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

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Abstract

Preeclampsia is a life-threatening pregnancy disorder that is widely believed 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 posttranslational 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 preeclamptic patients where it catalyzes the isopeptide modification of the angiotensin receptor AT1. To determine the role of elevated TG2 in preeclampsia, we employed a mouse model of preeclampsia based on injection of angiotensin receptor type 1 agonistic autoantibody (AT1-AA). A pathogenic role for TG2 in preeclampsia is suggested by in vivo experiments in which cystamine, a potent transglutaminase inhibitor, or siRNA-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-AA 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 the 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 GPCR stabilization by inhibiting ubiquitination-dependent degradation.

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Introduction

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 been long speculated that impaired placental development is an initial trigger to maternal symptoms[1-2]. Indeed, placental ischemia/hypoxia-induced pathogenic factors[3] including inflammatory cytokines[4-5], antiangiogenic factors[6-7], and AT1-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 remains undefined. Therefore, this study is aimed to identify novel placenta-related mediators in PE and the underlying mechanisms of disease pathogenesis.

Transglutaminases are a family of enzymes conducting posttranslational modification by forming inter- or intra-molecular 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 the maternal circulation[17]. Different from other transglutaminases, TG2 can also function as a high molecular G protein that couples the activation of certain GPCRs[18-20]. TG2 gene expression can be induced by TGF-β, TNF-α and IL-6[21-23], the inflammatory cytokines highly elevated in PE[3-5, 24]. The cytokine response of TG2 transcription is mediated by NF-κB and HIF-1α[23], two 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 mobilization[10] 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 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

receptors' ubiquitination-dependent degradation via isopeptide modification in stress conditions.

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 NT placentas (80±11 versus 40±5 milliunit/mg protein) (Figure 1A). To localize TG activity in NT/PE placentas, we probed placental sections for ε-(γ-glutamyl)-lysine isopeptides in immunohistochemistry (IHC) and immunofluorescent (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 two-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 preeclamptic 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 approximately two-fold higher than in NT placentas (Figure 1C and D), indicating that the increase in isopeptide modification at the preeclamptic syncytiotrophoblasts is likely due to the increased TG2 expression.

Since 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 milliunits/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 milliunits/ml), indicating that the elevated plasma TG activity in PE patients may be correlated with disease severity.

ε-(γ-glutamyl)-lysine Isopeptide modification on AT1 receptor is pronounced in PE placentas

As the major angiotension receptor and the in vivo target of AT1-AA, AT1 receptor is believed 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-IP assay in which AT1 receptor protein in NT/PE placental lysates was immunoprecipitated and its isopeptide level determined in 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. Additionally, in immunofluorescent staining, AT1 receptor (red) and isopeptide modification (green) could be 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 study[12] demonstrates that the transfer of purified AT1-AA or total IgG from PE patients into pregnant mice reproduces the key clinical features of PE, thereby establishing 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], infusion of IgG from PE women induces key PE clinical features including hypertension and proteinuria in pregnant mice, (Figure 3A and B). Similar to human studies, circulating TG activity was significantly elevated in the pregnant mice injected with PE IgG compared to the NT IgG-injected controls (Fig. 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 in Figure 3a) and proteinuria (from 106.5±37.8 to 38.5±6.9 ng albumin/mg creatinine in Figure 3B), as well as increased plasma TG activity (Fig. 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), while 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 is significantly down-regulated 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 if PE IgG could induce the interaction between AT1 receptors and TG2. More AT1 receptors were associated with TG2 in trophoblasts treated with PE IgG (Fig. 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 co-treated 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 (CHQ), indicating that AT1 receptors are mainly degraded through the ubiquitination/proteasome pathway[29].

