Elevation of (Pro)Renin and (Pro)Renin Receptor in Preeclampsia

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Condensation

(Pro)renin and (pro)renin receptor are elevated in patients with preeclampsia and in a rat model of preeclampsia.

OBJECTIVE

Preeclampsia (preE), a syndrome of hypertension, proteinuria, and edema, has many elusive triggers. The renin–angiotensin system has been implicated in preE pathogenesis. In this study, we test the hypothesis that (pro)renin levels are increased in preE patients and that levels of (pro)renin and (pro)renin receptor ((P)RR) are elevated in a rat model of preE.

METHODS

We recruited 30 preE and 43 normal pregnant consenting patients. We used normally pregnant rats (NP, n = 10) and pregnant rats receiving weekly injections of desoxycorticosterone acetate and whose drinking water was replaced with 0.9% saline (preE, n = 10). Plasma and placental levels of (pro)renin were assayed by ELISA. Placental and kidney (P)RR was measured both by immunoblotting and immunohistochemistry.

RESULTS

The mean plasma (pro)renin of 27.1 ± 5.2 in preE patients differs from that in patients without preE: 14.8 ± 5.2 ng Ang I/ml/hour (P < 0.0001). In rats, both plasma (NP: 22.7 ± 4.3 and preE: 49.2 ± 10.0 ng Ang I/ml/hour) and placental (NP: 152 ± 24 and preE: 302 ± 39 ng/g tissue) levels of (pro)renin were higher (P < 0.001) in preE compared to NP rats. (P)RR expression was greater (P < 0.05) in placental tissue of preE rats, while kidney (P)RR expression was similar.

CONCLUSION

Elevated levels of circulating (pro)renin have been observed in preE patients and in a rat model of preE. We also found the increased expression of placental (P)RR in preE rats.

Keywords: blood pressure; hypertension; placenta; preeclampsia; (pro)renin; (pro)renin receptor; renin–angiotensin system.

doi:10.1093/ajh/hpv019

Preeclampsia (preE) is a disorder affecting 3%–10% of pregnancies. PreE is frequently accompanied by reduced uterine placental perfusion often resulting in intrauterine growth restriction and release of vascular regulatory molecules from the placenta producing maternal systemic effects.1–3 PreE is a hypertensive disorder in pregnancy with multiple pathophysiologic triggers and pathophysiologic mechanisms.1,2,4 Methods for early diagnosis and treatment are not yet available, and prevention in women at increased risk is not currently possible.4

The renin–angiotensin system (RAS) plays a role in preE pathogenesis.5 In contrast to the downregulation of the circulating RAS in preE,6 Anton et al. demonstrated that Ang II and AT1 receptor expression levels are increased in the chorionic villi of women with preE.7 Anton et al. also demonstrated that the placental vascular bed is capable of producing Ang II.7 The increased concentration of Ang II may act directly on fetal chorionic villi vessels to increase resistance to blood flow and decrease maternal-fetal exchange of oxygen and nutrients.7,8

Pringle et al. assembled observations suggesting that the extent and timing of hypoxic challenges in pregnancy contribute to normal placental development.9 Alterations in this pattern may set the stage for dysfunctional perfusion later in pregnancy. Maternal circulating renin in human pregnancy is derived from a renal source which responds appropriately to renal-type physiological stimuli. However, the very high (pro)renin in maternal circulation is likely of ovarian and/or placental origin.10 (Pro)renin is the enzymatically inactive precursor of renin.11 There are actually 2 RAS operating at the placental boundary, one in the fetal placental tissue and the other in the maternal uterine vessels. A postulated mechanism by which the placenta may

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Initially submitted October 31, 2014; date of first revision December 7, 2014; accepted for publication January 30, 2015; online publication March 11, 2015.

