Zona occludens-2 protects against podocyte dysfunction induced by ADR in mice

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Zona occludens-2 (ZO-2) is a protein present at the tight junction and nucleus of epithelial cells. ZO-2 represses the transcription of genes regulated by the Wnt/β-catenin pathway. This pathway plays a critical role in podocyte injury and proteinuria. Here, we analyze whether the overexpression of ZO-2 in the glomerulus, by hydrodynamics transfection, prevents podocyte injury mediated by the Wnt/β-catenin pathway in the mouse model of adriamycin (ADR) nephropathy. By immunofluorescence and immunogold electron microscopy, we show that ZO-2 is present in mice glomerulus, not at the slit diaphragms where nephrin concentrates, but in the cytoplasm and at processes of podocytes. Our results indicate that in the glomeruli of mice treated with ADR, ZO-2 overexpression increases the amount of phosphorylated β-catenin, inhibits the expression of the transcription factor snail, prevents nephrin and podocalyxin loss, reduces podocyte effacement and massive fusions, restrains proteinuria, and supports urea and creatinine clearance. These results suggest that ZO-2 could be a new target for the regulation of hyperactive Wnt/β-catenin signaling in proteinuric kidney diseases.

ZO-2; podocyte; adriamycin; Wnt; nephrin; glomerulus; proteinuria

PODOCYTES ARE SPECIALIZED cells of the glomerulus that consist of cell bodies, major processes, and foot processes that interdigitate with those of neighboring cells (53). In contrast to other epithelial cells that have tight junctions (TJs) regulating the passage of ions and molecules through the paracellular pathway (20), podocytes exhibit slit diaphragms (SDs) (50). These structures bridge the space between neighboring foot processes and are responsible of the size selectivity of the glomerular filtration barrier. Epithelial TJs prevent the passage of molecules >8 Å (67), whereas the SDs allow passage of molecules up to 70 Å (53).

Podocytes arise from mesenchymal renal stem cells and during the “S-shaped body stage” of glomerular development, and they acquire a typical epithelial morphology with TJs located at the uppermost region of the lateral plasma membrane (38). Subsequently at the “capillary loop stage,” the cells develop foot processes and their TJs migrate down the lateral membrane, disappear, and are replaced by SDs. These structures contain unique membrane proteins like nephrin (52), podocin (3), and Nep1 (13); proteins present at adherens junctions including P-cadherin (51), FAT (31), β-catenin (51), p120 (40), and α-actinin 4 (41); and TJ proteins like MAGI-1 (27), JAM-A, occludin, cingulin (15), and ZO-1 (53).

Proteinuria is a defect in glomerular filtration common in various chronic kidney diseases, including diabetic nephropathy and focal segmental glomerulosclerosis (10). Massive proteinuria is found in animal models and patients with mutation or deletion of several SD-associated proteins like nephrin (37), nep1 (13), and podocin (3).

The Wnt signaling pathway plays a critical role in podocyte injury and proteinuria (11, 23). In this route, the extracellular signaling molecule Wnt acts through the cell surface receptor frizzled and the coreceptor LRP. In the absence of Wnt, β-catenin, a transcriptional activator and adaptor protein of the adherens junction, is present in a cytoplasmic complex with axin, APC, and the kinase GSK3β. The latter phosphorylates β-catenin leading to its degradation. Activation of the pathway triggers inaccessibility of β-catenin to ubiquitination and thus prohibits proteasomal degradation (42). The accumulation of β-catenin in the cytoplasm favors its translocation to the nucleus, where in association with LEF/TCF transcription factors it activates the transcription of target genes (49). In podocytes, the transient expression of Wnt1 or of a stabilized β-catenin induces the expression of snail, a key transcription factor, that represses nephrin expression and mediates podocyte epithelial-to-mesenchymal transition and dysfunction (11, 44, 60).

In the early stage of kidney development, Wnt4 (30) and Wnt9b (6) are highly expressed, whereas in adult kidney Wnt signaling is silenced (30). In contrast, in fibrotic kidneys after obstructive injury, Wnts and frizzled receptors are upregulated leading to the transcription of Wnt/β-catenin target genes (23). In addition, the incubation of podocytes with transforming growth factor, a potent cytokine upregulated in all kinds of chronic kidney diseases, induces Wnt1 expression, β-catenin activation, and it stimulates the expression of downstream target genes like snail (60).

