Increased urinary excretion of nephrin, podocalyxin, and βig-h3 in women with preeclampsia

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Wang Y, Zhao S, Loyd S, Groome LJ. Increased urinary excretion of nephrin, podocalyxin, and βig-h3 in women with preeclampsia. Am J Physiol Renal Physiol 302: F1084–F1089, 2012. First published February 1, 2012; doi:10.1152/ajprenal.00597.2011.—Emerging evidence has shown that podocyte injury and reduced specific podocyte protein expressions contribute to proteinuria in preeclampsia. We collected urine specimens from women with preeclampsia to study whether podocyte-specific protein shedding is associated with renal barrier dysfunction. Urine specimens from women with normal pregnancies and from pregnant women complicated by chronic hypertension were used for comparison. We determined soluble podocyte slit protein nephrin levels in the urine specimens. Podocalyxin, βig-h3, and VEGF concentrations were also measured. We found that nephrin and podocalyxin were barely detectable in the urine specimens from normal pregnant women and from women with chronic hypertension. In preeclampsia, urinary nephrin and podocalyxin concentrations were significantly increased and highly correlated to each other, r² = 0.595. Nephrin and podocalyxin were also correlated with urine protein concentrations. βig-h3 was detected in the urine specimens from women with preeclampsia, and it is highly correlated with nephrin and podocalyxin concentrations in preeclampsia. βig-h3 was undetectable in normal pregnancy and pregnancy complicated by chronic hypertension. Elevated VEGF levels were also found in women with preeclampsia compared with those of normal pregnancy and pregnancy complicated by chronic hypertension. These results provide strong evidence that podocyte protein shedding occurs in preeclampsia, and their levels are associated with proteinuria. The finding of urinary βig-h3 excretion in preeclampsia suggests that increased transforming growth factor activity might also be involved in the kidney lesion in this pregnancy disorder.

kidney lesion; glycoprotein shedding; TGF-β; proteinuria; pregnancy complication

NEWLY DEVELOPED HYPERTENSION and proteinuria after 20 wk of gestation are the two major clinical diagnostic criteria for preeclampsia, a hypertensive and multiple-system disorder unique to human pregnancy. Plasma protein leakage into the urine signifies renal/kidney lesions in this pregnancy disorder. Pathophysiologically, renal lesions are attributed to the glomerular endotheliosis characterized by glomerular endothelial cell swelling and subendothelial deposits of protein materials (5). Renal vasoconstriction is another contributing factor as part of the global systemic vasoconstriction in preeclampsia (9). Emerging evidence has revealed that other than glomerular endotheliosis, podocyte injury also plays a significant role in glomerular barrier dysfunction in preeclampsia (7, 8, 12, 27, 28). Supporting evidence includes (1) reduced podocyte-spe-
shedding of podocyte functional proteins is an indispensable podocyte injury in preeclampsia.

METHODS

Patient information. This study was approved by the Institutional Review Board (IRB) for human research at the Louisiana State University Health Sciences Center-Shreveport (LSUHSC-S), LA, and conducted in the Department of Obstetrics and Gynecology. A total of 34 pregnant women were recruited in this study. Among them, 8 were normotensive pregnant women, 6 had chronic hypertension, and 20 had preeclampsia. Normotensive pregnancy was defined as pregnancy with normal blood pressure (<140/90 mmHg) without medical and obstetric complications. Pregnancy complicated by chronic hypertension was defined as preexisting hypertension (>140/90 mmHg) before pregnancy and diagnosed before week 20 of gestation. Preeclampsia was defined as elevated blood pressure (>140/90 mmHg) on two separate occasions at least 6 h apart after 20 wk of gestation. Preeclampsia complicated by nephrotic syndrome and/or diabetes was excluded from the study.

Urinary sample process. Urine samples were collected after patient consent was obtained. Spot urine samples were collected in sterile containers. Freshly obtained urine specimens were centrifuged, aliquot, and stored at −70°C in a freezer until assay.

