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Ezrin Is Down-Regulated in Diabetic Kidney Glomeruli and Regulates Actin Reorganization and Glucose Uptake via GLUT1 in Cultured Podocytes

Q35 Anita A. Wasik,* Susanna Koskelainen,* Mervi E. Hyvönen,*† Luca Musante,* Eero Lehtonen,*‡ Kerttu Koskenniemi,§ Jukka Tienari,¶ Antti Vaheri,‖ Dontscho Kerjaschki,** Csaba Szalay,†† Csaba Révész,†† Pekka Varmanen,‡‡ Tuula A. Nyman,§§ Peter Hamar,†† Harry Holthöfer,¶¶ and Sanna Lehtonen*

Q1 Q2 Q3 *From the Departments of Pathology,* Virology,‖ and Bacteriology and Immunology, ¶¶ the Haartman Institute, the Children's Hospital,† the Laboratory Animal Centre,‡ the Departments of Veterinary Biosciences§ and Food and Environmental Sciences,‡‡ and the Institute of Biotechnology,§§ University of Helsinki, Helsinki, Finland; the Department of Pathology,¶ HUSLAB and Helsinki University Hospital, Helsinki and Hyvinkää, Finland; the Clinical Institute of Pathology,** Medical University of Vienna, Vienna, Austria; and the Institute of Pathophysiology,†† Semmelweis University, Budapest, Hungary*

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Q6 Address correspondence to Sanna Lehtonen, Department of Pathology, Haartman Institute, University of Helsinki, PO Box 21, 00014 Helsinki, Finland. E-mail: sanna.h.lehtonen@helsinki.fi.

Diabetic nephropathy is a complication of diabetes and a major cause of end-stage renal disease. To characterize the early pathophysiological mechanisms leading to glomerular podocyte injury in diabetic nephropathy, we performed quantitative proteomic profiling of glomeruli isolated from rats with streptozotocin-induced diabetes and controls. Fluorescence-based two-dimensional difference gel electrophoresis, coupled with mass spectrometry, identified 29 differentially expressed spots, including actin-binding protein ezrin and its interaction partner, NHERF2, which were down-regulated in the streptozotocin group. Knockdown of ezrin by siRNA in cultured podocytes increased glucose uptake compared with control siRNA-transfected cells, apparently by increasing translocation of glucose transporter GLUT1 to the plasma membrane. Knockdown of ezrin also induced actin remodeling under basal conditions, but reduced insulin-stimulated actin reorganization. Ezrin-dependent actin remodeling involved cofilin-1 that is essential for the turnover and reorganization of actin filaments. Phosphorylated, inactive cofilin-1 was up-regulated in diabetic glomeruli, suggesting altered actin dynamics. Furthermore, IHC analysis revealed reduced expression of ezrin in the podocytes of patients with diabetes. Our findings suggest that ezrin may play a role in the development of the renal complication in diabetes by regulating transport of glucose and organization of the actin cytoskeleton in podocytes. *(Am J Pathol 2014, ■: 1–13; http://dx.doi.org/10.1016/j.ajpath.2014.03.002)*

Diabetic nephropathy, one of the major complications of diabetes, develops in approximately one third of patients with diabetes and is the most common cause of renal Q7 replacement therapy worldwide.[1] The first sign of kidney injury is microalbuminuria that develops into overt albuminuria as nephropathy progresses. Histopathological hallmarks of diabetic nephropathy include mesangial expansion and glomerular sclerosis. By electron microscopy, podocyte foot process abnormalities and glomerular basement membrane thickening are observed.

Despite many studies, the pathophysiological features of diabetic nephropathy are still not completely understood. In patients with type 1 or type 2 diabetes, insulin resistance is associated with microalbuminuria.[2,3] Recent studies also indicate that podocyte dysfunction plays an important role in the development of diabetic nephropathy.[4] Interestingly, insulin signaling in podocytes is essential to maintain normal glomerular ultrafiltration, because deletion of the insulin receptor specifically from podocytes leads, under

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normoglycemic conditions, to albuminuria and histological changes resembling diabetic nephropathy.[5] In response to insulin, podocytes absorb glucose via glucose transporters GLUT4 and GLUT1,[6] and furthermore, treatment of cultured podocytes with insulin induces rapid remodeling of the actin cytoskeleton.[5] In many types of glomerular diseases, the integrity of the actin cytoskeleton in podocytes is altered, indicating that proper organization and regulation of the actin cytoskeleton are essential for podocyte structure and function.[7] This raises great interest in proteins that function in the regulation of dynamic actin organization as potential therapeutic targets in the treatment of glomerular diseases, including diabetic nephropathy.

To better understand the pathogenesis of diabetic nephropathy, we used fluorescence-based two-dimensional difference gel electrophoresis (2D-DIGE), coupled with mass spectrometry, to explore diabetes-induced changes in the protein expression profile of glomeruli isolated from rats with streptozotocin-induced diabetes and controls at an early stage of disease. Proteomic analysis indicated that ezrin, a member of the ezrin-radixin-moesin (ERM) family of actin-binding proteins,[8–10] and its interaction partner NHERF2 were down-regulated in the streptozotocin-treated rats. This is an interesting finding in terms of the maintenance of the proper organization of the actin cytoskeleton in glomerular cells, because ezrin and NHERF2 are important regulators of actin dynamics, and link membrane proteins to the underlying actin cytoskeleton.[8,9] In glomeruli, ezrin is expressed in podocytes,[11,12] and together with NHERF2, it connects the cell surface sialoprotein podocalyxin to actin.[13] Interestingly, an N-terminal fragment of ezrin has been shown to bind to advanced glycation end products in the kidneys of diabetic rats,[14] but the exact role of ezrin in podocyte injury in diabetic nephropathy has remained unclear.

Materials and Methods

Induction of Diabetes in Rats by Streptozotocin

Male Sprague-Dawley rats (Toxi-coop, Dunakeszi, Hungary), weighing 230 ± 10 g, were injected i.p. with 60 mg/kg streptozotocin (Sigma-Aldrich, St. Louis, MO) dissolved in citrate buffer, pH 4.5 (five rats) or with citrate buffer only (five rats). The development of diabetes was confirmed 1 week after streptozotocin injection by measuring postprandial blood glucose with Glucostix using Reflotron Plus Automat (Roche, Budaörs, Hungary), and at sacrifice 4 weeks after streptozotocin injection by oral glucose tolerance test. Urine was collected in diuresis cages, and urinary albumin was measured by a rat albumin-specific enzyme-linked immunosorbent assay kit (Immunology Consultants Laboratory Inc, Portland, OR). Animal procedures were approved by the Animal Care and Use Committee of Semmelweis University (Budapest, Hungary).

Electron Microscopy

Kidney samples were fixed in 1.5% glutaraldehyde, 3% paraformaldehyde, and 5% sucrose in 0.1 mol/L cacodylate buffer, pH 7.4, at room temperature for 2 hours, followed by postfixation in 1% osmium tetroxide in the same buffer for 1 hour. Samples were stained *en bloc* in 1% uranyl acetate in 10% ethanol for 1 hour, dehydrated in ethanol, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEM-1400 Transmission Electron Microscope (Jeol) equipped with a transmission electron microscopy charge-coupled device camera (Olympus Soft Imaging Solutions GmbH).

2D-DIGE Data

Glomeruli were isolated by graded sieving[15] from five individual streptozotocin-injected and five individual citrate-injected rats and lysed in 7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 30 mmol/L Tris-HCl, pH 8.0, and 0.2% SDS, sonicated 3 × 15 seconds, and centrifuged at 13,000 × *g* for 15 minutes. Protein concentrations were measured with the 2D Quant Kit (GE Healthcare, Chalfont St. Giles, UK). Glomerular lysate (50 μg) from rats with streptozotocin-induced diabetes or controls was labeled individually with Cy3 and Cy5, respectively, using CyDye DIGE Fluor minimal labeling kit (GE Healthcare), following the manufacturer's instructions. An internal standard (a pool of all samples) was labeled with Cy2. Isoelectric focusing was performed using linear, pH 3 to 10, 24-cm Immobiline DryStrips (GE Healthcare). The strips were equilibrated in 6 mol/L urea, 2% SDS, 1% dithiothreitol, 30% glycerol, and 75 mmol/L Tris-HCl, pH 8.8, followed by incubation in the same solution, but replacing dithiothreitol with 2.5% iodoacetamide. Proteins were resolved in 12% polyacrylamide gels at 10 W per gel for 16 hours and imaged with Typhoon 9400 (GE Healthcare). A comparison of the images was performed using DeCyder 2D 7.0 software (GE Healthcare). Reference gel was randomly selected from the control gels, and spots from the other gels were matched to those in the reference gel. The intensities of the spots were normalized by dividing each Cy3 or Cy5 spot volume with the corresponding Cy2 (internal standard) spot volume. Normalized intensities of matched spots were compared between the groups, and spots with intensity changes >1.5-fold with a CI >95% (Student's *t*-test analysis of variance analyses; $P < 0.05$) were considered differentially expressed and significant.