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influence maternal vascular tone is the placental production of (pro)renin. All components of RAS have previously been shown to be present in the placenta including (pro)renin and (pro)renin receptor ((P)RR). A growing body of evidence supports the existence of a local, intrinsically active RAS which appears to participate in the regulation of decidual vascular remodeling and uteroplacental blood flow. Several indirect lines of evidence indicate that upregulation of the RAS in the placenta might be important for preE pathogenesis. (Pro)renin can be activated in vivo proteolytically and/or nonproteolytically. A “handle” region on the receptor plays an important role in (pro)renin binding to (P)RR and its nonproteolytic activation. Decapeptides (10P–19P) known as “decoy” peptides can inhibit the binding of (pro)renin to (P)RR. The existence of this novel regulatory system that stimulates signal transduction and/or RAS activity is postulated. The activation mechanism by which this novel RAS participates in preE pathogenesis involves (pro)renin and its receptor. If this is true, blockade with a decoy peptide may effectively improve local placental perfusion and extend the duration of pregnancy, an important clinical outcome.

Recently, high circulating levels of soluble (P)RR were detected at delivery in patients with preE. However, the association of elevated (pro)renin and (P)RR with preE remains to be determined. In this study, we tested the hypothesis that levels of (pro)renin and (P)RR were increased in preE patients with preE and in a rat model of preE.

**METHODS**

**Study population**

Normal and preE pregnant patients were recruited from the Department of Obstetrics and Gynecology of Scott & White Healthcare in Temple, TX. This study was approved by the Scott & White Institutional Review Board.

PreE patients were identified with blood pressures (BP) >135/90 mm Hg after being normotensive prior to prepregnancy and with proteinuria (>300 mg/24 hours). The clinical status and assessments at the time of admission for maternal symptoms were used to establish the diagnosis of preE. Patients without preE symptoms during a similar gestational age interval were asked to participate in the study. We recruited 30 preE patients and 43 normal pregnant patients as controls. After obtaining informed written consent, heparinized blood samples were obtained and plasma analyzed for (pro)renin levels. Patient characteristics including BP, urinary protein, maternal age, gestational age at the time of blood collection, and maternal height and weight were obtained. None of these patients had diabetes mellitus during their pregnancies.

**Measurement of (pro)renin levels**

Plasma renin concentrations were estimated by angiotensin I immunosorbent assay ELISA kit (Ang I ELISA kit; Cayman Chemical, Ann Arbor, MI) after incubation of plasma with angiotensinogen under standard assay conditions as described previously. The renin concentrations were expressed as the concentration of Ang I in ng/ml/hour produced by renin under standard conditions. Total renin levels were determined with the Ang I ELISA, after activation of (pro)renin with 0.2 g/L trypsin for 1 hour. The (pro)renin activation in the presence of trypsin was stopped with 0.5 g/L soya bean trypsin inhibitor. By subtracting active renin from total renin, the (pro)renin concentration was obtained and expressed as ng of Ang I per ml/hour.

**Animal study**

Two groups of rats were studied: normal pregnant rats given tap water (NP, n = 10) and pregnant rats intraperitoneally injected initially with 12.5 mg of desoxycorticosterone acetate followed by weekly injections of 6.5 mg of desoxycorticosterone acetate and given 0.9% saline to drink (preE, n = 30). Systolic BP was measured by the tail-cuff method (IITC Inc., LifeScience Instruments, model 59, Waltham, MA). At 18–21 days of pregnancy, the rats were sacrificed, blood was drawn and 24-hour urine was collected. The pups were counted and the 24-hour urinary protein excretion was measured using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Organs were harvested including the placenta.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Normal pregnant (n = 43)</th>
<th>Preeclamptic (n = 30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>121 ± 10</td>
<td>166 ± 11</td>
<td>&lt;0.05a</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>71 ± 9</td>
<td>92 ± 12</td>
<td>&lt;0.05a</td>
</tr>
<tr>
<td>Urinary protein (mg/24 hours)</td>
<td>161 ± 24</td>
<td>457 ± 48</td>
<td>&lt; 0.05b</td>
</tr>
<tr>
<td>Age (years)</td>
<td>26.0 ± 4.2</td>
<td>23.5 ± 5.1</td>
<td>&lt;0.05a</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>30.9 ± 5.2</td>
<td>41.5 ± 8.6</td>
<td>&lt;0.05b</td>
</tr>
<tr>
<td>Gestational age at blood collection (weeks)</td>
<td>33.5 ± 3.5</td>
<td>35.5 ± 2.7</td>
<td>0.05a</td>
</tr>
<tr>
<td>Plasma prorenin (ng Ang I/ml/hour)</td>
<td>14.8 ± 5.2</td>
<td>27.1 ± 5.2</td>
<td>&lt;0.05a</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n = number of patients. Abbreviation: Ang I, angiotensin I.

