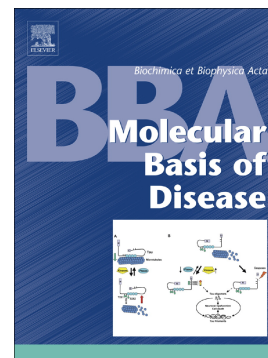


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Metformin overcomes high glucose-induced insulin resistance of podocytes by pleiotropic effects on SIRT1 and AMPK

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Abstract

Podocyte insulin sensitivity is critical for glomerular function, and the loss of appropriate insulin signaling leads to alterations and disorders featuring diabetic nephropathy. Energy-sensing pathways, such as AMP-dependent protein kinase (AMPK) and protein deacetylase SIRT1, have been shown to play an important role in insulin resistance. The absence of a stimulating effect of insulin on glucose uptake into podocytes after exposure to hyperglycemic conditions has been demonstrated to be related to a decreased level and activity of SIRT1 protein, leading to reduced AMPK phosphorylation.

The present work was undertaken to investigate metformin's ability to restore the insulin responsiveness of podocytes by regulating SIRT1 and AMPK activities.

Primary rat podocytes cultured with standard or high glucose concentrations for 5 days were transfected with siRNAs targeting SIRT1, AMPK α 1, or AMPK α 2. SIRT1 activity was measured by a fluorometric method. Insulin-stimulated changes in glucose uptake were used to detect insulin resistance. Podocyte permeability was measured by a transmembrane albumin flux assay to examine podocytes functioning.

Our results demonstrated that metformin activated SIRT1 and AMPK, prevented hyperglycemia-induced reduction of SIRT1 protein levels, ameliorated glucose uptake into podocytes, and decreased glomerular filtration barrier permeability. Furthermore, metformin activated AMPK in a SIRT1-independent manner, as the increase in AMPK phosphorylation after metformin treatment was not affected by SIRT1 downregulation. Therefore, the potentiating effect of metformin on insulin-resistant podocytes seemed to be dependent on AMPK, as well as SIRT1 activity, establishing multilateral effects of metformin action.

Keywords:

Podocyte, metformin, hyperglycemia, insulin resistance, SIRT1, AMPK

1. Introduction

Glomerular visceral epithelial cells (podocytes) play an important role in the development of diabetic nephropathy (DN), a complication associated with diabetes mellitus. These unique, highly specialized, and terminally differentiated cells constitute an essential and integral part of the glomerular filter and its most vulnerable component [1]. Podocytes have been the focus of extensive research on diabetes because of their importance in the development of DN, which affects up to 40% of patients with diabetes mellitus. An estimated 80% of countries will spend 5–13% of their national health expenditures on diabetes [2]. In the early onset of DN, a decreased podocyte number is observed. The end result is increased proteinuria, glomerular basement membrane (GBM) thickening, accumulation of mesangial matrix, and deterioration of renal function. As the disease progresses, more albumin leaks into the urine [3]. The crucial pathological feature of type 2 diabetes mellitus and metabolic syndrome is insulin resistance, often developing as a result of dysregulation of nutrient-responsive systems and disturbance of cellular homeostasis under diabetic conditions. Numerous studies have established that mammalian sirtuins, particularly SIRT1, regulate metabolic responses to changes in nutritional availability in multiple tissues, suggesting a key role of mammalian SIRT1 in the adequate cellular response to metabolic stress events, such as nutrient overload or deprivation [4]. Sirtuins are a family of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases/mono-ADP-ribosyltransferases first characterized in yeast, where they act as histone deacetylases [5]. The deacetylase reaction produces deacetylated protein, nicotinamide (NAM), and 2'-O-acetyl-ADP-ribose (AADPr). SIRT1 activity is regulated by changes in intracellular NAD⁺/NADH ratios [6]. Recent studies offer novel insight into the relationship between SIRT1, insulin resistance, and metabolic syndrome. SIRT1 activity is decreased in many cell types in diabetes, including adipocytes, hepatocytes, and myoblasts [7]. SIRT1 has been shown to play an important role in oxidative stress, mitochondrial dysfunction, and inflammation, which are hallmarks of insulin resistance and type 2 diabetes [8]. Evidence shows that decreased SIRT1 expression may contribute to muscle insulin resistance, as indicated in muscle biopsies obtained from type 2 diabetes patients [9]. Inhibition of SIRT1 induces insulin resistance in cultured insulin-sensitive cells and tissues, whereas SIRT1 activation leads to metabolic improvements, such as enhanced glucose utilization and insulin

sensitivity [10]. SIRT1 can activate AMP-activated protein kinase (AMPK) by deacetylating its upstream kinase LKB1, which promotes LKB1 translocation from the nucleus to the cytosol, where it is activated and phosphorylates and activates AMPK [11, 12]. Similarly, AMPK can activate SIRT1 by increasing the NAD^+/NADH ratio or the expression/activity of nicotinamide phosphoribosyltransferase (Nampt), the rate-limiting enzyme in NAD^+ biosynthesis [13]. Metformin is a biguanide drug commonly used to treat type 2 diabetes, decreasing hyperglycemia mostly by suppressing glucose production and release by the liver and increasing insulin-stimulated glucose uptake by peripheral tissues, such as muscles. Metformin decreases the rate of urine albumin excretion in patients with type 2 diabetes [14] and exerts beneficial effects in patients with impaired renal function [15]. Metformin's mechanism of action has been linked to the activation of AMPK [16]. We have shown that, in podocytes, metformin decreases the production of reactive oxygen species (ROS) through a reduction in NAD(P)H oxidase activity [17]. We have also demonstrated that metformin affects purinergic signaling through inhibition of ecto-ATPase, causing an increase in the extracellular ATP concentration and activation of P2 receptors with subsequent activation of AMPK and reduction in NAD(P)H oxidase activity [18]. Thus, metformin may act via multiple cellular targets to protect cell function. Recently, metformin was reported to indirectly induce hepatic SIRT1 through AMPK-mediated induction of nicotinamide phosphoribosyltransferase (Nampt) [19]. We previously reported that the exposure of podocytes to high glucose (HG) concentrations leads to decreased SIRT1 protein expression and activity, with concomitant reduction in AMPK phosphorylation levels and abolition of the stimulating effect of insulin on glucose uptake, suggesting that this mechanism may be involved in the development of insulin resistance in podocytes cultivated in the presence of HG concentrations, mimicking diabetic conditions [20]. In the current study, we examined the hypothesis that metformin modulates HG-induced insulin resistance in primary rat podocytes via activation of SIRT1 and AMPK.

2. Materials and methods

2.1. Chemicals

Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), with the exception of fetal bovine serum (FBS), which was from Gibco, Invitrogen (Carlsbad, CA, USA). (1,2- ^3H)-

deoxy-D-glucose and reagents for SDS-PAGE were purchased from MP Biochemicals (Solon, OH, USA), with the exception of the protein standard (Bio-Rad, Hertfordshire, UK) and protease inhibitor cocktail (Sigma-Aldrich). The reagents for small interfering RNA (siRNA) transfection were purchased from Santa Cruz Biotechnology. The Fluor de Lys[®] Fluorescent Assay System was purchased from Enzo Life Sciences (Farmingdale, NY, USA). The NAD⁺/NADH quantification kit was purchased from BioVision (Milpitas, CA, USA). All other reagents were purchased from Sigma-Aldrich.

2.2. *Antibodies*

Anti-SIRT1 and anti-phospho-AMPK α (Thr¹⁷²) were from Cell Signaling Technology (Danvers, MA, USA); anti-actin was from Sigma-Aldrich; anti-AMPK α 1, anti-AMPK α 2, and alkaline phosphatase–conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-Nampt (PBEF) was from Abcam (Cambridge, UK). Secondary antibody conjugated with Alexa Fluor 488 was from Thermo Fisher Scientific (Rockford, IL, USA).

