Urinary Podocyte Microparticles Identify Prealbuminuric Diabetic Glomerular Injury

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ABSTRACT

Microparticles (MPs) are small (0.1–1.0 μm) vesicles shed from the surface of cells in response to stress. Whether podocytes produce MPs and whether this production reflects glomerular injury are unclear. We examined MP formation in cultured human podocytes (hPODs) and diabetic mice. hPODs were exposed to cyclical stretch, high glucose (HG; 25 mM), angiotensin II, or TGF-β. Urinary podocyte MPs were assessed in three mouse models of diabetic nephropathy: streptozotocin (STZ)-treated, OVE26, and Akita mice. Cyclic stretch and HG increased MP release as assessed by flow cytometry (P < 0.01 and P < 0.05, respectively, versus controls). Inhibition of Rho-kinase (ROCK) with fasudil blocked HG-induced podocyte MP formation. STZ-treated (8 weeks) mice exhibited increased urinary podocyte MPs compared with age-matched nondiabetic mice. Similarly, 16-week-old OVE26 mice had elevated levels of urinary podocyte MPs compared with wild-type littermates (P < 0.01). In 1 week post-STZ–treated and 6- and 12-week-old Akita mice, urinary podocyte MPs increased significantly compared with those MPs in nondiabetic mice, despite normal urinary albumin levels. Our results indicate that podocytes produce MPs that are released into urine. Podocyte-derived MPs are generated by exposure to mechanical stretch and high glucose in vitro and could represent early markers of glomerular injury in diabetic nephropathy.

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Podocytes are critical to the maintenance of the glomerular filtration barrier (GFB), and their injury is associated with increased glomerular permeability.1 Diabetes is associated with GFB damage and subjects podocytes to injurious factors yielding podocyte injury and loss, ultimately resulting in increased glomerular permeability.2 This altered permeability manifests clinically as albuminuria: a signature of a compromised GFB and a hallmark of diabetic nephropathy. Both animal and human studies illustrate that changes to podocyte morphology followed by progressive podocyte loss are present early in diabetes and directly contribute to albuminuria.3–6 Although pharmacological intervention can delay progressive renal injury, early treatment is likely to provide the greatest therapeutic benefit, because podocytes possess limited regenerative capacity.7 Unfortunately, current indices of podocyte injury/loss (i.e., hypertrophy, detachment, and apoptosis) reflect established podocyte loss and, therefore, are less likely to facilitate early therapeutic intervention.1

One feature of stressed/damaged cells is the formation of extracellular vesicles shed from the plasma membrane called microparticles (MPs). MPs are extracellular vesicles distinguishable on the basis of size, mechanism of formation, and content.8 MPs (0.1–1.0 μm) are larger than exosomes (40–100 nm) and smaller than apoptotic bodies (> 1 μm). MPs are typified by externalized phosphatidylserine (identifiable using Annexin V staining) and the presence of surface antigens of the parent cell (identifiable using immunostaining).8,9 MPs can be detected in the plasma of healthy subjects and are putative biomarkers of disease. For example, endothelial MPs are increased in hypertension, diabetes, and CKD.8 Interestingly, although MPs are formed during the early stages of apoptosis, they also form during milder cell stress, such as endothelial cell activation or sublethal shear.10,11 In addition to their presence in plasma, MPs have been identified in urine.12,13 However, whether podocytes form MPs and whether such formation reflects...
glomerular injury are unclear. Accordingly, we examined podocyte MP production in diabetes.

To assess their properties, podocyte vesicles were isolated from the media of cultured human podocytes (hPODs) and mouse urine and characterized by nanoparticle tracking analysis (NTA) and flow cytometry. MPs from media were also examined by electron microscopy. NTA revealed that cultured hPODs released vesicles into media with a mean size of 214 nm (Figure 1A). Similarly, urine from 8-week-old streptozotocin (STZ)-treated mice contained vesicles with a mean size of 242 nm (Figure 1B). MPs were also isolated from media and urine and sorted by flow cytometry. For media, MPs were identified as events of 0.1–1.0 μm with positivity for Annexin V (Figure 1, C and D). For urine, MPs were gated for Annexin V positivity and then identified as 0.1- to 1.0-μm events that displayed immune-detectable podocalyxin (Figure 1, E and F). Isolated MP fractions from media were verified to possess characteristic size and morphology by electron microscopy (Figure 1, G and H).

