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Insulin stimulates glucose transport via protein kinase G type I alpha-dependent pathway in podocytes

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ABSTRACT

Podocyte resistance to the actions of insulin on glucose transport could contribute to the pathogenesis of diabetic podocytopathy (DP) via disturbances in cyclic-dependent protein kinase signaling. To determine whether cGMP-dependent protein kinase (PKG) is involved in the insulin regulation of glucose transport, we measured insulin-dependent glucose uptake into cultured rat podocytes under conditions of modified PKG activity using pharmacological (PKG activator or inhibitor) and biochemical (siRNA PKGIα, siRNA insulin receptor β) means. Our findings indicate the participation of PKG in insulin-stimulated transport and provide new insights into how PKG may trigger the resistance of glucose transport to insulin in DP.

1. Introduction

Podocytes are highly specialized cells that cover the glomerular capillaries, and they are a key part of the glomerular filtration barrier by contributing to the formation of the slit diaphragm [1]. Podocytes are uniquely sensitive to insulin and exhibit similarities to skeletal muscle and fat cells with respect to the kinetics of insulin-stimulated glucose uptake and the expression of glucose transporters [2–5]. Insulin signaling is initiated by binding to the insulin receptor (IR) with concomitant activation of tyrosine kinase activity in the IRβ subunits. Cumulative evidence suggests that podocyte function and insulin signaling are central to the development of diabetic nephropathy, but the underlying mechanism is unclear. Recently, Welsh et al. showed that the loss of insulin action on mouse podocytes causes changes in kidney structure and function that resemble the renal complications observed in human diabetes. In addition, insulin dynamically remodels the actin cytoskeleton of podocytes, which is critically important in maintaining the integrity of the glomerular filtration barrier. Actin reorganization results in changes in podocyte structure that is IR-dependent. Furthermore, IR stimulation results in the retraction of podocyte processes [6].

Much of insulin's downstream signaling that affects metabolic events involves the activation of phosphatidylinositol (PI) 3-kinase. Insulin activation causes the p85 subunit of PI3-kinase to dock to the tyrosine-phosphorylated IR substrates, IRS-1 and IRS-2. This interaction triggers a cascade of distal signaling responses [7]. PI3-kinase is also linked to activation of the protein kinase, Akt (protein kinase B), and further downstream to vesicular translocation of glucose transporter 4 (GLUT4) and the activation of glucose transport [8]. Another cell signaling pathway that appears to stimulate glucose uptake in skeletal muscle involves the nitric oxide/cyclic GMP pathway [9–11]. The stimulating effects of insulin and cGMP on glucose transport require activation of cGMP-dependent protein kinase G (PKG) in vascular smooth muscle cells [9].

Recently, we observed that the PKGIα isoform is expressed in cultured rat podocytes, and we have demonstrated that exposure to exogenous hydrogen peroxide or insulin induces PKGIα activation through the formation of interprotein disulfide bonds that link its two subunits [12,13]. We have also shown that insulin increases superoxide anion generation via the activation of NAD(P)H oxidase [13]. Our observations demonstrate that increased hydrogen peroxide concentration, PKGIα activation, actin reorganization, and changes in protein permeability across the podocyte filtration layer are functionally related. Other authors have shown a functional role of the microtubule and actin cytoskeletal systems in the mechanism underlying insulin action on GLUT4 traffic [14]. Actin motors likely coordinate the interaction between GLUT4 vesicles and microtubules, and both actin-based and microtubule-based motors are targets of PKG [15].

Our present study investigated whether the PKGI-dependent pathway can modulate insulin signaling and the glucose transport system in cultured rat podocytes. We also determined the role of

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PKGIα activation in the insulin induced enhancement of glucose transport and GLUT4 translocation. This signaling may play a role in the prevention of insulin resistance under conditions associated with oxidative stress.

2. Materials and methods

2.1. 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) 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 podocyte suspension 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. Phenotype and cell viability were determined using immunocytochemical methods as described previously. Briefly, podocyte-specific antibodies to Wilm's tumor-1 protein (WT-1; Biotrend Koeln, Germany) and synaptopodin (Progen, Heidelberg, Germany) were used to determine cell phenotype, and lactate dehydrogenase leakage was used to detect viability [5].