Adriamycin (ADR), an anthracycline antibiotic derived from the fungus Streptomyces peucetius, produces in rats (1) and BALB/c mice (7, 8) a nephrotic syndrome characterized by proteinuria, hypoalbuminemia, increased serum creatinine, and progressive renal injury. ADR activates Wnt/β-catenin signaling, whereas treatment with DKK1, a soluble factor that inhibits Wnt signaling, prevents nephrin and podocyalxin loss, reduces podocyte effacement, prevents podocyte injury mediated by the Wnt/β-catenin pathway, and the genetic ablation of β-catenin, respectively, ameliorate and protect against proteinuria...
ZO-2 PROTECTS AGAINST PODOCYTE DYSFUNCTION

A

Nephrin * ZO-2 * Merge *

Nestin * ZO-2 * Merge *

Scale: 15 µm

B

ZO-2 *

Nephrin

Scale: 230 nm

80 kV 50000

C

CTR

ZO-2

ADR

Scale: 15 µm

D

ZO-2

CTR

ADR

Actin

160 kDa

42 kDa

ZO-2

CTR

ADR

60

50

40

30

20

10

0

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ZO-2 protects against podocyte dysfunction

We recently showed in renal epithelial Madin-Darby canine kidney cells that ZO-2, and adaptor protein of the TJ, decreases Ser9 inhibitory phosphorylation of GSK-3β. In addition, ZO-2 overexpression in HEK293 and SW480 cells represses in a dose-dependent manner the activity of reporter genes driven by a promoter with artificial LEF/TCF binding sites or the Siamois promoter, whose activity is regulated by the Wnt/β-catenin pathway (56).

ZO-2, a member of the MAGUK (Membrane-associated guanylate kinase) protein family, functions as a scaffold for diverse molecular complexes (19). For example, it connects TJ integral proteins occludin (34) and claudins (33) to the actin cytoskeleton (64), and it associates to other adaptor proteins like ZO-1 (14) and cingulin (9) of the TJ, and to α-catenin (48) of the adherens junction, and to gap junction integral proteins connexins 36 (43) and 43 (54).

ZO-2 is needed for the polymerization of claudins into filaments at the TJ region (58), and for the establishment of a proper monolayer architecture (24). ZO-2 knockout mice embryos die shortly after implantation due to an arrest in early gastrulation (66), whereas ZO-2 chimeras obtained by injecting ZO-2−/− embryonic stem cells into wild-type blastocysts are viable (65), indicating that ZO-2 is essential for the extraembryonic tissue and not for the development of the embryo proper.

At the nucleus, ZO-2 is present at the nuclear matrix (35), exhibits a speckled distribution, colocalizes with SC-35 essential splicing factor (32), and associates with several transcription factors including Jun, Fos, C/EBP, and Myc (2). ZO-2 overexpression inhibits cyclin D1 (CD1) transcription (29) and protein expression and in consequence blocks cell cycle progression from G1 to S (56).

ZO-2 in sparse epithelial cells is present at the nucleus and the TJs (32). Entrance to the nucleus occurs at late G1 phase, thus explaining why in quiescent confluent cells ZO-2 is concentrated at the TJ (56).

The aim of this work is to explore whether ZO-2 is present in podocytes and can protect against proteinuria and podocyte dysfunction in a model of nephropathy induced by ADR in mice. Our results demonstrate that ZO-2 is present in podocytes, and in contrast to ZO-1 is absent from SD and instead colocalizes with nestin, a protein present in the cell body and primary process of podocytes. In the glomeruli of mice treated with ADR, ZO-2 overexpression increases the amount of phosphorylated β-catenin, inhibits the expression of the transcription factor snail, prevents nephrin and podocalyxin loss, and reduces podocyte effacement and proteinuria. Our findings suggest that ZO-2 could be a novel target for therapeutic intervention in a variety of kidney diseases modulated by the Wnt/β-catenin signaling.