Identification of Proteins by LC-MS/MS

Spots of interest were excised from a silver-stained 2D SDS-PAGE gel, in-gel digested with trypsin, and the resulting peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an Ultimate 3000 nano-LC (Dionex, Sunnyvale, CA) and a QSTAR Elite

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hybrid quadrupole time-of-flight MS (Applied Biosystems/MDS Sciex, Life Technologies, Carlsbad, CA) with nano-electrospray ionization, as described previously.[16] The LC-MS/MS data were searched with in-house Mascot through ProteinPilot 2.0 interface against the SwissProt database using the following criteria: rodent-specific taxonomy, trypsin digestion with one missed cleavage allowed, carbamidomethyl modification of cysteine as a fixed modification, and oxidation of methionine as a variable modification. All of the reported protein identifications were statistically significant ($P < 0.05$).

Cell Culture and Preparation of Cell Lysates

Mouse podocytes (kindly provided by Dr. Andrey Shaw, St. Louis, MO) were maintained and cell lysates were prepared in 1% Nonidet P-40 lysis buffer, as previously described.[17]

Antibodies

Antibodies used were mouse anti-ezrin (clone 3C12),[18] rabbit anti−phospho-ezrin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-NHERF2 (kindly provided by Dr. Peijian He, Emory University, Atlanta, GA), mouse anti-podocalyxin,[19] mouse anti-moesin (AbD Serotec, Oxford, UK), rabbit anti−phospho-cofilin-1 (Ser3; Cell Signaling Technology, Danvers, MA), rabbit anti−cofilin-1 and rabbit anti-GLUT4 (Abcam, Cambridge, UK), rabbit anti-GLUT1 (Millipore, Billerica, MA), rabbit anti-podocin, mouse anti-actin, and mouse anti−α-tubulin (Sigma-Aldrich). Actin stress fibers were visualized with tetrarhodamine isothiocyanate−phalloidin (Molecular Probes, Life Technologies, Carlsbad, CA).

Immunoblotting

Glomerular lysates of three individual streptozotocin-injected and three individual control rats were used for confirming the 2D-DIGE results. Glomerular lysates of 12-week-old six individual obese (fa/fa) and six individual lean (fa/+) Zucker rats (Crl:ZUC-Leprfa; Charles River Laboratories, Sulzfeld, Germany) were used for analyzing the expression level of ezrin in type 2 diabetes. Immunoblotting was performed as previously described,[20,21] and blots were quantified using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Indirect Immunofluorescence

Rat kidney cryosections were fixed with acetone and mouse podocytes with 4% paraformaldehyde in PBS and stained with primary antibodies, as described.[17] Detection was with AlexaFluor 555 donkey anti-rabbit and AlexaFluor 488 donkey anti-mouse IgGs (Molecular Probes). Samples were examined with a Zeiss Axioplan2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) or a Leica TCS SP8 MP CARS confocal microscope (Leica Microsystems, Wetzlar, Germany).

Silencing Ezrin by siRNA

Mouse podocytes were transfected with 150 nmol ON-TARGET plus SMARTpool mouse ezrin (L-046568-01-0005) or siCONTROL Non-Targeting Pool#2 (D-001206-14-05) siRNA (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Camarillo, CA) and used for experiments after 48 hours.

2-Deoxy-D-Glucose Uptake Assay

Glucose uptake of mouse podocytes was measured using 50 μmol/L (1 μCi/mL) 2-deoxy-D-[1, 2-^{3}H(N)]-glucose (PerkinElmer, Waltham, MA), as previously described.[17]

IHC of Human Kidney Samples

Kidney samples of renal cancer patients, with or without type 2 diabetes, were obtained from surgical nephrectomies at Helsinki and Uusimaa Hospital district, and were from the nonmalignant part of the kidney. Albuminuria, the clinical sign of diabetic nephropathy, was determined from the medical records. Tissue samples were fixed with formaldehyde and embedded in paraffin. Deparaffinized sections were stained with anti-ezrin antibodies, and VectaStain Elite ABC kit (Vector Laboratories, Burlingame, CA) and AEC (DakoCytomation, Glostrup, Denmark) were used for detection. Hematoxylin-counterstained slides were imaged using a Nikon Eclipse 800 microscope (Nikon), with the same microscope settings throughout the analysis. Glomeruli (six per sample) were analyzed from 13 patients with type 2 diabetes and 14 controls. The staining intensity of ezrin was visually graded by two researchers independently and blinded from the diabetes status. For histopathological analysis, the kidney samples were stained with PAS. The use of human material was approved by the local ethics committee.

Statistical Analysis

In all experiments, the differences between the groups were evaluated with the Student's *t*-test (Microsoft Excel). Sex frequencies were compared between cases and controls with the χ^2 test. Results are presented as means ± SD.

Results

Streptozotocin-Induced Diabetes Leads to Differential Expression of Glomerular Proteins

To characterize the early molecular changes associated with the development of diabetic kidney injury, we compared total soluble protein fractions of glomeruli isolated from rats

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with streptozotocin-induced diabetes and controls using 2D-DIGE coupled with mass spectrometry. To identify changes at an early stage of disease, we performed the analysis 4 weeks after induction of diabetes. The streptozotocin-injected rats used for the analysis were albuminuric and had high blood glucose compared with controls (Figure 1, A and B), but did not yet show ultrastructural changes in glomeruli (Figure 1, C and D). Lysates prepared from glomeruli isolated from five individual streptozotocin- and five citrate buffer–injected rats were labeled with Cy3 and Cy5, respectively, and an internal control (a pool of all glomerular samples prepared from diabetic and control rats) used for normalization was labeled with Cy2. Analysis with DeCyder software revealed 2274 spots that were present in all five gels. Of the 2274 spots, 29 exhibited a statistically significant (Student's *t*-test value ≤ 0.05) difference >1.5-fold between the diabetic and control rat glomerular samples. Fifteen spots were up-regulated (maximum, 3.16-fold) and 14 were down-regulated (maximum, 3.11-fold) in the diabetic kidney glomeruli (Supplemental Figure S1). Of the 29 differentially expressed spots, mass spectrometry identified multiple proteins in 17 spots, and a single protein in 12 spots, including actin binding and actin cytoskeleton organizing proteins, apoptosis-associated proteins, regulators of oxidative tolerance and DNA binding, and repair proteins (Supplemental Table S1).

Ezrin and NHERF2 Are Down-Regulated in the Glomeruli of Diabetic Rats

Although it is well documented that the organization of the actin cytoskeleton in podocytes is altered in several types of glomerular diseases,[7] little is known about the regulatory proteins that control actin dynamics in podocytes in diabetic nephropathy. We, therefore, chose cytoskeletal linkers ezrin and NHERF2[13] for further analysis. Ezrin and NHERF2 were both single identifications in the mass spectrometry, and showed 2.1- and 1.94-fold decreases in diabetic glomeruli (Figure 1, E–H, and Supplemental Table S1). Quantitative Western blot analysis confirmed that streptozotocin treatment decreased the amount of ezrin by 49% and NHERF2 by 42% compared with citrate-treated rat glomeruli (Figure 2, A and B). Phosphorylation of ezrin at threonine 567 was reduced by 49%, indicating that both the total amount of ezrin and that of the active ezrin are decreased in the glomeruli of rats with experimental diabetes. Also, podocalyxin, which is connected to actin through ezrin/NHERF2 complex,[15] was down-regulated by 35% in the glomeruli of streptozotocin-treated rats, confirming previous findings.[22] The expression of podocin remained unchanged (Figure 2, A and B). Immunostaining revealed decreased expression of ezrin, NHERF2, and phosphorylated ezrin (p-ezrin) in the glomeruli of diabetic rats (Figure 2, C–E and G–I), whereas podocin expression remained unchanged (Figure 2, F and J), further confirming the 2D-DIGE and quantitative Western blot results.