2.3. *Animals*

Experiments were conducted using primary cultures of rat podocytes and rats in accordance with EU Directive 2010/63/EU for animal experiments and a protocol approved by the Local Ethics Committee of the Medical University of Gdansk, Poland (No.3/2012). Female Wistar rats weighing 140 g were used for primary cultures of podocytes. Animals were maintained on a 12 hour light/dark cycle with free access to a regular pellet diet and tap water.

2.4. *Preparation and culture of rat podocytes*

Podocyte isolation was performed as described previously [21]. Briefly, female Wistar rats were anesthetized with 95 mg ketamine per kg body weight plus 5 mg xylazine per kg body weight *i.p.*. The final suspension of glomeruli in standard medium (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin) was obtained after excision, mincing, and pressing of the kidneys through a system of sieves with decreasing pore diameter (160, 106, and 53 μ m). Glomeruli were plated in 75 cm² 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 6 days. Outgrowing podocytes were trypsinized and the remaining glomerular cores removed with nylon mesh

(33- μ m pore size). Cells were seeded in 24-well plates or culture flasks and cultivated for 4–7 days. Experiments were performed with podocytes that had been cultured for no more than 20 days. The phenotype of the podocytes was determined as described previously [22] based on immunodetection methods using podocyte-specific antibodies against nephrin (ProSci Inc., Poway, CA, USA), podocin (Sigma-Aldrich), and podocalyxin (Sigma-Aldrich). Podocyte characteristics were maintained by all examined cells during incubation. Before experiments, cells were exposed for 5 days to standard glucose (SG) medium (RPMI 1640, 10% FBS, and 11.1 mM D-glucose) or HG medium (RPMI 1640, 10% FBS, and 30 mM D-glucose). L-glucose was used as an osmotic control for 30 mM D-glucose. AMPK activity in podocytes was modulated by its activator: metformin (2 mM, 5 days) or inhibitor: compound C (0.1 mM, 2 hours) [16].

2.5. *Measurement of glucose uptake*

Glucose uptake was measured as described previously [23] by the addition of 1 μ Ci/well of (1,2- 3 H)-deoxy-D-glucose diluted in non-radioactive glucose at a final concentration of 50 μ M with or without 300 nM insulin for 3 min. The plates were placed on ice and experimental medium removed from the cell layer to determine extracellular radioactivity. The cells were washed three times with ice-cold PBS and lysed by shaking in 0.05 M NaOH for 90 min at room temperature to measure intracellular radioactivity by liquid scintillation counting using MicroBeta2 Microplate Counter (Perkin Elmer, Waltham, MA, USA).

2.6. *Western blotting*

Podocytes were scraped into lysis buffer (1% Nonidet P-40, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 10% glycerol, and protease inhibitor cocktail). Samples were clarified by centrifugation at $9500 \times g$ for 20 min. Supernatants containing equal amounts of protein (20 μ g, measured by the Lowry method) were loaded onto a 10% SDS-PAGE gel, electrophoresed, and transferred onto a PVDF membrane. The membrane was blocked with 3% fat-free milk in Tris-buffered saline (20 mM Tris-HCl, 140 mM NaCl, and 0.01% NaN_3) and incubated overnight with primary antibodies. Next, secondary alkaline phosphatase-conjugated antibodies were used and proteins visualized by a staining reaction performed in the presence of 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue

tetrazolium. Densitometric quantification of bands was performed using Quantity One software (Bio-Rad).

2.7. Immunofluorescence

Podocytes were seeded on type-I collagen coated cover slips (Becton Dickinson Labware) and cultured in SG medium. Cells were immunostained according to a previously described protocol [22]. Single staining was achieved by incubating cells with primary mouse anti-SIRT1 antibody (1:50) and Alexa Fluor 488-conjugated secondary anti-mouse antibody (1:1000). Negative control cells were stained without primary antibodies. Proteins were visualized using a confocal laser scanning microscope (Leica SP8X) with a 63× lens. The exposure time and all other settings (gain, gamma, and intensity of the excitation) were the same in all cases, including the negative controls.

2.8. siRNA transfection

Podocytes were transfected with siRNA according to the manufacturer's instructions and as described previously [21]. Briefly, SIRT1 siRNA or non-silencing (scrambled) siRNA was diluted in Transfection Medium at a final concentration of 80 nM, mixed with siRNA Transfection Reagent, incubated for 30 min at room temperature, and added to the cells. After 7 h of incubation, an equal volume of RPMI 1640 supplemented with 2-fold higher serum and antibiotic concentrations was added for another 24 h. After transfection, cells were cultivated in SG medium for 5 days. Gene silencing was monitored at the protein level by Western blotting.

2.9. SIRT1 activity assay

SIRT1 activity was measured using the Fluor de Lys[®] Fluorescent Assay System according to the manufacturer's protocol. The procedure consisted of two reactions: 1) deacetylation of the Fluor de Lys[®]-SIRT1 Substrate (fluorogenic peptide encompassing residues 379-382 of p53, acetylated on lysine 382 coupled to an aminomethylcoumarin moiety) in the presence of cell lysate and 2) NAD⁺ sensitized the substrate to Fluor de Lys[®] Developer II (proteolytic factor), generating fluorescent aminomethylcoumarin. Fluorescence was measured using an LS-50B fluorescence spectrometer (Perkin Elmer, Waltham, MA, USA) at excitation and emission wavelengths of 360 and 450 nm, respectively. The reaction mixture without Fluor de Lys[®]-SIRT1 Substrate and NAD⁺ was used to

determine a background value. The amount of deacetylated product was quantified by comparisons to Fluor de Lys[®] Deacetylated Standard. Assays were performed in triplicate.

2.10. Determination of NAD⁺/NADH ratios

NAD⁺/NADH ratios were measured using the NAD⁺/NADH Quantification Colorimetric Kit (BioVision, Inc., Milpitas, CA, USA) as described previously [20]. Briefly, the NAD Cycling Enzyme Mix specifically recognizing NAD and NADH in an enzyme cycling reaction was used. Cell lysates were filtered through filters with a 10 kDa cut-off to remove enzymes that rapidly consume NADH. Total NAD (NAD and NADH) and NADH was quantified by comparisons to standard NADH.

2.11. Permeability assay

Transepithelial permeability to albumin was examined by measuring the diffusion of FITC-labeled BSA across the podocyte monolayer as described previously [24]. Briefly, podocytes were seeded on type IV collagen-coated cell culture inserts with a 3- μ m membrane pore size and placed in 24-well plates. Cells were used for experiments between 7 and 15 days post-seeding. Before experiments, podocytes were washed twice with PBS and medium on both sides of the insert, and the medium was replaced with serum-free RPMI 1640 (SFM). After 2 h, the medium in the upper compartment was replaced with fresh SFM, and the medium in the lower compartment was replaced with SFM containing 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 determined by measuring the absorbance at 490 nm using an ELx808 Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.12. Statistical analysis

All data are expressed as mean \pm SEM. Quantitative comparisons were analyzed using ANOVA followed by the Student–Newman–Keuls multiple comparison test. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of metformin on SIRT1 deacetylase activity and protein expression in podocytes

To evaluate the effect of metformin on SIRT1 enzymatic activity and protein levels in podocytes, cells were incubated in HG medium (30 mM glucose) and compared to cells cultured under control conditions in SG medium (11.1 mM glucose). Metformin increased SIRT1 activity in SG-cultured

podocytes by 16% (24.4 ± 0.7 vs. 18.5 ± 0.9 nmol of deacetylated product/mg protein, $P < 0.05$) and restored SIRT1 deacetylase activity in podocytes exposed to HG concentrations (47% increase, from 14.5 ± 0.7 to 21.3 ± 1.2 nmol of deacetylated product/mg protein, $P < 0.05$; Fig. 1A). In podocytes cultured in SG medium, treatment with compound C moderately decreased SIRT1 activity (11%, 16.1 ± 0.8 vs. 18.5 ± 0.9 nmol of deacetylated product/mg protein, $P < 0.05$) with no additional effect on deacetylase activity in podocytes incubated in the presence of HG concentrations. HG decreased SIRT1 protein levels by 25% (0.460 ± 0.048 vs. 0.613 ± 0.045 ; $P < 0.05$) compared to cells cultured under SG conditions (Fig. 1B). Metformin treatment increased SIRT1 protein in podocytes exposed to HG by 37% (to 0.648 ± 0.037 , $P < 0.05$). The effect of compound C on SIRT1 protein levels in podocytes cultured under SG or HG conditions was not significant. The degree of AMPK phosphorylation was decreased by 25% (0.570 ± 0.042 vs. 0.749 ± 0.086 ; $P < 0.05$) in the presence of HG concentrations (Fig. 1C). Metformin increased the degree of AMPK phosphorylation in podocytes cultured under SG and HG conditions, from 0.749 ± 0.086 to 1.15 ± 0.09 (54%) and from 0.570 ± 0.042 to 0.993 ± 0.087 (74%, $P < 0.05$), respectively.