The formation of MPs from podocytes was subsequently examined in media collected from hPODs exposed to high glucose (25 mM), subjected to cyclic mechanical stretch (a mimic of intraglomerular forces), or treated with angiotensin II (Ang II) or TGF-β (Figure 2). In the absence of stimulation, podocytes released MPs into media in a time-dependent fashion (Figure 2A). As assessed by flow cytometry, exposure of hPODs to high glucose increased MP formation at 24 hours (Figure 2B). hPODs subjected to cyclic stretch also produced more MPs than untreated controls (Figure 2B). Treatment with either Ang II (10−7 M) or TGF-β (5 ng/ml) did not significantly alter MP formation at 24 hours (Figure 2C). Because cytoskeletal reorganization is a key event in the formation of MPs, we examined whether Rho-kinase (ROCK) is involved in podocyte MP formation. Treatment with fasudil (10 μM) blocked high glucose–induced podocyte MP formation (Figure 2D).

To examine in vivo release of podocyte-derived MPs into urine, we used three mouse type 1 diabetes models (STZ-treated, OVE26, and Akita mice) as well as type 2 diabetic db/db mice. Consistent with previous reports, mice were hyperglycemic, and both OVE26 and STZ-treated mice exhibited early onset of albuminuria compared with nondiabetic controls (Figure 3A, Supplemental Tables 1 and 2). Akita mice were hyperglycemic but resistant to development of albuminuria to 12 weeks (Figure 3A, Supplemental Table 3). Podocyte-derived MPs were detected at significantly higher levels in urines of diabetic OVE26, STZ-treated, and Akita mice compared with nonpatients with diabetes (Figure 3C). Most interestingly, podocyte-derived MPs were detected in urines of STZ-treated mice after 1 week and Akita mice from 6 to 12 weeks of age when urinary albumin levels were not increased (Figure 3, A and C). Similar findings were obtained for 4-week-old nonalbuminuric db/db mice (db/db:...
Effects of ROCK inhibition on high glucose-induced podocyte MP formation

Figure 2. High glucose and mechanical stretch induce podocyte microparticle formation in vitro. (A) Cultured hPODs released MPs into media in a time-dependent fashion. Data are mean±SEM (n=3). *P<0.01 versus 0 hours; **P<0.001 versus 0 hours. (B) Effects of high glucose and cyclic stretch on MP formation by cultured hPODS. Cells were exposed to high glucose (25 mM D-glucose), normal glucose (control; 5 mM), or mannitol-treated high glucose and cyclic stretch for 24 hours. MPs were isolated from media by differential centrifugation, and Annexin V+ve MPs were quantified by flow cytometry. To account for differences in cell numbers, MP numbers were normalized to protein levels. Exposure to high glucose significantly increased podocyte MP formation compared with mannitol-treated or untreated controls at 24 hours. Similarly, cyclic stretch increased podocyte MP formation at 24 hours. However, the combination of both high glucose and cyclic stretch did not result in additive or synergistic effects on MP formation. Data are mean±SEM (n=5–8). *P<0.01 versus no stretch control; †P<0.01 versus no stretch mannitol treatment; ‡P<0.01 versus stretch control. (C) Effects of Ang II and TGF-β on MP formation by cultured hPODS. Cells were treated with Ang II (500 nM) or TGF-β (5 ng/ml) for 24 hours. Data are mean±SEM (n=3). (D) Effects of ROCK inhibition on high glucose-induced podocyte MP formation in vitro. Cells were exposed to high glucose, normal glucose, or mannitol-treated controls for osmolality in the presence and absence of the ROCK inhibitor fasudil (10 μM). *P<0.01 versus mannitol; †P<0.001 versus high glucose (n=3).

