 Cellular mechanisms responsible for the loss of capillary wall permselectivity in diabetic nephropathy are not well characterized. ZO-1 is a junctional protein involved in the assembly and proper function of a number of tight junctions and is also expressed at the junction of podocytes with the slit diaphragm. We investigated the effect of diabetes and high glucose concentration on the expression of ZO-1 in animal models of both type 1 and 2 diabetes and in rat glomerular epithelial cells. In diabetic animals, immunohistochemistry and Western blotting showed decreased expression of ZO-1 in glomeruli. Immunogold electron microscopy revealed redistribution of ZO-1 from the podocyte membrane to the cytoplasm in the diabetic animals. Exposure of rat glomerular epithelial cells to high glucose resulted in a decrease in the intensity of ZO-1 staining and redistribution of ZO-1 from the membrane to the cytoplasm, changes that are attenuated by blockade of the angiotensin II type 1 receptor. ZO-1 protein expression and serine and tyrosine phosphorylation of ZO-1 were also decreased in cells exposed to high glucose. These findings suggest that alterations in the content and localization of ZO-1 may be relevant to the pathogenesis of proteinuria in diabetes. Diabetes 55:894–900, 2006

Diabetic nephropathy is characterized by proteinuria and progressive fibrosis, resulting in a decline in kidney function in humans and experimental models of diabetes. Proteinuria is one of the most important prognostic risk factors for kidney disease progression (1). Cellular and molecular mechanisms underlying the loss of glomerular permselectivity in diabetic nephropathy and other proteinuric states are under intense investigation. Recent studies have identified mutations in the genes encoding podocyte structural proteins nephrin, podocin, CD2AP, neph-1, and α-actinin 4, which result in changes in glomerular permeability (2). ZO-1 α-τ, an 80–amino acid truncated isoform of the 225-kD tight junction protein ZO-1, is highly expressed within the podocyte in the cytoplasmic aspect of the foot process membrane, adjacent to the insertion of the slit diaphragm (3–5). It links some of the slit diaphragm proteins through its PDZ (PSD-95/discs-large/ZO-1) domains to the actin cytoskeleton. Thus, changes in the expression or properties of ZO-1 may accompany renal diseases associated with proteinuria, including diabetic nephropathy. Studies in humans and experimental animals highlighted the importance of poor glycemic control in the development of the functional and structural changes in the kidney during the evolution of diabetic nephropathy (1,6–10). Hyperglycemia and exposure of cultured cells to high glucose induce phenotypic modifications of cells that result in tissue injury (11–15). This study explored the expression of ZO-1 in two models of diabetes and the effect of glucose on ZO-1 expression and phosphorylation in cultured rat glomerular epithelial cells (GECs).

RESEARCH DESIGN AND METHODS

Animals were used in accordance with the guidelines for the care and use of laboratory animals set forth by the University of Texas. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing ~200–225 g were used. A total of 55 mg/kg streptozotocin (Sigma, St. Louis, MO) dissolved in citrate buffer (10 mmol/l, pH 4.5) or vehicle citrate buffer alone (controls) was administered through the tail vein under isofluorane inhalation anesthesia (Abbott, Abbott Park, IL). Control and diabetic rats, as well as adult 8-month-old db/db mice and control lean littermates (The Jackson Laboratory, Bar Harbor, ME), were placed in metabolic cages for urine collection. Urine protein was determined by Biuret assay, which detects intact urinary proteins, including albumin, as well as heavily degraded albumin fragments with molecular masses <10,000 Da (16). Urine creatinine was measured by modified Jaffé reaction. Proteinuria was expressed as milligram of protein per milligram of creatinine. Animals were killed by exsanguination under anesthesia. After harvesting, kidney cortex was snap frozen in liquid nitrogen and stored at −70°C.

Immunogold electron microscopy. Rat and mouse kidney cortex was finely chopped, fixed in 4% paraformaldehyde/0.2% picric acid, and embedded in LR white resin. Ultrathin sections were blocked with goat IgG and incubated with rabbit anti-ZO-1 (Zymed, South San Francisco, CA) followed by 15 nm colloidal gold conjugated goat anti-rabbit IgG (EBS, East Granby, CT). Grids were counterstained with 0.5% osmium and 1% uranyl acetate, and bound gold particles were visualized and photographed by electron microscopy.

Immunohistochemistry was performed in 4-μm-thick tissue sections, blocked with donkey IgG and incubated with polyclonal anti-ZO-1 followed by Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Sections were visualized and photographed with fluorescence or confocal microscopy. ZO-1 protein expression was quantified in 25 individual glomeruli in sections from four individual rats per group, using the Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD).

