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Tissue Transglutaminase Contributes to the Pathogenesis of Preeclampsia and Stabilizes Placental Angiotensin Receptor Type 1 by Ubiquitination-Preventing Isopeptide Modification

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Abstract—Preeclampsia is a life-threatening pregnancy disorder that is widely thought to be triggered by impaired placental development. However, the placenta-related pathogenic factors are not fully identified, and their underlying mechanisms in disease development remain unclear. Here, we report that the protein level and enzyme activity of tissue transglutaminase (TG2 or tTG), the most ubiquitous member of a family of enzymes that conducts post-translational modification of proteins by forming ϵ -(γ -glutamyl)-lysine isopeptide bonds, are significantly elevated in placentas of preeclamptic women. TG2 is localized in the placental syncytiotrophoblasts of patients with preeclampsia where it catalyzes the isopeptide modification of the angiotensin receptor type 1 (AT1). To determine the role of elevated TG2 in preeclampsia, we used a mouse model of preeclampsia based on injection of AT1-agonistic autoantibody. A pathogenic role for TG2 in preeclampsia is suggested by *in vivo* experiments in which cystamine, a potent transglutaminase inhibitor, or small interfering RNA-mediated TG2 knockdown significantly attenuated autoantibody-induced hypertension and proteinuria in pregnant mice. Cystamine treatment also prevented isopeptide modification of placental AT1 receptors in preeclamptic mice. Mechanistically, we revealed that AT1-agonistic autoantibody stimulation enhances the interaction between AT1 receptor and TG2 and results in increased AT1 receptor stabilization via transglutaminase-mediated isopeptide modification in trophoblasts. Mutagenesis studies further demonstrated that TG2-mediated isopeptide modification of AT1 receptors prevents ubiquitination-dependent receptor degradation. Taken together, our studies not only identify a novel pathogenic involvement of TG2 in preeclampsia but also suggest a previously unrecognized role of TG2 in the regulation of G protein-coupled receptor stabilization by inhibiting ubiquitination-dependent degradation. (*Hypertension*. 2014;63:353-361.) • [Online Data Supplement](#)

Key Words: angiotensin AT1 receptor ■ preeclampsia ■ pregnancy ■ protein processing, post-translational ■ renin-angiotensin system ■ transglutaminase 2 ■ ubiquitination

Preeclampsia (PE) is a gestation-specific syndrome with a high incidence of mother and infant morbidity and mortality worldwide. For years, its diagnosis was made solely by detecting the onset of hypertension and proteinuria.^{1,2} Although the underlying cause of PE remains largely unknown and the clinical management is limited, it has long been speculated that impaired placental development is an initial trigger to maternal symptoms.^{1,2} Placental ischemia/hypoxia-induced pathogenic factors,³ including inflammatory cytokines,^{4,5} antiangiogenic factors,^{6,7} and angiotensin receptor type 1 (AT1)-agonistic autoantibody (AA),⁸⁻¹² have been demonstrated to play a crucial role in the disease pathogenesis by recent studies. But the placenta-related pathogenic factors are still not fully identified, and underlying mechanisms remain undefined. Therefore, this study aimed to identify

novel placenta-related mediators in PE and the underlying mechanisms of disease pathogenesis.

Transglutaminases are a family of enzymes conducting post-translational modification by forming inter- or intramolecular ϵ -(γ -glutamyl)-lysine isopeptide bonds in a calcium-dependent manner.¹³ Metabolomic screening revealed that cystamine, an endogenous inhibitor of transglutaminases, was significantly less produced in cultured explants of PE placentas.¹⁴ Of note, tissue transglutaminase (TG2, tTG or G_h), the most ubiquitous transglutaminase, is enriched in placental syncytiotrophoblast layer,^{15,16} from which it is shed into maternal circulation.¹⁷ Different from other transglutaminases, TG2 can also function as a high-molecular-weight G protein that couples the activation of certain GPCRs.¹⁸⁻²⁰ TG2 gene expression can be induced by transforming growth factor- β , tumor

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necrosis factor- α , and interleukin-6,^{21–23} the inflammatory cytokines highly elevated in PE.^{3–5,24} The cytokine response of TG2 transcription is mediated by nuclear factor- κ B and hypoxia-inducible factor-1 α ,²³ 2 stress-induced transcription factors elevated in the hypoxic placentas of PE women as well.^{3,25} Although PE-associated conditions, including inflammation and hypoxia, are involved in the regulation of TG2 expression and enzyme activation,^{23,26} little is known about the exact role of TG2 in PE. In this study, we are intrigued by the colocalization between AT1 receptor^{27,28} and TG2^{15,16} in placental syncytiotrophoblasts, the capability of TG2 to couple with GPCRs,^{18–20} and an environment of hypoxia^{3,25} and calcium mobilization¹⁰ in PE placentas favoring the transamidation function of TG2.²⁶ Based on these facts, we show that TG2, which is significantly elevated in preeclamptic placentas, stabilizes the placental AT1 receptor, with isopeptide modification repressing its ubiquitination-dependent proteasome degradation. A pathological role for TG2 in PE is suggested by experiments presented below showing that cystamine, a potent TG inhibitor, or small interfering RNA

(siRNA) specific for TG2 significantly attenuate PE features in an experimental model of PE in pregnant mice. Cystamine treatment also prevented isopeptide modification of placental AT1 receptors in this model. Taken together, our results not only suggest a pathogenic role for TG2 in PE but also shed light on a novel and general mechanism of GPCR regulation, in which the high-molecular-weight G protein TG2/G_h prevents the ubiquitination-dependent degradation of receptors via isopeptide modification under stress conditions.

Methods and Materials

Please see Materials in the online-only Data Supplement.

Results

Enzyme Activity and Expression Level of TG2 Are Significantly Elevated in Preeclamptic Placentas

To evaluate the involvement of placental transglutaminase in PE, we initially found that the TG activity in PE placentas was approximately twice as high as in normotensive (NT) placentas (80 \pm 11 versus 40 \pm 5 mU/mg protein; Figure 1A).

