Online supplementary materials for
Tissue transglutaminase contributes to the pathogenesis of preeclampsia and stabilizes placental angiotensin receptor AT1 by ubiquitination-preventing isopeptide modification

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Methods and Materials

Patients
PE patients admitted to Memorial Hermann Hospital were diagnosed by the faculty of the Department of Obstetrics, Gynecology and Reproductive Sciences, the University of Texas Health Science Center at Houston based on the definition set by the National High Blood Pressure Education Program Working Group Report. The criteria of patient selection, including no previous history of hypertension, are based on previous publications¹. Control pregnant women selected in this study have a normotensive and uncomplicated pregnancy ending with a normal term delivery. The research protocol on human subjects in this study is approved by the Institutional Committee for the Protection of Human Subjects. Informed consent was obtained from all the human subjects. The patients’ detailed information is summarized in Table 1 of supplementary data.

Cell cultures
Human trophoblast cell line HTR-8/SVneo was maintained in RPMI 1640 with 10% FBS and 1% antibiotics. Stable Chinese hamster cell (CHO) lines expressing WT or Q315A AT1 receptor and TG2 were maintained in RPMI 1640 with 10% FBS, 500 µg/ml G418, 500 µg/ml hygromycin, and 1% antibiotics. Experimental protocols were carried out in serum-free RPMI 1640 media.

All the lines continually displayed proper cellular morphology consistent with published literature regarding the authenticity of these cell lines. All the cells in the study were maintained in a mycoplasma-free environment in which the mycoplasma contamination was prevented by periodic treatment with a mycoplasma removal agent from AbD Serotec. The mycoplasma-free environment was confirmed with InvivoGen Mycoplasma Detection Kit.
Immunohistochemistry (IHC) and immunofluorescence (IF) dual-staining
Paraffin embedded sections of human placenta were immunostained for TG2 (1:100, mouse source, Abcam, MA) or ε-(γ-glutamyl)-lysine isopeptide (1:50, mouse source, Abcam, MA) with VECTASTAIN ABC-AP Immunohistochemistry (IHC) and immunofluorescence (IF) dual-staining kit (Vector Labs, CA) following manufacturer’s instruction. The immunohistochemical staining (red) was quantified with Image-Pro Plus software (Media Cybernetics, MD).

Double Immunofluorescence staining
The simultaneous immunofluorescence staining for human placental AT1 receptor (1:100, rabbit source, Santa Cruz, CA) and ε-(γ-glutamyl)-lysine isopeptide (1:50, mouse source, Abcam, MA) was performed according to double immunofluorescence simultaneous staining protocol (Abcam, MA). After primary antibody staining, Alexa 568(red)-conjugated donkey anti-rabbit antibody (1:1000, Invitrogen, CA) and Alexa 488(green)-conjugated donkey anti-mouse antibody (1:1000, Invitrogen, CA) were employed to stain anti-AT1 receptor antibody and anti-ε-(γ-glutamyl)-lysine isopeptide antibody on the human placental sections, respectively. Finally, coverslips were mounted in DAPI-containing mounting medium (Vector Labs, CA).

In vitro transglutaminase assay
TG activity in human and mouse samples was determined with in vitro TG activity assay kits (Covalab, France; Sigma-Aldrich, MO) following manufacturers’ instructions.

Introduction of cystamine and tTG siRNA to PE mouse model
PE mouse model was established by retro-orbital sinus injection of 800 µg IgG purified from PE patients’ plasma on Embryonic Day 13 and 14 into pregnant mice as previously described. To repress TG activity in PE mice, 2.24 mg cystamine dihydrochloride (Sigma-Aldrich, MO) dissolved in 0.15 ml sterile PBS was injected retro-orbitally into pregnant mice on Embryonic Day 13 and 14 together with 800 µg NT/PE IgG. In the following days till Embryonic Day 18, cystamine-injected mice were also fed with drinking water containing cystamine dihydrochloride (0.9 gram/liter). To knockdown TG2 expression in PE mice, 200 µg murine TG2 siRNA (Sigma-Aldrich, MO) embedded in nanoparticles (Altogen, NV) was prepared and injected together with PE IgG into each pregnant mouse following manufacturers’ instructions.

Blood pressure and proteinuria measurements
Blood pressure and proteinuria in PE IgG-injected mice, PE IgG-injected mice treated with cystamine, or PE IgG-injected mice co-injected with TG2 siRNA were measured as described before.

Immunoprecipitation
Tissues or cells were lysed in lysis buffer (1XTBS, 1% Triton X-100, 5 mM EDTA), protease inhibitor cocktail (Sigma-Aldrich, MO), and phosphatase inhibitor cocktail (Sigma-Aldrich, MO) with proper sonification or homogenization. Then, 500 µl cell or tissue lysates were incubated overnight at 4 degree with 50 µl antibody-bound Protein G or Protein A Sepharose High Performance beads (GE Healthcare Life Sciences) prepared according to manufacturer’s instruction and rocked gently. After immunoprecipitation, the beads were washed 4 times with 1XTBS, and boiled with 50 µl 2X Laemmli buffer for Western blot analysis.