The AT1 receptor cytoplasmic tail has only one glutamine (position 315, or Q315) that was previously shown to be the FXIIIa transglutaminase-mediated AT1 receptor crosslinking site[30]. Thus, to assess the effect of TG2-mediated isopeptide modification on AT1 receptor ubiquitination, we established stable CHO cell lines overexpressing TG2 along with either WT or mutant AT1 receptor in which glutamine at position 315 is replaced with alanine (Q315A). Compared with WT AT1 receptor, the Q315A mutant is less abundant and resistant to TG2-mediated isopeptide modification (Figure 4E and F). However, when the ubiquitination/proteasome pathway was blocked by proteasome inhibitor MG132, the Q315A mutant showed a similar overall cellular abundance to WT AT1 receptors, and the poly-ubiquitination level of the Q315A mutant (and its associated proteins), as well as its mono-ubiquitination level are much higher than those of WT AT1 receptor (Figure 4G). Reciprocally, in the anti-ubiquitin immunoprecipitated products, more mono-ubiquitinated AT1 receptor was identified from the Q315A mutant cells, and was not changed with Ang II stimulation (Figure 4H). In contrast the WT AT1 receptor showed reduced monoubiquitination following Ang 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 receptor by repressing ubiquitination-dependent degradation.

Discussion

In this study we are the first to identify significantly increased enzyme activity and protein expression of tissue transglutaminase in PE placentas. Intrigued by the colocalization among TG2, isopeptides, and AT1 receptor, we furhter 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 posttranslationally 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.

We report here a significant increase in plasma TG activity in PE patients. The increased TG is unlikely to be FXIIIa transglutaminase because the usage of EDTA as anti-coagulant in plasma collection results in the cleavage of FXIIIa transglutaminase by thrombin[31], our data not shown. Therefore, the increase in plasma TG activity probably results from increased circulating TG2 in microparticles 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 TNF and IL-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.

Tissue transglutaminase is found to be 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 intra-peritoneal administrative methods in mice and has been shown to successfully control the development of Hungtington 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. Due to 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 believe 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], and [26]. Uniquely, the transamidation activity of tissue transglutaminase is inhibited by the binding of cellular energy-carrying nucleotides including GTP, GDP, and ATP[13, 26] and [40]. Under normal physiological conditions, cells maintain an intracellular environment of low calcium and high GTP concentrations, which inhibits TG2's transamidation function[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 tissue transglutaminase and AT1 receptors in PE IgG-treated HTR cells. We also observed a significant increase in isopeptide modification of AT1 receptors at the sycytiotrophoblasts of PE placentas. Through 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. Indeed, AT1-AAs in the circulation of PE patients 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 observation[30] about the inability of TG2 to crosslink AT1 receptor into dimers, and suggests that the modification may be intra-molecular as documented on 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. Since the degradation of AT1 receptors is mainly through the ubiquitin-proteasome pathway[29], we examined the protein level and ubiquitination status of wild-type and Q315A mutant AT1 receptors. We observed that in CHO 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 are the presence of several lysine residues in the C-terminal tail for ubiquitination. Protein ubiquitination, a process resulting in protein degradation, utilizes ATP to form a high-energy glycine-lysine isopeptide bond between ubiquitin and its acceptor[44], while 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 Ang II sensitivity and hypertension.

Taken together, our results in this study suggest a vicious cycle for the pathogenesis of PE in which increased tissue transglutaminase 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 tissue transglutaminase contributes to the disease pathogenesis and causes the pathological accumulation of AT1 receptor 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. And the identification of other TG2 modification targets will help us better understand the disease nature as well. Moreover, the existence of the conditions (e.g. 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 two processes compete for the same lysine in AT1 receptor cytoplasmic tail with proteomic approaches. Using 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 could surprisingly stabilize GPCRs via ubiquitination-preventing isopeptide modification in certain stress conditions.

Methods and Materials

Please see online supplementary materials

Acknowledgments

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Novelty and Significance

What is new?

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

What is relevant?

1. The pathogenic involvement of tissue transglutaminase in preeclampsia has been demonstrated for the first time in this study.
2. The finding of TG2-mediated AT1 receptor stabilization in this study may also suggest a novel GPCR regulatory mechanism in which TG2/G_h, a high molecular weight G protein, could even stabilize its coupling GPCRs through ubiquitination-preventing isopeptide modification in stress or pathological conditions favoring the transamidation function of TG2.

