with 0.1% Triton-X for 3 min and then blocked with PBSB solution [phosphate-buffered saline (PBS) plus 2% FBS, 2% bovine serum albumin (BSA) and 0.2% fish gelatin] for 1 h. After blocking, cells were incubated with anti-VASP (1:30) and anti-PKGγ (1:15) antibodies in PBSB at 4°C for 1.5 h. The primary antibodies were incubated with blocking peptide to eliminate non-specific staining. Next, the cells were washed three times with cold PBS and incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:750) or Alexa Fluor 546 (1:750). Actin was stained using Alexa Fluor 633 phalloidin (1:200). Specimens were imaged using a confocal laser scanning microscope (Leica SP8X) with a 63× oil immersion lens.

**Permeability assay**

Transepithelial permeability to albumin was investigated by measuring the diffusion of fluorescein isothiocyanate (FITC)-labelled BSA (Sigma-Aldrich) across the podocyte monolayer as described previously by Oshima et al. (35, 36), with minor modifications. Briefly, podocytes (25 × 10^3 cells/well) were seeded on 3-µm membrane pore size cell culture inserts coated with type IV collagen (Corning) and placed in 24-well plates. Transwell permeability experiments were conducted on differentiated cells between 7 and 15 days post-seeding. Before the experiments, podocytes were washed twice with PBS and medium on both sides of the insert and the medium replaced with serum-free RPMI-1640 medium (SFM). After 2 h, the medium in the upper compartment was replaced with fresh SFM supplemented with 1 mg/ml FITC-albumin. After 1 h incubation, the solution from the upper chamber was transferred to a 96-well plate and the absorbance of FITC-albumin measured at 490 nm using an EL808 Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA).

**Statistical analysis**

Statistical analyses were performed using the unpaired t-test and one-way ANOVA, followed by the Student–Newman–Keuls test to determine significance. Values are reported as means ± SEMs. Significance was set at P < 0.05.

**Results**

Short-term incubation with insulin increases PKGγ-dependent VASP phosphorylation in cultured rat podocytes

Insulin-mediated PKGγ activation greatly affects podocyte functioning (15, 37, 38). Therefore, we questioned whether insulin has an impact on pVASP Ser239. The expression of total VASP did not change significantly after administration of insulin or 8-Br-cGMP (Fig. 1A), but we found that podocytes treated with insulin (300 nM, 5 min) or PKG activator 8-Br-cGMP (100 µM, 5 min) had significantly elevated basal levels of phosphorylated VASP Ser239, by 23% (0.86 ± 0.06 versus 0.69 ± 0.04, n = 9, P < 0.05, Fig. 1B) and 174% (1.89 ± 0.24 versus 0.69 ± 0.04, n = 9, P < 0.05, Fig. 1B), respectively. Pre-incubation of podocytes with PKG inhibitor Rp-8-Br-cGMPS abolished the effect of insulin on VASP phosphorylation (0.57 ± 0.04 versus 0.86 ± 0.06, n = 8–9, P < 0.05, Fig. 1B). Moreover, immunofluorescent staining of podocytes to visualize localization of PKGγ and VASP confirmed that both insulin and 8-Br-cGMP increased the interaction of these two proteins.

![Fig. 1. The effects of PKG modulators on VASP expression and its phosphorylation level in podocytes. Podocytes were treated with insulin (INS; 300 nM, 5 min), PKG activator 8-Br-cGMP (100 µM, 5 min), and/or PKG inhibitor Rp-8-Br-cGMPS (Rp-8; 100 µM, 20 min pre-incubation). Densitometric analysis of the corresponding bands was performed, and values are presented as the ratio of band intensity for VASP to actin (A) and pVASP Ser239 to VASP (B). Values are the mean ± SEM, n = 9–10, *P < 0.05.](image-url)
proteins and changed their localization from perinuclear to a more even distribution in the cell (Fig. 2). These results suggest that PKGIα activation is required for insulin-induced pVASP Ser239, and phosphorylation at this site alters PKGIα and VASP localization in podocytes.

**Long-term incubation with insulin and HG increase VASP expression in cultured rat podocytes**

Insulin and HG mediate PKGIα activation in podocytes (18). We hypothesized that VASP expression is augmented under hyperinsulinaemic and hyperglycaemic conditions. Podocytes were cultured in standard glucose (SG, 11 mM) or HG (30 mM) medium in the presence or absence of insulin (300 nM) for 5 days. Insulin and HG increased the level of total VASP protein by 35% (0.83 ± 0.03 to 1.12 ± 0.08, n = 4, P < 0.05) and 29% (0.83 ± 0.03 to 1.07 ± 0.07, n = 4, P < 0.05), respectively (Fig. 3A). Immunofluorescence experiments confirmed the increased PKGIα and VASP colocalization in podocytes cultured under hyperinsulinaemic conditions (from 25.78% to 38.65%, n = 3, P < 0.05, Fig. 3B). In addition, long-term incubation of podocytes with insulin led to an enhancement of the immunofluorescent signal and perinuclear localization of PKGIα and VASP (Fig. 3B). Under hyperglycaemic conditions, colocalization of PKGIα and VASP significantly increased, correlating with a more even distribution of these proteins in podocytes compared with SG (Fig. 3B).
To confirm that PKGIα interacted with VASP under SG and HG conditions, podocytes were treated with Rp-8-Br-cGMPS (50 μM), a PKG inhibitor, for 5 days. Rp-8-Br-cGMPS decreased the amount of VASP protein by 32% (0.72 ± 0.05 to 0.49 ± 0.02, n = 3, P < 0.05) and 48% (0.87 ± 0.03 to 0.45 ± 0.06, n = 3, P < 0.05) compared with SG.