Materials and Methods

Mouse model of ADR nephropathy. Male Balb/c mice, with an initial body weight of 20 g, were used. Animal care and treatment were conducted in conformity with the institutional guidelines and international laws and policies in compliance with NOM-062-ZOO-1999. Animals were housed in a constant temperature room with a 12:12-h dark-light cycle and fed a standard diet. Nephrosis was induced in 8-wk-old male BALB/c mice by a single injection of 10 mg/kg body wt of ADR through the tail vein.

Hydrodynamics-based gene transfection. For the overexpression of ZO-2 in mice glomeruli, we employed the empty vector pGW1, full-length canine ZO-2, introduced into the cytomegavirus expression plasmid pGW1 (pGW1-HA-ZO-2) and kindly provided by Ronald Javier (Baylor College of Medicine, Houston, TX), or a construct previously reported by us (2) of the middle segment of ZO-2 (His/Max-3PSG-ZO-2) that contains the third PDZ domain and the SH3 and guanylate kinase domains of the molecule. In some experiments, we employed a hairpin oligonucleotide cloned into the vector pRNAT-H1.1/Neo against mouse β-catenin (shRNA β-catenin) that had been previously tested for its capacity to silence β-catenin expression (62). These constructs were administered to the animals by a hydrodynamic-based gene transfer technique previously described (45). Briefly, 25 μg of plasmid DNA (≈30 μl) in 2 ml of saline solution (cat. no. MIR 5240; TransIT-QR Delivery Solution, Mirus, Madison, WI) were injected in less than 7 s via the tail vein with a 3-ml syringe with a G27 needle.

ZO-2 gene transfer was done on days −3, 3, and 5, considering as day 0 when ADR was injected. Animals were killed on day 7.

Immunofluorescence. Kidneys from control mice, ADR treated and transfected with the empty vector pGW1 or ADR treated and transfected with the full-length ZO-2 construct (pGW1-ZO-2) or the middle segment of ZO-2 (His/Max-3PSG-ZO-2), were excised and washed with PBS. Cubes of 0.5 cm/side were cut and immediately immersed for 2 min in 2-methylbutylane (Aldrich M3,263–1; Milwaukee, WI), which was previously cooled in liquid nitrogen. The cubes were then transferred for 10 min to liquid nitrogen. Next, 3-μm sections were cut in a Leica CM 1510 cryostat (Wetzlar, Germany) and mounted on gelatin-coated slides that were then kept frozen at −70°C. For the immunofluorescence experiments, the sections were fixed for 30 min with 3% paraformaldehyde and subsequently incubated for 5 min at −20°C in acetone and for 10 min at room temperature in 1% (vol/vol) Triton X-100. Then, the tissue sections were washed thrice with PBS, blocked for 1 h at room temperature with 1% (w/vol) IgG-free albumin (1331-A, Research Organics, Cleveland, OH), and incubated overnight at 4°C with any of the following primary polyclonal antibodies: goat anti-nephrin (cat. no. SC-19000; Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:50), goat anti-nestin (cat. no. SC-19001; Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:100), mouse anti-zona occludens (ZO)-2 PROTECTS AGAINST PODOCYTE DYSFUNCTION