Measurements of nephrin, podocalyxin, βig-h3, and VEGF. Urine concentrations of nephrin, podocalyxin, βig-h3, and VEGF were measured by ELISA. ELISA kits for nephrin, podocalyxin, and βig-h3 were purchased from Exocell (Philadelphia, PA). An ELISA kit for VEGF was from R&D Systems, (Minneapolis, MN). Assays were carried out according to the manufacturers’ instructions. Urine samples were diluted with dilution buffer provided by the ELISA kits in a ratio of 1:10 for nephrin assay and 1:2 for podocalyxin and βig-h3 assays. The range of the standard curve was 0.078–5 ng/ml for nephrin, 0.5–60 ng/ml for podocalyxin, and 0.078–5 ng/ml for βig-h3. The range of the standard curve for VEGF assay was 1.95–1,000 pg/ml. An aliquot of a 100-μl sample without dilution was applied to the assay. For each assay, all samples were measured in duplicate on the same assay day. Within-assay variations were <7% for all the assays.

Measurements of urine protein and creatinine levels. Total urinary protein and creatinine concentrations were measured in all urinary samples. Urine protein concentrations were measured by the Bradford method. Creatinine concentrations were measured using urinary creatinine ELISA kits (Arbor Assays, Ann Arbor, MI). The range of the urinary creatinine standard curve was 0.02–20 mg/dl. All samples were diluted 1:20 and measured in duplicate.

Statistical analysis. Clinical demographic data are presented as means ± SD. Statistical analysis was performed with a Mann-Whitney test, unpaired t-test, and ANOVA using the computer software program StatView (SAS Institute, Cary, NC). Fisher’s protected least significant difference test or a Student-Newman-Keuls test was used as a post hoc test. A probability level <0.05 was considered statistically significant.

RESULTS

Patient clinical data. The clinical information including maternal age, racial status, blood pressure, body mass index (BMI), gestational age at urine sample collection and delivery, and delivery mode was obtained by chart review and is shown in Table 1. There were no significant differences in maternal age, gestational age at urine sample collection, and delivery among the three groups. Maternal systolic and diastolic blood pressure were significantly higher in the preeclamptic and chronic hypertension groups than in the normal pregnancy group. BMI was different between the preeclamptic and the chronic hypertension groups, but not between the preeclamptic and the normal pregnancy groups. The cesarean section rate was higher in the preeclamptic and chronic hypertension groups than in the normal group.

Increased urine concentrations of nephrin, podocalyxin, and βig-h3 in preeclampsia. Urine concentrations of nephrin and podocalyxin are shown in Table 2. Both nephrin and podocalyxin levels were significantly higher in the preeclamptic than in the normal pregnancy groups: nephrin, 1,226 ± 286 vs. 86 ± 22 ng/ml, P < 0.01; and podocalyxin, 195 ± 48 vs. 29 ± 9 ng/ml, P < 0.05, respectively. Nephrin, but not podocalyxin, levels were also significantly higher in the preeclamptic than in the chronic hypertension group (nephrin: 164 ± 63 ng/ml and podocalyxin: 96 ± 36 ng/ml, P = 0.23). The correlations between urine nephrin and podocalyxin levels in normal pregnancy, pregnancy complicated with chronic hypertension, and pregnancy complicated with preeclampsia are shown in Fig. 1. Urine nephrin and podocalyxin levels are highly correlated in

Table 1. Demographic data of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Normal Pregnancy (n = 8)</th>
<th>Chronic Hypertension (n = 6)</th>
<th>Preeclampsia (n = 20)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, yr</td>
<td>29 ± 6</td>
<td>32 ± 10</td>
<td>25 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>Black</td>
<td>3</td>
<td>6</td>
<td>16</td>
<td>NA</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td></td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>123 ± 10</td>
<td>156 ± 20</td>
<td>158 ± 13</td>
<td>P &lt; 0.01*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>67 ± 10</td>
<td>97 ± 16</td>
<td>95 ± 7</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>2.579 ± 709</td>
<td>P &lt; 0.01†</td>
</tr>
<tr>
<td>BMI</td>
<td>37 ± 12</td>
<td>50 ± 16</td>
<td>36 ± 9</td>
<td>P &lt; 0.01‡</td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine collection</td>
<td>30 ± 10</td>
<td>32 ± 4</td>
<td>31 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>At delivery</td>
<td>36 ± 3</td>
<td>34 ± 5</td>
<td>31 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Delivery mode</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>C-section</td>
<td>5</td>
<td>6</td>
<td>16</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are means ± SD. BMI, body mass index; NS, not significant; NA, not analyzed. *Preeclampsia vs. normal pregnancy. †Preeclampsia vs. normal pregnancy and chronic hypertension. ‡Preeclampsia vs. chronic hypertension.