Knockdown of Ezrin Induces Dynamic Remodeling of the Actin Cytoskeleton in Podocytes

Because ERM proteins are important modulators of F-actin organization[10,23] and dynamic actin cytoskeleton is essential for the normal structure and function of podocytes, we analyzed the effect of ezrin knockdown on actin cytoskeleton organization in podocytes. Introduction of ezrin siRNA into podocytes reduced the level of ezrin by 60% compared with the control siRNA-transfected cells (Figure 3, A and B). Another member of the ERM family, moesin, was not

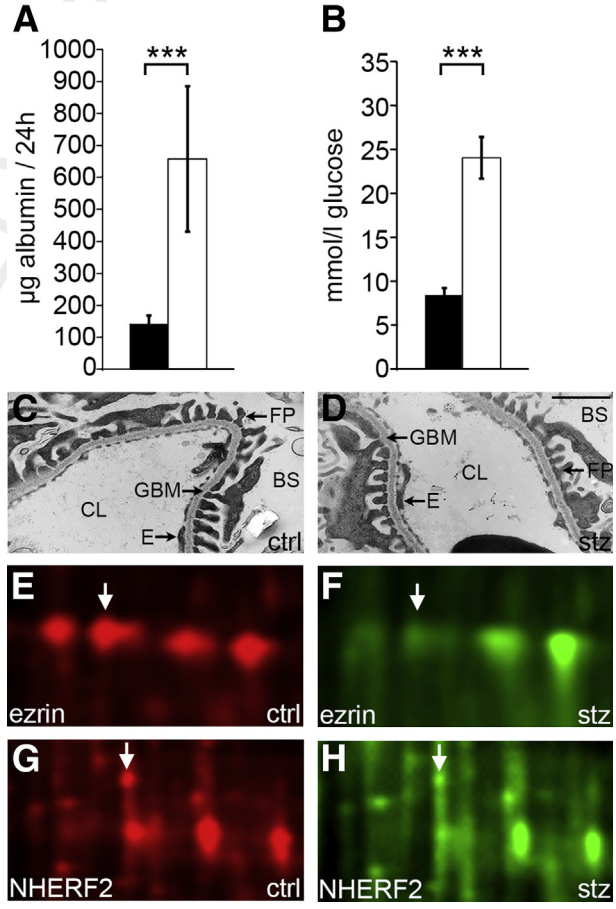

Figure 1 Streptozotocin (stz)–treated rats are hyperglycemic and albuminuric, and show differentially regulated proteins in glomeruli by 2D-DIGE. Urinary albumin (**A**) and blood glucose (**B**) levels are elevated in streptozotocin-treated rats (black bars) compared with citrate-injected controls (white bars) at sacrifice 4 weeks after induction of diabetes. Electron microscopy of control (ctrl; **C**) and streptozotocin-treated (**D**) rats reveals normal glomerular structure in the streptozotocin-treated rats 4 weeks after induction of diabetes. Selected regions of the 2D-DIGE gel showing the spots representing ezrin (**arrows**, **E** and **F**) and NHERF2 (**arrows**, **G** and **H**). **F** and **H:** Both proteins are down-regulated in the glomeruli of diabetic rats. ***$P \leq 0.001$ using the Student's *t*-test. Scale bar = 1 µm (**C** and **D**). BS, Bowman's space; CL, capillary lumen; E, glomerular endothelial cells; FP, podocyte foot process; GBM, glomerular basement membrane.

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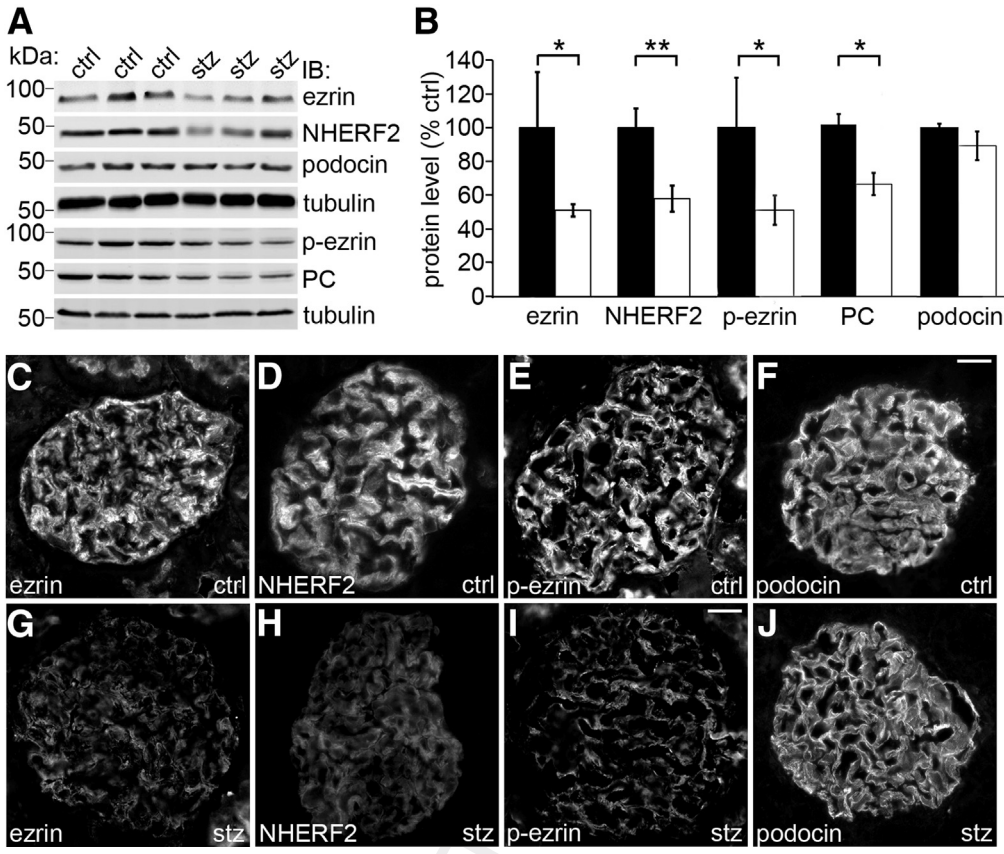

Figure 2 Streptozotocin (stz)—induced diabetes leads to reduction of ezrin, NHERF2, phosphorylated ezrin, and podocalyxin in glomeruli. **A:** Ezrin, NHERF2, p-ezrin, and podocalyxin (PC) are down-regulated in glomeruli of streptozotocin-induced diabetic rats by 49%, 42%, 49%, and 35%, respectively. Expression of podocin remains unchanged. Tubulin is included as a loading control (ctrl). **B:** Quantification of ezrin, NHERF2, p-ezrin, podocalyxin, and podocin levels in **A**. Immunofluorescence images of glomeruli of control (black bars in **B**; **C**—**F**) and streptozotocin-treated (white bars in **B**; **G**—**J**) rats stained for ezrin, NHERF2, p-ezrin, and podocin. Ezrin (**C**), NHERF2 (**D**), and p-ezrin (**E**) are down-regulated in streptozotocin-treated rats (**G**, **H**, and **I**, respectively). **F** and **J:** The expression of podocin remains unchanged. In **A** and **B**, 50 μg of glomerular lysate from three individual control and three streptozotocin-induced diabetic rats was separated by SDS-PAGE and immunoblotted with ezrin, NHERF2, p-ezrin, podocalyxin, podocin, and tubulin IgGs. **C**—**J:** Rat kidney cryosections were fixed with acetone, labeled with ezrin, NHERF2, p-ezrin, and podocin IgGs, and examined by fluorescence microscopy. $*P \leq 0.05$, $**P \leq 0.01$ using the Student's *t*-test. Scale bar = 20 μm. Q29

affected by ezrin knockdown (Figure 3, A and B). Staining of control siRNA-treated cells for ezrin revealed ezrin in the cytoplasm (Figure 3C), and phalloidin, visualizing filamentous actin, showed prominent actin stress fibers (Figure 3D). Depletion of ezrin by siRNA (Figure 3E) led to reduction of actin stress fibers and accumulation of actin in the cortical region of podocytes (Figure 3F).