Fig. 1. Adriamycin (ADR) nephropathy diminishes the expression of zona occludens (ZO)-2 at podocytes in the glomerulus. A: ZO-2 and nestin colocalize at the glomerulus. Kidney slices from 8-wk-old mice were processed by immunofluorescence, with antibodies against ZO-2 (red) and nestin (green) or nestin (green). Nephrin and nestin stain the glomeruli and are absent from the renal tubules while ZO-2 is present in both places. Nestin and ZO-2 colocalize at the glomeruli while the distribution pattern of ZO-2 and nephrin is distinct as shown in the merged images. Arrows, glomeruli; asterisks, renal tubules. Bars = 15 μm. B: immunogold electron microscopy reveals the presence of ZO-2 at podocyte foot processes. Kidney sections were stained with anti-ZO-2 (left) and anti-nephrin (right) polyclonal antibodies, followed by immunogold labeling and electron microscopy. Labeling with 15-nm gold particles reveals the presence of ZO-2 at podocyte foot processes, whereas nephrin 10-nm gold label concentrates at the slit diaphragm (SDs) between podocyte foot processes. Arrow, SD; arrowhead, foot processes. *p < 0.05, Student’s t-test.
rabbit anti-ZO-1 (cat. no. 61–7300; Zymed, San Francisco, CA; dilution 1:50), and rabbit anti-ZO-2 (cat. no. 71–1400; Zymed; dilution 1:50); and the mouse monoclonal antibodies anti-nestin (cat. no. MAB353; Millipore, Temecula, CA) and anti-podocalyxin/gp135 (a generous gift of Dr. George Ojakian from New York State University; dilution 1:50). After three washes for 5 min with PBS at room temperature, the following corresponding secondary antibodies were employed: donkey anti-goat IgG coupled to Alexa Fluor 488 (cat. no.
A10155; Invitrogen, Carlsbad, CA; dilution 1:100), chicken anti-rabbit IgG coupled to Alexa-Fluor 594 (cat. no. A21422; Invitrogen; dilution 1:100), and goat anti-mouse IgG coupled to FITC (cat. no. 62–6511; Invitrogen; dilution 1:100). The immunofluorescence was examined using a multiphotonic confocal microscope (Leica TCS-SP5 MO PANDEM, Wetzlar, Germany). Images were processed with Leica Microsystems LAS AF Lite software.

For the colocalization experiments of nestin and ZO-2, the sections, after being fixed for 30 min with 3% paraformaldehyde, were subsequently incubated for 20 min at 90°C with 10 mM sodium citrate, and for 20 min at room temperature with 10 mM sodium citrate as previously described (46). Then, the tissue sections were washed with PBS, blocked with albumin, and processed as described above.

**Immunoelectron microscopy.** Small pieces of the mice kidney cortex were fixed with 4% paraformaldehyde (cat. no. 30525–89-4; Electron Microscope Science, Haffield, PA) and 0.1% glutaraldehyde (cat. no. 111–30-8; Electron Microscope Science) in 0.1 M PBS, pH 7.4. The samples were dehydrated and embedded in LR White resin (cat. no. 14381; Electron Microscope Sciences). Ultrathin sections were placed on carbon/formvar-coated nickel grids. The grids were floated on droplets of PBS for 10 min and then on 10% (vol/vol) whole goat serum for 1 h. Next, the grids were washed with droplets of PBS for a total of 15 min. Finally, the grids were floated on a drop of the anti-rabbit IgG conjugated to 15-nm gold particles (cat. no. 25112; Electron Microscope Science) in 0.1 M PBS, pH 7.4 in a moist chamber. The grids were then washed with droplets of PBS for a total of 15 min. After which each grid was floated on 10 μl of antibodies against nephrin or ZO-2 (dilution 1:5) overnight at 4°C in a moist chamber. The grids were then washed with droplets of PBS for a total of 15 min. Finally, the grids were floated on a drop of the anti-rabbit IgG conjugated to 15-nm gold particles (cat. no. 25112; Electron Microscope Science). The grids were then washed for 15 min with PBS and for 10 min with distilled water. Next, the grids were stained for 1 min with 2% uranyl acetate (cat. no. 541–09-3; Electron Microscope Science) and for 30 s with lead citrate (cat. no. 512–26-5; Electron Microscope Science). Stained grids were then washed with droplets of PBS for a total of 15 min, after which each grid was floated on 10 μl of antibodies against antibodies against nestin (red) and HA (green). In pGW1-transfected mice (first panel), the expression of ZO-2 resembles that present in control animals (46). Then, the tissue sections were washed with PBS, the material that remained on the top of the sieve, which contained the glomeruli, was collected in cold PBS and centrifuged for 10 min at 1,200 rpm. The supernatant was decanted and the pellet containing the glomeruli was resuspended in PBS.