Immunoblotting of mouse glomerular lysates. Mouse glomeruli were isolated using Dynabeads perfusion technique as previously described (17) and solubilized in radioimmunoprecipitation assay buffer (20 mmol/l Tris × HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l Na2VO4, 1 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1%
TABLE 1
Rat model of type 1 diabetes

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Control</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>Time</td>
<td>3 weeks</td>
</tr>
<tr>
<td></td>
<td>12 weeks</td>
<td>68.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>24 weeks</td>
<td>67.0 ± 1.0</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>Time</td>
<td>3 weeks</td>
</tr>
<tr>
<td></td>
<td>12 weeks</td>
<td>451.0 ± 10.0</td>
</tr>
<tr>
<td></td>
<td>24 weeks</td>
<td>521.0 ± 13.0</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001 compared with control (Student’s t test). †P < 0.05.

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TABLE 2
Mouse model of type 2 diabetes (8 months old)

<table>
<thead>
<tr>
<th>Control</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>144 ± 14</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>33.3 ± 0.8</td>
</tr>
<tr>
<td>Urine protein/creatinine (mg/mg)</td>
<td>96.9 ± 8.9</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001 compared with control (Student’s t test). †P < 0.05.

RESULTS
Glomerular expression of ZO-1 in diabetes. Diabetic rats exhibited weight loss and proteinuria compared with controls (Table 1). Diabetic mice exhibited weight gain and proteinuria compared with controls (Table 2). Immunofluorescence and confocal microscopy showed a decrease in ZO-1 expression in diabetic rat glomeruli compared with controls as early as 1 week of diabetes (Fig. 1). Similarly, there was a decrease in the expression of ZO-1 in glomeruli of diabetic mice (Fig. 1). Immunoblotting of mouse glomerular lysates also demonstrated a decrease in glomerular ZO-1 expression in diabetic mice (Fig. 1).

Localization of ZO-1 by immunogold electron microscopy. In control mice, immunogold electron microscopy showed a normal pattern of distribution of ZO-1 in the close proximity to the membrane of the glomerular podocyte foot processes adjacent to the slits diaphragm (Fig. 2A). In db/db mice, ZO-1 was redistributed from the lateral membrane of the podocyte foot processes to the cytoplasm (Fig. 2B). Similar redistribution of ZO-1 was observed in diabetic rats (Fig. 2D and E), which also exhibited areas of widening and effacement of foot processes (Fig. 2E).

Effect of high glucose on ZO-1 expression in rat glomerular epithelial cells. Immunofluorescence microscopy showed normal staining of ZO-1 in the cell membrane of rat GECs grown in physiological glucose concentration (5 mmol/l) for 24 h (Fig. 3A). Incubation of GECs with high glucose concentration (25 mmol/l) resulted in decreased intensity of ZO-1 staining in the cell membrane, with increased cytoplasmic localization (Fig. 3B). This effect was at least partially reversed by the ARB ZD 7155 (Fig. 3C).

Immunoblotting of rat GEC lysates demonstrated a decrease in ZO-1 expression in cells grown in 25 mmol/l glucose compared with cells grown in 5 mmol/l glucose (Fig. 4A). This was not due to an osmotic effect because mannitol (25 mmol/l) had no effect on expression of ZO-1 (Fig. 4B).

Effect of high glucose on ZO-1 phosphorylation. Immunoprecipitation with anti–ZO-1, followed by immunoblotting with phosphoserine or phosphotyrosine antibodies, showed reductions in both serine (Fig. 5A) and tyrosine (Fig. 5B) phosphorylation of ZO-1 in cells grown in 25 mmol/l glucose environment compared with cells grown in 5 mmol/l glucose environment at 24, 48, and 72 h.

DISCUSSION
In this study, we demonstrate a decrease in the expression of ZO-1 in glomeruli of rats with type 1 and type 2 diabetes associated with redistribution of ZO-1 from the cell membrane to the cytoplasm of the podocyte foot processes. Exposure of rat GECs to high glucose concentration also results in decreased expression and phosphorylation of ZO-1 and its redistribution from the cell membrane to the cytoplasm of the podocyte foot processes.

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The Student’s t test. P < 0.05 was considered significant.

NP-40). Equal amounts of protein were electrophoresed on 7.5% polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk, 0.2% Tween in PBS and incubated with rabbit polyclonal anti–ZO-1, followed by horseradish peroxidase–conjugated goat anti-rabbit IgG. Reactive bands were detected by chemiluminescence.