1365±860 versus db/m: 2±1 podocalyxin+ve MPs/mg creatinine; P<0.05). Urinary podocyte MP levels correlated with albuminuria in OVE26 (P<0.001), STZ-treated (P<0.001), and Akita (P<0.05) mice (Figure 3D). Finally, we assessed urinary podocyte MPs using a separate podocyte surface marker (podoplanin) and found that levels of podoplanin+ve MPs were increased in 16-week-old OVE26 mice (Figure 4).

The principle finding of the present study is that diabetic stress conditions induce formation of MPs from podocytes and that podocyte MPs identify the earliest stages of diabetic renal injury in mice. Both high glucose and mechanical stretch were potent stimuli for podocyte MP formation in vitro, whereas stress factors, such as Ang II and TGF-β, were without effect.

It is increasingly clear that extracellular vesicles are present in urine and may be of significant clinical use. Exosomes, although distinct from MPs, have been identified in urine samples and proposed as tools for the noninvasive identification of renal pathology. Using electron microscopy, Pascual et al. identified 100- to 200-nm urinary vesicles containing the complement receptor-1 consistent with podocyte MPs. Subsequent studies have further confirmed the presence of MPs in human urine. Nevertheless, the precise MP populations present in urine, stimuli responsible for their formation, and their use as biomarkers of renal injury remain unclear. Our data suggest that podocytes represent a source of urinary MPs. Notably, MPs of podocyte origin represented only a small fraction of all MPs identified in urine. The origins of remaining MPs are unclear, although the tubular epithelium represents one potential source.

Although total urinary MPs were variable, podocyte MPs showed considerably more promise as biomarkers of glomerular injury. Podocyte MPs offer several advantages over traditional biomarkers of glomerular injury. First, podocytes are a primary cellular target in diabetic nephropathy, and MPs offer direct insight into their health. Second, podocytes are terminally differentiated cells with a limited capacity for regeneration. Thus, early identification of podocyte injury may allow for more optimal therapeutic intervention in diabetes. Third, although detection of urinary podocytes (podocyturia) reflects progressive glomerular injury, it typically follows the onset of albuminuria. In contrast, MPs have been reported to reflect ongoing but potentially reversible cell stress, which could allow for the identification of at-risk podocytes before irreversible injury/death.

Several stimuli for MP formation have been identified, including lipopolysaccharide, thrombin, and proinflammatory
cytokines. Our data identify both high glucose and mechanical stretch as potent stimuli for MP release. Others have shown that high glucose increases MP formation in mesangial cells and that mechanical stretch induces MP formation from platelets and endothelial cells. Interestingly, although Ang II induces MP formation in endothelial cells, it did not induce MP formation in cultured podocytes. Thus, the stimuli that govern MP formation may differ between these cell types.
types. Nevertheless, hyperglycemia and increased glomerular capillary pressure are hallmarks of diabetic nephropathy, and it is conceivable that these processes contributed to formation and release of podocyte MPs in our diabetic mice. Podocyte MP formation was also ROCK-dependent, because treatment with fasudil blocked high glucose-induced podocyte MP formation. These data are consistent with our previous reports identifying ROCK as a determinant of endothelial cell stress that may predict subsequent podocyte loss and GFB damage. Unfortunately, in our OVE26 mice, albuminuria was present at the earliest times for which it was possible to obtain urine samples. Conversely, in Akita mice, which display a later onset of albuminuria, we observed progressive increases in urinary podocyte MP levels at times when we were unable to detect albuminuria. In addition, there was a strong correlation between urinary albumin levels and podocyte MPs in STZ-treated, OVE26, and Akita mice, further supporting the notion that the presence of podocyte MPs in urine is a sensitive indicator of underlying podocyte stress and injury.

In summary, our study provides strong evidence that the appearance of podocyte-derived MPs in urine is a sensitive and early indicator of podocyte damage in diabetes. Although observations from the present study require additional investigation in clinical samples, the present study highlights the potential for use of podocyte MPs in earlier diagnosis of glomerular injury and identification of diabetic individuals at risk of developing nephropathy.