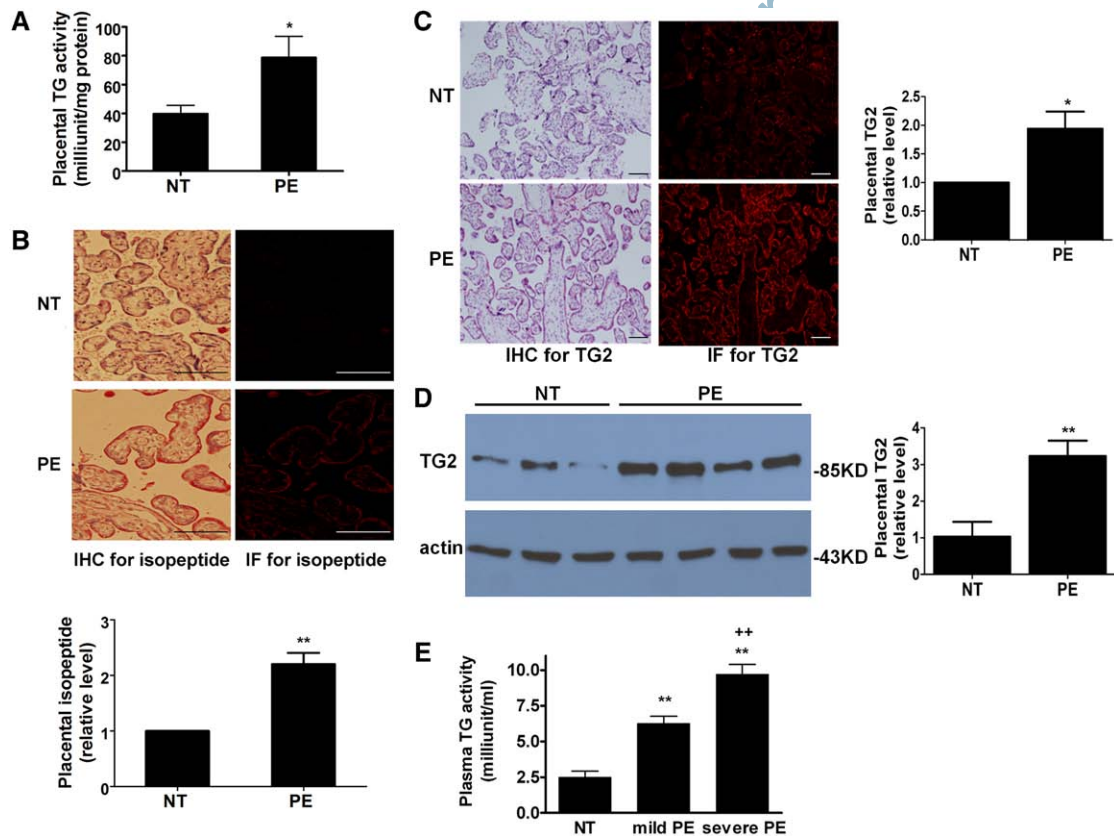


Figure 1. Placental TG activity and tissue transglutaminase (TG2) are increased in preeclampsia (PE). **A**, TG activity is significantly increased (\approx 2-fold) in PE placental lysates ($*P < 0.05$ for PE vs normotensive [NT] placentas; $n = 3$ NT and PE placentas). **B**, Increased level of ϵ -(γ -glutamyl)-lysine isopeptide is mainly localized to the syncytiotrophoblasts of human PE placentas by immunohistochemistry/immunofluorescence (IHC/IF) dual staining. The quantification of color signal also shows a 2-fold increase in PE placentas ($**P < 0.01$ PE placenta vs NT placenta; the color signal level of NT placenta is arbitrarily assigned as 1 in each staining pair; $n = 5$ pairs of randomly chosen NT and PE placental slides stained). **C**, Pronounced expression of TG2 is localized at the syncytiotrophoblasts of human PE placentas by IHC/IF dual staining. The quantification of color signal (bar graph) demonstrates an \approx 2-fold increase of TG2 in PE ($*P < 0.05$ NT placenta vs PE placenta; the color signal level of NT placenta is arbitrarily assigned as 1 in each staining pair; $n = 4$ pairs of randomly chosen NT and PE placental slides stained). **D**, In PE, the level of placental TG2 is \approx 3-fold higher than in NT as measured in Western blot with human placental lysates after normalization with actin level ($**P < 0.01$ NT vs PE; $n = 3$ in NT group, 4 in PE group). **E**, Compared with NT controls (2.5 \pm 0.4 mU/mL), a higher level of plasma TG activity is identified in either mild PE (6.3 \pm 0.5 mU/mL) or severe PE (9.7 \pm 0.7 mU/mL) with *in vitro* TG activity assay (Sigma-Aldrich, MO; $**P < 0.01$ NT [$n = 25$] vs mild PE [$n = 25$] or severe PE [$n = 25$]; $**P < 0.01$ mild PE vs severe PE). Data in Figure 1 represent mean \pm SEM from indicated experiments.

To localize TG activity in NT/PE placentas, we probed placental sections for ϵ -(γ -glutamyl)-lysine isopeptides using immunohistochemistry (IHC) and immunofluorescence (IF) dual staining. Compared with NT placentas, a higher level of ϵ -(γ -glutamyl)-lysine isopeptide was localized to the syncytiotrophoblasts of PE placentas (Figure 1B). Similar to the TG activity assay result, a 2-fold increase in ϵ -(γ -glutamyl)-lysine isopeptide level was observed in PE placentas (Figure 1B). Thus, these studies indicate that elevated TG activity leads to increased isopeptide modification in pre-eclamptic syncytiotrophoblasts.

Subsequently, we determined the specific TG responsible for the increase in ϵ -(γ -glutamyl)-lysine isopeptide in preeclamptic syncytiotrophoblasts. Consistent with previous studies,^{15,16} TG2 was localized in the placental syncytiotrophoblasts in our IHC/IF dual staining (Figure 1C). Both Western blotting and IHC/IF dual staining show that in PE placentas the level of TG2 is \approx 2-fold higher than in NT placentas (Figure 1C and 1D), indicating that the increase in isopeptide modification at the preeclamptic syncytiotrophoblasts is likely because of the increased TG2 expression.