aComparison using Student’s t-test.

bComparison using Mann–Whitney U-test.
Measurement of (pro)renin levels in placental tissue

The placental tissue was minced and 1 g of tissue was suspended in 5 ml ice-cold lysis buffer and homogenized. The homogenates were centrifuged at 10,000 rpm for 10 minutes and the supernatants were used for analysis of the (pro)renin levels. (Pro)renin levels were measured in the supernatant from placental tissue by methods similar to those employed for plasma.\(^{22}\)

Measurement of (P)RR expression in placental tissue

(P)RR expression was measured by gel electrophoresis of the placental homogenate followed by detection with immunoblotting (western blotting). An equal amount of protein in each placental sample was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. Membranes were blocked in 20 mM Tris (pH 7.6), 250 mM NaCl containing 5% bovine serum albumin and probed with a mouse anti-ATP6IP2 antibody (Abcam, Cambridge, MA). Following incubation with peroxidase- or alkaline phosphatase-conjugated donkey anti-mouse secondary antibody (Santa Cruz, Dallas, TX), proteins were visualized with a chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). The intensity of the bands was measured by scanning video densitometry using the phosho-imager, Storm 860, (GE Healthcare) and the ImageQuant TL software version 2003.2 (GE Healthcare). The intensities related to ATP6IP2 antibody binding was normalized with those of β-actin.

Immunohistochemistry of placenta and kidney samples from preE and NP rats

Kidney and placenta samples were frozen in Optimal Cutting Temperature compound and cut on the Cryostat as 20-micron-thick slices. Tissue slices were put on positively charged slides. Slides were incubated at 37 °C for 15 minutes and washed in phosphate-buffered saline (PBS) for 5 minutes. Slides were placed in 0.01% hydrogen peroxide for 20 minutes and washed in PBS for 5 minutes. A hydrophobic pen was used to circle tissue sections and 5% goat serum was added to the circled tissue sections for 2 hours. Slides were incubated in a humidified box and the anti-ATP6IP2 antibody (Abcam) which is a specific for (P)RR was added in 1% goat serum. Negative control samples were placed.

Table 2. BP, urinary protein excretion, number of pups, plasma and placental (pro)renin levels in NP and preE rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>NP (n = 10)</th>
<th>PreE (n = 10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (mm Hg)</td>
<td>106.2 ± 5.1</td>
<td>93.3 ± 9.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Final BP (mm Hg)</td>
<td>95.9 ± 2.8</td>
<td>135.0 ± 5.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary protein excretion (mg/24 hours)</td>
<td>123.1 ± 6.2</td>
<td>149.5 ± 9.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Number of pups</td>
<td>14.4 ± 0.8</td>
<td>11.7 ± 0.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma prorenin (ng Ang I/ml/hour)</td>
<td>22.7 ± 4.3</td>
<td>49.2 ± 10.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Placental prorenin (ng/g tissue)</td>
<td>152 ± 24</td>
<td>302 ± 39</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n = number of rats; P values from Student’s t-test.
Abbreviations: BP, blood pressure; NP, normally pregnant rats; preE, preeclampsia.