Cofilin-1 is an actin-modulating protein that severs and depolymerizes F-actin and enhances the dynamics of actin filaments.[24–26] Because of the key role of cofilin-1 in regulating the organization of the actin cytoskeleton in podocytes,[27,28] we hypothesized that cofilin-1 could also be involved in ezrin-mediated actin reorganization. We found that cofilin-1 and phosphorylated, inactive cofilin-1 (p-cofilin-1) localize in the cytoplasm in control siRNA-transfected podocytes (Figure 3, G and H), whereas both proteins were partially translocated to the cell periphery in ezrin-depleted podocytes (Figure 3, I and J). This indicates that depletion of ezrin induces partial relocalization of cofilin-1 to the plasma membrane to mediate remodeling of the cortical actin cytoskeleton.

Insulin Stimulates Ezrin Phosphorylation and Translocation to the Cell Periphery

To study the role of ezrin in regulating podocyte function, we used cultured mouse podocytes that express ezrin endogenously (Figure 3). In other cell types, the activity of ezrin is dynamically regulated in response to various signals, including glucose, leading to translocation of the active, phosphorylated ezrin to the plasma membrane[29] to function as a linker of the membrane proteins to the underlying actin cytoskeleton. In starved conditions, ezrin and p-ezrin were predominantly distributed in the cytoplasm of podocytes (Figure 4, A and C), and insulin stimulation led [F4] to partial translocation of both to the plasma membrane (Figure 4, B and D).

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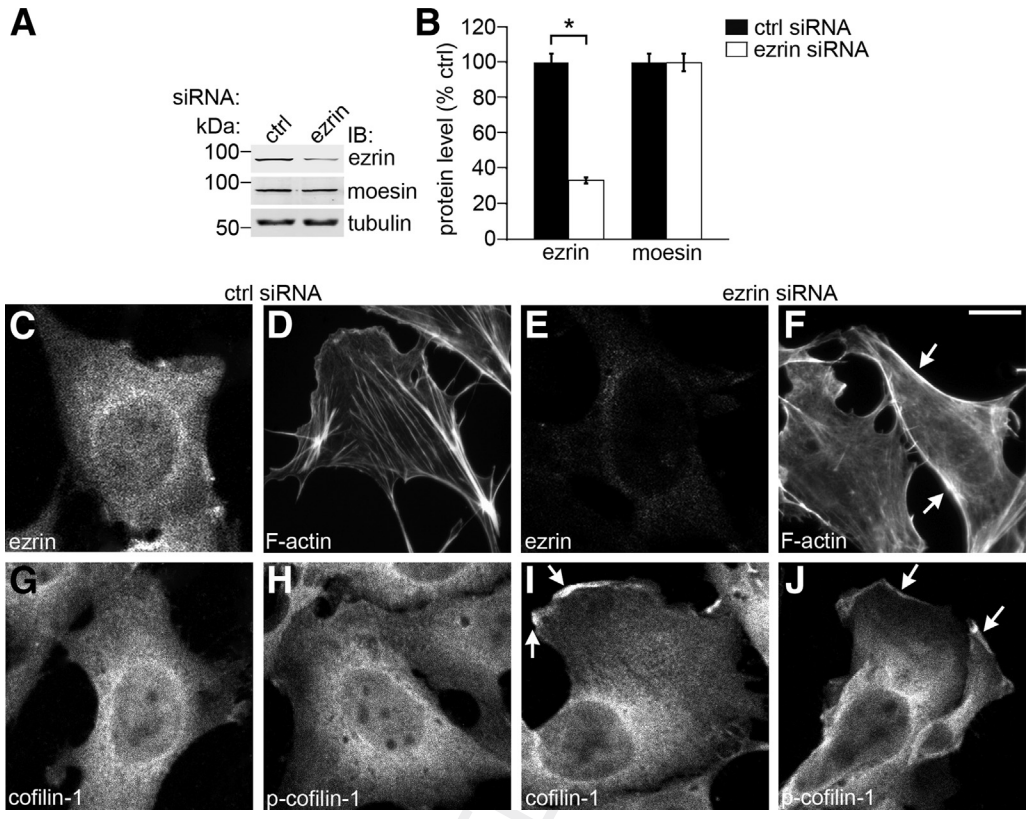

Figure 3 Knockdown of ezrin increases remodeling of cortical actin in podocytes. **A:** Ezrin siRNA leads to 60% reduction of ezrin expression in mouse podocytes. Moesin expression is not affected by ezrin knockdown. Tubulin is included as a loading control (ctrl). **B:** Quantification of the level of ezrin and moesin in **A**. Labeling of mouse podocytes for ezrin (**C** and **E**), F-actin (**D** and **F**), cofilin-1 (**G** and **I**), or p-cofilin-1 (**H** and **J**). In control siRNA-treated cells, ezrin localizes in the cytoplasm (**C**), actin stress fibers are present (**D**), and cofilin-1 (**G**) and p-cofilin-1 (**H**) are distributed in the cytoplasm. Knockdown of ezrin by siRNA leads to reduction of ezrin (**E**) and reduction of actin stress fibers and accumulation of cortical actin underneath the plasma membrane (**arrows**, **F**). Cofilin-1 (**I**) and p-cofilin-1 (**J**) are partially translocated to the cell periphery (**arrows**). Cultured mouse podocytes were treated with 150 nmol/L ezrin or control siRNA. In **A** and **B**, 50 μg of podocyte lysates was separated by SDS-PAGE and immunoblotted with ezrin, moesin, and tubulin IgGs. In **C**–**J**, cells were fixed with paraformaldehyde, labeled with ezrin, cofilin-1 and p-cofilin-1 IgGs, or phalloidin, and examined by conventional (**D** and **F**) or confocal (**C**, **E**, and **G**–**J**) microscopy. Data show the means ± SD of three independent experiments. $*P \leq 0.05$ using the Student's *t*-test. Scale bars: 20 μm (**C**, **E**, and **G**–**J**); 30 μm (**D** and **F**).

Knockdown of Ezrin Increases Glucose Uptake of Podocytes

Knockdown of ezrin increases the dynamic reorganization of the actin cytoskeleton (Figure 3), a prerequisite for insulin-mediated glucose uptake.[30] This, together with the finding that ezrin functions in the release of insulin granules by regulating exocytosis in β-cells,[29] suggests that, in podocytes, ezrin could regulate the trafficking of glucose transporters and glucose uptake. To determine whether ezrin regulates glucose transporter trafficking in podocytes, we measured 2-deoxy-D-glucose uptake in mouse podocytes transfected with ezrin siRNA and found that glucose uptake was increased by 53% in ezrin-depleted podocytes compared with control siRNA-transfected cells (set to 100%) under basal conditions (Figure 5A). In serum-starved podocytes, knockdown of ezrin increased glucose uptake by 31% compared with serum-starved, control, siRNA-transfected cells (set to 100%) (Figure 5B). Stimulation of control siRNA-transfected podocytes with insulin increased glucose uptake by 110% (Figure 5B). However, in ezrin siRNA-transfected podocytes, insulin increased glucose uptake only by 76% compared with serum-starved, control siRNA-transfected cells (Figure 5B). These data indicate that depletion of ezrin increases glucose uptake of podocytes, but insulin-stimulated glucose uptake is significantly inhibited by knockdown of ezrin.

Cofilin-1 Is Involved in Ezrin-Mediated Cortical Actin Remodeling

We next searched for the mechanism of how ezrin participates in the regulation of glucose uptake in podocytes. Because cofilin-1–mediated actin reorganization is involved in the translocation of GLUT4 storage vesicles to the cell surface,[31] we analyzed both actin organization and cofilin-1/p-cofilin-1 localization in control and ezrin knockdown podocytes stimulated with insulin (Figure 6). In control siRNA-transfected podocytes, insulin stimulation induced remodeling of the actin cytoskeleton, as visualized by

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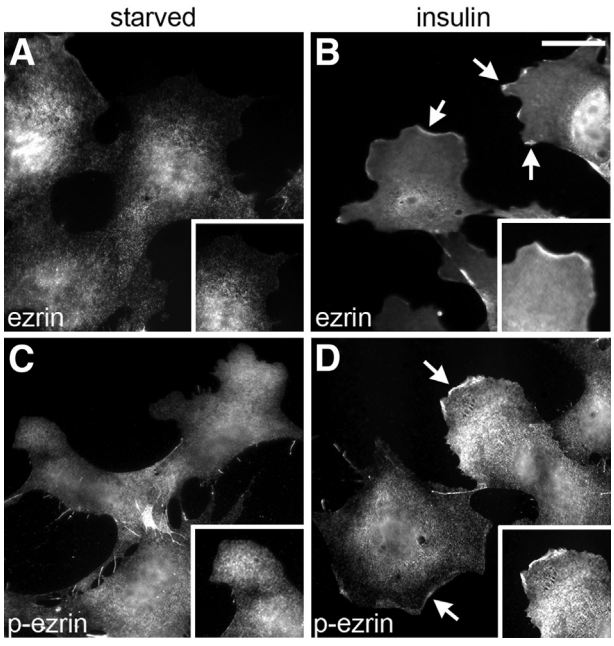