**Western blot.** Western blots were performed as previously described (39) using the following primary polyclonal antibodies: goat anti-nephrin IgG (cat. no. SC-19000; Santa Cruz Biotechnology; dilution 1:50), rabbit Ig anti-snail (cat. no. ab63371; Abcam, Cambridge, MA; dilution 1:300), rabbit Ig anti-nitrotyrosine (cat. no. 06–284; Millipore; dilution 1:300), rabbit Ig anti-β catenin phosphorylated in S33 and S37 (cat. no. 486000; Invitrogen, Caramarillo, CA; dilution 1:300), and a mouse monoclonal antibody against actin (a generous gift of Dr. José Manuel Hernández, Department of Cell Biology, Cinvestav, México). As secondary antibodies, the following peroxidase conjugates were used: goat Ig anti-mouse IgG (cat. no. 62–6420; Zymed Laboratories, Grand Island, NY; dilution 1:5,000), and donkey Ig anti-goat IgG (cat. no. V805; Promega, Madison, WI; dilution 1:3,000), followed by a chemiluminescence detection system (cat. no. RNP 2132; ECL+PLUS, GE Health Care, Piscataway, NJ).

**Statistical analysis.** All values are presented as means ± SE. Comparisons between groups were performed using Student’s t-test for unpaired comparisons. A P value <0.05 was considered statistically significant.

**RESULTS**

**ZO-2 colocalizes with nestin at the glomerulus.** Our first aim was to determine whether ZO-2 is present at the glomerulus and to study its localization. In Fig. 1A, we can observe by immunofluorescence the presence of ZO-2 at mice glomeruli and renal tubules (middle). However, it is noteworthy that ZO-2 does not colocalize with nephrin (top left and right), a protein specifically located at the SD of glomerular podocytes (52) and absent from renal tubules. The distribution pattern of ZO-2 at the glomerulus strongly suggested it is present in podocytes. To confirm this issue, we...
c ontained the glomeruli with anti-ZO-2 and anti-nestin antibodies. Nestin is an intermediate filament protein whose expression in adult kidney is restricted to podocytes cytoplasm and processes (55). In Fig. 1A, bottom, we observe a clear colocalization of nestin and ZO-2, thus confirming the presence of ZO-2 in podocytes.

To determine more accurately the subcellular localization of ZO-2 in podocytes, we performed an immunogold labeling experiment on thin sections of kidney tissue. Figure 1B, left, shows that with the anti-ZO-2 antibody, the 15-nm-diameter gold particles concentrate at the foot processes of podocytes observed by TEM. In contrast, when the anti-nestin antibody was used (Figure 1B, right), the 10-nm gold particles concentrate at the SD as previously reported (52).

**ADR nephropathy reduces the expression of ZO-2 in the glomerulus.** Next, we analyzed what happens with ZO-2 in the mice model of nephropathy induced by ADR. Figure 1C shows how the expression of ZO-2 detected by immunofluorescence is significantly reduced in the glomerulus after 5 days of treatment with ADR compared with controls. To confirm these results, a Western blot analysis was done on extracts from isolated glomeruli, like the ones shown in Fig. 1D, top, revealing a pronounced decrease in ZO-2 expression in ADR-treated glomeruli (Fig. 1D, bottom). These results thus suggest that the decreased expression of ZO-2 might contribute to the pathogenesis of ADR-induced nephropathy.

**Hydrodynamics-based gene transfection of ZO-2 induces the overexpression of ZO-2 protein in mice glomeruli.** We then explored whether ZO-2 blocks Wnt/β-catenin signaling and prevents podocyte injury induced by ADR in mice.

To overexpress ZO-2 in mice glomeruli, we employed a hydrodynamics-based gene transfection assay (45). We injected the DNA in a volume of saline solution (2 ml) that is 33% higher than the total blood volume of mice. As a result, injected DNA accumulates in the inferior vena cava as the injection rate exceeds the cardiac output. This generates a high hydrostatic pressure in the inferior vena cava that forces the flow of DNA into tissues linked to it like the kidney.

Figure 2A (first panel) shows how 3 days after transfecting the mice with the empty vector pGW1, the expression of ZO-2 at the glomeruli displays a similar pattern to that present in untransfected animals (Fig. 1A). In pGW1-HA-ZO-2-transfected animals, an abundant expression of exogenous ZO-2, detected with an antibody against HA, is present at the glomerulus (Fig. 2A, third panel, arrowhead), but absent from the tubules, where only endogenous ZO-2 is present (Fig. 2A, third panel, arrow). HA staining is negative in vector (Fig. 2A, second panel) and in mock transfected (Fig. 2A, fourth panel) kidneys. It should be mentioned that in each hydrodynamics transfection assay ~35% of glomeruli became transfected.