Fig. 3. The influence of insulin and HG on VASP expression in rat podocytes. Insulin (INS, 300 nM) increased VASP levels in podocytes exposed to SG (11 mM) or HG (30 mM) (A). Colocalization of PKGIα and VASP in podocytes cultured in SG or HG medium in the presence or absence of insulin (300 nM, 5 days) (B). Densitometric analysis was performed to establish the amount of VASP protein under the different conditions. Actin was used as a loading control. Quantitative analysis of protein colocalization was performed using LAS AF 3.3.0 software. The pixel intensities were quantified and evaluated by Pearson’s correlation to derive the colocalization rate (%). Values are the mean ± SEM, n = 3–4, *P < 0.05 compared with SG.
PKGIα may mediate enhanced VASP expression and alter VASP functioning in podocytes by affecting its phosphorylation and localization under hyperinsulinaemic and hyperglycaemic conditions.

Downregulation of PKGIα expression in podocytes disturbs VASP phosphorylation at Ser239

Phosphorylation at Ser239 of VASP is a useful marker for monitoring both cGMP-dependent and cGMP-independent activation of PKG (39). To determine whether increased VASP Ser239 phosphorylation is a consequence of PKGIα activation, we transfected podocytes with PKGIα siRNA, reducing the quantity of this protein by 40% (0.76 ± 0.04 versus 0.46 ± 0.04, n = 9, P < 0.05) under SG conditions and 48% (0.88 ± 0.06 versus 0.46 ± 0.02, n = 9, P < 0.05) under HG conditions (Fig. 5A). In total, 49.5% transfection efficiency was obtained with the PKGIα siRNA (Fig. 6B).

Our results show that PKGIα regulates the VASP phosphorylation state. PKGIα gene silencing decreased the basal level of pVASP Ser239 by 47% (0.93 ± 0.02 versus 0.43 ± 0.06, n = 4, P < 0.05; Fig. 5B) and the basal level of VASP by 10% (1.37 ± 0.03 versus 1.24 ± 0.03, n = 5, P < 0.05; Fig. 5C). Rp-8-Br-cGMPS treatment also significantly reduced the quantity of pVASP Ser239 (Fig. 5B).

The data also indicate that HG-dependent activation of PKGIα results in increased pVASP Ser239. This effect was abolished by both PKGIα gene silencing (0.88 ± 0.06 versus 0.46 ± 0.02, n = 9, P < 0.05) and Rp-8-Br-cGMPS (1.09 ± 0.06 versus 0.77 ± 0.04, n = 3–4, P < 0.05, Fig. 5B). Moreover, we found different evidence of a mutual interaction between PKGIα and VASP activity. We observed that downregulation of PKGIα expression induced a 20% decrease in the level of VASP protein (1.52 ± 0.06 versus 1.22 ± 0.04, n = 9, P < 0.05, Fig. 5C) under HG conditions.

This observation raises the question of whether increased phosphorylation of VASP determines a PKGIα-mediated decrease in pMLC in podocytes. To investigate this in more detail, we examined the level of pMLC phosphorylation under the same experimental conditions as in the pVASP Ser239 experiments above. Podocytes with reduced PKGIα gene expression and podocytes treated with Rp-8-Br-cGMPS had the same level of pMLC as control cells cultured under SG conditions (Fig. 5D). Under HG conditions, the pMCL level was diminished by 21% (0.81 ± 0.03 versus 0.64 ± 0.03, n = 10–11, P < 0.05). In addition, inhibition of PKGIα activity by PKGIα siRNA or Rp-8-Br-cGMPS significantly increased the phosphorylation of MLC (Fig. 5D).

These results indicate a possible mechanism by which HG-dependent activation of the PKGIα/VASP signalling pathway leads to diminished pMLC and contributes to limited podocyte contractility.

Effect of insulin and HG on PKGIα/VASP signalling in podocytes with downregulated VASP expression

We previously showed that insulin and HG contribute to a reduction in the pMLC pool in a PKGIα-dependent manner (18). Based on our findings that PKGIα exhibits a regulatory effect on VASP expression, we examined whether VASP is involved in insulin- and HG-dependent regulation of PKGIα expression and the contractile apparatus in podocytes.