Because TG2 in placental syncytiotrophoblasts could be shed into circulation in microparticles,¹⁷ we checked whether the increase in TG2 in PE placentas is associated with an elevated circulating TG activity. As shown in Figure 1E, we found significantly higher levels of plasma TG activity in both mild and severe PE than in NT samples (6.3 ± 0.5 and 9.7 ± 0.7 versus 2.5 ± 0.4 mU/mL, respectively). The plasma TG activity in severe PE was further elevated relative to mild PE (9.7 ± 0.7 versus 6.3 ± 0.5 mU/mL), indicating that the elevated plasma TG activity in patients with PE may be correlated with disease severity.

ϵ -(γ -glutamyl)-Lysine Isopeptide Modification on AT1 Receptor Is Pronounced in PE Placentas

As the major angiotensin receptor and the *in vivo* target of AT1-AA, AT1 receptor is thought to be involved in the pathogenesis of PE. Interestingly, AT1 receptors are highly expressed in placental syncytiotrophoblasts^{27,28} where elevated TG2 and isopeptide modification is found in PE. Thus, based on these findings and in a view of the fact that TG2 couples to certain GPCRs,^{18–20} we chose to investigate whether the enhanced TG2 activity results in an increase in isopeptide modification of the colocalized AT1 receptor in PE placentas. To assess this possibility, all the proteins with ϵ -(γ -glutamyl)-lysine isopeptides in human NT/PE placentas were immunoprecipitated and probed for AT1 receptor. Compared with NT controls, AT1 receptors were readily identified in the immunoprecipitated products from PE placental lysates (Figure 2A), indicating that the isopeptide modification of AT1 receptor was significantly increased in PE placentas. This finding was confirmed with a reciprocal co-immunoprecipitation assay in which AT1 receptor protein in NT/PE placental lysates was immunoprecipitated and its isopeptide level determined using Western blotting. As shown in Figure 2B, a higher level of AT1 receptor with isopeptide modification is characterized in the anti-AT1 receptor pulldown products from PE placental lysates. In addition, in immunofluorescent staining, AT1 receptor (red) and isopeptide modification (green) could be

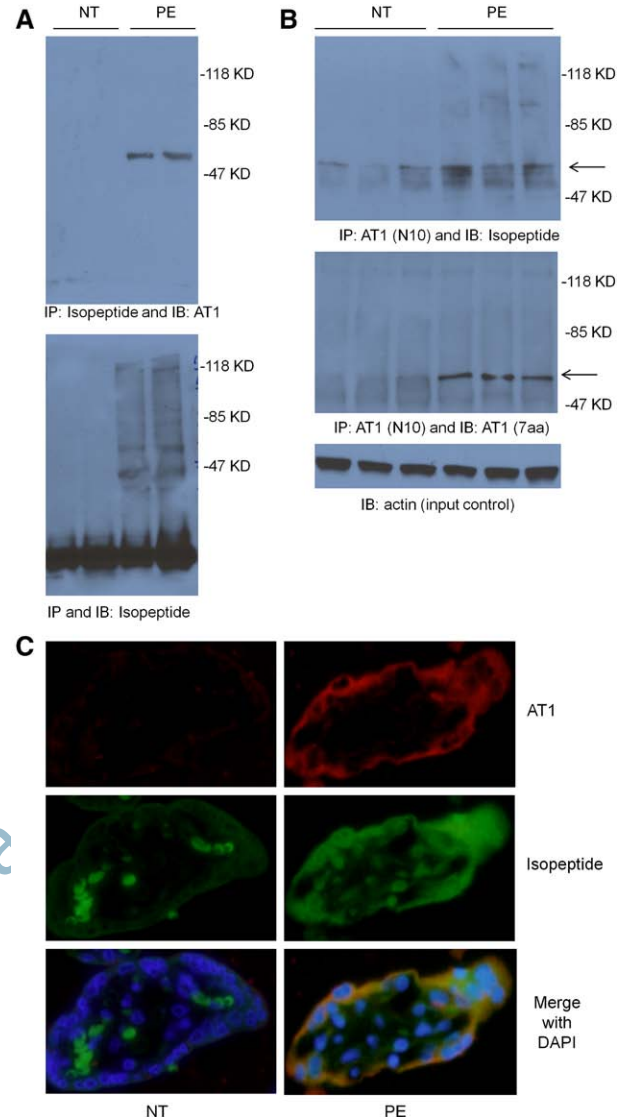


Figure 2. Angiotensin receptor type 1 (AT1) is modified with ϵ -(γ -glutamyl)-lysine isopeptide in preeclamptic (PE) placentas. **A**, After immunoprecipitation of proteins with ϵ -(γ -glutamyl)-lysine isopeptide modification, AT1 receptor was identified by Western blot in immunoprecipitated products from human PE placental lysates, not normotensive (NT) placental lysates ($n=2$ randomly chosen patient samples in each category). **B**, After immunoprecipitation, more AT1 receptor with isopeptide modification was observed in PE placental lysates by Western blot ($n=3$ randomly chosen patient samples in each category). **C**, AT1 receptor (red) and ϵ -(γ -glutamyl)-lysine isopeptide (green) colocalize (yellow) in the syncytiotrophoblasts of human PE placenta, accompanied with higher levels of AT1 receptor and proteins with isopeptide modification. Staining result is representative in 4 pairs of randomly chosen NT and PE placental slides. DAPI indicates 4',6-diamidino-2-phenylindole.

colocalized (yellow) at the microvillous membrane of human PE placentas (Figure 2C), a finding consistent with the Co-IP results and suggesting the specific involvement of TG2 in AT1 receptor modification.