2.2. Preparation the membrane and cytosolic fraction

Podocytes were washed twice with ice-cold PBS and homogenized in a lysis buffer (30 mM Tris, pH 7.5, 10 mM EGTA, 5 mM EDTA, 1 mM DTT, 250 mM sucrose) in the present of a protease inhibitor cocktail (Sigma–Aldrich). The lysates were centrifuged at 9500×*g* for 10 min at 4 °C and supernatant was ultracentrifuged at 60,000×*g* for 40 min at 4 °C. Afterwards supernatant was used as a cytosolic fraction and pallet was resuspended with lysis buffer (without sucrose), solubilized with 1% Triton X-100 (membrane fraction). The protein expression of GLUT4 (membrane and cytosolic fraction) was further examined by Western blot analysis.

2.3. 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 in 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-Akt 1/2/3 (1:600, Santa Cruz Biotechnology), anti-p-Akt 1/2/3 (Ser^{473}) (1:600, Santa Cruz Biotechnology), anti-insulin Rβ (1:200, Santa Cruz Biotechnology), anti-p-insulin Rβ ($Tyr^{1150/1151}$) (1:200, Santa Cruz Biotechnology), anti-PKGIα (1:400, Santa Cruz Biotechnology), and anti-actin (1:3000, Sigma–Aldrich). To detect primary antibodies bound to the membrane it was incubated for 2 h with the appropriate alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit IgG-AP, goat anti-mouse IgG-AP, Santa Cruz Biotechnology). Protein bands were detected using the colorimetric 5-bromo-4-chloro-3-indolylphasphate/nitroblue tetrazolium (BCIP/NBT) system. Band density was measured quantitatively using the Quantity One program (Bio-Rad). Protein content was measured with the Lowry method.

2.4. RNA interference and cell transfection

A small interfering RNA (siRNA) that targeted PKGIα and a control, nonsilencing siRNA (scrambled siRNA, negative control) were 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 the cells cultivated in antibiotic-free RPMI 1640 supplemented with 10% FBS. The cells were transfected with siRNAs and the siRNA Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. Briefly, the PKGIα 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 added to the Transfection Medium, mixed gently, and added to the cells; after 7 h, growth medium containing 2-fold higher FBS and antibiotics was added. The cells were incubated for an additional 24 h. After transfection, gene silencing was monitored at the protein level by Western blotting.

2.5. Immunofluorescence

Podocytes were seeded on type-I collagen-coated coverslips (Becton Dickinson Labware, Beckton, UK) and cultured in RPMI 1640 supplemented with 10% FBS. Cells were fixed in PBS containing 2% formaldehyde for 10 min at room temperature. The coverslips were placed on ice, and the cells were permeabilized with 0.3% Triton-X 100 for 3–4 min and then blocked with PBSB solution (PBS containing 2% FBS, 2% BSA, and 0.2% fish gelatin) for 60 min. After blocking, the cells were incubated with anti-GLUT4 antibody in PBSB (1:100) at 4 °C for 1 h. To evaluate non-specific staining, a control reaction was prepared, where the primary antibodies were omitted from the PBSB in the final step. Next, the cells were washed three times with cold PBS and incubated with Cy3-conjugated anti-rabbit (1:100) secondary antibodies for 45 min. 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 under a fluorescence microscope (Olympus IX51).

2.6. Measurement of glucose uptake

Podocytes were seeded at a density of 3×10^4/well on type I collagen-coated 24-well plates. Cells were preincubated with serum-free RPMI 1640 medium for 2 h and then exposed to 100 μM Rp-8-cGMPS or buffer for 20 min. Measurements were started with the addition of 1 μCi 2-deoxy-[1,2-^{3}H]glucose (2-DG) diluted in non-radioactive glucose (final concentration 50 μM) with 100 μM H_2O_2, 300 nM insulin or 100 μM 8-Br-cGMP. The 2-DG uptake was determined over a 3-min period at 37 °C. Next, the plates were placed on ice and the experimental medium rapidly removed from above the cell layer to determine the extracellular radioactivity. The podocyte layer was then washed three times with ice-cold PBS and lysed by shaking for 90 min in 0.05 mM NaOH at room temperature. The intracellular and extracellular radioactivity was determined by liquid scintillation counting (Wallac 1409). The protein content was measured using a modified Bradford method. In

each experiment glucose transport was calculated from the mean of at least triplicate determinations.