Western blot
The procedure for Western blot analysis used in this study has been described previously. Western blot results in this study are representative of multiple determinations.

Cloning and mutagenesis
The cDNA encoding human tissue transglutaminase was PCR-amplified from a human placental cDNA library with the primers ATATATAAGCTTATGGCCAGAGCTGGTC (5’) and ATATAACTCGAGCCTTATGGCGGCGGCAATG (3’), and then cloned into pCDNA3.1/Hygro (+) plasmid at HindIII and XhoI sites. The cDNA encoding human AT1 receptor was PCR-amplified from a ready cDNA plasmid (Origene, MD) with the primers GCGGCCAGCTTATGATTCTCAACTCTTCTTCT (5’) and GAGACGCTCGAGTCACTCAACCTCAAAACA (3’), and then cloned into pCDNA3/G418 (+) plasmid at HindIII and XhoI sites. The cDNA encoding Q315A AT1 receptor mutant was generated by Quickchange Mutagenesis (Stratagene/Agilent, CA) with the primers TTTCTCGCGCTTCTAAAATATATTCCCCCA (5’) and TAGAAGCGCGAGAAATATCTTTTAAATT (3’). All the above clones were confirmed with DNA sequencing (Genewiz, NJ).

Statistical analysis
Data were expressed as the mean ± SEM. GraphPad Prism was employed to determine the statistical significance of the data. Student’s t tests (paired or unpaired as appropriate) were applied in two-group analysis. The means of multiple groups were compared by the one-way ANOVA, followed by a Tukey multiple comparisons test. A value of \(P<0.05\) was chosen as the threshold of statistical significance. The statistical method is justified as appropriate as reported before.

Antibody List
AT1 receptor antibody (7aa, goat source) was specially ordered from Bethyl Labs, Texas with the 7-aa epitope of angiotensin II receptor type 1 agonistic autoantibody (AT1-AA) (AFHYEQ) as the immunogen, which is from the second extracellular loop of the receptor. Previously, others have successfully
generated and purified AT1 receptor antibody from rabbits with the same method⁵.

AT1 receptor antibody (N10, rabbit source) is from Santa Cruz with catalog # sc-1173 whose applications in human and mouse have been validated by Supplementary References #6 and the numerous citations provided by the company.

TG2 antibody (clone CUB7402) is from Abcam with catalog # ab2386 whose applications in human and mouse have been validated in Supplementary References #7, #8, and #9.

ε-(γ-glutamyl)-lysine isopeptide antibody (clone 81D1C2) is from Abcam with catalog # ab422 whose applications in IB, IF, and IHC have been validated in Supplementary References #10, #11, and #8, respectively.

Beads conjugated with anti-ε-(γ-glutamyl)-lysine isopeptide IgM (81D4) is from Abcam/Covalab whose application in IP has been validated in Supplementary References #12.

Ubiquitin antibody (clone P4D1, mouse monoclonal) is from Santa Cruz with catalog # sc-8017 whose applications in IB and IP have been well validated by the numerous citations provided by the company.

Supplementary references:


Table S1: Clinical characteristic features of human subject

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NT</th>
<th>Mild PE</th>
<th>Severe PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Age</td>
<td>25.92±1.14</td>
<td>27.31±0.95</td>
<td>26.75±1.27</td>
</tr>
<tr>
<td>Race (%)</td>
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<td>White</td>
<td>White</td>
</tr>
<tr>
<td>African American</td>
<td>50</td>
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<td>54</td>
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<tr>
<td>White</td>
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<td>38</td>
<td>29</td>
</tr>
<tr>
<td>Hispanic</td>
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<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Gravity</td>
<td>2.15±0.32</td>
<td>2±0.19</td>
<td>2.5±0.32</td>
</tr>
<tr>
<td>BMI</td>
<td>31.88±1.36</td>
<td>35.89±1.61</td>
<td>36.02±1.94</td>
</tr>
<tr>
<td>Weeks gestational age</td>
<td>38.88±0.20</td>
<td>36.44±0.32</td>
<td>34.28±0.74</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>119.65±2.36</td>
<td>144.76±2.70*</td>
<td>163.46±2.84**</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71.04±1.85</td>
<td>84±1.95*</td>
<td>97.86±2.45**</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>N/A</td>
<td>520.3±56.64</td>
<td>1122.74±218.29*</td>
</tr>
</tbody>
</table>

This table demonstrates that the blood pressure and proteinuria are significantly elevated in mild and severe preeclamptic (PE) women versus normotensive (NT) pregnant women. The value in each category is indicated as mean ± SEM. *P<0.001 vs normotensive pregnant women; ** P<0.001 vs mild PE pregnant women.