To verify that exogenous ZO-2 is expressed in podocytes, we costained pGW1-HA-ZO-2-transfected glomeruli with nestin. In Fig. 2B, we observe the colocalization of nestin and exogenous ZO-2, thus confirming the presence of the latter in podocytes. Figure 2C demonstrates by Western blot how in isolated glomeruli the transfection of pGW1-HA-ZO-2 increases the amount of ZO-2 protein expressed. Taken together, these results show that hydrodynamics-based gene transfection is an efficient method for delivering ZO-2 into mice kidney.

ZO-2 protects against podocyte dysfunction in a model of nephropathy induced by ADR in mice. To assess whether ZO-2 prevents podocyte injury induced by ADR, we administered this antibiotic to mice that overexpress ZO-2. Figure 2D depicts the treatment scheme followed in mice transfected with pGW1 or pGW1-HA-ZO-2 and treated with ADR. Figure 2E shows how ADR treatment in mice transfected with the empty vector pGW1 significantly decreases the expression of nephrin and podocalyxin in glomeruli compared with mock transfected animals without ADR treatment. In contrast, nephrin and podocalyxin were largely preserved in the glomeruli of mice transfected with pGW1-HA-ZO-2. Podocalyxin, a protein that distributes on the sides and tops of foot processes, was tested due to its negative charges that maintain the SD open allowing normal kidney function (36). To confirm the effect of ZO-2 on nephrin expression, a Western blot was performed in isolated glomeruli showing that ZO-2 transfection impedes nephrin reduction induced by ADR (see Fig. 5A).

Since ZO-2 transfection protects against ADR-induced loss of glomerular proteins like podocalyxin and SD components like nephrin and ZO-1, we next analyzed whether ZO-2 preserves the ultrastructure of podocyte foot processes. Figure 3 shows that in glomeruli transfected with the empty vector pGW1 and treated with ADR, there is an extensive loss of podocyte architecture as the foot processes efface, display massive fusions, and SDs disappear. These ultrastructural lesions are largely prevented in the glomeruli of mice transfected with pGW1-HA-ZO-2.

We next analyzed whether ZO-2 maintains the glomerular filtration rate, estimated on the basis of urea and creatinine clearance. Figure 4A shows a decrease in urea clearance induced by ADR that is more pronounced in animals transfected with the empty vector than in those receiving pGW1-HA-ZO-2. Figure 4B depicts the decrease in creatinine clearance detectable in the group of mice treated with ADR and transfected with the empty vector pGW1, while creatinine clearance was maintained in ADR-treated mice transfected with pGW1-HA-ZO-2.
In a similar fashion, ZO-2 protects against proteinuria. In this experiment, a nonfunctional middle segment of ZO-2 named 3PSG (35, 56) was employed to test the specificity of ZO-2 action in the Wnt pathway. In addition, to inhibit the Wnt pathway we employed a shRNA against mouse β-catenin. Figure 4C shows that on day 7 after ADR injection, proteinuria was induced in all mice. However, in the pGW1-HA-ZO-2 and β-catenin shRNA-transfected mice, proteinuria was less intense than in mice transfected with the empty vector while no change is observed in mice transfected with pGW1-HA-ZO-2. C: proteinuria was induced in all mice treated with ADR; however, a more severe form is present in mice transfected with the empty vector pGW1 or with the middle segment of ZO-2 (3PSG) than in those receiving HA-ZO-2 or a β-catenin shRNA. D: Western blot shows the decreased expression of total β-catenin in isolated glomeruli treated with ADR and transfected by hydrodynamics with β-catenin shRNA compared with those transfected with the empty vector pGW1. *P < 0.01; **P < 0.001 Student’s t-test. The results correspond to 3 independent experiments.