3.2. *Effect of metformin on redox state and Nampt protein expression in podocytes*

HG concentrations decreased the NAD^+/NADH ratio by 18% (1.03 ± 0.06 vs. 1.25 ± 0.05 , $P < 0.05$; Fig. 2A). Treatment with metformin resulted in a decreased NAD^+/NADH ratio in podocytes under both SG (0.960 ± 0.036 vs. 1.25 ± 0.05 , $P < 0.05$) and HG (0.747 ± 0.044 vs. 1.03 ± 0.06 , $P < 0.05$) conditions. No significant effect of compound C on the podocyte NAD^+/NADH ratio was observed. The Nampt protein level was not changed in podocytes cultured in HG medium. After treatment with metformin, Nampt protein was increased under both SG and HG conditions, by 19% (0.857 ± 0.057 vs. 0.723 ± 0.053 , $P < 0.05$) and 26% (0.851 ± 0.037 vs. 0.676 ± 0.075 , $P < 0.05$), respectively (Fig. 2B). The Nampt level in cells treated with compound C was not changed.

3.3. *Effects of metformin on SIRT1 and AMPK α subunit levels in podocytes with downregulated expression of SIRT1 or AMPK α 1/AMPK α 2*

Transfection of podocytes with siRNA targeting SIRT1 decreased the protein amount by 27% (0.568 ± 0.036 vs. 0.775 ± 0.026 , $P < 0.05$, Fig. 3A). Metformin increased the level of SIRT1 protein in cells with downregulated SIRT1 expression (74% increase, from 0.568 ± 0.036 to 0.987 ± 0.101 ,

$P<0.05$). A stimulating effect of metformin on the SIRT1 protein level was also observed in podocytes with downregulated AMPK α 1 or AMPK α 2 expression. Metformin increased the level of SIRT1 protein by 30% (from 0.771 ± 0.041 to 1.01 ± 0.05 , $P<0.05$) and 31% (from 0.785 ± 0.070 to 1.03 ± 0.04 , $P<0.05$) in podocytes with downregulated AMPK α 1 and AMPK α 2, respectively. Transfection with AMPK α 1 or AMPK α 2 siRNA resulted in a 30% decrease in protein levels (0.635 ± 0.074 vs. 0.887 ± 0.088 , $P<0.05$) for AMPK α 1 (Fig. 3B), and a 40% decrease (0.525 ± 0.053 vs. 0.898 ± 0.111 , $P<0.05$) for AMPK α 2 (Fig. 3C). No effects of metformin on AMPK α 1 or AMPK α 2 protein levels were observed in podocytes transfected with SIRT1, AMPK α 1, or AMPK α 2 siRNA.

3.4. *Effects of metformin on SIRT1 deacetylase activity and AMPK phosphorylation in podocytes with downregulated expression of SIRT1 or AMPK α 1/AMPK α 2*

After transfection of podocytes with siRNA targeting SIRT1 or AMPK α 1, but not AMPK α 2, a decrease in SIRT1 deacetylase activity was observed (Fig. 4A). Enzyme activity was decreased by 28% (from 21.7 ± 0.6 to 15.7 ± 0.6 , $P<0.05$) and 18% (to 17.2 ± 0.5 nmol of deacetylated product/mg protein, $P<0.05$) for SIRT1 and AMPK α 1 siRNA, respectively. Metformin treatment increased SIRT1 deacetylase activity in SIRT1, AMPK α 1, or AMPK α 2 siRNA transfected cells by 40% (21.5 ± 0.7 vs. 15.7 ± 0.6 , $P<0.05$), 45% (24.9 ± 0.4 vs. 17.2 ± 0.5 , $P<0.05$), and 22% (26.0 ± 0.3 vs. 20.1 ± 1.0 nmol of deacetylated product/mg protein, $P<0.05$), respectively. Transfection of podocytes with SIRT1 and AMPK α 1 siRNA resulted in decreased AMPK phosphorylation (40%, from 0.907 ± 0.046 to 0.547 ± 0.044 and 0.548 ± 0.029 , respectively, $P<0.05$, Fig. 4B). AMPK α 2 siRNA transfection had no effect on AMPK phosphorylation. Metformin significantly increased the phosphorylation level of AMPK, by 45% (from 0.907 ± 0.046 to 1.32 ± 0.06 , $P<0.05$) for podocytes transfected with scrambled siRNA and 127% (from 0.547 ± 0.044 to 1.24 ± 0.11 , $P<0.05$), 113% (from 0.548 ± 0.029 to 1.17 ± 0.03 , $P<0.05$), and 43% (from 0.883 ± 0.068 to 1.26 ± 0.14 , $P<0.05$) for cells transfected with siRNA targeting SIRT1, AMPK α 1, or AMPK α 2, respectively.

3.5. *Intracellular distribution of SIRT1 protein in podocytes treated with metformin*

Immunofluorescence confirmed the presence of SIRT1 in primary rat podocytes (Fig. 5). The staining signal was perinuclear and diffused within the cytoplasm of the cell body and processes of podocytes cultivated under both SG and HG conditions. After metformin treatment there was no change in the

intracellular distribution of SIRT1, which was observed in the podocyte cell body as well as in the foot processes, however the staining signal was less evenly diffused and clusters of granules containing SIRT1 protein were observed within the cytoplasm.

3.6. *Effect of metformin on basal and insulin-dependent glucose uptake into podocytes with downregulated expression of SIRT1 or AMPK α 1/AMPK α 2*

The effect of metformin on basal and insulin-stimulated glucose uptake into podocytes with SIRT1 and AMPK α 1/AMPK α 2 expression downregulated by appropriate siRNAs was compared to cells transfected with scrambled siRNA. Control cells were insulin responsive, and we observed an insulin-dependent increase in glucose uptake of 37% (2.69 ± 0.14 vs. 1.97 ± 0.07 nmol/min/mg protein, $P < 0.05$, (Fig. 6A). In contrast, SIRT1 siRNA and AMPK α 1/AMPK α 2 siRNA transfection abolished the insulin effect on glucose uptake (Fig. 6B-D). After metformin treatment, an increase in glucose uptake was observed in scrambled, SIRT1, and AMPK α 1/AMPK α 2 siRNA-transfected cells, with an increase of 50% (2.93 ± 0.35 vs. 1.97 ± 0.07 , $P < 0.05$), 30% (2.54 ± 0.11 vs. 2.00 ± 0.14 , $P < 0.05$), 31% (2.82 ± 0.24 vs. 2.15 ± 0.10 , $P < 0.05$), and 39% (2.83 ± 0.20 vs. 2.04 ± 0.12 nmol/min/mg protein, $P < 0.05$), respectively. Similar effects were observed in non-transfected cells cultured under SG or HG conditions (Fig. 6E and F). Insulin stimulated glucose uptake in cells cultured in SG medium, from 1.86 ± 0.17 to 2.70 ± 0.31 nmol/min/mg protein ($P < 0.05$), but had no stimulating effect on glucose uptake into cells exposed to HG conditions. Metformin increased glucose uptake into cells cultivated in SG or HG medium (from 1.86 ± 0.17 to 2.81 ± 0.25 nmol/min/mg protein, $P < 0.05$ and from 1.97 ± 0.12 to 3.03 ± 0.45 nmol/min/mg protein, $P < 0.05$, respectively). An additional corroborant effect of metformin on insulin-dependent glucose uptake was not observed.