Cell culture. Rat GECs courtesy of Dr. Jeffrey I. Kreisberg (18) were grown in Dulbecco’s modified Eagle’s medium. Cells were serum deprived for 24 h and then exposed to 5 mmol/l glucose, 25 mmol/l glucose, and 25 mmol/l glucose plus 20 nmol/l angiotensin II receptor blocker (ARB) ZD 7155 (Tocris, Ellisville, MO) for 24 h. Cells were stained using polyclonal anti–ZO-1 as primary antibody and goat Cy3-labeled anti-rabbit IgG as secondary antibody (19).

Immunoprecipitation and immunoblotting of cell lysates. Confluent GECs were serum deprived for 24 h and then exposed to 5 mmol/l glucose, 25 mmol/l glucose, or 25 mmol/l mannitol for 24, 48, and 72 h. Immunoprecipitation and immunoblotting were performed as described (20). Cells were solubilized with radiolabeling immunoprecipitation assay buffer, and equal amounts of protein were electrophoresed on 7.5% polyacrylamide gels. Polyclonal anti–ZO-1 was used as primary antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG was used as secondary antibody.

For immunoprecipitation, 0.5 μg polyclonal anti–ZO-1 per 100 μg protein was used and equal amounts of protein were electrophoresed on 7.5% polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked and incubated with either polyclonal anti–ZO-1, polyclonal anti–phospho-serine (Zymed) or monoclonal anti–phospho-tyrosine (Upstate, Lake Placid, NY), followed by horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse IgG. Reactive bands were detected by chemiluminescence.

Statistics. Data were expressed as means and SE and comparisons made by the Student’s t test. P < 0.05 was considered significant.
This translocation of ZO-1 is attenuated by an AT1 receptor blocker. These findings may be relevant to the pathogenesis of proteinuria in diabetic states. Diabetic nephropathy is associated with structural alterations that contribute to the increased permeability of the glomerular capillary wall to macromolecules (21–25). The cellular mechanisms leading to loss of permselectivity during progression of diabetic nephropathy are not well characterized. The ultimate selective barrier for the majority of plasma proteins resides in the slit diaphragm, a modified adherens junction bridging the gaps between the interdigitating foot processes of adjacent podocytes (26–29). The slit diaphragm consists of p-cadherin, nephrin, and Neph1 assembled into a zipper-like isoporous filter structure (30). Other proteins, such as ZO-1, α-actinin 4, CD2AP, and podocin are also expressed in the podocyte adjacent to the slit diaphragm (31,32). There is evidence that diabetes is associated with modifications of some of these podocyte junctional proteins, likely in response to activation of the renin-angiotensin system (24,33). A recent study showed that nephrin is downregulated in the podocyte in type 1 and type 2 diabetes, a change reversible by confocal microscopy and immunoblotting.

FIG. 1. Diabetes induces a decrease in glomerular expression of ZO-1. Confocal microscopy showing decreased ZO-1 staining in glomeruli of type 1 diabetic rats (B, D, and F) compared with controls (A, C, and E). Immunofluorescence microscopy in an additional group of rats showing decreased glomerular ZO-1 staining in type 1 diabetic rats (H, J, and L) compared with controls (G, I, and K). The histogram represents means ± SE of 25 individual glomeruli in sections from four rats in each group. Results are means ± SE. *P < 0.05. There is also decreased ZO-1 expression in glomeruli of type 2 diabetic mice (N) compared with controls (M), by confocal microscopy and immunoblotting.
with blockade of the renin-angiotensin system. Changes in the nephrin gene and protein expression are associated with podocyte ultrastructural abnormalities, including reduced presence of electron-dense filamentous structures within the slit diaphragm (25).