Figure 4 Ezrin and phosphorylated ezrin translocate to the cell periphery in response to insulin. In serum-starved cells, ezrin (**A**) and p-ezrin (**C**) localize in the cytoplasm. Insulin stimulation leads to translocation of ezrin (**B**) and p-ezrin (**D**) to the cell periphery (**arrows**). Cultured mouse podocytes were starved for 20 hours and treated with 20 nmol/L insulin for 15 minutes or left untreated. Cells were fixed with paraformaldehyde, labeled with ezrin and p-ezrin IgGs, and examined by fluorescence microscopy. Scale bars: 20 μm (**B**); 7.5 μm (**insets**).

diminished stress fibers and increased cortical actin detected with phalloidin (Figure 6, A and B). In ezrin siRNA-transfected podocytes that were serum starved, phalloidin indicated the presence of cortical actin (Figure 6C), but insulin stimulation reduced the cortical actin and increased the number of stress fibers (Figure 6D). This indicates that in control podocytes, insulin stimulation increases cortical actin reorganization, and that knockdown of ezrin reduces this insulin-induced cortical actin remodeling.

Next, we analyzed whether the localization of cofilin-1 and p-cofilin-1 reflects the dynamic changes observed in the organization of the actin cytoskeleton on ezrin knockdown and insulin stimulation. In control siRNA-transfected podocytes, insulin induced partial translocation of cofilin-1 and p-cofilin-1 from their cytoplasmic location (Figure 6, E and I) to the cell periphery (Figure 6, F and J). In serum-starved, ezrin-depleted podocytes, cofilin-1 (Figure 6G) and p-cofilin-1 (Figure 6K) were partially observed in the periphery of podocytes, but in insulin-stimulated, ezrin-depleted podocytes, both proteins were found only in the cytoplasm (Figure 6, H and L). This indicates that cofilin-1 is involved in the ezrin-mediated remodeling of cortical actin.

Insulin Regulates Phosphorylation of Cofilin-1 in Podocytes

Short-term treatment of muscle cells with insulin leads to dephosphorylation of cofilin-1.[31] Treatment of podocytes with insulin for 15 minutes similarly reduced the level of dephosphorylated cofilin-1 by 80% (Figure 7, A–C). On the other hand, culturing podocytes in the presence of insulin for 48 hours increased the level of inactive, phosphorylated cofilin-1 by 36.5% (Figure 7, D–F). Thus, the effect of insulin on the activity of cofilin-1 is determined by the acute or chronic nature of the exposure, either activating or inactivating it, respectively.

Knockdown of Ezrin Induces GLUT1 Translocation to the Plasma Membrane

We further explored whether depletion of ezrin affects the trafficking of glucose transporters in podocytes. In control siRNA-transfected podocytes, GLUT1 was observed in the cytoplasm in basal and starved conditions (Figure 8, A and E), and insulin induced translocation of GLUT1 to the plasma membrane (Figure 8I). In ezrin-depleted podocytes, however, GLUT1 was observed at the plasma membrane in basal and starved conditions (Figure 8, B and F) and after insulin stimulation (Figure 8J). In both control and ezrin siRNA-transfected podocytes, GLUT4 was observed in the cytoplasm in basal and starved conditions (Figure 8, C, D, G, and H), and insulin induced its translocation to the plasma membrane (Figure 8, K and L). These data suggest that depletion of ezrin increases glucose uptake by enhancing translocation of GLUT1 to the plasma membrane.

Phosphorylation of Cofilin-1 Is Increased in Glomeruli of Diabetic Rats

Because actin dynamics are altered in podocyte injury and cofilin-1 plays an important role in regulating actin dynamics

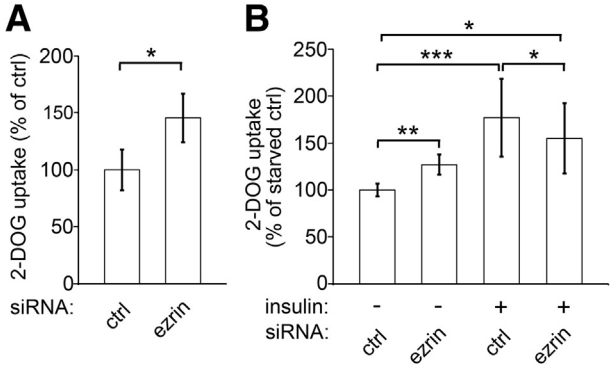

Figure 5 Knockdown of ezrin increases glucose uptake in podocytes. **A:** Depletion of ezrin increases the glucose uptake activity of mouse podocytes by 53% compared with the control (ctrl) siRNA-transfected cells (set to 100%) under basal conditions. **B:** Knockdown of ezrin increases the glucose uptake activity of mouse podocytes by 31% in serum-starved cells (ezrin siRNA, without insulin). Glucose uptake activity of the control siRNA-transfected and serum-starved cells is set to 100% (control siRNA, without insulin). After insulin stimulation, the increase in glucose uptake is 110% in control siRNA-transfected cells (control siRNA, with insulin), and 76% in ezrin siRNA-transfected cells (ezrin siRNA, with insulin). Data are the means ± SD of three independent experiments. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ using the Student's *t*-test.

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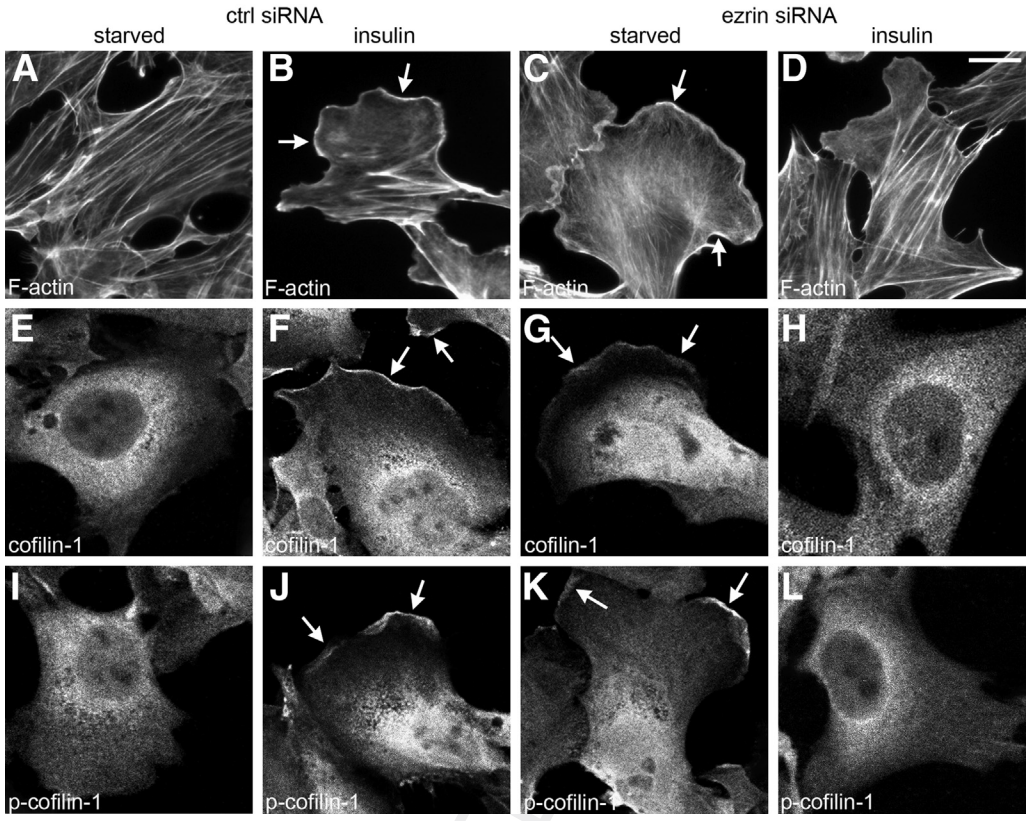