3.7. *Effect of metformin on podocyte permeability to albumin after downregulation of SIRT1 protein expression*

HG concentrations increased podocyte permeability to albumin by 37% (122 ± 7 vs. 88.9 ± 5.0 μ g/ml, $P < 0.05$) compared to cells cultivated in SG medium (Fig. 7A). Metformin significantly sealed the podocyte monolayer, decreasing its permeability to albumin by 40% (53.0 ± 5.7 vs. 88.9 ± 5.0 μ g/ml, $P < 0.05$) and 46% (65.3 ± 6.3 vs. 122 ± 7 μ g/ml, $P < 0.05$) under SG and HG conditions, respectively. The albumin permeability of cells cultured in SG medium was increased after treatment with AMPK

inhibitor (20%, 90.6 ± 2.9 vs. 75.6 ± 2.2 $\mu\text{g/ml}$, $P < 0.05$, Fig. 7B), with no further effect of compound C on the permeability of HG-cultivated podocytes.

We evaluated the effect of SIRT1 protein downregulation on podocyte permeability and found that, in cells transfected with SIRT1 siRNA and cultured under SG conditions, the permeability to albumin was increased by 25% (109 ± 4 vs. 87.5 ± 6.3 $\mu\text{g/ml}$, $P < 0.05$, Fig. 7C). Metformin treatment improved podocyte permeability with a decrease of 33% (58.5 ± 1.6 vs. 87.5 ± 6.3 $\mu\text{g/ml}$, $P < 0.05$) and 15% (93.3 ± 8.2 vs. 109 ± 4 $\mu\text{g/ml}$, $P < 0.05$) for scrambled and SIRT1 siRNA transfected podocytes, respectively.

4. Discussion

Several studies have linked the insulin-signaling pathways with podocyte function. At first, glomerular podocytes were shown to possess a heterogeneous glucose transport system consisting of facilitative glucose transporters (GLUTs), and that the insulin response of podocytes occurs with an increase in PI3K and MAPK signaling, resulting in increased glucose uptake via the facilitative glucose transporters GLUT1 and GLUT4 [22, 25]. Growing evidence indicates that the insulin resistance of podocytes may be an important initiator for many of the pathological processes observed in DN [26, 27].

A previous study revealed that exposure of primary rat podocytes to HG concentrations attenuates the insulin responsiveness of podocytes in association with decreased AMPK phosphorylation levels, resulting in the abolition of insulin-stimulated glucose uptake into these cells [21]. We also demonstrated previously that exposure of podocytes to hyperglycemic conditions increases the albumin permeability of a podocyte monolayer [24] and that HG-mediated induction of insulin resistance in podocytes is associated with downregulation of SIRT1 protein, decreased enzymatic activity, and a subsequent decrease in AMPK phosphorylation [20]. The SIRT1 level positively correlates with insulin sensitivity [28] and is directly or indirectly involved in insulin signaling [29]. SIRT1 downregulation was demonstrated to be involved in the pathogenesis and development of DN, and was correlated with the level of proteinuria [30]. As a major regulator of cellular metabolism, AMPK was also involved in improved insulin sensitivity and glucose homeostasis [31]. Downregulation of AMPK in response to HG exposure was first shown to occur in skeletal muscle

cells [32]. The same effect, with a parallel decrease in SIRT1 activity, was observed in cultured HepG2 cells; SIRT1 inhibitor NAM decreased both SIRT1 and AMPK activity, whereas incubation with quercetin, a SIRT1 activator, increased both activities [33]. These studies led to examining a possible link between SIRT1 and AMPK, finding that AMPK and SIRT1 positively regulate each other and share many common target molecules [34]. Several studies suggest that SIRT1 functions as a regulator of AMPK activity through AMPK upstream LKB1 kinase [35, 36], though AMPK suppression was also shown to be caused by a decrease in SIRT1 activity due to decreased AMPK-mediated Nampt expression [37, 38]. The precise interactions between SIRT1 and AMPK in podocytes remain unclear, but our previous results indicated that the decreased AMPK phosphorylation in HG-cultured cells occurs in a SIRT1-dependent manner. AMPK phosphorylation was reduced in SIRT1-depleted cells and the stimulating effect of insulin on AMPK phosphorylation was suppressed, suggesting that SIRT1 is involved in the regulation of AMPK activity in podocytes [20].

Metformin, a biguanide derivative, is a commonly used drug in the treatment of type 2 diabetes, as it suppresses endogenous glucose output and increases peripheral insulin sensitivity [39, 40]. In recent years, attention has been concentrated on AMPK in the context of metformin's action [16, 41], but data on the activation of SIRT1 by metformin have also been published. Metformin has been reported to activate hepatic SIRT1 through AMPK-mediated induction of Nampt [19]. In the current study, we observed a stimulating effect of metformin on the enzymatic activity of SIRT1 in podocytes. We also demonstrated that HG concentrations do not alter metformin's ability to activate SIRT1 in podocytes. Moreover, metformin prevented hyperglycemia-induced reductions in SIRT1 protein levels in these cells. In parallel, metformin increased AMPK phosphorylation in podocytes incubated in control or hyperglycemic medium. As a result of metformin action, HG-induced impairment of glucose uptake into podocytes was ameliorated. Therefore, despite AMPK activation, increasing SIRT1 protein levels and activity could be an important mechanism by which metformin overcomes insulin resistance in HG-exposed podocytes.

Accumulating data have shown that metformin acts mainly through AMPK, increasing the AMP/ADP and/or ADP/ATP ratios [16]. This effect may be reinforced by a decrease in the cytosolic

NAD⁺/NADH ratio [42, 43]. It was reported that metformin decreased NAD⁺/NADH ratio in liver to inhibit glucose production via a novel direct target, mitochondrial glycerol-3-phosphate dehydrogenase [44]. Decreased AMPK activity and Nampt and SIRT1 expression have also been observed in the white adipose tissue of *db/db* mice, and metformin has been shown to increase AMPK activity and restore Nampt and SIRT1 levels [37]. Our results show that the NAD⁺/NADH ratio decreased in podocytes exposed to hyperglycemic conditions, which is in line with the decreased NAD⁺/NADH ratio observed in mesangial cells grown in HG medium [45]. Metformin diminished the NAD⁺/NADH ratio in podocytes exposed to both SG and HG concentrations. These data are also supported by other studies demonstrating that metformin decreases the intracellular NAD⁺/NADH ratio [46]. Our data suggest that the reduced NAD⁺/NADH ratio in metformin-treated podocytes could result from SIRT1 activation and subsequent NAD⁺ consumption. The Nampt protein level was increased in podocytes treated with metformin, presumably to compensate for the NAD⁺ deficit. Our study demonstrated that the stimulatory effects of metformin on SIRT1 protein level and activity were not affected by transfection with SIRT1 siRNA. Metformin restored the decreased SIRT1 protein levels resulting from transfection and activated SIRT1 in cells transfected with scrambled or SIRT1 siRNA. SIRT1 protein expression did not change after downregulation of AMPK catalytic subunits AMPK α 1 or AMPK α 2. However, SIRT1 enzymatic activity was decreased in AMPK α 1-depleted podocytes, suggesting that crosstalk between SIRT1 and AMPK prefers the AMPK α 1 isoform. In contrast, SIRT1-AMPK crosstalk has not been shown to favor either AMPK isoform in skeletal muscle cells [47]. The same research group provided evidence of preferential activation of AMPK α 1 by metformin, but metformin also significantly increased AMPK phosphorylation and the activities of both AMPK α 1 and AMPK α 2 in skeletal muscle cells, where AMPK activation is associated with increased rates of glucose uptake [48]. We found that the degree of AMPK phosphorylation is significantly reduced in podocytes with suppressed expression of SIRT1. Our results are in agreement with those obtained for retinal capillary endothelial cells with SIRT1 knocked down, which have reduced AMPK activation [49]. Our results suggest that metformin activates AMPK independent of SIRT1, because the increase in the degree of AMPK phosphorylation after metformin treatment was not affected by SIRT1 downregulation. A similar effect of metformin was observed in HepG2 cells