2.7. Statistical analysis

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

3. Results

3.1. Role of PKGI in insulin-stimulated glucose uptake in podocytes

The cultured rat podocytes were insulin-sensitive, and insulin stimulation increased glucose uptake by approximately 50% (Fig. 1). The PKG activators H_2O_2 and 8-Br-cGMP induced a significant increase in glucose uptake in podocytes to 2.35 ± 0.22 nmol/min/mg protein ($P < 0.05$) and 1.87 ± 0.20 nmol/min/mg protein ($P < 0.05$), respectively, from a basal value of 1.37 ± 0.11 nmol/min/mg protein (Fig. 1). To determine the role of PKGI in insulin-induced glucose uptake, we evaluated the effect of PKGI inhibitor Rp-8-cGMPS. Rp-8-cGMPS attenuated the insulin- or H_2O_2-induced increase in glucose uptake. This finding suggests that PKGI plays a critical role in the actions of insulin on glucose transport into podocytes (Fig. 1). Rp-8-cGMPS alone had no significant effect on basal glucose uptake.

3.2. Insulin enhances signaling pathways via PKGI activation

We investigated the effect of insulin on the phosphorylation of proteins involved in insulin signal transduction. Exposing the podocytes to 300 nM insulin resulted in rapid phosphorylation of IR (0.34 ± 0.05 vs. 1.25 ± 0.16, $P < 0.05$) and Akt (1.25 ± 0.15 vs 1.74 ± 0.16, $P < 0.15$). Both PKGI activators, H_2O_2 and 8-Br-cGMP (100 μM, 3 min) increased the levels of phosphorylated IR by approximately 418% and 141%, respectively (Fig. 2A, $P < 0.05$) and phosphorylated Akt by approximately 98% and 55%, respectively (Fig. 2B, $P < 0.05$). We hypothesized that insulin may induce changes in insulin receptor phosphorylation via PKGI activation. To test this hypothesis, podocytes were incubated with insulin or H_2O_2 in the presence of the PKGI inhibitor, Rp-8-cGMPS (100 μM, preincubation 20 min). Rp-8-cGMPS significantly reduced the effect of insulin on IR phosphorylation by roughly 25% (1.25 ± 0.16 vs 0.94 ± 0.14, Fig. 2A, $P < 0.05$) and abolished the effect of insulin on Akt phosphorylation (Fig. 2B, $P < 0.05$). We observed a similar effect in the presence of H_2O_2. Rp-8-cGMPS alone had no significant effect on IR or Akt phosphorylation. These results suggest that insulin and H_2O_2 regulate the insulin signaling pathway via PKGI activation in cultured rat podocytes.

Insulin stimulation is associated with the translocation of GLUT4 transporter to the cell membrane; therefore, we investigated the cellular distribution of GLUT4 in the present of PKGI inhibitor (Rp-8-cGMPS, 100 μM, 20 min). As shown in Fig. 3A and B insulin markedly enhanced GLUT4 transporter content in membrane fraction, about 77% ($P \leqslant 0.05$), and decrease in cytosolic fraction, about 32% ($P \leqslant 0.05$). Pre-incubating podocytes with PKGI inhibitor abolished this effect.

Moreover, immunofluorescence experiments showed that, similar to insulin, PKGI activators caused substantial changes in the subcellular localization of the GLUT4 transporter in cultured rat podocytes (Fig. 3C). The intensity of GLUT4 immunostaining increased close to the cell surface. Pre-incubating with PKGI inhibitor also abolished this effect.