In Vivo Pathogenic Role of TG2 in PE

Our recent study¹² demonstrates that the transfer of purified AT1-AA or total IgG from patients with PE into pregnant mice reproduces the key clinical features of PE, thereby establishing

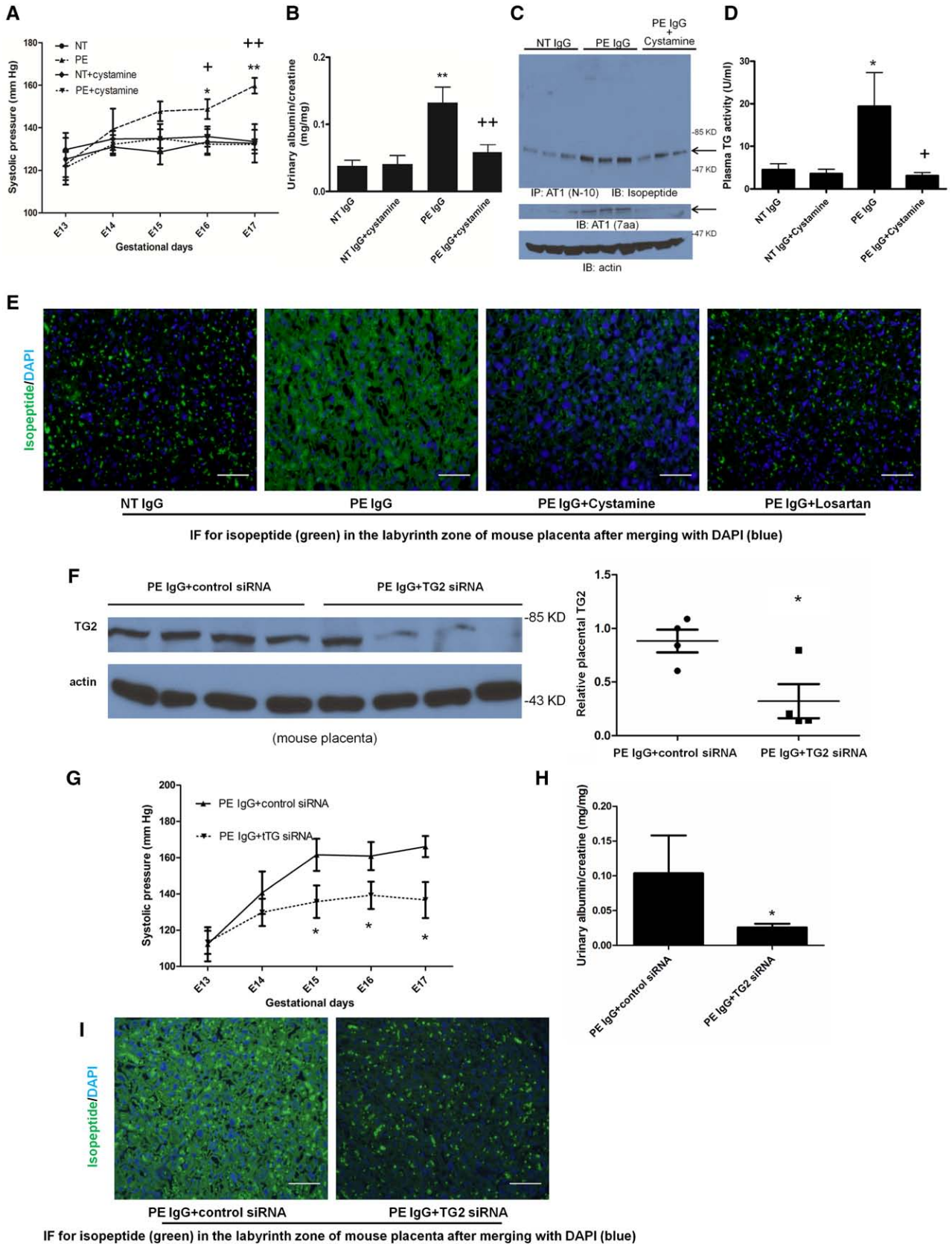


Figure 3. Cystamine and small interfering RNA (siRNA)-mediated tissue transglutaminase (TG2 or tTG) knockdown alleviate placental isopeptide modifications and clinical features of preeclampsia (PE) in autoantibody-induced mouse model. **A**, The increase in systolic blood pressure is prevented by TG inhibitor cystamine in PE mice ($*P<0.05$, $**P<0.01$ normotensive [NT] IgG vs PE IgG; $*P<0.05$, $**P<0.01$ PE IgG vs PE IgG+cystamine; $n=6$ mice in each group). **B**, Proteinuria in PE mice is also ameliorated by cystamine treatment ($**P<0.01$ NT IgG vs PE IgG; $**P<0.01$ PE IgG vs PE IgG+cystamine; $n=6$ mice in each group). **C**, Accumulation and isopeptide modification of AT1

Figure 3. (Continued) receptors are inhibited by cystamine treatment. After the mouse placental lysates were precleared with blank protein A beads, AT1 receptor in the lysates was immunoprecipitated with rabbit anti-AT1 receptor antibody (N10; Santa Cruz), and subsequently, its isopeptide modification level was determined by Western blot probing for isopeptide (ab422; Abcam). The placental AT1 receptor is assessed using Western blot directly with goat anti-AT1 receptor antibody (7aa; Bethyl laboratories). **D**, Cystamine treatment represses PE IgG-induced increase in plasma TG activity in pregnant mice. Murine plasma collected with EDTA as anticoagulant on E18 was assayed using *in vitro* TG activity assay (Covalab, France; * $P < 0.05$, NT IgG vs PE IgG; * $P < 0.05$, PE IgG vs PE IgG+cystamine; $n = 4$ mice in PE IgG group, 6 mice in other groups); **E**, Overall ϵ -(γ -glutamyl)-lysine isopeptide modification (green in immunofluorescent [IF] staining) in the placental labyrinth zone of PE mice is downregulated by cystamine treatment, as by losartan ($n = 6$ mice in each group). **F**, TG2 is significantly reduced in placentas of PE mice injected with TG2 small interfering RNA (siRNA). The protein level of TG2 in mouse placentas collected on embryonic day 18 was evaluated by Western blot analysis. After normalization with actin levels, the quantification shows that the TG2 level is significantly lower in the placentas of PE mice injected with TG2 siRNA (* $P < 0.05$ PE IgG+control siRNA vs PE IgG+TG2 siRNA; $n = 4$ mice in each group). **G**, tTG siRNA thwarted blood pressure increase in PE mice from E15 to E17 (* $P < 0.05$ PE IgG+control siRNA [$n = 6$ mice] vs PE IgG+tTG siRNA [$n = 5$ mice]). **H**, Proteinuria is also alleviated in TG2 siRNA-injected PE mice (* $P < 0.05$ PE IgG+control siRNA vs PE IgG+TG2 siRNA; $n = 4$ mice in each group). **I**, Accumulation of ϵ -(γ -glutamyl)-lysine isopeptide modification (green in IF staining) is repressed in the placental labyrinth zone of PE mice injected with TG2 siRNA ($n = 6$ mice in PE IgG+control siRNA group, 5 mice in PE IgG+TG2 siRNA group).