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also highly correlated with nephrin, normals pregnancies (7.29 pg/mL) compared with those in normal pregnancies (7.29 ± 1.93 pg/mL) and pregnancy complicated by chronic hypertension (3.41 ± 1.43 pg/mL).

**Table 2. Urine concentrations of nephrin, podocalyxin, βig-h3, and VEGF**

<table>
<thead>
<tr>
<th></th>
<th>Normal Pregnancy (n = 8)</th>
<th>Chronic Hypertension (n = 6)</th>
<th>Preeclampsia (n = 20)</th>
<th>P Value (Preeclampsia vs. Normal)</th>
<th>P Value (Preeclampsia vs. Chronic Hypertension)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrin, ng/ml (range)</td>
<td>86 ± 22 (5–216)</td>
<td>164 ± 63 (16–433)</td>
<td>1,226 ± 286 (55–5297)</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Podocalyxin, ng/ml (range)</td>
<td>29 ± 9 (4–83)</td>
<td>96 ± 36 (22–245)</td>
<td>195 ± 48 (2–648)</td>
<td>&lt;0.05</td>
<td>0.225</td>
</tr>
<tr>
<td>βig-h3, ng/ml (range)</td>
<td>0</td>
<td>8 ± 2 (0–24)</td>
<td>609 ± 8.30 (22.20–157.13)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urine creatinine, mg/dl (range)</td>
<td>72.94 ± 8.46 (35.48–106.69)</td>
<td>128.94 ± 28.22 (46.77–248.52)</td>
<td>1,894 ± 339 (93–6316)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Podocylaxin/creatinine (range)</td>
<td>107 ± 20 (14–202)</td>
<td>129 ± 28 (47–249)</td>
<td>305 ± 65 (7–944)</td>
<td>&lt;0.05</td>
<td>0.056</td>
</tr>
<tr>
<td>Podocalyxin/creatinine (range)</td>
<td>39 ± 11 (7–87)</td>
<td>91 ± 23 (34–179)</td>
<td>16 ± 4 (0–50)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>βig-h3/creatinine (range)</td>
<td>0</td>
<td>0</td>
<td>16 ± 4 (0–50)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>VEGF, pg/ml (range)</td>
<td>7.29 ± 1.93 (1.14–15.76)</td>
<td>3.41 ± 1.43 (0.23–6.47)</td>
<td>13.25 ± 3.12 (1.00–47.96)</td>
<td>0.357</td>
<td>0.060</td>
</tr>
</tbody>
</table>

Values are means ± SE.

the preeclamptic and chronic hypertension groups, but not in the normal pregnancy group.

Urineary concentrations of βig-h3, creatinine, and VEGF are presented in Table 2. βig-h3 was detected in the urine specimens from women with preeclampsia but not in the specimens from women with normal pregnancies and pregnancy complicated by chronic hypertension. There was no difference in urine creatinine levels between normal pregnancy and preeclampsia. Urinary creatinine levels were higher in specimens from pregnant women with chronic hypertension than in preeclampsia. VEGF concentrations were elevated in the preeclamptic group (13.25 ± 3.12 pg/ml) compared with those in normal pregnancies (7.29 ± 1.93 pg/ml) and pregnancy complicated by chronic hypertension (3.41 ± 1.43 pg/ml).

**Correlations of urine nephrin and podocalyxin concentrations with protein and βig-h3 levels in preeclampsia.** Nephrin is a podocyte slit diaphragm-specific protein, and podocalyxin is a major component of the glycocalyx on podocytes. To determine the relationships among nephrin, podocalyxin, and urine protein levels in preeclampsia, correlations of nephrin and podocalyxin with urine protein levels were analyzed. As shown in Fig. 2, A and B, urine nephrin concentrations are highly correlated with urine protein concentrations, \( y = 257.35x - 297.90, r^2 = 0.746 \). Similarly, urine podocalyxin concentrations are also correlated with urine protein concentrations, \( y = 31.12x + 10.64, r^2 = 0.390 \). These results suggest that increased podocyte nephrin and podocalyxin shedding is associated with disrupted renal barrier function in preeclampsia.