3.3. PKGIα is involved in insulin-stimulated glucose transport

Next, we investigated the role of PKGIα and IRβ in the regulation of glucose transport. We introduced small-interfering RNA (siRNA) into podocytes to knock down PKGIα or IRβ protein expression. Podocytes transfected with PKGIα siRNA or IRβ siRNA exhibited a significant reduction in PKGIα (44%) or IRβ (42%)

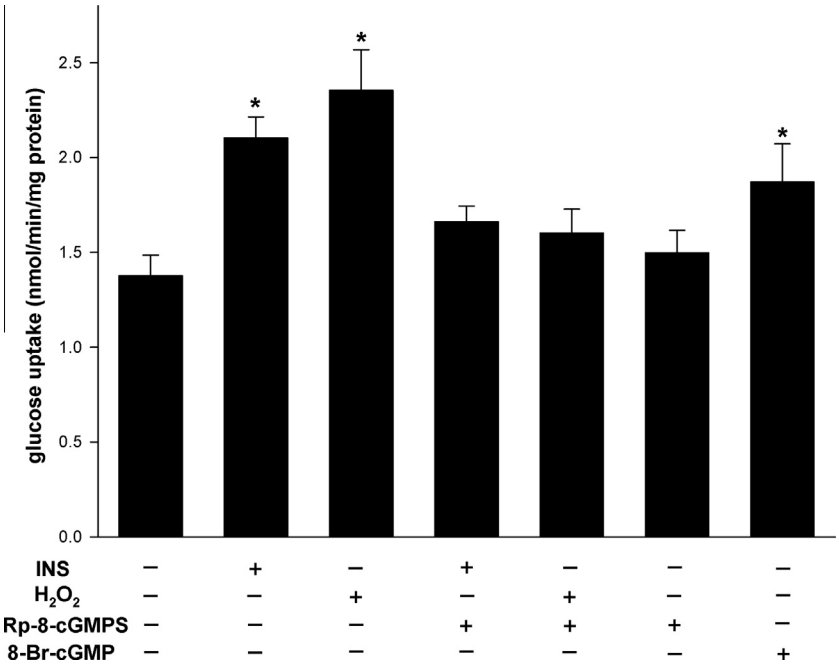

Fig. 1. Effect of PKGI inhibitor on insulin-stimulated glucose uptake into cultured rat podocytes. Cells were pre-incubated with Rp-8-cGMPS or buffer for 20 min. Uptake was measured after the addition of 1 μCi of [1,2-^{3}H]-deoxy-D-glucose diluted in non-radioactive glucose to final concentration of 50 μM and 300 nM insulin, 100 μM H_2O_2 or 100 μM 8-Br-cGMP for 3 min. Values are the mean ± SEM ($n = 4$), $^*P < 0.05$ compared to control.