CONCISE METHODS

Animal Models
All mice were housed at University of Ottawa animal care facilities and provided free access to food and water on a 12:12-hour light:dark cycle. Protocols were approved by the University of Ottawa Animal Ethics Committee and performed according to the recommendations of the Canadian Council for Animal Care.

STZ-Induced Diabetic Mice
Male mice (FVB background, 8-weeks) were injected intraperitoneally with five low doses of STZ (50 mg/kg per day; Sigma-Aldrich, St. Louis, MO) or sodium-citrate vehicle over 5 consecutive days to induce type 1 diabetes. Blood glucose levels were monitored weekly from hind limb blood samples. Mice were monitored for up to 8 weeks post-STZ injection.

OVE26 Mice
OVE26 mice carry a calmodulin transgene under the insulin promoter that results in β-cell destruction, severe diabetes, and features of both diabetic nephropathy and cardiomyopathy. Male OVE26 mice on an FVB background were monitored for up to 16 weeks of age.

Akita Mice
Wild-type (Ins2 WT/WT) C57BL/6] and diabetic heterozygous Akita (Ins2 WT/C96Y) male mice were purchased from The Jackson Laboratory. Akita mice develop insulin-dependent diabetes and display features of early diabetic nephropathy. Male mice were monitored up to 12 weeks of age.

db/db Mice
Male db/db mice (C57BLKS/J background) and their control heterozygous littermates (db/m) were purchased from The Jackson Laboratory.

Urinary Albumin Excretion
Spot urines were collected, and urinary albumin excretion was determined using the albumin to creatinine ratio on morning spot urines using a murine albumin ELISA (Bethyl Laboratories, Montgomery, TX) and a creatinine assay kit (Exocell).

Cell Culture
A conditionally immortalized hPOD line was obtained from Moin Saleem (University of Bristol, Bristol, UK) and cultured using the methods described. Briefly, cells were grown on collagen I–coated culture plates (0.1 mg/ml; Sigma-Aldrich) in RPMI-1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 100 μg/ml normocin (Cedarlane Laboratories Ltd., Hornby, ON, Canada), and penicillin-streptomycin solution (1:100; Invitrogen). Podocytes were propagated at 33°C in the presence of 10 U/ml recombinant human γ-IFN (Invitrogen). For induction of podocyte differentiation, cells were maintained at 38°C for 14 days in the absence of γ-IFN. For high-glucose experiments, cultured, fully differentiated cells were maintained in serum-free media for 24 hours and then exposed to
normal d-glucose (5 mM), high d-glucose (25 mM), or high mannitol (20 mM+5 mM d-glucose; osmotic control) for 2–72 hours. Stimulation was stopped by removing the media and adding ice cold PBS. All experiments were performed in fully differentiated podocytes.

**Mechanical Stretch**
For studies involving mechanical stretch, differentiated podocytes were seeded onto six-well collagen I-coated stretch plates (Flexcell Int., Hillborough, NC) for 3 days. Plates were mounted onto vacuum-based loading docks of the Flexcell FX-4000T apparatus (Flexcell Int.) as described with modification.28 Podocytes were incubated in RPMI containing 0.5% FBS and subjected to 10% equibiaxial elongation at a frequency of 0.5 Hz for 24 hours. Nonstretched cells (control) were exposed to identical experimental conditions but without mechanical stretch.

**Microparticle Isolation**
**Cell Culture Studies**
Media were collected from cultured hPODs, and MPs were isolated as described with slight modification.22 Briefly, samples were centrifuged at 2500 × g for 20 minutes at 20°C to obtain media free of cells and apoptotic bodies. MPs were then isolated from cell-free media by centrifugation at 20,000 × g for 20 minutes at 20°C, the MP-containing pellet was collected, and the supernatant, which contains exosomes, smaller vesicles, and soluble factors, was discarded. The MP-containing pellet was resuspended in Annexin V binding buffer for flow cytometric analysis.