a valuable humanized PE animal model favoring TG2 activation. To determine whether increased TG activity contributes to PE pathogenesis, we inhibited TG activity with the well-established TG inhibitor cystamine in our PE mouse model induced by injection of IgG (containing AT1-AA) from PE women.¹² Similar to previous studies,¹² injection of IgG from PE women induces key PE clinical features including hypertension and proteinuria in pregnant mice (Figure 3A and 3B). Similar to human studies, circulating TG activity was significantly elevated in the pregnant mice injected with PE IgG compared with the NT IgG-injected controls (Figure 3D). Compared with pregnant mice injected with PE IgG alone, cystamine treatment attenuated the key clinical features of preeclampsia, including hypertension (from 159.5 ± 5.6 to 132.6 ± 2.7 mmHg as shown in Figure 3A) and proteinuria (from 106.5 ± 37.8 to 38.5 ± 6.9 ng albumin/mg creatinine as shown in Figure 3B), as well as increased plasma TG activity (Figure 3D). NT IgG-injected mice with or without cystamine treatment retained the baseline values for these parameters. Using this PE mouse model, we saw increased AT1 receptor with isopeptide modification in placentas (Figure 3C), whereas cystamine treatment abolished the modification and accumulation of placental AT1 receptors (Figure 3C). The efficiency of cystamine in preventing isopeptide modification in the placental labyrinth zone (the counterpart of human placental syncytiotrophoblasts) of PE IgG mice was further confirmed by isopeptide immunostaining (Figure 3E). These results suggest that placental AT1 receptor accumulation is a pathological consequence of isopeptide modification caused by increased placental TG activity. Taken together, the data from our PE animal model indicate that the elevated TG activity is required for autoantibody-induced PE features in pregnant mice.

As a broad-spectrum inhibitor of transglutaminases, cystamine is not sufficient to identify the specific TG contributing to the PE features in our mouse model. To specifically assess the role of TG2 in the pathogenesis of PE, siRNA-embedded nanoparticles were injected into pregnant mice on embryonic day 13 (E13) and E14, together with PE IgG, to knockdown TG2 expression in PE mice. As shown in Figure 3F, on E18, placental expression of TG2 was significantly downregulated in PE mice injected with TG2 siRNA compared with control siRNA-injected PE mice. Correspondingly, a significant attenuation of blood pressure increase was observed in TG2 siRNA-injected PE mice from E15 to E17 (Figure 3G). Proteinuria, another important PE feature, was also attenuated in TG2 siRNA-injected PE mice (Figure 3H). TG2

knockdown repressed the accumulation of isopeptide modification in the placental labyrinth zone of PE IgG-injected mice as well (Figure 3I). Taken together, our *in vivo* data confirm the essential role of TG2 in the development of PE.

TG2-Mediated Isopeptide Modification Stabilizes AT1 Receptor by Repressing Ubiquitination-Dependent Degradation

Elevated AT1 receptor isopeptide modification was associated with increased receptor levels in human PE placentas (Figure 2) and in placentas of PE IgG-injected mice (Figure 3C). To investigate the mechanism and consequences of AT1 receptor modification, we used the established human trophoblast cell line, HTR-8/SVneo. Treated cells were initially tested to see whether PE IgG could induce the interaction between AT1 receptors and TG2. More AT1 receptors were associated with TG2 in trophoblasts treated with PE IgG (Figure 4A) in comparison with HTR cells treated with NT IgG, suggesting a promotional effect of AT1-AA on AT1 receptor and TG2 interaction in PE. Next, we checked the protein level and isopeptide modification of AT1 receptors in treated trophoblasts. Similar to human PE placentas, the level of AT1 receptor protein and the extent of isopeptide modification were increased in PE IgG-treated trophoblasts. These features were largely eliminated in cells cotreated with the transglutaminase inhibitor cystamine (Figure 4B) or the AT1 receptor antagonist losartan (Figure 4C), suggesting that the accumulation of AT1 receptors in PE IgG-treated trophoblasts is through AT1-AA-induced isopeptide modification.

To elucidate the molecular mechanism underlying TG2-mediated AT1 receptor accumulation, we first investigated the receptor's degradation pathway in HTR cells. As shown in Figure 4D, AT1 receptors showed a rapid cellular accumulation in cells treated with the proteasome inhibitor MG132 but not in cells treated with the lysosome inhibitor chloroquine, indicating that AT1 receptors are mainly degraded through the ubiquitination/proteasome pathway.²⁹

The AT1 receptor cytoplasmic tail has only 1 glutamine (position 315 or Q315) that was previously shown to be the factor XIIIa transglutaminase-mediated AT1 receptor crosslinking site.³⁰ Thus, to assess the effect of TG2-mediated isopeptide modification on AT1 receptor ubiquitination, we established stable Chinese hamster ovary cell lines overexpressing TG2 along with either wild-type (WT) or mutant AT1 receptor in which glutamine at position 315 is replaced with alanine (Q315A).