Therefore, we used siRNA to downregulate VASP expression in podocytes. Based on obtained results
(Fig. 6A), the VASP siRNA Duplex A was used. We observed an \(~35\%\) decrease in VASP protein (1.11 ± 0.05 to 0.72 ± 0.03; \(n = 11–13\), \(P < 0.05\), Fig. 6A) in podocytes. Taking into consideration the fact, that podocytes are difficult to transfect as they constitute primary cell line, the \(~35\%\) knockdown of
the VASP expression was considered as satisfactory for further research.

Insulin or HG alone caused increase in the level pVASP Ser239 by 21% (Fig. 7A). The combined effect of insulin and HG on these proteins was not additive. Furthermore, VASP siRNA transfection drastically decreased the level of pVASP Ser239, by 69% in the presence of SG and 77% in the presence of insulin and/or HG (Fig. 7A).

Next, we measured the effect of insulin and/or HG on PKGIα expression after downregulation of VASP using siRNA. The transfection of podocytes with VASP siRNA reduced the effect of both insulin and HG on VASP expression (Fig. 7B). Incubation with insulin and/or HG induced an increase in PKGIα expression in podocytes (Fig. 7C). Interestingly, transfection of podocytes with VASP siRNA substantially reduced the effect of insulin or HG on PKGIα expression, by 24% (1.75 ± 0.05 versus 1.34 ± 0.06, n = 14, P < 0.05) and 32% (1.9 ± 0.08 versus 1.3 ± 0.06, n = 14–15, P < 0.05), respectively (Fig. 7C). Notably, VASP downregulation by siRNA significantly diminished the basal PKGIα expression in podocytes (Fig. 7C).

Given the observed effects of insulin and/or HG on PKGIα-dependent phosphorylation of VASP at Ser239, we predicted that the expression of proteins involved in regulating the podocyte contraction apparatus should be restored to a basal level in podocytes transfected with VASP siRNA. We assayed that insulin or HG decreased the basal phosphorylation of MLC (1.49 ± 0.04) by ~13% (Fig. 7D). The combined exposure to insulin and HG had no additive effect on pMLC. In accordance with our predictions, VASP siRNA administration increased pMLC expression by ~23% in the presence of insulin, 21% in the presence of HG, and 39% in the presence of both insulin and HG (Fig. 7D). These results suggest that insulin and HG regulated the podocyte contraction apparatus by signalling to pMLC through activation of the PKGIα/VASP signalling pathway.

Insulin and HG affect actin cytoskeleton organization through a VASP-dependent mechanism in cultured rat podocytes

Because insulin and HG signalling and VASP function are associated with actin filament formation, dynamics, and molecular organization, we hypothesized that VASP mediates actin cytoskeleton reorganization in rat podocytes cultured under hyperinsulinaemic or hyperglycaemic conditions. Podocytes seeded on coverslips were transfected with VASP siRNA and cultured in SG or HG medium in the presence or absence of insulin. Morphological changes of cells were captured using a fluorescence microscope. The F-actin of the podocytes exposed to SG conditions was distributed as prominent bundles that traversed the cell along the axis of the podocyte (Fig. 8). Insulin- and HG-treated cells demonstrated increased F-actin immunostaining in cortical regions, grouped together along the cell periphery, but insulin and HG had only a slight effect on intracellular F-actin staining. The effects of insulin and HG on F-actin network organization were abolished by siRNA-mediated knockdown of VASP expression (Fig. 8).
Fig. 7. Effect of VASP gene silencing on PKGIα-dependent pMLC. Cells were transfected with VASP siRNA and cultured under SG or HG conditions in the presence or absence of insulin (INS, 300 nM, 5 days). The band intensities for pVASP Ser239 (A), VASP (B), PKGIα (C) and pMLC (D) are reported as the ratio to the actin band intensity. Values are the mean ± SEM, n = 9–15. *P < 0.05 compared with SG scrambled siRNA, # P < .05 compared with appropriate scrambled siRNA.
Insulin and HG increase permeability of cultured rat podocytes to albumin through a VASP-dependent mechanism

In light of recent findings that insulin and HG increase podocyte permeability to albumin in a PKG1ε-dependent manner (18), we hypothesized that VASP is part of this signalling pathway. Both insulin and HG markedly increased podocyte permeability. The transmembrane flux for albumin markedly increased 43.7% in podocytes incubated with insulin. Moreover, under HG conditions, albumin permeation was significantly elevated, by 40% (Fig. 9). The combined action of insulin and HG had no additional effect on albumin permeability (Fig. 9).

To characterize the role of VASP in the regulation of podocyte permeability to albumin, we knocked down VASP protein expression using siRNA. Cells transfected with VASP siRNA exhibited a significant reduction in podocyte permeability to albumin under both hyperinsulinaemic (32.53 ± 2.72 versus 55.56 ± 3.02 μg/ml, n = 13–14, P < 0.05) and hyperglycaemic (32.83 ± 2.43 versus 52.18 ± 4.43 μg/ml, n = 12–14, P < 0.05) conditions (Fig. 9). These results suggest that insulin- and HG-triggered PKG1ε/VASP
signalling to the actin cytoskeleton, resulting in its reorganization and increases permeability to albumin across the podocyte filtration barrier.