Figure 6 Knockdown of ezrin reduces insulin-induced cortical actin reorganization in podocytes by affecting cofilin-1 localization. Labeling of mouse podocytes for F-actin, cofilin-1, and p-cofilin-1. In starved, control, siRNA-treated podocytes, actin stress fibers are present (**A**), and cofilin-1 (**E**) and p-cofilin-1 (**I**) are distributed in the cytoplasm. Insulin stimulation of control siRNA-transfected podocytes leads to accumulation of F-actin to the leading edge (**arrows**, **B**) and partial translocation of cofilin-1 (**F**) and p-cofilin-1 (**J**) to the cell periphery (**arrows**). In starved cells, knockdown of ezrin by siRNA leads to reduction of actin stress fibers and accumulation of F-actin in the cortical region (**arrows**, **C**). Cofilin-1 (**G**) and p-cofilin-1 (**K**) are partially translocated to the cell periphery (**arrows**). **D:** Insulin stimulation of ezrin-depleted podocytes partially restores actin stress fibers. Cofilin-1 (**H**) and p-cofilin-1 (**L**) localize in the cytoplasm. Cultured mouse podocytes were treated with 150 nmol/L ezrin or control siRNA, starved and either stimulated with 20 nmol/L insulin for 15 minutes or left unstimulated, fixed with paraformaldehyde, labeled with cofilin-1 and p-cofilin-1 IgGs and phalloidin, and examined by conventional (**A**–**D**) or confocal (**E**–**L**) microscopy. Scale bar = 20 μm (**D**). Q31

in podocytes,[27,28] one could expect that the amount of p-cofilin-1 is altered in glomeruli of streptozotocin-treated rats. Quantitative Western blot analysis showed that the level of total cofilin-1 remained unchanged, but p-cofilin-1 [F9] increased in the glomeruli of the diabetic rats (Figure 9). This indicates that cofilin-1 is inactivated (phosphorylated) in glomeruli in experimental diabetes.

Expression of Ezrin Is Reduced in Glomeruli of Obese Zucker Rats and in Glomeruli of Patients with Type 2 Diabetes

Next, we investigated the expression level of ezrin using 12-week-old obese Zucker rats and their age-matched lean controls. Obese Zucker rats are insulin resistant and slightly diabetic because of a mutation in the leptin receptor gene, and they develop albuminuria by 40 weeks of age.[32,33] Quantitative Western blot analysis revealed that the expression level of ezrin is decreased by 20% in glomeruli of obese Zucker rats when compared with lean Zucker rats (Supplemental Figure S2).

The expression of ezrin was also studied by immunohistochemistry (IHC) in human kidney samples obtained from nephrectomies. The staining intensity of ezrin was visually graded in glomeruli from 13 patients with type 2 diabetes and 14 controls. The patients did not have clinical nephropathy, and histopathological analysis revealed no signs diagnostic of diabetic nephropathy. The mean age was similar between the groups: 69.2 ± 9.6 years for patients with diabetes and 69.5 ± 12.2 years for controls ($P = 0.95$, Student's t-test). The sex distribution did not differ significantly between the groups: there were 8 men and 5 women in the group of patients with diabetes and 11 men and 3 women in the control group ($P = 0.33$, χ^2 test). The expression of ezrin was significantly lower in glomeruli of patients with diabetes (Figure 10). [F10].

Discussion

Diabetic nephropathy is a devastating complication of diabetes and may ultimately progress to end-stage renal disease. The pathophysiological mechanisms of diabetic

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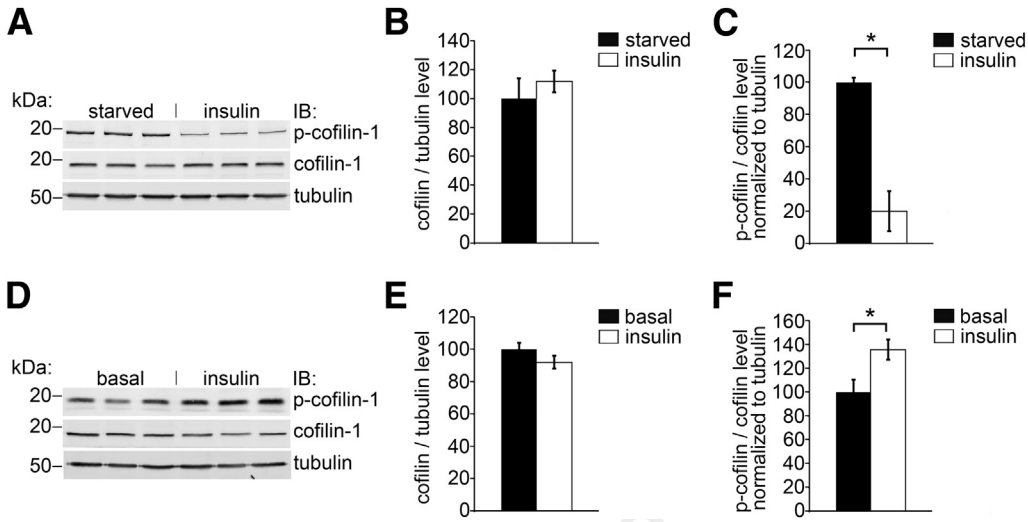

Figure 7 Insulin regulates phosphorylation of cofilin-1 in podocytes. **A:** The p-cofilin-1 is down-regulated in mouse podocytes by 80% after 15 minutes of insulin stimulation. The total cofilin-1 level remains unchanged. Tubulin is included as a loading control. **B:** Quantification of total cofilin-1 level in **A**. **C:** Quantification of p-cofilin-1 level normalized to cofilin-1 in **A**. **D:** The p-cofilin-1 is up-regulated in mouse podocytes by 36.5% after 48 hours of insulin stimulation. The total cofilin-1 level remains unchanged. Tubulin is included as a loading control. **E:** Quantification of total cofilin-1 level in **D**. **F:** Quantification of p-cofilin-1 level normalized to cofilin-1 in **D**. In **A**–**C**, mouse podocytes were starved or starved and stimulated with 20 nmol/L insulin for 15 minutes. In **D**–**F**, mouse podocytes were cultured without (basal) or with 20 nmol/L insulin for 48 hours. Cell lysates (50 μg) were separated by SDS-PAGE and immunoblotted with p-cofilin-1, cofilin-1, and tubulin IgGs. Data show the means ± SD of three independent experiments. *$P \leq 0.05$ using the Student's *t*-test.

nephropathy have been under extensive study, and the results indicate an important role for podocyte injury in this process. We performed quantitative proteomic profiling of glomeruli isolated from rats with streptozotocin-induced diabetes and controls to identify differentially expressed proteins that could be associated with the development of podocyte injury. The major changes observed were among proteins involved in apoptosis, regulation of oxidative tolerance, and organization of the actin cytoskeleton, all processes known to be involved in podocyte function or injury and to participate in the development of diabetic nephropathy.[34,35]

The cytoskeletal proteins down-regulated in the diabetic glomeruli included ezrin and its interaction partner, NHERF2. Ezrin and NHERF2 are important regulators of podocyte function because they link podocalyxin, the major sialoprotein of podocytes, to the actin cytoskeleton.[13] However, the exact role of ezrin in podocyte injury in diabetic nephropathy has not been investigated before. In the nonphosphorylated, inactive conformation, the N- and C-termini of ezrin self-associate, but phosphorylation of threonine 567 leads to unfolding of the conformation, thus exposing the binding sites to F-actin and the plasma membrane, and activation of ezrin.[36] Herein, we report that glomeruli of streptozotocin-treated rats show lowered levels of total and, concomitantly, threonine 567–phosphorylated ezrin. We also found that the expression of ezrin is reduced in glomeruli of insulin-resistant and slightly diabetic obese Zucker rats and in podocytes of patients with type 2 diabetes without clinical nephropathy or histopathological signs of diabetic nephropathy. Supporting our findings, phosphorylation of ezrin has been reported to be reduced in skeletal muscle of obese patients with type 2 diabetes.[37] These data together indicate that down-regulation of ezrin and/or its activity may be involved in the development of diabetic complications.