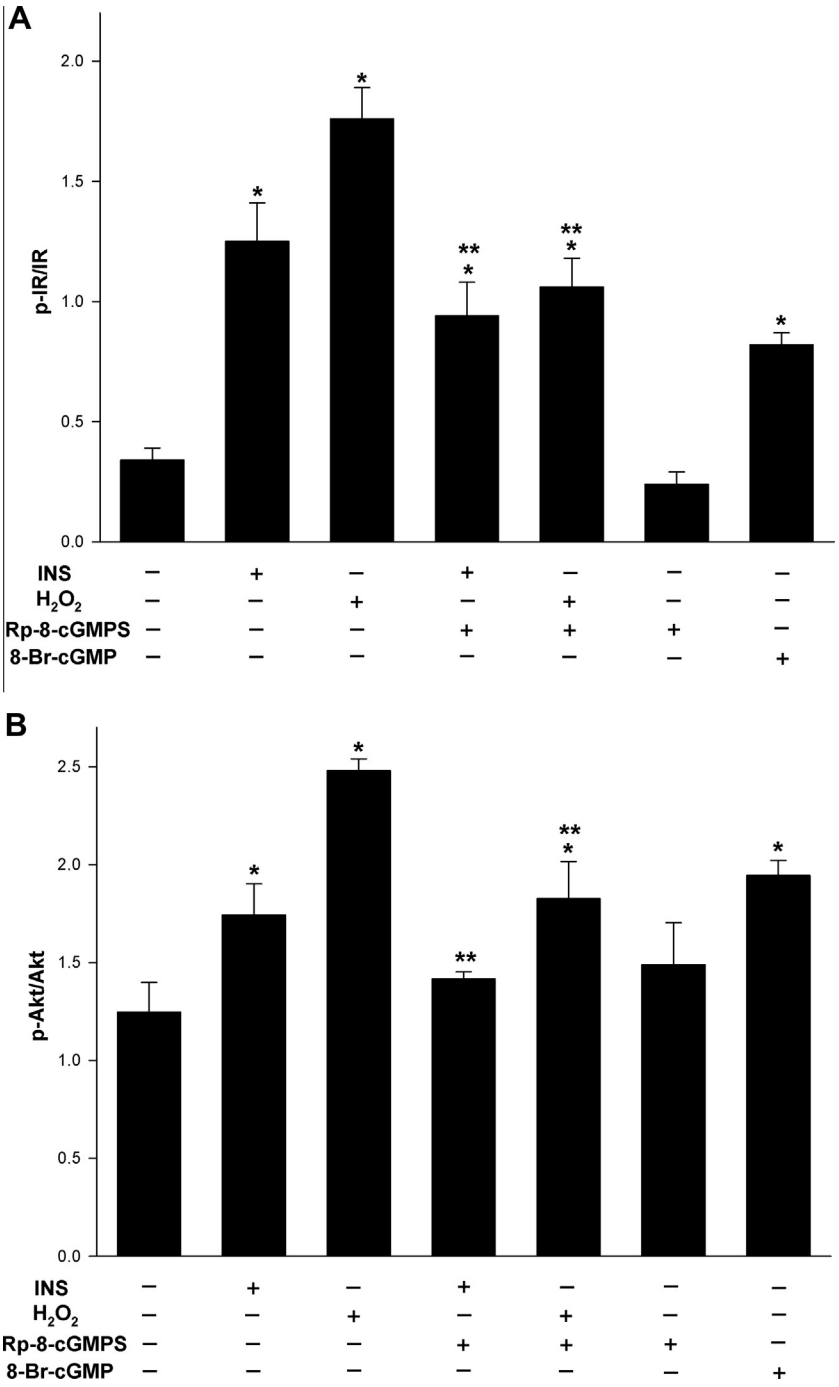

Fig. 2. Insulin enhances insulin signaling pathways via PKG activation. Cells were pre-incubated with Rp-8-cGMPS (100 μM for 20 min), and then incubated with insulin (300 nM, 3 min), H_2O_2 (100 μM, 3 min) or 8-Br-cGMP (100 μM, 3 min). The cell lysates (20 μg) were analyzed by Western blotting. Quantitative densitometric analysis was used to determine the ratio of (A) p-IRβ ($Tyr^{1150/1151}$) to IRβ and (B) p-Akt1/2/3 (Ser^{473}) to Akt1/2/3. Values are the mean ± SEM ($n = 4$), $^{*}P < 0.05$ vs. control, $^{**}P < 0.05$ vs. the appropriate control with insulin or H_2O_2.

protein expression compared to podocytes transfected with scrambled siRNA (Fig. 4A and B).

We found that downregulation of PKGIα attenuated insulin- and H_2O_2-induced increases in glucose uptake. This finding indicates that PKGIα plays a critical role in this process (Fig. 4C). A similar effect was observed with the downregulation of IRβ (Fig. 4F). Moreover, downregulation of IRβ abolished 8-Br-cGMP-dependent increases in glucose uptake.

4. Discussion

The overall conclusion of our present observations is a novel mechanism in which insulin significantly increases glucose uptake into podocytes via the activation of protein kinase G type Iα.