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ZO-2 inhibits ADR-induced changes on β-catenin and snail expression. We next analyzed the effect of ZO-2 on the Wnt/β-catenin signaling pathway. The Western blot of isolated glomeruli shown in Fig. 5A reveals that in ADR-treated animals transfected with the empty vector or with the 3PSG ZO-2 segment, the amount of β-catenin phosphorylated on S33 and S37 diminishes while the expression of snail and total β-catenin increases, compared with mock transfected mice and to ADR-treated animals transfected with pGW1-HA-ZO-2. These results hence demonstrate that ZO-2 overturns the expression of key elements of the Wnt/β-catenin signaling pathway. Since recent studies demonstrate that this pathway is regulated by reactive oxygen species via nucleoredoxin (16), we asked whether ZO-2 could inhibit the oxidative/nitrosative stress triggered by ADR. Figure 5B shows that the increase in nitrotyrosine (NT), a marker of peroxynitrite-induced injury and nitrosative stress, induced by ADR treatment, does not diminish in the glomeruli of mice transfected with ZO-2. Taken together, these results indicate that ZO-2 inhibits the Wnt/β-
The name ZO-2 reveals the TJ localization of the protein in epithelial cells. However, work performed by us and others demonstrated that in proliferating cells, ZO-2 is also present at the nucleus (32, 57). Here, we demonstrated by immunofluorescence and gold immunolabeling that ZO-2 is expressed in the podocytes of the glomerulus. Since ZO-2 amino acid sequence has a 68% (21) homology to ZO-1 and these proteins exhibit some redundant functions such as the induction of claudin polymerization at the TJ (58), we expected to find ZO-2 at the SD where ZO-1 and nephrin are located (52, 53). However, our results demonstrate that ZO-2 is absent from SD and instead is present in the cytoplasm and podocyte processes in colocalization with nestin.

ZO-2 has multiple protein-protein interaction domains including PDZ, SH3, and guanylate kinase (GuK) domains and a PDZ binding motif. These characteristics allow ZO-2 to function as a cytoplasmic or nuclear scaffold that can bring together diverse proteins including intercellular junction proteins, sig-

**DISCUSSION**

The name ZO-2 reveals the TJ localization of the protein in epithelial cells. However, work performed by us and others demonstrated that in proliferating cells, ZO-2 is also present at the nucleus (32, 57). Here, we demonstrated by immunofluorescence and gold immunolabeling that ZO-2 is expressed in the podocytes of the glomerulus. Since ZO-2 amino acid sequence has a 68% (21) homology to ZO-1 and these proteins exhibit some redundant functions such as the induction of claudin polymerization at the TJ (58), we expected to find ZO-2 at the SD where ZO-1 and nephrin are located (52, 53). However, our results demonstrate that ZO-2 is absent from SD and instead is present in the cytoplasm and podocyte processes in colocalization with nestin.

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**Fig. 5.** ZO-2 inhibits the Wnt/β-catenin signaling pathway by a mechanism independent of oxidative stress. A: Western blot showing how full-length ZO-2 but not the middle 3PSG segment of ZO-2 prevents ADR-induced changes on the glomerular expression of phosphorylated β-catenin, total β-catenin, snail, and nephrin (left). Densitometric analysis of 3 independent experiments (right). *P < 0.01; **P < 0.001 Student’s t-test. B: ZO-2 does not prevent the increase of nitrotyrosine (NT), a marker of oxidative stress, in glomeruli of animals treated with ADR. Representative Western blot done from isolated glomeruli derived from control and animals treated for 5 days with ADR with pGW1 or pGW1-HA-ZO-2 transfection (left). Actin was used as a loading control. Densitometric analysis of 3 independent experiments (right).
naling proteins, and factors involved in gene transcription (for a review, see Refs. 20, 21). ZO-2 downregulates the transcription of CD1 gene via an E-box, through the association to c-myc transcription factor (29), decreases the cellular amount of CD1 protein, and blocks the transition from G1 to the S phase of the cell cycle, without producing an increase in cellular apoptosis or necrosis (56). These observations support the proposal of ZO-2 as a tumor suppressor protein. This idea was initially raised after observing the high degree of identity that ZO amino acid sequences have with the tumor suppressor protein Dlg (63). The concept was then reinforced after observing that 1) ZO-2 expression is silenced in multiple cancerous tissues, including pancreatic adenocarcinoma, prostate adenocarcinoma, colon cancer, breast cancer, testicular in situ carcinoma, lung squamous carcinoma, and lung adenocarcinoma (for reviews, see Refs. 21 and 22) and 2) that ZO-2 is the target of viral oncoenic proteins (18).