Discussion

This study revealed a new mechanism for the regulation of podocyte barrier permeability through PKG1ζ-dependent phosphorylation of VASP at Ser239 under hyperinsulinaemic and hyperglycaemic conditions. Activation of the PKG1ζ/VASP pathway may disturb the permeability of the glomerular filtration barrier and mediate the development of DN. The proposed mechanism is shown in Fig. 10.

Short incubation with insulin rapidly increased PKG1ζ-dependent pVASP Ser239 in podocytes. Next, long-term incubation with insulin and HG induced a decrease of VASP Ser239 phosphorylation via PKG1ζ-dependent manner in cultured rat podocytes. VASP mediated reorganization of the actin cytoskeleton in podocytes cultured under hyperinsulinaemic or hyperglycaemic conditions, and insulin and HG increased podocyte barrier permeability through stimulation of VASP signalling pathways.

Podocytes regulate glomerular filtration barrier permeability via their contractile properties, which are closely related to dynamic actin cytoskeleton reorganization. A high concentration of insulin- and glucose-mediated podocyte injury, which altered FP actin organization, resulting in increased glomerular barrier permeability (18, 40). We showed that both insulin and HG mediate regulation of the contractile apparatus and filtration barrier permeability through activation of PKG1ζ signalling pathways in cultured rat podocytes (18, 37). This study confirmed the pivotal role of PKG1ζ in regulation of the contractile apparatus in podocytes under hyperinsulinaemic and hyperglycaemic conditions. Furthermore, for the first time, we demonstrated that VASP interacts with PKG1ζ and is involved in the regulation of actin organization and albumin permeability in cultured rat podocytes in response to insulin and HG.

It is thought that insulin induces a dynamic reorganization of podocyte FPs after meal to deal with transiently elevated filtration load (12). Our recent studies showed that insulin induces PKG1ζ activation and increases permeability of both podocytes and glomeruli (14, 37). Therefore, we examined the contribution of insulin in PKG1ζ-dependent phosphorylation of VASP at Ser239 in podocytes. We demonstrated that short exposure of cells to either insulin or cGMP analogue 8-Br-cGMP quickly increases pVASP Ser239, and this effect was abolished by the PKG inhibitor. Moreover, immunofluorescent staining confirmed that the short-term incubation of podocytes with insulin significantly increases the interaction between PKG1ζ and VASP. These results confirmed that phosphorylation of VASP at Ser239 is PKG1ζ specific.

Based on our findings that disulphide activation of PKG1ζ is a relevant factor that increases podocyte permeability to albumin during hyperinsulinaemia (14) and hyperglycaemia (19), we researched the effect of insulin and HG on VASP expression, its phosphorylated state and localization in podocytes. This study demonstrated that insulin and HG augment VASP expression and change its localization in podocytes. Perhaps diabetic conditions alter the VASP phosphorylation state, which in turn may change VASP localization and affect actin filament dynamics. The level of pVASP Ser239 protein was also significantly elevated by insulin and HG, which is consistent with increased expression of PKG1ζ under the same experimental conditions. Inhibition of PKG1ζ activity by specific gene silencing or Rp-8-Br-cGMPS decreased the amount of total VASP and its phosphorylated form in podocytes, confirming the interaction between VASP and PKG1ζ. We also found that podocyte transfection with VASP siRNA decreased pVASP Ser239 under hyperinsulinaemic and hyperglycaemic conditions. Surprisingly, VASP gene silencing significantly diminished the basal level of PKG1ζ protein in insulin- and HG-stimulated podocytes, suggesting
that a negative feedback regulatory mechanism between VASP and PKG\(\alpha\) exist. In another study, HG treatment resulted in an opposite effect on pVASP Ser239 in VSMCs. However, the experiment incorporated different cell types, duration of incubation with HG, and assumed impairment of cGMP-mediated pVASP Ser239 through oxidative stress (41). In podocytes, oxidative stress activates PKG\(\alpha\) independently of cGMP signalling (17).

PKG\(\alpha\) is implicated in the regulation of the podocyte contractile apparatus via MLC phosphatase-dependent dephosphorylation of MLC at Ser19 (16, 17). In this study, we demonstrated that insulin and HG regulate the podocyte contraction apparatus through activation of the PKG\(\alpha\)/VASP signalling pathway, siRNA-mediated knockdown of VASP abolished the effect of insulin and HG on pMLC. Therefore, active PKG\(\alpha\) phosphorylated VASP at Ser239, resulting in signal transduction to the contractile apparatus, and may promote dephosphorylation of MLC under diabetic conditions. In VSMCs, relaxation was also determined by PKG/VASP signalling, including increased pVASP Ser239 (42, 43). Studies performed on mouse VASP-deficient cardiac fibroblasts showed that basal pMLC is significantly increased (22).