Insulin induces cortical actin remodeling and glucose uptake in cultured podocytes.[5,6] We found that knockdown of ezrin induces dynamic remodeling of cortical actin in cultured podocytes under basal conditions, but diminishes the insulin-induced dynamics of cortical actin. Consistently, knockdown of ezrin in podocytes by siRNA increased glucose uptake under basal conditions. After insulin stimulation, glucose uptake was still enhanced, but remained lower, in ezrin-depleted podocytes than in control siRNA-transfected podocytes. These data indicate that, despite the reduced insulin responsiveness, as measured by cortical actin reorganization, podocytes still absorb more glucose when the expression of ezrin is reduced. The increase in glucose uptake was due to enhanced trafficking of constitutive glucose transporters, demonstrated by translocation of GLUT1 to the plasma membrane in ezrin-depleted podocytes. In muscle cells, remodeling of the cortical filamentous actin is required for GLUT4 translocation.[30] Despite reduced cortical actin remodeling, we observed translocation of GLUT4 to the plasma membrane after insulin stimulation in ezrin-depleted podocytes. This may be due to the partial down-regulation of ezrin achieved by siRNA transfections. Excessive uptake of glucose into cells may activate several pathways that lead to podocyte injury,[38] suggesting that a reduced level of ezrin, leading to increased uptake of glucose, may be harmful for podocytes. Surprisingly, however, podocyte-specific overexpression of GLUT1 in diabetic mice protects against glomerulopathy.[39]

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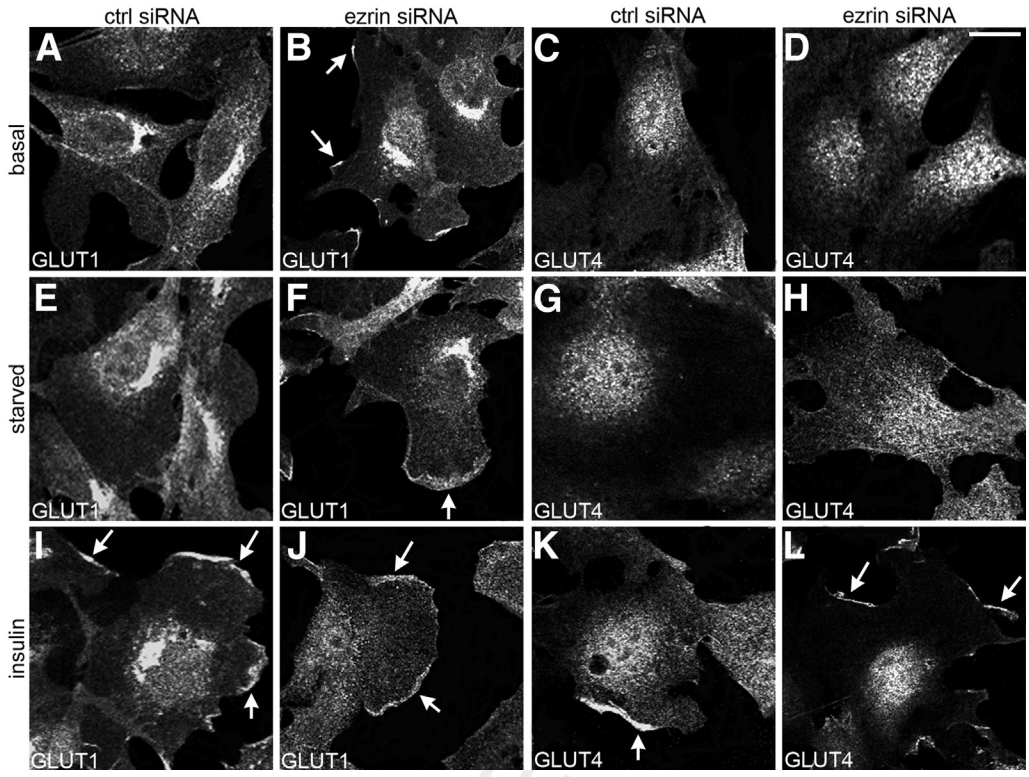

Figure 8 Ezrin depletion induces translocation of GLUT1 to the plasma membrane. Labeling of mouse podocytes for GLUT1 (**A**, **B**, **E**, **F**, **I**, and **J**) and GLUT4 (**C**, **D**, **G**, **H**, **K**, and **L**). In basal and starved, control (ctrl), siRNA-treated podocytes, GLUT1 (**A** and **E**) and GLUT4 (**C** and **G**) are distributed in the cytoplasm. Insulin stimulation of control siRNA-transfected podocytes leads to the translocation of GLUT1 (**arrows**, **I**) and GLUT4 (**arrows**, **K**) to the plasma membrane. In basal and starved podocytes, knockdown of ezrin by siRNA leads to the translocation of GLUT1 (**arrows**, **B** and **F**) to the plasma membrane. GLUT4 (**D** and **H**) remains distributed in the cytoplasm. In insulin-stimulated, ezrin-depleted podocytes, GLUT1 (**arrows**, **J**) and GLUT4 (**arrows**, **L**) translocate to the plasma membrane. Cultured mouse podocytes were treated with 150 nmol/L ezrin or control siRNA, starved, and stimulated with 20 nmol/L insulin for 15 minutes or left unstimulated, fixed with paraformaldehyde, labeled with GLUT1 and GLUT4 IgGs, and examined by confocal microscopy. Scale bar = 20 μm (**D**). Q32

Further studies are required to clarify the precise role of ezrin in modulating glucose uptake activity *in vivo* and the concomitant effect on the function of podocytes.

Reduced expression of ezrin in the glomerular podocytes of diabetic rats may modulate the function of podocytes via the interaction of ezrin with podocalyxin. NHERF2 and ezrin form a complex with podocalyxin and link it to the actin cytoskeleton.[13] Altered organization of the foot processes in rats after experimentally induced proteinuria associates with disruption of this complex and uncoupling of podocalyxin from the actin cytoskeleton.[13] In a similar manner, a reduced level of ezrin and NHERF2 in the podocytes of streptozotocin-induced diabetic rats may disrupt the association of podocalyxin and the actin cytoskeleton and, thus, impair the structural integrity of podocyte foot processes. Podocalyxin was also down-regulated by 35% in the glomeruli of streptozotocin-treated rats, possibly exaggerating the effect on actin cytoskeleton.

Our data show that ezrin-dependent remodeling of actin involves the actin-severing protein cofilin-1. Knockdown or mutation of cofilin-1 disturbs the filtration barrier in zebrafish,[28] and mice with podocyte-specific deletion of cofilin-1 develop proteinuria by 3 months of age, indicating that cofilin-1 is essential for maintaining the architecture of mature podocytes.[27] Furthermore, cofilin-1–mutant podocytes fail to recover after injury, indicating that cofilin-1 is essential for the structural changes of the actin cytoskeleton during recovery from podocyte injury.[27] Phosphorylation of cofilin-1 on serine 3 leads to its inactivation, resulting in reduced binding to actin and depolymerizing activity.[40,41] Our finding of phosphorylated cofilin-1, the inactive form of the protein, at the cell membrane of cultured podocytes after insulin stimulation may appear controversial, because one would expect the dynamic organization of cortical actin to require cofilin-1 in its active, dephosphorylated form. However, a similar observation was previously made in human epidermoid carcinoma KB cells in which cofilin and p-cofilin were observed at the plasma membrane after insulin stimulation.[42] The authors suggested that p-cofilin at the membrane could be residual phosphorylated protein waiting to be dephosphorylated, or its presence could reflect a rapid phosphorylation/dephosphorylation cycle of cofilin to reactivate the protein for its actin-severing function.[42]

We found that the effect of insulin on cofilin-1 phosphorylation in cultured podocytes depends on the length of the stimulation. Short-term insulin treatment of podocytes induced cofilin-1 dephosphorylation similarly as in muscle cells.[31] However, long-term insulin treatment increased the