[50]. We found that metformin-induced activation of AMPK in podocytes occurred regardless of the AMPK α 1/AMPK α 2 protein level. AMPK α 1 and AMPK α 2 have different expression patterns in various tissues, and they have been shown to control different metabolic actions [51]; thus, the cellular effects of AMPK activation may depend on whether the AMPK α 1 or AMPK α 2 isoform is stimulated. Notably, several metabolic actions of metformin have been demonstrated to occur independent of AMPK activation, such as the downregulation of glucose-6-phosphatase expression and inhibition of respiratory complex I, or are mediated by p38 MAPK- and PKC-dependent mechanisms [52, 53]. Our previous results revealed that, in podocytes, downregulation of AMPK α 1 or AMPK α 2 protein expression abolishes the stimulating effect of insulin on glucose uptake, suggesting that both isoforms play an important role in increasing glucose uptake in response to insulin stimulation. We demonstrated that one subunit is not sufficient to maintain insulin effects in podocytes when the second subunit is attenuated [21]. The current study confirmed our previous results, and we also demonstrated that metformin is able to increase glucose uptake into podocytes with downregulated AMPK α 1 or AMPK α 2 protein expression. We confirmed that SIRT1 downregulation results in the suppression of insulin action on glucose uptake into podocytes, and demonstrated that metformin treatment reverses this effect. Similar results were observed in SIRT1-depleted adipocytes. SIRT1 knockdown inhibited insulin-stimulated glucose transport, and treatment with activators of SIRT1 increased the insulin effect on glucose uptake [54].

Our previous study demonstrated that hyperglycemia increases the albumin permeability of podocytes due to activation of the Nox4 subunit of NADPH oxidase and dimerization of PKG1 α in podocytes [24]. Because we demonstrated that SIRT1-AMPK crosstalk is involved in HG-dependent impairment of insulin responsiveness in podocytes [20], we examined the albumin permeability of podocytes in cells depleted of SIRT1 and showed that the decrease in SIRT1 protein levels in podocytes may contribute to increased albumin leakage through the glomerular filtration barrier observed in DN. Our results reveal that metformin treatment improved the glomerular filtration barrier tightness, decreasing the albumin permeability of podocytes with downregulated SIRT1 protein expression, which may be associated with restoration of the SIRT1 protein level and increase in its activity. SIRT1 activation by

resveratrol was shown to attenuate DN, decreasing HG-induced VEGF expression in podocytes and its secretion in the cultured podocyte media, an effect that was attenuated by knocking down SIRT1 [55]. In summary, the present study suggests that metformin activated SIRT1 and AMPK in podocytes via different signaling pathways. The degree of AMPK phosphorylation was related to SIRT1 activity, as it decreased after SIRT1 downregulation by siRNA. However, metformin presumably activated AMPK independent of SIRT1; the increase in AMPK phosphorylation after metformin treatment was not affected by SIRT1 downregulation. Potentiation of the effect of metformin treatment on insulin-dependent glucose uptake in podocytes seemed to be dependent on AMPK, as well as SIRT1 activity, establishing metformin as a compound with pleiotropic effects of action. Metformin simultaneously activating SIRT1 and AMPK may improve the insulin resistance of podocytes and prevent diabetes-related complications.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

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Figure legends

Fig. 1. Effects of metformin (MTF) on SIRT1 deacetylase activity and protein amount in primary rat podocytes. (A) Cells were cultured in the presence of standard glucose (SG) or high glucose (HG) and treated with MTF or compound C (CC) to measure SIRT1 deacetylase activity or (B) SIRT1 protein expression. (C) The degree of AMPK phosphorylation was determined under the same conditions. Representative immunoblots for quantitative densitometric analysis of SIRT1 protein expression (panel B) or AMPK phosphorylation (panel C) are included. Actin was used as a loading control. Values are presented as mean \pm SEM (n=4–6). *P<0.05 vs. SG, **P<0.05 vs. HG.

Fig. 2. The effect of metformin (MTF) and compound C (CC) on redox state and Nampt protein expression in podocytes cultured in the presence of standard glucose (SG) or high glucose (HG). (A) Measurement of NAD⁺/NADH. (B) To determine Nampt protein levels, densitometric quantification of the corresponding bands was performed and expressed as the ratio to actin. Values are presented as mean \pm SEM (n=5). *P<0.05 vs. control in SG, **P<0.05 vs. control in HG.

Fig. 3. Effects of metformin (MTF) on SIRT1 and AMPK α subunit (AMPK α 1 and AMPK α 2) expression in podocytes transfected with small-interfering RNA targeting SIRT1 or AMPK α 1/AMPK α 2. After transfection, cells were cultivated in standard glucose (SG) medium in the presence of MTF. (A) SIRT1, (B) AMPK α 1, and (C) AMPK α 2 proteins were quantified relative to actin. (D) Representative immunoblots for SIRT1 and AMPK α 1/AMPK α 2 protein expression analysis by quantitative densitometry. Values are presented as mean \pm SEM (n=6). *P<0.05 vs. scrambled siRNA control, **P<0.05 vs. appropriate control.

Fig. 4. Effects of metformin (MTF) on SIRT1 deacetylase activity and degree of AMPK phosphorylation in podocytes transfected with small-interfering RNA targeting SIRT1 or AMPK α 1/AMPK α 2. After transfection, cells were cultivated in standard glucose (SG) medium in the presence of MTF. (A) SIRT1 deacetylase activity. (B) degree of AMPK phosphorylation in podocytes transfected with SIRT1 siRNA or AMPK α 1/AMPK α 2 siRNA. (C) Representative immunoblots for AMPK phosphorylation analysis by quantitative densitometry. Blotting was performed with an anti-

actin antibody as a protein loading control. Values are presented as mean \pm SEM (n=5-6). *P<0.05 vs. scrambled siRNA control, **P<0.05 vs. appropriate control.

Fig. 5. Changes in the intracellular localization of SIRT1 after metformin (MTF) treatment. Confocal imaging of the immunofluorescent distribution of anti-SIRT1 reactivity in podocytes cultivated in standard glucose (SG) or high glucose (HG) medium in the presence of MTF.

Fig. 6. The effect of metformin (MTF) on insulin-dependent glucose uptake into podocytes with downregulated SIRT1, AMPK α 1, or AMPK α 2 protein expression. (A) Cells were transfected with scrambled small-interfering RNA, (B) siRNA targeting SIRT1, (C) siRNA targeting AMPK α 1, or (D) siRNA targeting AMPK α 2. After transfection, cells were cultivated in standard glucose (SG) medium. (E) Non-transfected cells were incubated in SG or (F) high glucose (HG) medium in the presence of MTF. Glucose uptake measurements began with the addition of 1 μ Ci of (1,2- 3 H)-deoxy-D-glucose diluted in non-radioactive glucose to a final concentration of 50 μ M and 300 nM insulin. Glucose uptake was measured for 3 min. Values are presented as mean \pm SEM (n=8). *P<0.05 vs. basal glucose uptake.

Fig. 7. The effect of metformin on albumin permeability across a podocyte monolayer. (A) Podocytes were cultivated in standard glucose (SG) or high glucose (HG) medium in the presence of metformin (MTF) or (B) compound C (CC). (C) Cells were transfected with scrambled siRNA RNA or SIRT1 siRNA, and then cultivated in SG medium in the presence of MTF. Results are presented as mean \pm SEM (n=4-6). *P<0.05 vs. control in SG; **P<0.05 vs. control in HG.