Podocyte function as main regulator of glomerular filtration barrier permeability relies on dynamic reorganization of the actin cytoskeleton. More than 100 proteins are involved in controlling actin dynamics (44), including VASP, which acts as the actin filament elongation factor (28). In VSMCs, knockdown of VASP diminished cell contractility, which was associated with limited elongation of actin filaments (29). In this study, we demonstrated that insulin and HG induce filamentous actin (F-actin) reorganization by affecting VASP function. siRNA-mediated knockdown of VASP prevented the accumulation of F-actin close to the plasma membrane in podocytes cultured in medium supplemented with a high concentration of insulin or glucose. Recent studies have shown that Ena/VASP proteins reduce F-actin branching, and actin filaments are longer (45). These results suggest that VASP signalling to the actin cytoskeleton may be part of a significant dysfunctional pathway in insulin-resistant states. In addition, podocytes incubated with nephrotic plasma have increased VASP phosphorylation and enhanced cell migration, which is associated with dynamic actin remodelling (33). In another study, authors suggested that NO-induced pVASP Ser239 leads to drastically decreased F-actin in cell processes of PTECs, retraction of lamellipodia, and cell rounding (46). Thus, the opposite effect of VASP phosphorylation on cell migration may be cell type-dependent and differ due to activators and activated pathways.

Improper podocyte cytoskeletal reorganization causes not only insulin resistance, but also increased albumin permeability. Our findings indicate that VASP is involved in the insulin- and HG-dependent increase in podocyte permeability to albumin. Downregulation of VASP expression greatly reduced the albumin permeability induced by high concentrations of both insulin and glucose. Insulin and HG had no synergistic effect on podocyte permeability to albumin. It may indicate that the main role in insulin- and HG-dependent regulation of podocyte permeability plays PKG\(\alpha\)/VASP pathway. Combined usage of insulin and HG had no additional effect also on PKG\(\alpha\) protein expression and on the phosphorylation of MLC. Moreover, these experimental conditions did not affect further increase of pVASP Ser239. Piwkowska et al. (18) have previously showed that simultaneous treatment of podocytes with insulin and HG does not have additive effects on PKG\(\alpha\) and MLC activation or expression, compared with both insulin and HG alone. However, they hypothesized that insulin- and HG-dependent activation of PKG\(\alpha\) may be responsible, at least partially, for an increased podocyte permeability to albumin. Studies conducted on brain endothelial cells have shown that increased pVASP Ser239 is associated with an increased blood-brain barrier permeability under hypoxic conditions (47). Davis et al. (47) has also suggested that VASP phosphorylation followed hypoxia is mediated by activation of vascular endothelial growth factor (VEGF) and its receptor VEGFR2. In podocytes, insulin stimulates the production of VEGF-A (48), which is an important autocrine factor that preserves the integrity of the glomerular filtration barrier through a modification of podocyte structure and function (49, 50). Unfortunately, the role of VASP phosphorylation in changes of podocyte permeability induced by VEGF-A is largely unknown. In pulmonary artery endothelial cells, VASP phosphorylation has a protective effect on barrier permeability (51). However, this effect is mediated by cGMP, which is thought to indicate a protective influence on endothelial barrier function. In hyperglycaemic conditions, the concentration of cGMP decreased (52–54), and this may be a cause of podocyte injury. Moreover, Hohenstein et al. (55) showed that, in podocytes, VASP expression is significantly increased during crescentic nephritis and correlates with a loss of podocytes. The data suggest that enhanced VASP expression may play an important role in podocyte pathophysiology and impair the function of the glomerular filtration barrier. Unfortunately, the underlying mechanism in the regulation of podocyte permeability is less understood and could involve changes in actin organization mediated by VASP phosphorylation. Therefore, the precise regulatory mechanisms between VASP and PKG\(\alpha\) and the role of VASP phosphorylation by PKG in the control of podocyte function under diabetic conditions will require further study.

In conclusion, the results suggest that the insulin- and HG-induced increase in podocyte barrier permeability may be due to activation of PKG\(\alpha\)/VASP signalling to the actin cytoskeleton. This mechanism may be responsible for increased glomerular filtration barrier permeability, resulting in albuminuria in patients suffering from elevated insulin or glucose.

**Funding**

This work was supported by the Polish National Science Centre (grant number 2014/14/E/NZ4/00358 to A.P.).
References