The first major finding of the present study indicate that PKG activators significantly increases glucose uptake into cultured rat podocytes. PKGI is a homodimer comprised of two identical

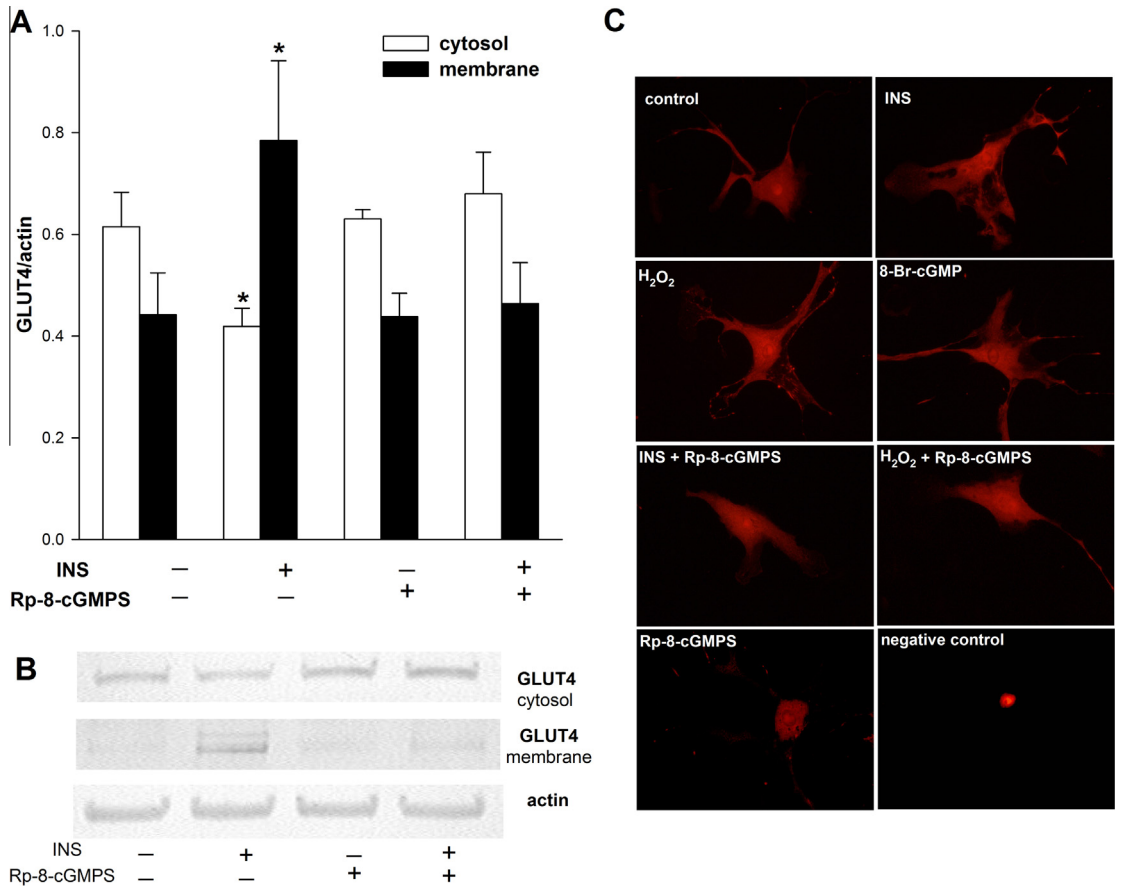

Fig. 3. The effect of PKGI signaling pathways on expression and translocation of the GLUT4 transporter. Results of Western blot analysis of cytosolic and membrane fractions. Values are the means ± SEM ($n = 4$). $^{*}P \leqslant 0.05$ vs. control (A). Representative immunoblots for cytosolic and membrane fractions (B). Rat podocytes seeded onto coverslips were preincubated with Rp-8-cGMPS (100 μM for 20 min) and then incubated for 3 min in the absence or presence of 300 nM insulin, 100 μM H_2O_2 or 100 μM 8-Br-cGMP. Cells were then immunostained with anti-GLUT4 antibody as indicated in the Section 2 (C).

subunits, which are found as two isoforms, Iα and Iβ, that differ in the first 90–100 residues of the N terminus [16]. Dimerization of two PKGI subunits increases its catalytic activity, and thus, its biological action. This oxidation-induced activation of PKGI represents an alternative regulatory mechanism in addition to the classical activation, which involves nitric oxide and cGMP [17]. Our previous work provided evidence that hydrogen peroxide induces the formation of an interprotein disulfide bridge between two PKGIα subunits, which leads to its activation in the absence of cGMP binding [12]. Recently, we also demonstrated that insulin causes disulfide oxidation in PKGIα, which is consistent with its ability to generate ROS [13].