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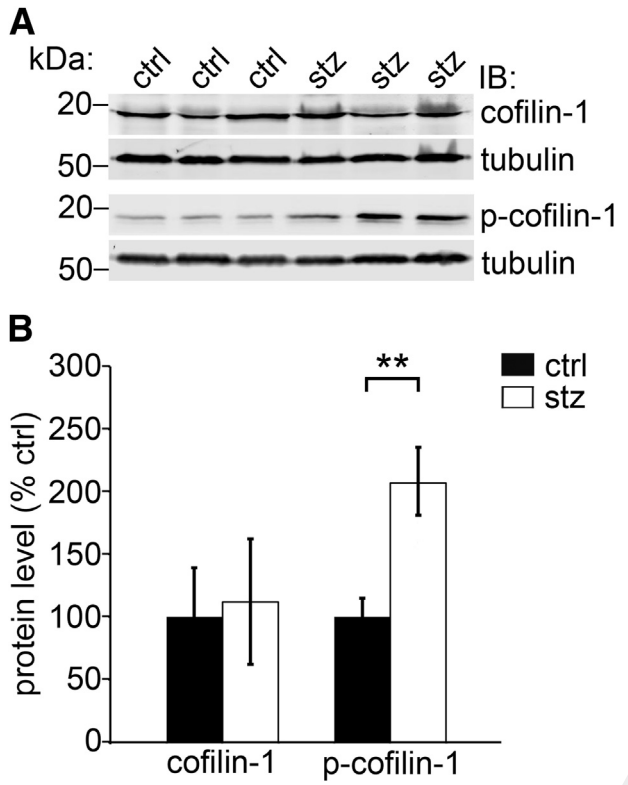

Figure 9 Phosphorylation of cofilin-1 is increased in glomeruli of diabetic rats. **A:** Immunoblotting for cofilin-1 shows that streptozotocin (stz)—induced diabetes does not affect the level of total cofilin-1 in glomeruli. The p-cofilin-1 is up-regulated in glomeruli of streptozotocin-induced diabetic rats. Tubulin is included as a loading control (ctrl). **B:** Quantification of the levels of cofilin-1 and p-cofilin-1 in **A**. Glomerular lysates (50 μg) from three individual control and three streptozotocin-induced diabetic rats were separated by SDS-PAGE and immunoblotted with cofilin-1, p-cofilin-1, and tubulin IgGs. Data show the means ± SD. $^{**}P \leq 0.01$ using the Student's *t*-test.

level of inactive, phosphorylated cofilin-1. This may lead to disturbed actin remodeling and disturbances in podocyte function. Also, high glucose and TGF-β, both associated with diabetic nephropathy,[38,43] induce inactivation of cofilin-1.[28,44] This is consistent with our data indicating that in diabetic rat glomeruli, cofilin-1 is inactivated by phosphorylation. Similarly, cofilin-1 was previously shown to be phosphorylated in patients with glomerular diseases affecting podocytes, possibly reducing the ability of podocytes to respond to changes in the glomerular pressure.[28] Another study found that phosphorylation of cofilin-1 is reduced early in puromycin aminonucleoside-induced nephrotic glomeruli.[27] The differences in the results previously described may be due to different species and diseases or disease models, the stage of disease progression, or, as previously discussed, active recycling of cofilin-1 between its active and inactive forms. Nevertheless, our data indicate an important role for the ezrin/cofilin-1 axis in maintaining the functional organization of the actin cytoskeleton in cultured podocytes.

Collectively, we show that a reduced level of ezrin increases cortical actin dynamics and, mediated by the constitutive glucose transporter GLUT1, glucose uptake in podocytes. A previous study found that increased phosphorylation of ezrin, induced by angiotensin II, enhances cortical actin dynamics.[45] This, together with our data, suggests that the role of ezrin in regulating actin dynamics

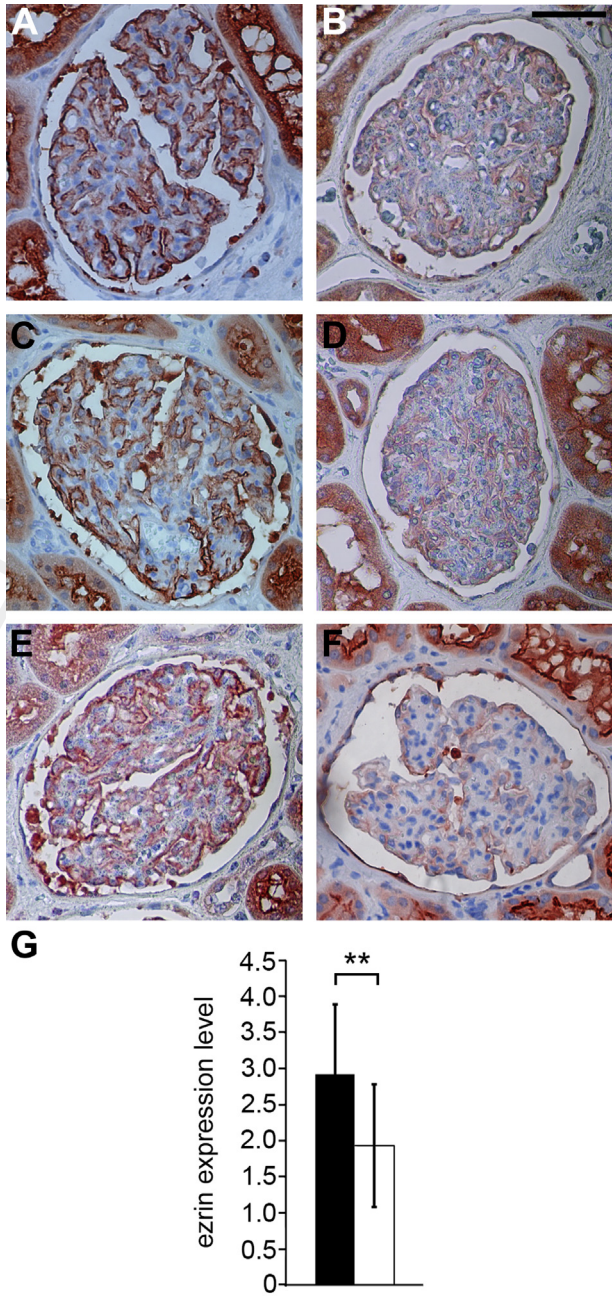

Figure 10 The expression of ezrin is reduced in glomeruli of patients with diabetes. Immunoperoxidase staining for ezrin in glomeruli of controls (**A**, **C**, and **E**) and patients with diabetes (**B**, **D**, and **F**). **G:** Quantitation of ezrin staining intensity visually graded in samples from 13 patients with diabetes (white bars) and 14 controls (ctrl; black bars) by two researchers independently and blinded from the diabetes status showing that ezrin is expressed at a lower level in glomeruli of patients with diabetes. Six glomeruli were analyzed from each sample. Paraffin sections were processed for immunoperoxidase staining, as described in *Materials and Methods*, and labeled with anti-ezrin IgG. Data show the means ± SD. $^{**}P \leq 0.01$ using the Student's *t*-test. Scale bar = 50 μm (**B**).

and podocyte function is complex, and the level and/or activity of ezrin needs to be carefully regulated, depending on the state of the cells and environmental stimuli. Furthermore, we found that ezrin-induced cortical actin remodeling involves the actin severing and depolymerizing protein cofilin-1. Further studies are required to define whether ezrin regulates the dynamics of cofilin-1 *in vivo*, and whether the effect is direct or via other proteins. Also, the specific role of cofilin-1 in regulating the trafficking of glucose transporters and glucose uptake in podocytes both *in vitro* and *in vivo*, together with ezrin and/or other partners, warrants further studies. In conclusion, our data showing reduced expression of ezrin in podocytes in both experimental nephrosis in rats and in patients with diabetes suggest that down-regulation of ezrin may associate with the development of the kidney complication in diabetes.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2014.03.002*.

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Supplemental Figure S1 Representative 2D-DIGE gel. Glomerular lysate samples (50 μg) from individual rats with streptozotocin-induced diabetes or controls and an internal standard were labeled with Cy3, Cy5, and Cy2, respectively. Labeled samples were coseparated on a 24-cm (pH 3 to 10) linear IPG strip, followed by separation in 12% SDS-PAGE. After scanning with a Typhoon 9400 scanner, the gel was stained with silver. Of the 29 differentially expressed spots, 14 were down-regulated and 15 were up-regulated in the diabetic kidney glomeruli. Significantly down-regulated spots are numbered with blue, and up-regulated ones are numbered with red. Identified proteins are listed in Supplemental Table S1

Supplemental Figure S2 Ezrin is down-regulated in glomeruli of obese Zucker rats by 20%. **A:** Tubulin is included as a loading control. **B:** Quantification of ezrin levels in **A**. Glomerular lysate samples (50 μg) from six individual lean and six obese Zucker rats were separated by SDS-PAGE and immunoblotted with ezrin and tubulin IgGs. $*P \leq 0.05$ using a Student's *t*-test.