1. Han, S.H. and Susztak, K. (2014) The hyperglycemic and hyperinsulinemic combo gives you diabetic kidney disease immediately. Focus on ‘Combined acute hyperglycemic and hyperinsulinemic clamp induced pro-inflammatory responses in the kidney’. *Am. J. Physiol. Cell. Physiol.* 306, C198–199
2. Lin, J.S., and Susztak, K. (2016) Podocytes: the weakest link in diabetic kidney disease? *Curr. Diab. Rep.* 16, 45
3. Pavenstädt, H., Kriz, W., and Kretzler, M. (2003) Cell biology of the glomerular podocyte. *Physiol. Rev.* 83, 253–307
4. Li, J.J., Kwak, S.J., Jung, D.S., Kim, J.J., Yoo, T.H., Ryu, D.R., Han, S.H., Choi, H.Y., Lee, J.E., Moon, S.J., Kim, D.K., Han, D.S., and Kang, S.W. (2007) Podocyte biology in diabetic nephropathy. *Kidney Int.* 72, S36–42
5. Xue, R., Gui, D., Zheng, L., Zhai, R., Wang, F., and Wang, N. (2017) Mechanistic insight and management of diabetic nephropathy: recent progress and future perspective. *J. Diabetes Res.* 2017, 1–7
6. Neal, C.R. (2015) Podocytes ... what’s under yours? (Podocytes and foot processes and how they change in nephropathy). *Front. Endocrinol.* 6, 9
7. Garg, P. (2018) A review of podocyte biology. *Am. J. Nephrol.* 47, 3–13
8. Mundel, P. and Reiser, J. (2010) Proteinuria: an enzymatic disease of the podocyte. *Kidney Int.* 77, 571–580
9. Reiser, J., Oh, J., Shirato, I., Asanuma, K., Hug, A., Mundel, T.M., Honey, K., Ishidoh, K., Kominami, E., Kreidberg, J.A., Tomino, Y., and Mundel, P. (2004) Podocyte migration during nephrotic syndrome requires a coordinated interplay between cathepsin L and z3 integrin. *J. Biol. Chem.* 279, 34827–34832
10. W Wolf, G., Chen, S., and Ziyadeh, F.N. (2010) From glycemic and hyperinsulinemic clamp induced protein kinase G type I z subunits in a Nox4-dependent manner. *Exp. Cell Res.* 320, 144–152
11. Ellis, E.N., Steffes, M.W., Chavers, B., and Mauer, S.M. (1987) Observations of glomerular epithelial cell structure in patients with type I diabetes mellitus. *Kidney Int.* 32, 736–741
12. Welsh, G.I., Hale, L.J., Eremina, V., Jeansson, M., Maezawa, Y., Lennon, R., Pons, D.A., Owen, R.J., Satchell, S.C., Miles, M.J., Caunt, C.J., McArdle, C.A., Pavenstädt, H., Tavaré, J.M., Herzenberg, A.M., Kahn, C.R., Mathieson, P.W., Quaggin, S.E., Saleem, M.A., and Coward, R.J.M. (2010) Insulin signaling to the glomerular podocyte is critical for normal kidney function. *Cell Metab.* 12, 329–340
13. Piwkowska, A., Rogacka, D., Jankowski, M., Dominiczak, M.H., Stepińska, J.K., and Angielski, S. (2010) Metformin induces suppression of NAD(P)H oxidase activity in podocytes. *Biochem. Biophys. Res. Commun.* 393, 268–273.
14. Piwkowska, A., Rogacka, D., Kasztan, M., Angielski, S., and Jankowski, M. (2013) Insulin increases glomerular filtration barrier permeability through dimerization of protein kinase G type Iz subunits. *Biochim. Biophys. Acta* 1832, 791–804
15. Rogacka, D., Audzeyenka, I., Rachubik, P., Rychowski, M., Kasztan, M., Jankowski, M., Angielski, S., and Piwkowska, A. (2017) Insulin increases filtration barrier permeability via TRPC6-dependent activation of PKGIz signaling pathways. *Biochim. Biophys. Acta* 1863, 1312–1325
16. Butt, E., Geiger, J., Jarchau, T., Lohmann, S.M., and Walter, U. (1993) The cGMP-dependent protein kinase gene–protein, and function. *Neurochem. Res.* 18, 27–42
17. Piwkowska, A. (2017) Role of protein kinase G and reactive oxygen species in the regulation of podocyte function in health and disease. *J. Cell. Physiol.* 232, 691–697
18. Piwkowska, A., Rogacka, D., Audzeyenka, I., Angielski, S., and Jankowski, M. (2015) Combined effect of insulin and high glucose concentration on albumin permeability in cultured rat podocytes. *Biochem. Biophys. Res. Commun.* 461, 383–389
19. Piwkowska, A., Rogacka, D., Audzeyenka, I., Angielski, S., and Jankowski, M. (2014) High glucose increases glomerular filtration barrier permeability by activating protein kinase G type Iz subunits in a Nox4-dependent manner. *Exp. Cell Res.* 320, 144–152
20. Surks, H.K., Mochizuki, N., Kasai, Y., Georgescu, S.P., Tang, K.M., Ito, M., Lincoln, T.M., and Mendelsohn, M.E. (1999) Regulation of myosin phosphatase by a specific interaction with cGMP–dependent protein kinase I. *Science* 286, 1583–1587
21. Dippold, R.P., and Fisher, S.A. (2016) Myosin phosphatase isoforms as determinants of smooth muscle contractile function and calcium sensitivity of force production. *Microcirculation* 21, 239–248
22. Galler, A.B., Garcia Arquizonios, M.I., Baumgartner, W., Kuhn, M., Smoleski, A., Simm, A., and Reinhard, M. (2006) VASP-dependent regulation of actin cytoskeleton rigidity, cell adhesion, and detachment. *Histochem. Cell Biol.* 125, 457–474
23. Gammaway, S., Hauser, W., Kobsar, A., Glazova, M., and Walter, U. (2001) Distribution, cellular localization, and postnatal development of VASP and Mena expression in mouse tissues. *Histochem. Cell Biol.* 116, 535–543
24. Trichet, L., Sykes, C., and Plastino, J. (2008) Relaxing the actin cytoskeleton for adhesion and movement with Ena/VASP. *J. Cell Biol.* 181, 19–25
25. Tojkander, S., Gateva, G., Husain, A., Krishnan, R., and Lappalainen, P. (2015) Generation of contractile actomyosin bundles depends on mechanosensitive actin filament assembly and disassembly. *Elife* 4, e06126
26. Rottner, K., Behrendt, B., Small, J.V., and Wehland, J. (1999) VASP dynamics during lamellipodia protrusion. *Nat. Cell Biol.* 1, 321–322
27. Benz, P.M., Blume, C., Seifert, S., Wilhelm, S., Waschke, J., Schuh, K., Gertler, F., Münzel, T., and Renné, T. (2009) Differential VASP phosphorylation controls remodeling of the actin cytoskeleton. *J. Cell Sci.* 122, 3954–3965
28. Harbeck, B., Hüttelmaier, S., Schlüter, K., Jockusch, B.M., and Illenberger, S. (2000) Phosphorylation of the vasodilator-stimulated phosphoprotein coordinates its interaction with actin. *J. Biol. Chem.* 275, 30817–30825
29. Kim, H.R., Graceffa, P., Ferron, F., Gallant, C., Boczkowska, M., Dominguez, R., and Morgan, K.G. (2010) Actin polymerization in differentiated vascular smooth muscle cells requires vasodilator-stimulated phosphoprotein. *Am. J. Physiol. Cell Physiol.* 298, C559–C571
30. Smolenski, A., Bachmann, C., Reinhard, K., Höning-Liedl, P., Jarchau, T., Hoschuetzky, H., and Walter, U. (1998) Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239
phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. J. Biol. Chem. 273, 20029–20035.