To determine the role of PKGI in insulin-induced glucose uptake, we evaluated the effect of Rp-8-cGMPS, an inhibitor of PKG. We found that PKG inhibition attenuated insulin- and hydrogen peroxide-induced stimulation of glucose uptake. A similar effect was observed after knocking down PKGIα protein expression with small-interfering RNA. Other authors also showed that in cultured human vascular smooth muscle cells (hVSMCs), which predominantly express PKGIα, the stimulating effects of insulin on glucose transport require the PKG activation [9]. However, in contrast to hVSMCs, pretreating podocytes with PKG inhibitor does not lead to significant changes in basal glucose transport suggesting differential regulation of basal glucose transport in smooth muscle cells and podocytes. The potential mechanism of PKG action on glucose transport involves intracellular distribution of GLUT4. We showed that insulin markedly enhanced GLUT4 transporter content in membrane fraction. Pre-incubating podocytes with PKGI inhibitor abolished this effect. The immunofluorescence experiments also showed that PKGI activators cause changes in the subcellular localization of the GLUT4 transporter in podocytes, similar to insulin. The intensity of GLUT4 immunostaining increased close to the cell surface. Podocytes pre-incubated with PKGI inhibitor abolished this effect but further experiments using confocal microscopy should be performed. Other authors have also shown that PKG plays a central role in the translocation of intracellular vesicles containing GLUT4 to the plasma membrane [9].

Several reports provided evidence for the participation of the cGMP-dependent pathway in insulin-stimulated glucose transport. The acute administration of zaprinast (cGMP phosphodiesterases inhibitor) enhances insulin-mediated muscle glucose uptake [18]. Insulin also enhances the phosphorylation and activity of endothelial nitric oxide synthase in cultured endothelial cells [19]. On the other hand, systemic infusion of nitric oxide synthase inhibitor (L-NAME) blocks 50% of the insulin-mediated increase in glucose uptake by muscle in rats [20].

The second major finding of the present study indicate that PKG activators regulate insulin signaling pathways in podocytes. Both activators, H_2O_2 and 8-Br-cGMP increase phosphorylation of IR and Akt. The effect of insulin on IR phosphorylation was significantly reduced in the presence of PKG inhibitor Rp-8-cGMPS. We hypothesized that insulin may induce changes in IR phosphorylation through activation of PKGI. Moreover, the effect of insulin on Akt phosphorylation was abolished in the presence of Rp-8-cGMPS. It is possible that PKG may be a target for pharmacological intervention in a number of conditions characterized by disturbances