βig-h3 is a secreted protein induced by TGF-β. Interestingly, βig-h3 was detected in urine specimens from women with preeclampsia but not from normal pregnant women and pregnant women with chronic hypertension. These results suggest that increased βig-h3 excretion could be an indicator of TGF-β involvement in the renal/kidney lesion in preeclampsia. As shown in Fig. 2, C and D, urine βig-h3 concentrations are also highly correlated with nephrin, \( y = 117.07x + 260.34, r^2 = 0.515 \), and podocalyxin, \( y = 18.57x + 41.69, r^2 = 0.463 \), concentrations in preeclampsia.

**DISCUSSION**

In this study, we determined nephrin, podocalyxin, and βig-h3 concentrations in urine specimens from three pregnant groups: normal pregnant women, pregnant women with chronic hypertension, and those with preeclampsia. We found that urine nephrin and podocalyxin levels are significantly higher in women with preeclampsia than those in normal pregnant women and in pregnant women with chronic hypertension. Nephrin is a specific podocyte slit diaphragm protein (20), and it plays an essential role in maintaining renal filtration barrier functionality. Podocalyxin is a sialoglycoprotein, which is the major component of the glycocalyx on podocytes. Increased urine secretions of nephrin and podocalyxin in preeclampsia demonstrate podocyte injury and shedding of podocyte functional proteins in this pregnancy disorder.

Nephrin is a 180 kDa transmembrane protein expressed by glomerular podocytes and predominately localized at the glomerular slit diaphragm (20). The glomerular slit diaphragm is a modified adhesion junction and controls glomerular permselectivity of plasma proteins (17, 19). Immunoelectron microscopy studies have revealed that nephrin is present in junctions with a ladderlike structure between podocytes (18). Reduced nephrin expression was found in kidney biopsy specimens from women with preeclampsia and in autopsy specimens from women who died from preeclampsia (7, 27). Reduced nephrin expression in shed podocytes further supports increased nephrin shedding in preeclampsia (28). We previously reported that reduced nephrin expression was associated with decreased and altered expression and distribution of podoplanin and polarity proteins PARD-3 and PARD-6 in the shed podocytes from women with preeclampsia (28). These findings indicate that reduced nephrin expression is associated with increased nephrin shedding, suggesting that urinary nephrin concentration could be an index of podocyte injury in preeclampsia. The correlation of urinary nephrin with proteinuria concentrations in preeclampsia further supports this notion.

Several studies have shown that podocyte glycoproteins are reduced in preeclampsia, such as podoplanin and GLEPP-1 (27, 28). In the present study, we also found increased urinary podocalyxin levels in preeclampsia. Podocalyxin is the major sialoglycoprotein in glomerular podocytes (13). It is a negatively charged 140-kDa glycoprotein that not only coats the cell surface but also coats the foot processes of podocytes; thus it functions to keep adjacent foot processes separated and the filtration barrier open. Podocalyxin is not only present on podocytes but also on endothelial cells, platelets, and parietal epithelial cells (22). Although podocalyxin is not podocyte specific, it is probably the most frequent marker protein used for podocyte diagnosis in the urine (3). The urine podocalyxin level has been used as a readout in many renal diseases, including membranous nephropathy, focal segmental glomerulosclerosis, lupus nephritis, and diabetic nephropathy; and the
level of podocalyxin reflects the disease activity in these kidney disorders (3). Increased urinary podocalyxin levels found in our study are consistent with the positive podocalyxin expression in shed podocytes in preeclampsia (8). Moreover, we also found that urine podocalyxin levels are highly correlated with urine nephrin levels in the chronic hypertension and preeclampsia groups, even though the nephrin and podocalyxin levels are very low in patients with chronic hypertension.

Another important finding of our study is the discovery of kidney secretion of βig-h3 in women with preeclampsia. In this study, βig-h3 was detected in all, but one, urine specimens in the preeclamptic group. It is undetectable in urinary specimens from normal pregnant women and from pregnant women with chronic hypertension, which suggests that secretion of βig-h3 could be a unique feature of kidney lesions in preeclampsia.