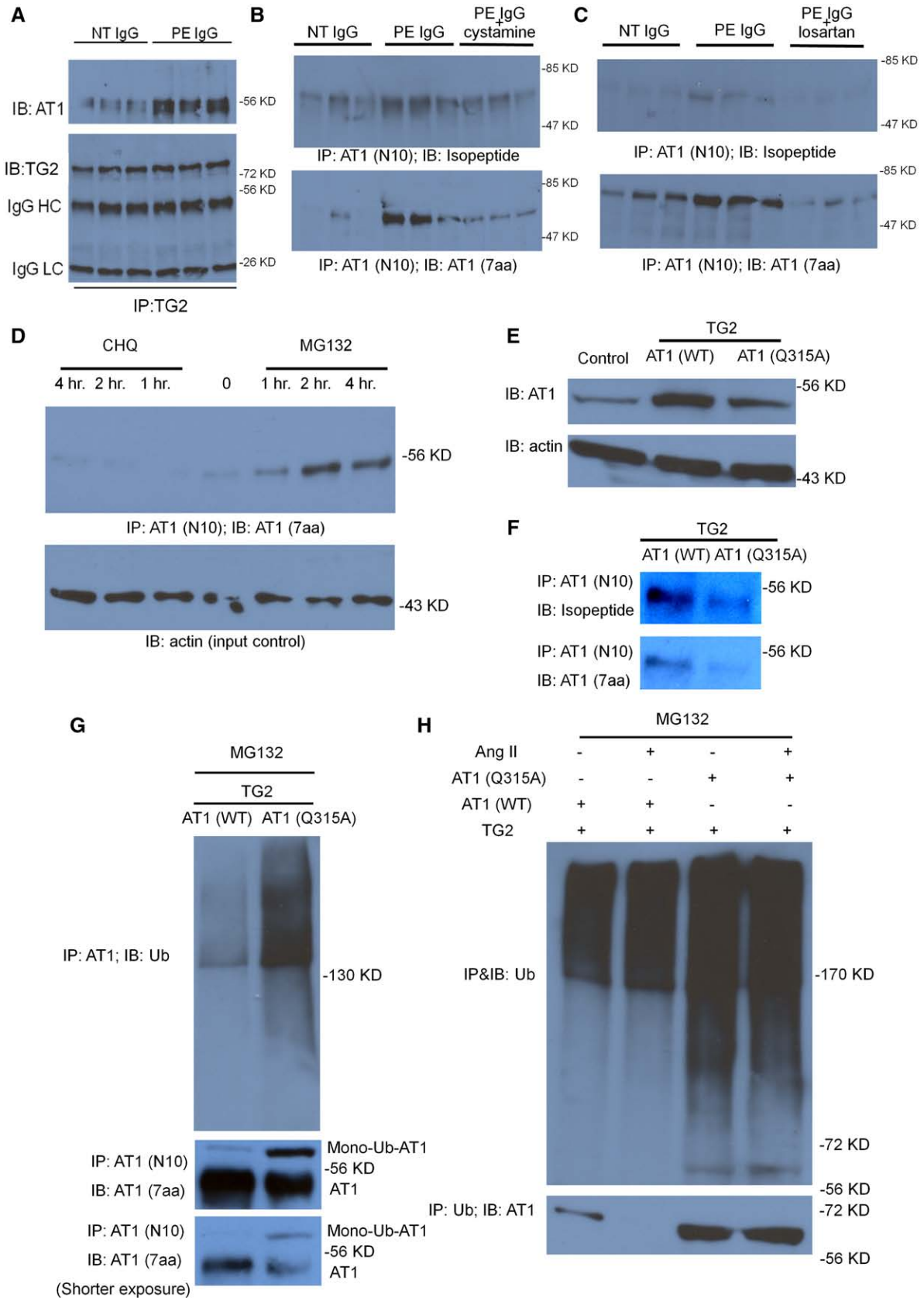


Figure 4. Tissue transglutaminase (TG2)-mediated isopeptide modification on Q315 of the AT1 receptor stabilizes the receptor via preventing ubiquitination. **A**, Preeclamptic (PE) IgG promotes the interaction between AT1 receptors and TG2 in human trophoblast cell line HTR-8/SVneo cells. Compared with normotensive (NT) IgG (100 μg), the interaction between AT1 receptors and TG2 was enhanced after 4-hour treatment with PE IgG (100 μg) at 37°C. TG2 and its associating proteins were immunoprecipitated, and the presence/abundance of AT1 receptors in the pull-down products was assessed by Western blot (n=3 independent experiments). **B**, TG inhibitor

Figure 4. (Continued) cystamine (500 $\mu\text{mol/L}$) repressed PE IgG (100 μg)-induced accumulation and isopeptide modification of AT1 receptors. After 4-hour treatment at 37°C, AT1 receptors in the treated cells were immunoprecipitated with rabbit anti-AT1 receptor antibody (N10; Santa Cruz), and the isopeptide modification and AT1 receptor level of the pulldown products were evaluated in Western blot with mouse anti-isopeptide antibody (ab422; Abcam) and goat anti-AT1 receptor antibody (7aa; Bethyl Laboratories), respectively (n=3 independent experiments). **C**, AT1 receptor antagonist losartan (5 $\mu\text{mol/L}$) prevents PE IgG (100 μg)-induced accumulation and isopeptide modification of AT1 receptor in trophoblast cell line HTR-8/SVneo cells as well (n=3 independent experiments). The IP procedure was identical to B. **D**, AT1 receptor accumulates rapidly in MG132 (20 $\mu\text{mol/L}$, proteasome inhibitor)-treated but not chloroquine (CHQ; 200 $\mu\text{mol/L}$, a lysosome inhibitor)-treated, trophoblasts. AT1 receptor in the treated cells was concentrated by immunoprecipitation with rabbit anti-AT1 receptor antibody (N10; Santa Cruz), and then the level of enriched AT1 receptor was determined by Western blot analysis with goat anti-AT1 receptor antibody (7aa; Bethyl Laboratories). Q315A AT1 receptor mutant is less abundant in cells (**E**) and resistant to TG2-mediated isopeptide modification (**F**; n=3 independent experiments). The IP and Western blot procedure in F was similar to B. **G**, Polyubiquitination and monoubiquitination levels of immunoprecipitated AT1 receptor are elevated in Q315A mutant cells treated with proteasome inhibitor MG132 (20 $\mu\text{mol/L}$; n=3 independent experiments). **H**, Reciprocally, increased monoubiquitinated AT1 receptor is identified in the ubiquitinated proteins of Q315A mutant cells. The increase in monoubiquitinated AT1 receptor Q315A mutant was not changed by angiotensin II (100 nmol/L) treatment that decreased the level of monoubiquitinated wild-type AT1 receptor.