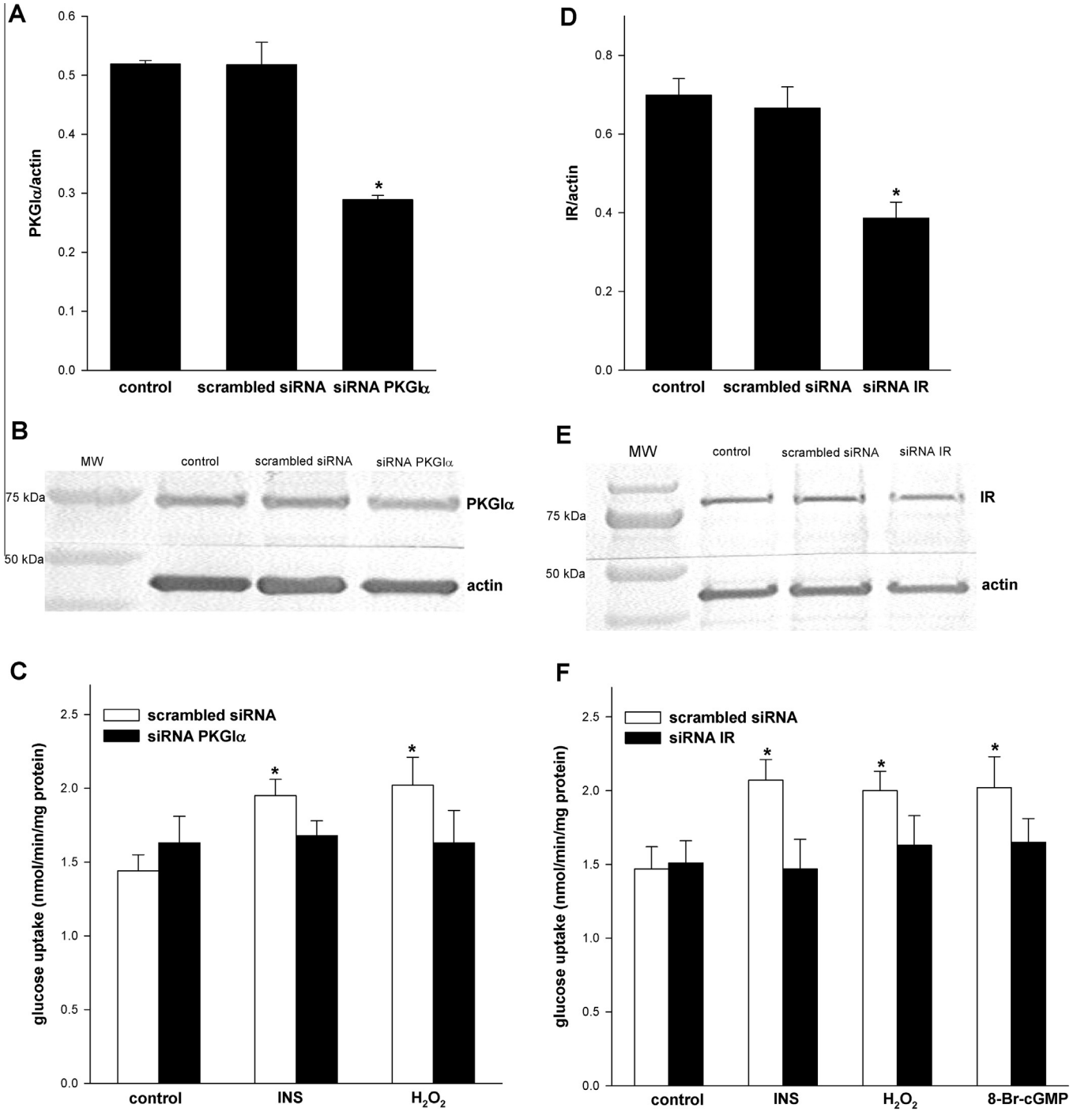

Fig. 4. The effect of PKGIα and IRβ gene silencing on insulin-stimulated glucose uptake into cultured rat podocytes. The effect of PKGIα or IRβ small interfering RNA (siRNA) and scrambled siRNA on PKGIα (A) and IRβ (D) proteins expression. Densitometry of the PKGIα and IRβ bands were normalized to the actin band. Values are the mean ± SEM of four independent experiments. $^{*}P < 0.05$ versus transfection with scrambled siRNA or non-transfected podocytes (control). Representative immunoblots show PKGIα, IRβ and actin expression in homogenates from transfected and non-transfected podocytes (B and E). The effect of downregulation of PKGIα (C) or IRβ (F) on glucose uptake. Uptake was measured after the addition of 1 μCi of [1,2-^{3}H]-deoxy-D-glucose diluted in non-radioactive glucose to a final concentration of 50 μM and 300 nM insulin, 100 μM H_2O_2 or 100 μM 8-Br-cGMP for 3 min. The values are the mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared to control.

in insulin signaling. In conclusion, this study provides evidence that, in podocytes, protein kinase G type I α modulates the insulin signaling pathway and glucose transport.

Acknowledgments

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