Figure 1. (a) Receiver operating characteristic (ROC) curve analysis for the association of serum prorenin levels (ng Ang I/ml/hour) with preeclampsia (preE) diagnosed using established clinical criteria. A threshold value greater than 21 ng Ang I/ml/hour has a sensitivity of 90% and specificity of 93% for preE in this series of 73 patients. The area under the curve is 0.95 with a 95% CI of 0.88–0.99 which is different from 0.5 (P < 0.0001). Thirty patients had preE and 43 NP (normally pregnant rats) in this series. (b) Comparison of (pro)renin levels for 30 patients with between 28 and 39 weeks of pregnancy and those for 43 normally pregnant within the same gestational age interval. Those with PreE differed from those without (P < 0.0001 using Student’s t-test). There was no significant relationship of (pro)renin levels with gestational age in either group using linear regression analyses (P > 0.10). Regression lines are shown with 95% CIs.
in the humidified box without the antibody and only 1% goat serum. The humidified box was placed in the refrigerator overnight. Slides were washed in PBS three times for 30 minutes. The secondary antibody was added in 1% goat serum to slides for 2 hours. Then slides were again washed in PBS for 3 × 30 minutes. Diaminobenzidine was added to the slides for less than 30 seconds then slides were washed in water. Slides were then dipped 10 times in 50% alcohol, 70% alcohol, 90% alcohol, 95% alcohol, and xylene consecutively. Finally, dibutyl phthalate xylene mountant was added to the slides and a coverslip was placed over the tissue.

Statistical methods

Heterogeneity of variance of patient characteristics and assay values were examined with Levene’s test. For those measurements with heterogeneous variance, Mann–Whitney U- tests were used for comparisons of the 2 patient groups. Otherwise, Student’s t-tests were used to compare groups. Variation in (pro)renin measurements associated with gestational age in normal pregnancies and those with preE symptoms were examined using linear regression methods (Statistica, Statsoft, Tulsa, OK). A receiver operating characteristic (ROC) curve was developed for the (pro)renin measurements to define the optimal threshold criterion value (MedCalc, MedCalc Software, Belgium). The area under curve value for the ROC was examined for the difference from 0.5. The criterion value closest to the upper left-hand point on the ROC plot was taken as the optimal threshold for this dataset. The sensitivity and specificity for the optimal threshold criterion was reported for the relationship to diagnosis of preE.

Data from the NP rats were compared to preE rats using Student’s t-test. A P value of less than 0.05 was considered significant.

RESULTS

Patient data

Mean characteristics for the patient population, comparing the preE to normally pregnant patients, are provided in Table 1. As expected, pregnant patients with symptoms of preE differed from those with normal pregnancies in variables related to these symptoms. Specifically, mean systolic BP for the preE patients differed (P < 0.0001) from those of normal patients (166 ± 11 and 121 ± 10 mm Hg, respectively). The mean diastolic BP differed (P < 0.0001) (92 ± 12 and 71 ± 9 mm Hg, respectively). The mean urinary protein levels differed (P < 0.0001) (457 ± 48 mg/24 hours in the preE group and 160 ± 24 mg/24 hours in the NP group). Patient body mass index differed (P < 0.0001) between groups with 33.1 ± 5.2 kg/m² for patients with normal pregnancies and 41.5 ± 8.6 kg/m² for patients with preE. The patients with preE averaged to be about 2 weeks further along in pregnancy than those with normal pregnancies, but the range of gestations for both groups was 28–39 weeks of gestation.