Compared with WT AT1 receptor, the Q315A mutant is less abundant and resistant to TG2-mediated isopeptide modification (Figure 4E and 4F). However, when the ubiquitination/proteasome pathway was blocked by proteasome inhibitor MG132, the Q315A mutant showed a similar overall cellular abundance of WT AT1 receptors, and the polyubiquitination level of the Q315A mutant (and its associated proteins) and its monoubiquitination level are much higher than those of WT AT1 receptor (Figure 4G). Reciprocally, in the anti-ubiquitin immunoprecipitated products, more monoubiquitinated AT1 receptor was identified from the Q315A mutant cells and was not changed with angiotensin II stimulation (Figure 4H). In contrast, the WT AT1 receptor showed reduced monoubiquitination after angiotensin II stimulation, presumably as a result of isopeptide modification by TG2. In summary, our findings here support a working model that TG2-mediated isopeptide modification stabilizes AT1 receptors by repressing ubiquitination-dependent degradation.

Discussion

In this study, we are the first to identify significantly increased enzyme activity and protein expression of TG2 in PE placentas. Intrigued by the colocalization among TG2, isopeptides, and AT1 receptor, we further found a higher level of AT1 receptor with isopeptide modification in syncytiotrophoblasts of PE placentas. Based on these findings, we proceeded to demonstrate that AT1-AA-induced isopeptide modification stabilized AT1 receptor in trophoblasts. Inhibition of TG activity by cystamine or siRNA-mediated TG2 knockdown ameliorated PE IgG-induced disease features in pregnant mice and reduced placental AT1 receptor isopeptide modification. Mechanistic studies revealed that isopeptide modification on Q315 of the AT1 receptor inhibited its ubiquitination-dependent degradation. Overall, our findings demonstrate a novel mechanism underlying increased AT1 receptor accumulation in PE placentas, in which TG2-mediated isopeptide modification at Q315 stabilizes the receptor post-translationally by repressing its ubiquitin-dependent proteasome degradation. The TG2-mediated stabilization of AT1 receptor may also suggest a general mechanism for the regulation of GPCR stability by TG2.

Here, we report a significant increase in plasma TG activity in patients with PE. The increased TG is unlikely to be factor XIIIa transglutaminase because the use of EDTA as anticoagulant in plasma collection results in the cleavage of factor XIIIa transglutaminase by thrombin.³¹ Therefore, the increase in plasma TG activity probably results from increased circulating

TG2 shed from PE placental syncytiotrophoblasts.¹⁷ The elevated TG activity in the circulation of PE women may also be a result of transcriptional induction by elevated tumor necrosis factor and interleukin-6.^{21–23} The strong positive correlation between plasma TG activity and disease severity suggests that plasma TG activity may serve as a novel biomarker of PE.

TG2 is involved in multiple cardiovascular diseases, including spontaneous hypertension,³² atherosclerosis,³³ and cardiac hypertrophy.³⁴ We show here that elevated transglutaminase activity is associated with PE in humans and a mouse model. To test whether elevated TG2 contributes to the disease pathogenesis, we treated the autoantibody-induced PE mouse model with the transglutaminase inhibitor cystamine or TG2-specific siRNA. Cystamine is a potent competitive TG inhibitor with well-established oral and intraperitoneal administrative methods in mice and has been shown to successfully control the development of Huntington disease in animal models.^{35–38} However, as a broad-spectrum TG inhibitor,³⁹ cystamine lacks specificity to determine the exact TG responsible for features of PE in the autoantibody-injected pregnant mice. To overcome this disadvantage, we specifically knocked down TG2 in PE mice with siRNA specific for TG2. As a result of the dual roles of TG2 as a transglutaminase and a G protein,²⁶ the siRNA knockdown method is not sufficient to distinguish its TG function from its GTPase function in PE pathogenesis. Thus, we think that the combination of cystamine-mediated TG activity repression and siRNA-mediated TG2 knockdown is necessary to delineate the critical role of TG2 as the transamidation enzyme in PE pathogenesis.

Transglutaminases modify proteins in a calcium-dependent manner by catalyzing the formation of an isopeptide bond between the γ -carboxamide group of a glutamine residue and the ϵ -amino group of a lysine residue.^{13,23, 26} Uniquely, the transamidation activity of TG2 is inhibited by the binding of cellular energy-carrying nucleotides, including GTP, GDP, and ATP.^{13,26,40} Under normal physiological conditions, cells maintain an intracellular environment of low calcium and high GTP concentrations, which inhibits the transamidation function of TG2.²⁶ TG2 activation is usually associated with pathological conditions as shown here and in many other studies. Although atherosclerosis is associated with factor XIIIa transglutaminase-mediated crosslinking of AT1 receptors into dimers,³⁰ little is known about the exact mechanism underlying the role of TG2 in hypertension-related diseases. Here, for the first time we revealed the pronounced interaction between TG2 and AT1 receptors in PE IgG-treated HTR cells. We also

observed a significant increase in isopeptide modification of AT1 receptors at the syncytiotrophoblasts of PE placentas. By treating our PE mouse model and PE IgG-treated HTR cells with AT1 receptor antagonist losartan or transglutaminase inhibitor cystamine, we further demonstrated that AT1-AA-induced receptor activation increases the level of AT1 receptor isopeptide modification. AT1-AAs in the circulation of patients with PE can easily contact syncytiotrophoblasts and thereby induce calcium mobilization,¹⁰ favoring the activation of TG2 in an environment of hypoxia-induced ATP/GTP depletion.²⁶ Interestingly, the lack of a significant change in molecular weight of AT1 receptor after TG2-mediated isopeptide modification is consistent with previous observation³⁰ about the inability of TG2 to crosslink AT1 receptor into dimers and suggests that the modification may be intramolecular as documented in other proteins^{41,42} or a result of the incorporation of a small primary amine.⁴³