βig-h3 was originally identified in and produced by a human lung adenocarcinoma cell line (A549) after being induced by
TGF-β in culture (23). βig-h3 is known in the literature as a TGF-β-induced protein (TGFBIp). Studies have shown that βig-h3 is an extracellular matrix protein that can be induced by TGF-β in many cell types, including epithelial cells, fibroblasts, and keratinocytes (25). It mediates cell adhesion to collagen, laminin, and fibronectin through its interaction with different β-integrins. βig-h3 can be secreted, functioning to support cell adhesion and spreading (24). In lymphatic endothelial cells, βig-h3 could modulate cell adhesion and migration by binding to the β3-integrin (10). In colon cancer cells, it was found to promote cell extravasation and tumor metastasis by inducing the dissociation of VE-cadherin junctions between endothelial cells via activation of the integrin αVβ5-Src signaling pathway (16). It was also reported that the C-terminal fragment of βig-h3 was required for apoptosis in human cancer cells (26). In addition, overexpression of βig-h3 was found in various tumors in different organs including the kidney and brain (11). Increased urinary concentrations of βig-h3 have also been reported in type-2 diabetes mellitus (4) and in chronic cyclosporine nephrotoxicity (14). To our knowledge, our study is the first to determine the urinary level of βig-h3 in preeclampsia. Although the reason for kidney secretion of βig-h3 in preeclampsia is not known, the detection of βig-h3 in urinary specimens suggests increased TGF-β1 bioactivity in the kidney during the disease process in preeclampsia. Increased urinary βig-h3 secretion correlated with increased podocyte shedding of nephrin and podocalyxin further supports the concept of TGF-β/βig-h3 involvement in the renal/kidney dysfunction in this pregnancy disorder.

We also measured VEGF concentrations in the urine specimens. Although VEGF levels were not statistically significant between the study groups, VEGF levels were found higher in the preeclamptic group than in the normal pregnancy and pregnancy complicated by chronic hypertension groups. Podocytes express and produce VEGF. VEGF produced by podocytes plays critical roles in the development of glomerular endothelial fenestrations and maintenance of glomerular endothelial cell function (21). Mice with a homozygous deletion in podocyte-specific VEGF fail to develop a filtration barrier (6). We previously reported enhanced VEGF expression in the kidney biopsy specimens from women who had preeclampsia (27). Although it is not known whether increased VEGF secretion is a consequence of increased VEGF expression or a compensatory mechanism of podocytes to produce more VEGF to maintain glomerular endothelial function, our data support the hypothesis that endogenous production of VEGF from renal tissue might contribute to the elevated urine excretion of VEGF in preeclamptic patients (2). One concern raised is the specificity of podocyte protein shedding in preeclampsia since podocyte injury is common in kidney diseases such as minimal change, membrane or lupus nephritis, and other nephrotic syndromes. Currently, the information on nephrin and podocalyxin measurement in urine specimens in kidney diseases is sparse, but expressions of nephrin and podocalyxin have been frequently used as markers for urinary podocyte identification (22). Despite this, transient podocyte detachment in patients with preeclampsia could differentiate this pregnancy disorder from those of nephrotic syndrome in nonpregnant subjects. Studies to determine whether shedding of soluble podocyte-specific protein occurs before clinical proteinuria in pregnant women shall answer this question.

In summary, this is the first study that simultaneously determined urinary concentrations of nephrin and podocalyxin and kidney excretion of βig-h3 in normal pregnancy, pregnancy complicated by chronic hypertension, and pregnancy complicated by preeclampsia. Although the sample size is small in the groups of normal pregnancy and pregnancy complicated by chronic hypertension, results obtained from this study demonstrate that podocyte injury/podocyte protein shedding contributes to and/or is a consequence of kidney lesions in preeclampsia. First, increased urinary secretion of nephrin and podocalyxin indicates that not only foot process slit protein but also cell surface glycoprotein is shed from injured podocytes in preeclampsia; second, the correlation of nephrin and podocalyxin with proteinuria further demonstrates the association of podocyte injury with glomerular barrier dysfunction in preeclampsia; and last but not least, the urinary excretion of βig-h3 in preeclampsia provides evidence of increased TGF-β activity in the kidney during preeclampsia. Although the reason for urinary excretion of βig-h3 is not known, the finding of urinary excretion of βig-h3 in preeclampsia, but not in normal pregnancy and pregnancy complicated by chronic hypertension, suggests that βig-h3 might be a useful surrogate biomarker of kidney dysfunction in preeclampsia. Further studies of podocyte marker protein excretion correlated with altered kidney TGF-β/βig-h3 pathway signaling warrants the unraveling of TGF-β/βig-h3 roles in the pathophysiology of kidney/renal lesions in preeclampsia and of other kidney involved diseases.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.W., S.L., and L.J.G. provided conception and design of research; Y.W. and S.Z. analyzed data; Y.W., S.Z., and L.J.G. performed experiments.

REFERENCES