(Pro)renin levels are elevated in plasma of preE patients compared to those with normal pregnancies

The mean plasma level of (pro)renin of 27.1 ± 5.2 (mean with SD) ng Ang I/ml/hour in the preE group significantly (P < 0.0001) differs from that in the normally pregnant group (14.8 ± 5.2 ng Ang I/ml/hour) (Table 1). Figure 1a presents the ROC curve for the relationship of (pro)renin levels to preE in this dataset. The area under the curve for this ROC was 0.95 with 95% CI of 0.88–0.99 and the optimal threshold for identifying patients with preE was a value of (pro)renin greater than 21 ng of Ang I/ml/hour which had a 90% sensitivity and 93% specificity for preE. Figure 1b shows the comparison between the patients with and without preE symptoms. (Pro)renin levels are shown at the gestational ages when samples were collected. There were no significant gestational age relationships for (pro)renin in patients with preE (P = 0.10) and in those without preE (P = 0.13) using linear regression analyses.

Figure 2. (a) The (pro)renin receptor ((P)RR) was measured in the placental tissue from the 2 groups of animals by western blotting, running the homogenate from the placental tissues from NP (normally pregnant rats) and preE (preeclampsia) rats in gel followed by detecting with immunoblotting using anti-ATP6IP2 antibody. The animals were: NP (n = 10) and pregnant + desoxycorticosterone acetate + saline (preE) (n = 10). The placental (P)RR was significantly upregulated in preE rats compared to NP (<0.05). The results presented are the mean ± SE. A blot from a representative experiment is shown in the figure. (b) The (P)RR was measured in the kidney tissue from NP and preE rats by western blotting using anti-ATP6IP2 antibody. There was no difference in the expression of (P)RR in NP and preE kidneys.
BP, urine, and blood analyses of rat study

Data for BP measurements, the urinary excretion of protein, and number of pups are presented in Table 2. BP in NP animals did not change over the course of the experiments (18–20 days). BP rose in the preE compared to NP ($P < 0.0001$). The preE animals showed a statistically significant ($P < 0.03$) increase in protein excretion when compared with the NP group. The mean number of pups for the preE group was significantly lower than those for the NP group ($P < 0.03$). These data reproduce the observations made concerning the BP and protein excretion obtained in previous studies of this animal model of preE.1,24

Both the circulatory and placental levels of (pro)renin are increased in preE rats

Both the plasma and placental (pro)renin concentrations were significantly higher in preE rats compared to NP rats (plasma (pro)renin for NP: 22.7 ± 4.3 and PreE: 49.2 ± 10.0 ng Ang I/ml/hour; placental (pro)renin for NP: 152 ± 24 and PreE: 302 ± 39 ng/g tissue) (Table 2).

(P)RR was upregulated in the placental tissue of preE rats

As shown in Figure 2a, The placental (P)RR was upregulated ($P < 0.05$) in preE rats compared to NP.

(P)RR was unchanged in the kidney tissue of preE rats

(P)RR expression in kidney tissue of preE and NP rats were similar (Figure 2b).

Immunohistochemistry of kidney and placental tissues of NP and preE rats

Immunohistochemistry was used to localize the sites for (P)RR in rat kidney and placenta (Figure 3c,d). Labeled cells in the kidney were associated with the vessels of the glomerular capsule. Labeled cells in the placenta were clustered rather than distributed uniformly. Negative controls without primary antibody (not shown) did not demonstrate staining in either of these regions of the kidney or placenta.

DISCUSSION

The present study demonstrates that plasma (pro)renin levels are significantly elevated in preE patients and in a rat model of preE. Both the placental (pro)renin and (P)RR levels are significantly upregulated in the preE rat model. Using ROC methods, we found that (pro)renin levels greater than 21 ng of Ang I/ml/hour are associated with preE. These findings are similar with those reported previously.5,25–27 Several investigators have demonstrated the presence of (pro)renin28 and its mRNA in the human placenta.29,30 It has been shown that (pro)renin concentrations are higher in human umbilical venous blood than in arterial blood. This observation provides additional evidence that (pro)renin is likely of placental origins.31 There was no significant relationship between plasma (pro)renin levels and gestational age in our study. However, we evaluated patients in the second half of pregnancy. We suspect that (pro)renin levels can precede the onset of preE symptoms as Ringholm et al. found high (pro)
renin concentrations as early as 8 weeks of gestation in women had type I diabetes mellitus and later developed preE.  