Fig. 8. Proposed mechanism of metformin action in restoring insulin responsiveness of podocytes exposed to high glucose concentration.

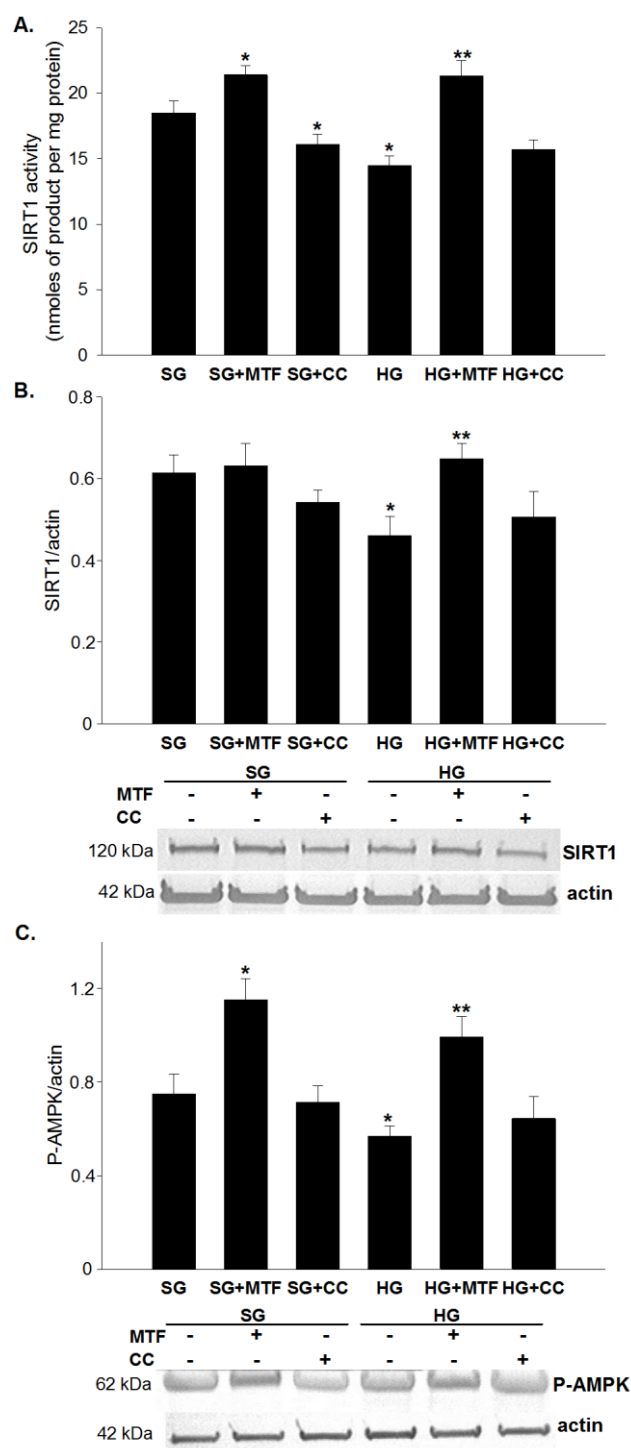


Figure 1

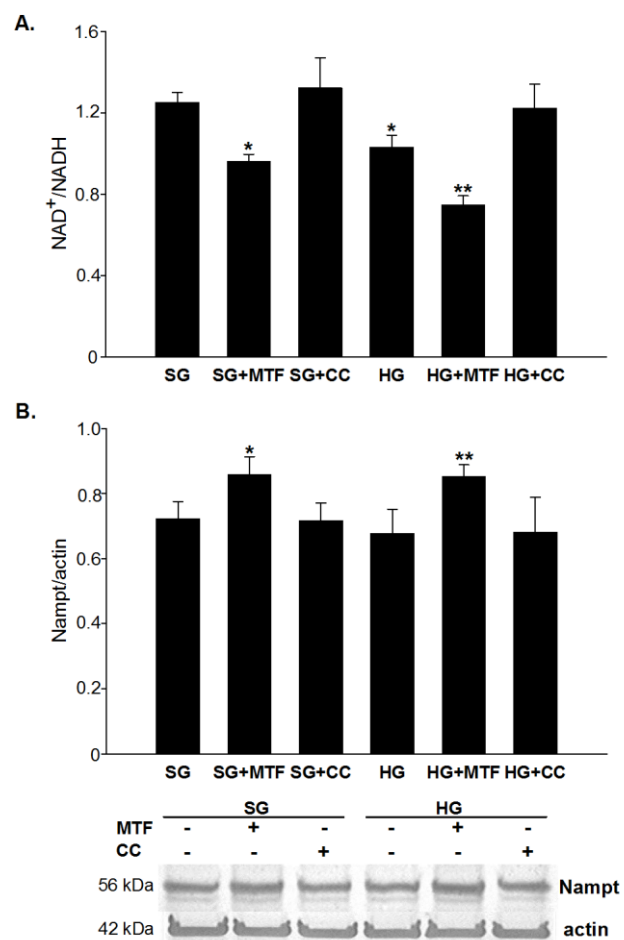


Figure 2

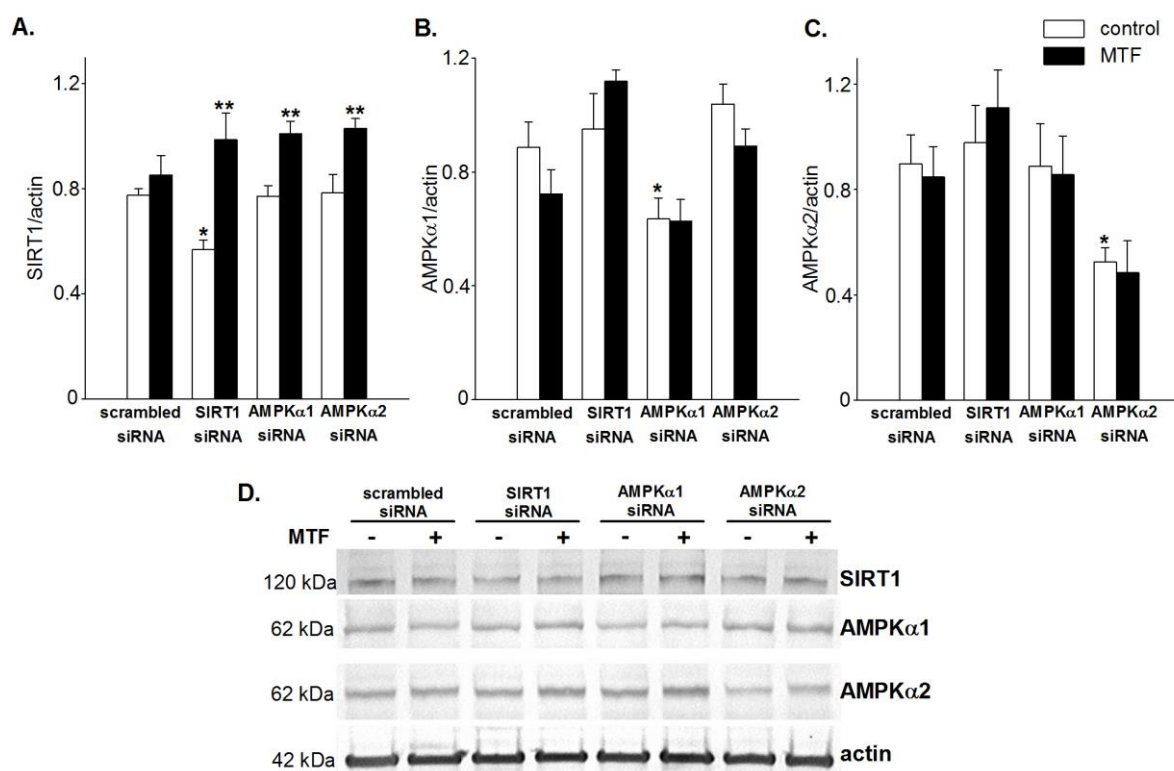


Figure 3

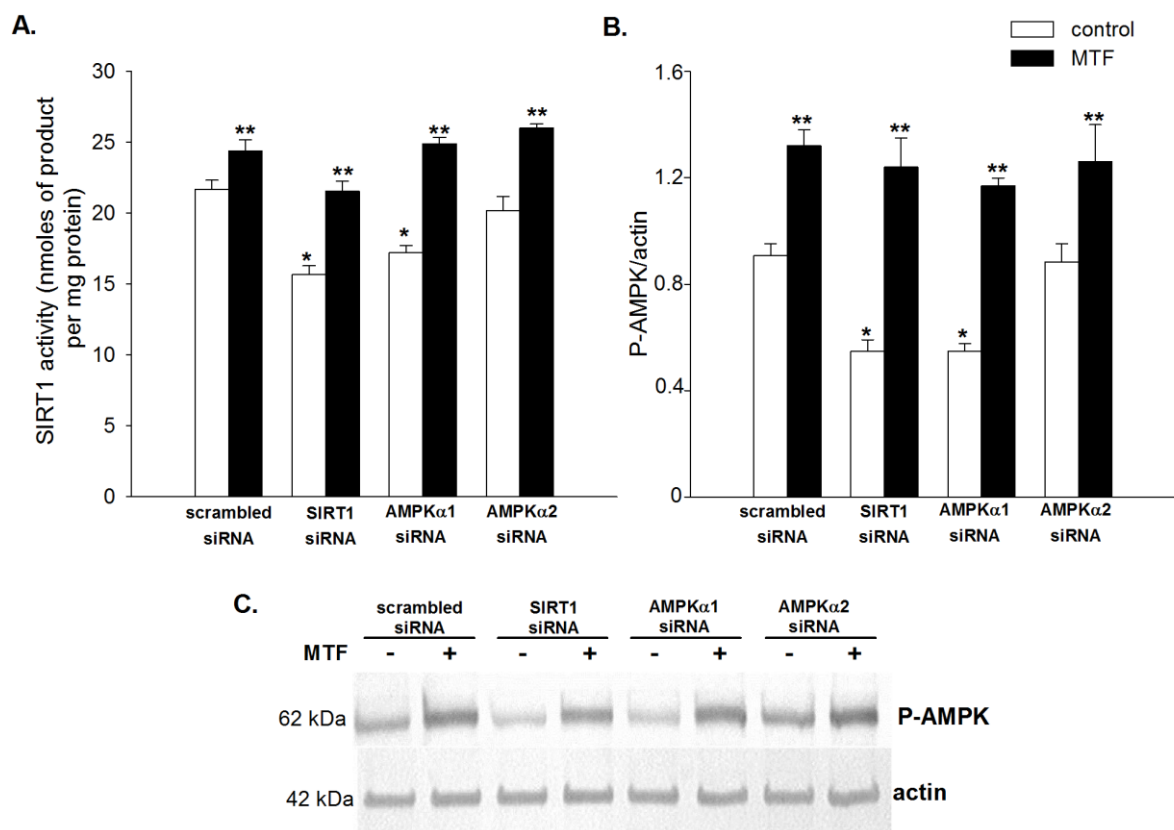