In Vivo Studies
Urine samples (>100 μl) were transferred to microcentrifuge tubes, and MPs were isolated by differential centrifugation as above. Samples were centrifuged at 2500 × g for 20 minutes at 20°C, the supernatant was transferred to fresh microcentrifuge tubes, and MPs were isolated by centrifugation at 20,000 × g for 20 minutes (20°C). The MP-containing pellet was resuspended in Annexin V binding buffer before flow cytometry.

**Flow Cytometric Detection of Microparticles**
MPs were quantified using a MoFlo Fluorescence Activated Cell Sorter as described.23 An Alexa-647–labeled Annexin V (0.5 μg/ml; Biolegend, San Diego, CA) was used to all identify events as MPs. For urine samples, a phycoerythrin (PE)-conjugated podocalyxin antibody (1:200; R&D Systems) was used to identify MPs specifically as podocytes in origin. In some experiments, a PE-conjugated podoplanin antibody (1:100; Biolegend) was alternatively used to identify podocyte origin. As a negative control, a subpopulation of MPs was resuspended in Annexin V binding buffer lacking calcium and containing a PE-conjugated IgG control antibody. MPs were defined as particles of <1.0 μm and >0.1 μm in size that exhibited significantly more fluorescence than their negative controls. Using this approach, exosomes are not quantified because of their small size (not seen by cytometer) and lack of Annexin V positivity.29 To account for differences in cell numbers, media samples were normalized to MPs per milligram of protein. Similarly, to account for differences in urine volume, urine samples were normalized to urinary creatinine levels (Creatinine Companion Kit; Exocell).

**Electron Microscopy**
MPs were isolated from media of cultured hPODs and visualized by electron microscopy as described.22,23

**NTA**
Sizing and enumeration of extracellular vesicles were achieved by NTA using a Nanosight LM10 instrument (NanoSight Limited, Amesbury, UK) equipped with NTA 2.3 software.30 NTA is a light-scattering technique that uses video analysis for sizing and enumeration of extracellular vesicles.30 Media or urine samples were collected and diluted in PBS to a particle concentration within the range of 2–20 × 10^9/ml (optimal working range of the system). An approximately 300-μl sample was loaded into the sample chamber, and videos of 60 seconds were recorded for each sample with a shutter speed of approximately 30 milliseconds and a camera gain between 250 and 650. Settings for software analysis were detection threshold, 30–50; blur, 5 × 5; and minimum expected particle size, auto. Size distributions are presented as the average and SEM of five to six video recordings per sample.

**Statistical Analyses**
Results are expressed as mean ± SEM and were analyzed by using a Kruskal–Wallis test and one- or two-way ANOVA with a Bonferroni post-test as appropriate. For parametric statistical tests, data were verified to not deviate from Gaussian distributions with a Kolmogorov–Smirnov test. All statistical analyses were performed with GraphPad Prism 4.0 (GraphPad Software, Inc.). P < 0.05 was considered significant.

**ACKNOWLEDGMENTS**
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**DISCLOSURES**
None.

**REFERENCES**
7. Quaggin SE, Keidberg JA: Development of the renal glomerulus: Good neighbors and

Supplemental Table 1: Whole animal data for OVE26 mice

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<td>Plasma Glucose (mM)</td>
<td>6.8±1.1</td>
<td>28.6±3.6*</td>
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<td>Urinary Albumin/Creatinine (mg/g)</td>
<td>24±11</td>
<td>1285±352**</td>
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WT: wild type mice, * P<0.05, **P<0.01 vs. age matched WT control

Supplemental Table 2: Whole animal data for STZ mice

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<td>Control</td>
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<td>Plasma Glucose (mM)</td>
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<td>21.2±1.4*</td>
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<td>Urinary Albumin/Creatinine (mg/g)</td>
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STZ: streptozotocin-treated mice * P<0.05, **P<0.01 vs age-matched control mice

Supplemental Table 3: Whole animal data for Akita mice

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<td>Akita</td>
<td>WT</td>
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<tr>
<td>Plasma Glucose (mM)</td>
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<td>28.6±1.6**</td>
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**P<0.01 vs age-matched Wild-type, -: Not measured