ZO-1 is a 225-kD adaptor protein of the membrane-associated guanylate kinase family containing five PDZ domains, an SH3 domain, and a guanylate domain (34). In the podocyte foot process, ZO-1 colocalizes and associates with other proteins such as F-actin, α-actinin 4, nephr-1, and α and β catenins (30). ZO-1 is thought to anchor the slit diaphragm to the actin cytoskeleton (34). In tight junctions from different epithelia, alterations of ZO-1 are associated with increased paracellular permeability (35). Our in vivo studies in type 1 and type 2 diabetes models demonstrate that there is a decrease in glomerular expression of ZO-1, associated with redistribution of ZO-1 from the podocyte membrane to the cytoplasm. The functional consequences of these alterations remain speculative. The decreased expression of ZO-1 was seen as early as 1 week after diabetes induction. However, since diabetic animals exhibited proteinuria even at this early stage of diabetes, it is difficult at present to determine whether ZO-1 alterations are a cause or a consequence of proteinuria. Alterations in ZO-1 have been implicated in the pathogenesis of proteinuria (36). In a genetic rat model of proteinuria, the proteinuric state was associated with ultrastructural changes with redistribution of ZO-1 from the podocyte foot process membrane to the cytoplasm. Blockade of the renin-angiotensin system abrogated the proteinuria and prevented ZO-1 redistribution in the podocyte (36).

Hyperglycemia is a major causative factor for diabetes complications. High glucose concentration leads to cellular defects that result in tissue injury. Indeed, the in vitro

FIG. 2. Translocation of ZO-1 from the podocyte cell membrane to the cytoplasm. Immunogold electron micrograph in mice showing that ZO-1 localizes preferentially to the membrane of the podocyte foot process, adjacent to the slit diaphragm in controls (C, arrows). In diabetic mice, ZO-1 assumes primarily cytoplasmic localization (B, arrows). Similar findings were present in rats with type 1 diabetes (C, control; D, diabetes). Note the widening and effacement of podocyte foot processes (E, arrowhead).
studies in cultured rat GECs show that high glucose concentration decreases expression of ZO-1 and results in the redistribution of the protein from the membrane to the cytoplasm. The ARB ZD 7155 markedly attenuated these changes in ZO-1, suggesting that angiotensin II at least partially mediates the alterations of ZO-1 observed in the in vivo models. It is interesting to note that exposure of retinal endothelial cells to high glucose results in similar alterations in ZO-1 (37).

Posttranslational modification of proteins including podocyte proteins alter their physical properties and function (38). In our study, diabetes was associated with ultrastructural changes of ZO-1 in glomeruli. Furthermore, exposure of rat glomerular epithelial cells to high glucose resulted in a decrease in serine and tyrosine phosphorylation of ZO-1. Posttranslational modifications of proteins and their functional consequences are likely to be tissue specific. In bovine retinal endothelial cells in vitro, tyrosine phosphorylation of ZO-1 is associated with increased permeability through the paracellular pathway (39). ZO-1 also has serine phosphorylation sites (40), and serine/threonine phosphorylation of ZO-1 is associated with increased paracellular permeability in rabbit nasal epithelium (41). The type of injury may also influence ZO-1 phosphorylation. For example, there is enhanced tyrosine phosphorylation of ZO-1 in glomeruli of rats treated with protamine sulfate (42).

The functional consequences of our findings remain speculative. The translocation of ZO-1 from the foot process membrane to the cytoplasm may lead to modifications of other podocyte junctional proteins, resulting in dissociation of the slit diaphragm from the actin cytoskeleton and loss of permselectivity to proteins. In rat GECs, high glucose alters the structure of filamentous f-actin, the

FIG. 3. High glucose decreases the expression of ZO-1 in the cell membrane, with redistribution to the cytoplasm. Immunofluorescence microscopy showing linear ZO-1 staining along the cell membranes in rat GECs grown in 5 mmol/l glucose environment (A and C). The intensity of ZO-1 staining is decreased in the membranes of cells grown in a 25-mmol/l glucose environment, where the staining adopts a cytoplasmic pattern (B and D). The ARB ZD 7155 (20 nmol/l) markedly attenuates the high glucose–induced alterations in ZO-1 (E).

FIG. 4. High glucose results in decreased expression of ZO-1. Immunoblot showing decreased expression of ZO-1 in rat GECs grown in 25 mmol/l compared with a 5-mmol/l glucose environment (A) over time, independent of an osmotic effect (B).
disruption of which leads to proteinuria (12). In summary, diabetes causes decreased expression and translocation of ZO-1 from the podocyte cell membrane to the cytoplasm. This effect is likely mediated by high glucose and angiotensin II. We suggest that high glucose enhances glomerular capillary barrier permeability, at least in part by modifying the expression and localization of the junctional protein ZO-1.

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FIG. 5. High glucose results in decreased serine (A) and tyrosine (B) phosphorylation of ZO-1. There is decreased serine and tyrosine phosphorylation over time in cells grown in a 25- vs. 5-mmol/l glucose environment. Lysates exposed to beads alone do not show a band in the 225-kD region (C).
ZO-1 AND DIABETIC NEPHROPATHY