WNT/β-catenin signal transduction is involved in many pathological conditions including colorectal cancer, mela-noma, and kidney diseases. We previously showed that ZO-2 blocks the inhibitory phosphorylation of GSK-3β at Thr286 and has a repressive effect not only on an artificial promoter regulated by LEF/TCF sites but also on a natural target of the Wnt/β-catenin signaling pathway, such as Siamois (56), that regulates the early dorsal axis specification in embryos (5). In addition, we demonstrated by qRT-PCR that the endogenous expression of Axin-2, a Wnt target gene, is inhibited by ZO-2 overexpression (56). These observations prompted us to study whether ZO-2 could block the development of kidney disease triggered by the Wnt signal transduction pathway.

Here, we worked with the model of ADR treatment in mouse since the participation of the Wnt/β-catenin signaling pathway in podocyte dysfunction and albuminuria has been clearly established (11). Our interest was to test the impact of ZO-2 overexpression on podocytes. Therefore, we employed the hydrodynamics gene transfection assay, as previous observations demonstrated that it allows the efficient delivery of cDNA constructs for hepatocyte growth factor (12) and interleukin-10 (26) to glomeruli, in mice and rats, respectively. Our results show that exogenous ZO-2 is expressed at podocytes in the glomerulus, thus confirming the efficiency of the assay for cDNA delivery to the glomerulus, but not to the renal tubules (12, 26).

By immunofluorescence microscopy, we showed that ZO-2 overexpression preserves nephrin and podocalyxin expression after ADR treatment. This is important as podocalyxin is needed for the maintenance of opened podocyte slits (36), and nephrin is an essential protein of the SD, whose absence or decreased expression is linked to podocyte dysfunction (37, 59). The protective action of ZO-2 against ADR injury is further confirmed by observing that the ultrastructure of the podocyte foot processes is maintained in pGW1-HA-ZO-2-transfected animals.

In agreement with these structural results, podocyte dysfunction evaluated by proteinuria was reduced by ZO-2 overexpression, but not by the expression of a nonfunctional segment of ZO-2. However, it cannot be ruled out that ZO-2 overexpression elsewhere in the body might indirectly contribute to and affect the course of the renal disease.

The results of urea and creatinine clearance reinforce the vision of ZO-2 acting as a protector of ADR-induced injury. However, it is notable that the absolute values are about fourfold greater for urea than for creatinine clearance. This is a strong indication that the plasma creatinine measurements by Jaffe reaction overestimated the true plasma creatinine concentration by at least fourfold, which is expected due to the presence of noncreatinine chromagens in serum (61).

The ability of ZO-2 to block the Wnt/β-catenin pathway appears to be the key for understanding why ZO-2 prevents damage to the glomeruli induced after ADR treatment. Our previous results showed for the first time that ZO-2 in epithelial cells blocks the inhibitory phosphorylation of GSK-3β at Thr286 and inhibits the transcription of gene targets of the Wnt/β-catenin pathway (56). In accordance, here we observed that the proteinuria induced by ADR diminishes upon transfection of ZO-2 or a shRNA against β-catenin. In line with this, transfection of ZO-2 induces an increased glomerular content of phosphorylated β-catenin and a decreased expression of snail.

Interestingly, ZO-2 inhibition of the Wnt/β-catenin signaling pathway occurs by a mechanism independent of redox regulation, as no change in the content of the oxidative stress marker NT was observed. However, since ZO-2 is a scaffold protein, it remains to be determined whether it forms a complex with Axin-APC-GSK-3β that favors β-catenin phosphorylation, ubiquitination, and degradation, as has been observed for WTX, another protein with tumor suppressor activity, encoded by a gene mutated in Wilms tumors (47).

In summary, these results demonstrate that ZO-2 protects against podocyte dysfunction induced by ADR. This action is regulated by ZO-2-mediated inhibition of the Wnt/β-catenin signaling pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