In this study, while investigating the TG2-mediated AT1 receptor isopeptide modification, we also observed an increased level of AT1 receptor in PE placentas. To study this correlation, we generated a modification-deficient AT1 receptor mutant (Q315A) whose cytoplasmic tail glutamine residue (site of TG modification) was mutated to alanine. Because the degradation of AT1 receptors is mainly through the ubiquitin-proteasome pathway,²⁹ we examined the protein level and ubiquitination status of WT and Q315A mutant AT1 receptors. We observed that in Chinese hamster ovary cells overexpressing TG2, the Q315A mutant showed an increased ubiquitination and decreased protein accumulation compared with WT AT1 receptor, indicating that the isopeptide modification at Q315 of the receptor's cytoplasmic tail prevents its ubiquitination-dependent proteasome degradation. Consistent with this model is the presence of several lysine residues in the C-terminal tail for ubiquitination. Protein ubiquitination, a process resulting in protein degradation, uses ATP to form a high-energy glycine-lysine isopeptide bond between ubiquitin and its acceptor,⁴⁴ whereas TG2-mediated modification, a process resulting in protein stabilization, forms glutamine-lysine isopeptide bonds in a low ATP/GTP environment that activates TG2.²⁶ Our data suggest a competing relationship between AT1 receptor ubiquitination and TG2-mediated modification. Our findings may also provide an explanation for the contribution of proinflammatory cytokines in hypertension. Specifically, increased proinflammatory cytokines stimulate TG2 production that results in increased isopeptide modification and stabilization of AT1 receptors, and the increased level of AT1 receptors in turn contributes to increased angiotensin II sensitivity and hypertension.

Taken together, our results in this study suggest a vicious cycle for the pathogenesis of PE in which increased TG2 activity in syncytiotrophoblasts stabilizes AT1 receptor to generate a long-lasting and amplified disease signal in PE placentas. Our findings also raise the possibility that TG2-mediated modification may be a general mechanism for the regulation of GPCR stability.

Perspectives

In this study, we demonstrated that elevated TG2 contributes to the disease pathogenesis and causes the pathological accumulation of AT1 receptors in PE placentas. To further elucidate

the underlying mechanism, future work will be first focused on determining the pathogenic significance of TG2-mediated placental AT1 receptor stabilization. In addition, the identification of other TG2 modification targets will help us better understand the disease nature as well. Furthermore, the existence of the conditions (eg, excessive calcium mobilization and hypoxia) favoring TG2 activation in other hypertensive diseases also suggests the potential therapeutic applications of TG2 inhibition. Mechanistically, we revealed that TG2-mediated isopeptide modification stabilizes AT1 receptor by inhibiting ubiquitination-dependent degradation. Because of the mutually opposite functional consequences between AT1 receptor ubiquitination and TG2-mediated isopeptide modification, it will be of great interest to investigate whether these 2 processes compete for the same lysine in AT1 receptor cytoplasmic tail. Using the AT1 receptor as the model, our data also suggest a novel and general mechanism of GPCR regulation, in which the high-molecular-weight G protein TG2/G_h stabilizes GPCRs via ubiquitination-preventing isopeptide modification in certain stress conditions.

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Disclosures

None.

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Novelty and Significance

What Is New?

- In preeclamptic placentas, tissue transglutaminase (TG2) level and activity are significantly elevated, resulting in increased isopeptide modification of angiotensin receptor type 1 (AT1) in syncytiotrophoblasts.
- Cystamine (broad-spectrum TG inhibitor) or TG2 small interfering RNA (specific protein knockdown) attenuated AT1-agonistic autoantibody-induced hypertension and proteinuria in pregnant mice.
- TG2-mediated isopeptide modification stabilizes AT1 receptor via preventing ubiquitination-dependent receptor degradation.

What Is Relevant?

- The pathogenic involvement of TG2 in preeclampsia has been demonstrated for the first time in this study.

- The finding of TG2-mediated AT1 receptor stabilization in this study may also suggest a novel GPCR regulatory mechanism in which TG2/G_q, a high-molecular-weight G protein, could stabilize GPCRs through ubiquitination-preventing isopeptide modification in stress or pathological conditions favoring the transamidation function of TG2.

Summary

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

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 μ g IgG purified from PE patients' plasma on Embryonic Day 13 and 14 into pregnant mice as previously described². 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 μ g 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 μ g 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 before².

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 μ l cell or tissue lysates were incubated overnight at 4 degree with 50 μ l 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 μ l 2X Laemmli buffer for Western blot analysis.

Western blot

The procedure for Western blot analysis used in this study has been described previously³. 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 ATATATAAGCTTATGGCCGAGGAGCTGGTC (5') and ATATAACTCGAGCCTTAGGCGGGGCCAATG (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 GCGGCCAAGCTTATGATTCTCAACTCTTCT (5') and GAGACGCTCGAGTCACTCAACCTCAAACA (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 TTTCTCGCGTTCTAAAATATATCCCCCA (5') and TAGAAGCGCGAGAAAATATCTTTTAAATTT (3'). All the above clones were confirmed with DNA sequencing (Genewiz, NJ).

Statistical analysis

Data were expressed as the mean \pm 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 before².

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) (AFHYESQ) as the immunogen, which is from the second extracellular loop of the receptor⁴. 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.

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Table S1: Clinical characteristic features of human subject

Parameter	NT	Mild PE	Severe PE
n	25	25	25
Age	25.92±1.14	27.31±0.95	26.75±1.27
Race(%) African American	50	48	54
White	35	38	29
Hispanic	15	14	18
Gravity	2.15±0.32	2±0.19	2.5±0.32
BMI	31.88±1.36	35.89±1.61	36.02±1.94
Weeks gestational age	38.88±0.20	36.44±0.32	34.28±0.74
Systolic BP (mmHg)	119.65±2.36	144.76±2.70*	163.46±2.84**
Diastolic BP (mmHg)	71.04±1.85	84±1.95*	97.86±2.45**
Proteinuria (mg/24h)	N/A	520.3±56.64	1122.74±218.29 ⁺

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.

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