31. Barzik, M., Kotova, T.I., Higgs, H.N., Hazelwood, L., Hanein, D., Gertler, F.B., and Schafer, D.A. (2005) Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins. J. Biol. Chem. 280, 28653–28662.

32. Defawe, O.D., Kim, S., Chen, L., Huang, D., Kenagy, R.D., Brenn, T., Walter, U., Daum, G., and Clowes, A.W. (2010) VASP phosphorylation at serine239 regulates the effects of NO on smooth muscle cell invasion and contraction of collagen. J. Cell. Physiol. 222, 230–237.

33. Harris, J.J., McCarthy, H.J., Ni, L., Wherlock, M., Kang, H.G., Wetzels, J.F., Welsh, G.I., and Saleem, M.A. (2013) Active proteases in nephritic plasma lead to a podocin-dependent phosphorylation of VASP in podocytes via protease activated receptor-1. J. Pathol. 229, 660–671.

34. Jankowski, M., Piwkowska, A., Rogacka, D., Audzeyenka, D., Janaszak-Jasiecka, A., and Angielski, S. (2011) Expression of membrane-bound NPP-type ecto-phosphodiesterases in rat podocytes cultured at normal and high glucose concentrations. Biochem. Biophys. Res. Commun. 416, 64–69.

35. Oshima, T., Laroux, F.S., Conte, L.L., Morize, Z., Kawachi, S., Bauer, P., Grisham, M.B., Specian, R.D., Carter, P., Jennings, S., Granger, D.N., Joh, T., and Alexander, J.S. (2001) Interferon-γ and interleukin-10 reciprocally regulate endothelial junction integrity and barrier function. Microvasc. Res. 61, 130–143.

36. Piwkowska, A., Rogacka, D., Jankowski, M., Kocbuch, K., and Angielski, S. (2012) Hydrogen peroxide induces dimerization of protein kinase G type Iα subunits and increases albumin permeability in cultured rat podocytes. J. Cell. Physiol. 227, 1004–1016.

37. Piwkowska, A., Rogacka, D., Audzeyenka, I., Kasztan, M., Angielski, S., and Jankowski, M. (2015) Insulin increases glomerular filtration barrier permeability through PKGα-dependent mobilization of BKCa channels in cultured rat podocytes. Biochim. Biophys. Acta 1852, 1599–1609.

38. Piwkowska, A., Rogacka, D., Angielski, S., and Jankowski, M. (2014) Insulin stimulates glucose transport via protein kinase G type Iα-dependent pathway in podocytes. Biochem. Biophys. Res. Commun. 446, 328–334.