Figure 4

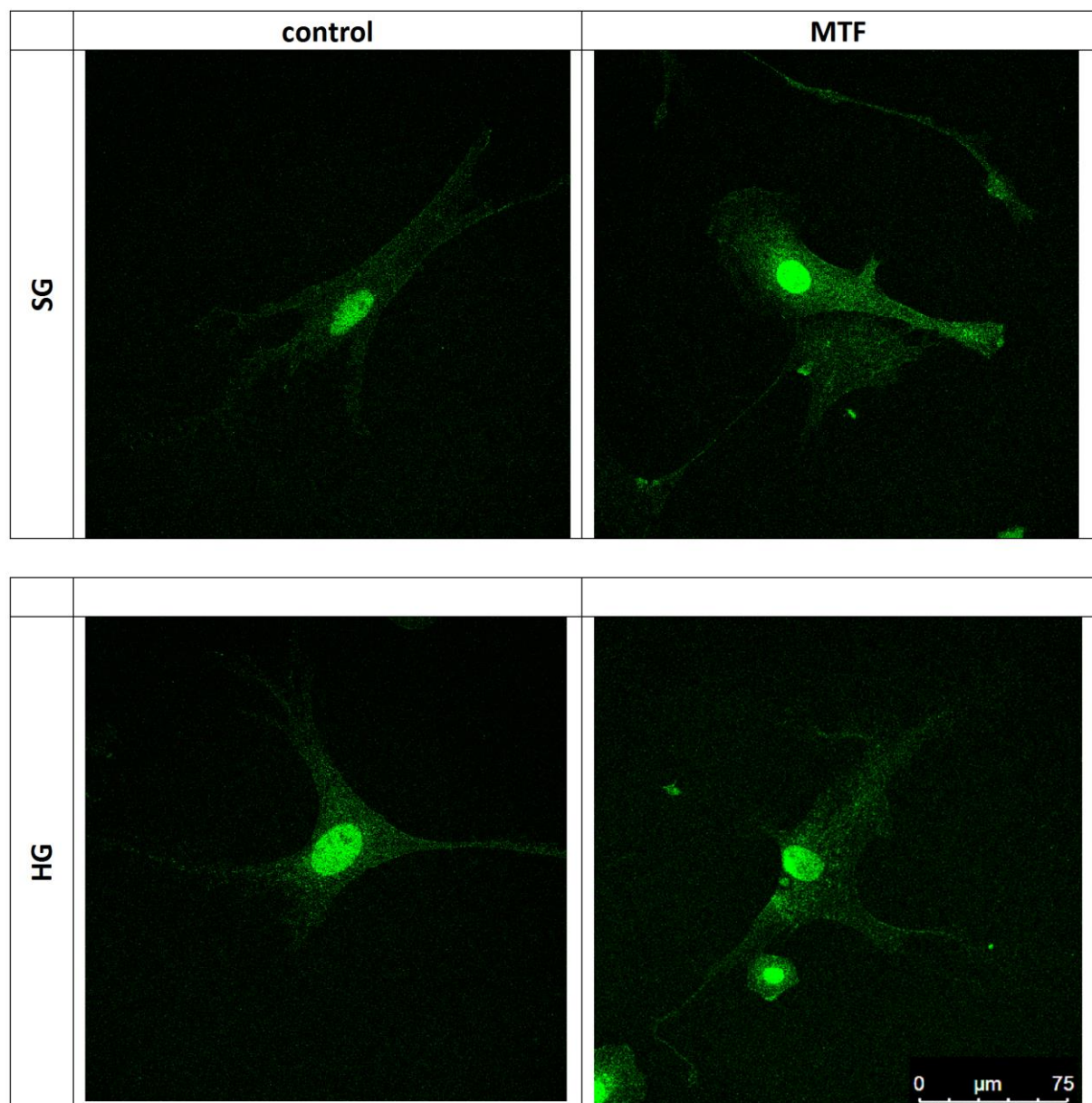


Figure 5

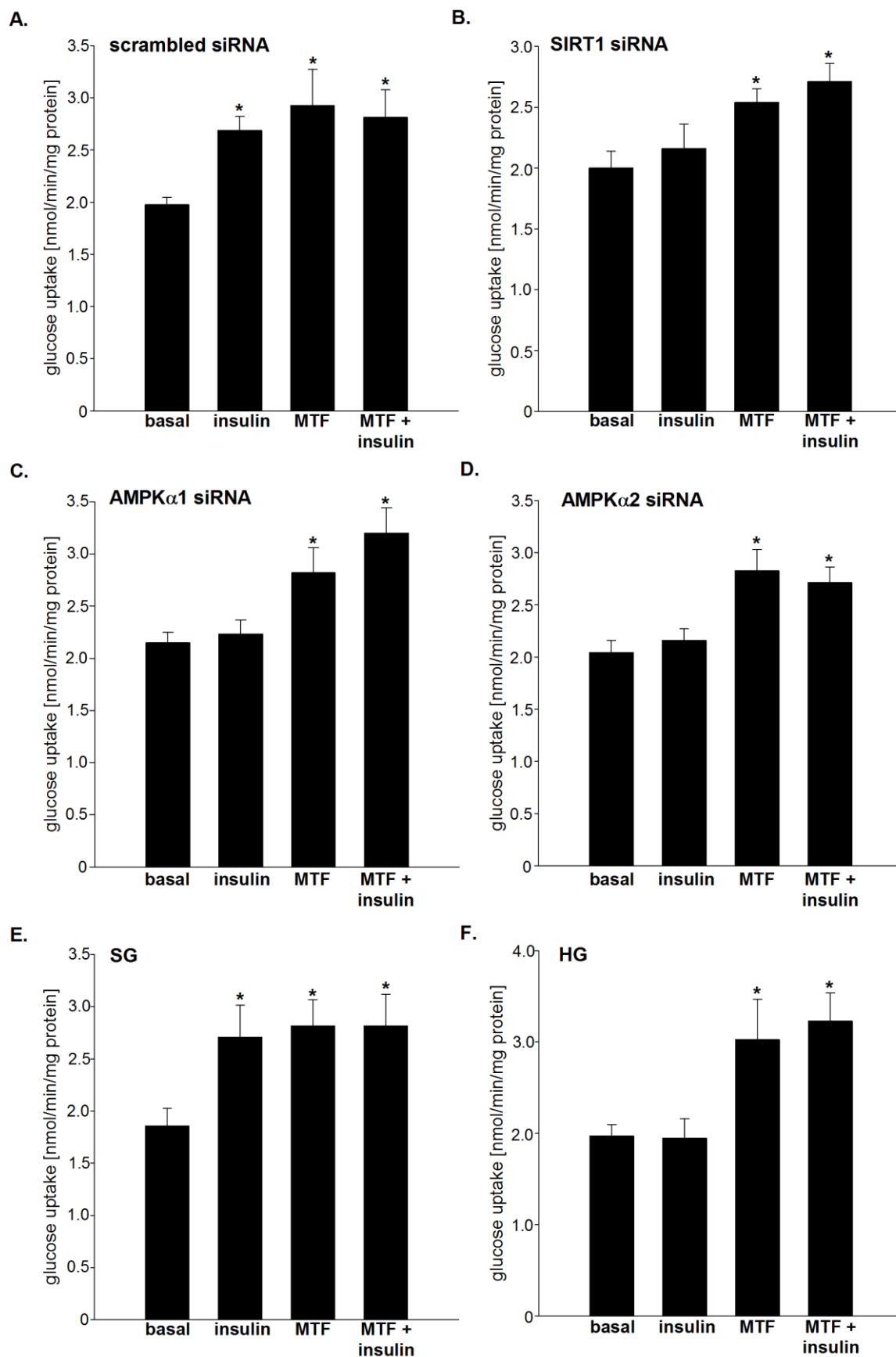


Figure 6

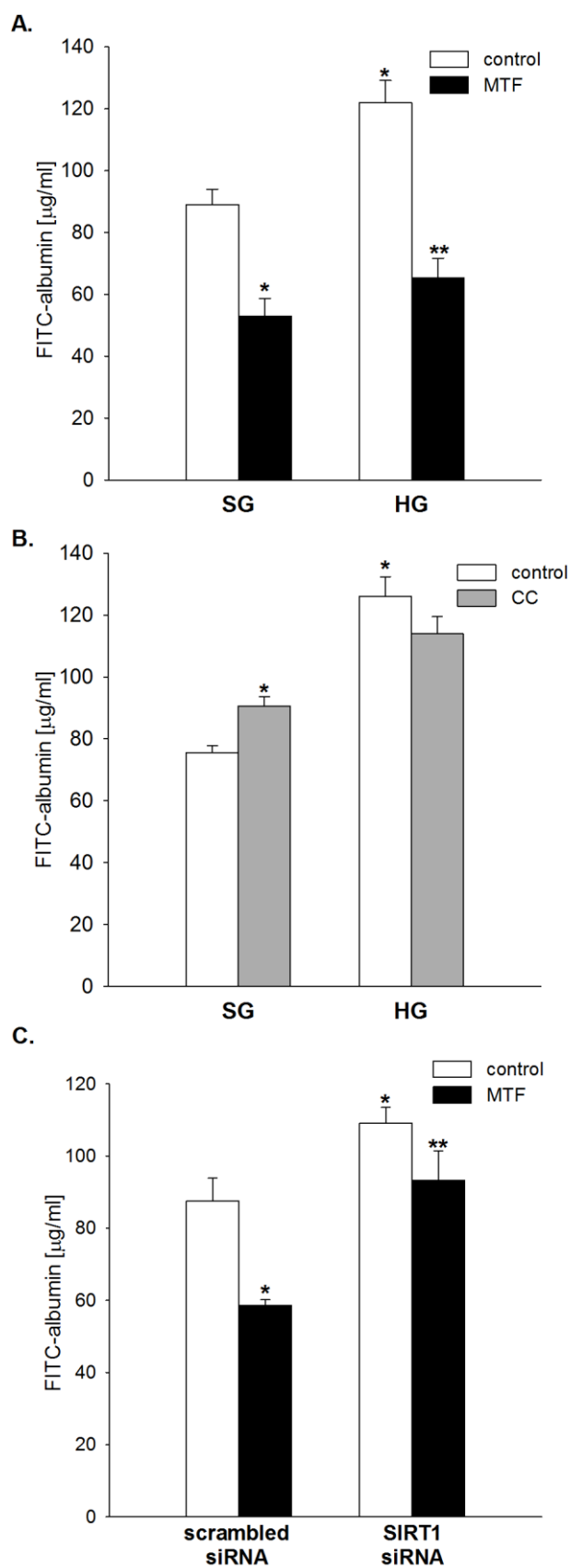


Figure 7

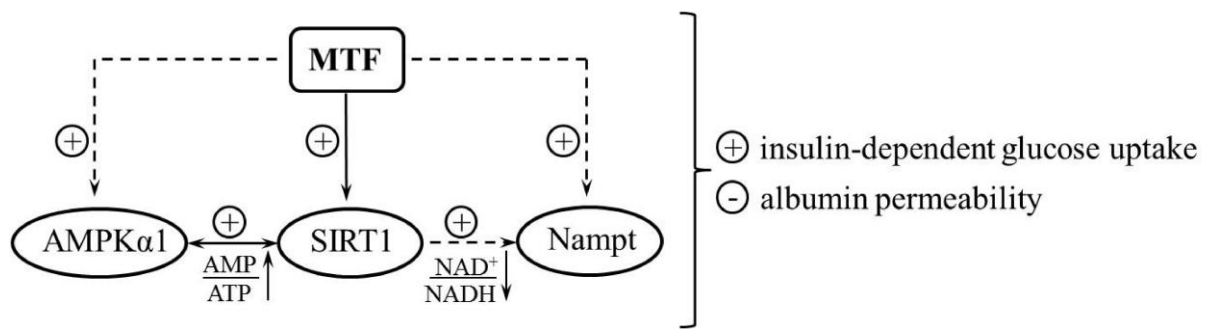


Figure 8

Highlights

- Metformin prevents hyperglycemia-reduced SIRT1 protein level
- SIRT1 activity in podocytes is increased after metformin treatment
- Metformin improves high glucose-induced impairment of glucose uptake into podocytes
- SIRT1 activity is decreased in AMPK α 1- but not AMPK α 2-depleted podocytes
- Metformin overcomes insulin resistance by multilateral effects on SIRT1 and AMPK