Recently, Watanabe et al. found high concentrations of soluble (P)RR in the plasma of preE patients.  

The higher expression of the (P)RR in the placental tissues of preE rats in the present study along with similar (P)RR expression in rat kidneys supports the notion that the high concentration of soluble (P)RR in the plasma of preE patients is likely of placental origin. Findings from previous studies and the present study suggest that uteroplacental components including the placenta are a major source of soluble (P)RR in pregnant women. In our study, placental (P)RR expression was found to be higher in preE rats compared to NP which extends our previous report of alterations of RAS components in this rat model of preE. This observation is consistent with those obtained in human preE.

It has been demonstrated that the human placental (pro)renin RAS plays an important role in the normal trophoblast invasion and angiogenesis. The (pro)renin-RAS has various effects in human uterine tissues, either directly by (P)RR-mediated cell signaling, or indirectly via Ang II as shown in Figure 4a. The overexpression of (P)RR in animal studies caused the slow progression of nephropathy and hypertension including tissue RAS activation. Population-based studies suggested that (P)RR gene polymorphisms contribute to BP levels, thus the (P)RR is associated with the development of hypertension.

The expression of (P)RR is higher in preE rats compared to NP. We suggest that increased levels of both (pro)renin and (P)RR may contribute to development of hypertension in preE rats and in human preE patients. It has been demonstrated that (P)RR plays a vital role in embryonic and fetal development and plasma (pro)renin levels are increased during pregnancy. The (P)RR at the maternal-fetal interface of placenta is involved in the tissue RAS activation and could activate (pro)renin in maternal plasma thus leading to the activation of the circulating RAS. These cumulative effects may cause the hypertension in preE which is depicted in our working model (Figure 4b). A novel component of this research on (P)RR associated RAS modulation is that (pro)renin binding to its receptor may not only facilitate angiotensin generation locally but also activate second messenger pathways within the vascular cells. If true, it implies that (pro)renin can also act as an agonist independent of Ang II generation. It has been reported that (pro)renin induced p42/p44 (ERK1/2) MAPK activation and transforming growth factor β1 release in mesangial cells. These effects were attenuated following siRNA knockdown of the receptor. The decoy peptide, the handle region (peptide) blocker of (pro)renin-(P)RR binding, prevented diabetic nephropathy in rat models.

This study provides for the first report of the elevation of plasma (pro)renin in preE women and a rat model of preE. Though it shows a correlation in plasma (pro)renin in humans and placental (P)RR in preE rat models, it does not prove that these elements contribute to the etiology of preE as the patients were evaluated after the onset of the condition. A larger serial study is now underway to measure serum (pro)renin levels throughout gestation and prior to diagnosis of preE. Several others mentioned in this discussion have made connections with detrimental secondary signaling from (P)RR. Further in vitro studies outlining (P)RR signaling and clarifying that it is the source for multiple routes of pathogenic signaling could be accomplished with use of decapptide inhibitors. In vivo studies of decapptide therapy with preE rat models could also confirm or disprove the pathogenesis of (P)RR activity while providing insight to a potential therapy and improved prognosis for preE, for which there are currently limited treatment options.

**FUNDING**

Funding for this work was provided by Scott, Sherwood and Brindley Foundation (R4047UDDI to M.N.U.) and the Noble Centennial Endowment for Research in Obstetrics and Gynecology (R4500KUEH to T.J.K.), Scott & White Healthcare, and from the Scott & White Research & Education Division (R9240UDDI), Temple, TX.
ACKNOWLEDGMENTS

The authors thank Glen Cryer for assistance with manuscript preparation. Findings were presented at the 33rd Annual Meeting of the Society for Maternal-Fetal Medicine on February 11–16, 2013 in San Francisco, CA, USA. Reprints will not be available.

DISCLOSURE

The authors declared no conflict of interest.

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