39. Neo, B.H., Patel, D., Kandhi, S., and Wolin, M.S. (2013) Roles for cytosolic NADPH reductase in regulating pulmonary artery relaxation by thiol oxidation-elicited subunit dimerization of protein kinase G1α. Am. J. Physiol. Circ. Physiol. 305, H1330–343.

40. Rozy, A.G., and Coward, R.J.M. (2018) The evolving importance of insulin signaling in podocyte health and disease. Front. Endocrinol 9, 693.

41. Russo, I., Viretto, M., Doronzo, G., Barale, C., Mattiello, L., Anfossi, G., and Trovati, M. (2014) A short-term incubation with high glucose impairs VASP phosphorylation at serine 239 in response to the nitric oxide/cGMP pathway in vascular smooth muscle cells: role of oxidative stress. Biomed. Res. Int. 2014, 1–9.

42. Davel, A.P., Victorio, J.A., Delbin, M.A., Fukuda, L.E., and Rossoni, L.V. (2015) Enhanced endothelium-dependent relaxation of rat pulmonary artery following β-adrenergic overstimulation: involvement of the NO/cGMP/VASP pathway. Life Sci. 125, 49–56.

43. Schäfer, A., Burkhardt, M., Vollkommer, T., Bauersachs, J., Münzel, T., Walter, U., and Smolen ski, A. (2003) Endothelium-dependent and -independent relaxation and VASP serines 157/239 phosphorylation by cyclic nucleotide-elevating vasodilators in rat aorta. Biochem. Pharmacol. 65, 397–403.

44. Insall, R.H., and Mackesy, L.M. (2009) Actin dynamics at the leading edge: from simple machinery to complex networks. Dev. Cell 17, 310–322.

45. Bear, J.E., Svitkina, T.M., Krause, M., Schafer, D.A., Loureiro, J.J., Strasser, G.A., Maly, I.V., Chaga, O.Y., Cooper, J.A., Borisy, G.G., and Gertler, F.B. (2002) Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell 109, 509–521.

46. Lindsay, S.L., Ramsey, S., Atichison, M., Renné, T., and Evans, T.J. (2007) Modulation of lamellipodial structure and dynamics by NO-dependent phosphorylation of VASP Ser239. J. Cell Sci. 120, 3011–3021.

47. Davis, B., Tang, J., Zhang, L., Mu, D., Jiang, X., Biran, V., Vexler, Z., and Ferriero, D.M. (2010) Role of vasodilator stimulated phosphoprotein in VEGF induced blood-brain barrier permeability in endothelial cell monolayers. Int. J. Dev. Neurosci. 28, 423–428.

48. Hale, I.J., Hurcombe, J., Lay, A., Santamaria, B., Valverde, A.M., Saleem, M.A., Mathieson, P.W., Welsh, G.I., and Coward, R.J. (2013) Insulin directly stimulates VEGF-A production in the glomerular podocyte. Am. J. Physiol. Renal. Physiol. 305, F182–188.

49. Advani, A. (2014) Vascular endothelial growth factor and the kidney: something of the marvellous. Curr. Opin. Nephrol. Hypertens. 23, 87–92.

50. Logue, O.C., McGowan, J.W.D., George, E.M., and Bidwell, G.L. (2016) Therapeutic angiogenesis by vascular endothelial growth factor supplementation for treatment of renal disease. Curr. Opin. Nephrol. Hypertens. 25, 404–409.

51. Wojciak-Stothard, B., Torondel, B., Zhao, L., Renné, T., and Leiper, J.M. (2009) Modulation of Rac1 activity by ADMA/DDAH regulates pulmonary endothelial barrier function. Mol. Biol. Cell 20, 33–42.

52. Wang, S., Shiva, S., Poczatek, M.H., Darley-Usmar, V., and Murphy-Ullrich, J.E. (2002) Nitric oxide and cGMP-dependent protein kinase regulation of glucose-mediated thrombospondin 1-dependent transforming growth factor-β activation in mesangial cells. J. Biol. Chem. 277, 9880–9888.

53. Jia, T., Wang, Y.N., Zhang, J., Hao, X., Zhang, D., and Xu, X. (2019) Cinaciguat in combination with insulin induces a favorable effect on implant osseointegration in type 2 diabetic rats. Biomed. Pharmacother. 118, 109216.

54. Zhao, L.R., Du, Y.J., Chen, L., Liu, Z.G., Pan, Y.H., Liu, J.F., and Liu, B. (2014) Quercetin protects against high glucose-induced damage in bone marrow-derived endothelial progenitor cells. Int. J. Mol. Med. 34, 1025–1031.

55. Hohenstein, B., Kasperek, L., Kobelt, D.J., Daniel, C., Gambaryan, S., Renné, T., Walter, U., Amann, K.U., and Hugo, C.P.M. (2005) Vasodilator-stimulated phosphoprotein-deficient mice demonstrate increased platelet activation but improved renal endothelial preservation and regeneration in passive nephrotoxic nephritis. J. Am. Soc. Nephrol. 16, 986–996.