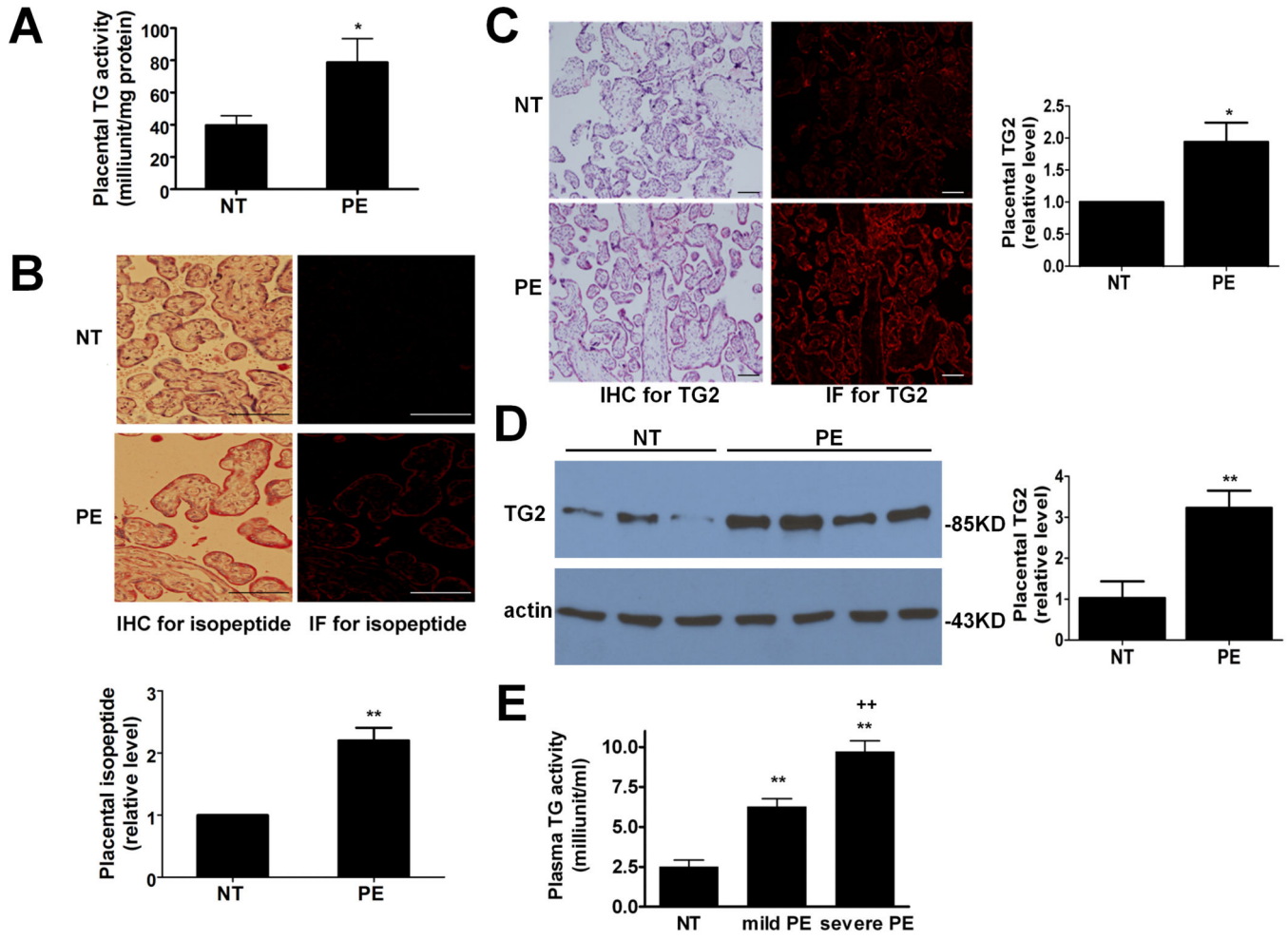

Figure 1. Placental TG activity and tissue transglutaminase (TG2) are increased in PE

(A) TG activity is significantly increased (approximately two-fold) in PE placental lysates (**P* value for PE versus 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 IHC/IF dual staining. The quantification of color signal also shows a two-fold increase in PE placentas (***P*<0.01 PE placenta versus 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 tissue transglutaminase (TG2) is localized at the syncytiotrophoblasts of human PE placentas by IHC/IF dual staining. The quantification of color signal (bar graph) demonstrates an approximately two-fold increase of TG2 in PE (**P*<0.05 NT placenta versus 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 approximately three-fold higher than in NT as measured in Western blot with human placental lysates after normalization with actin level (***P*<0.01 NT versus PE, n=3 in NT group, 4 in PE group). **(E)** Compared with NT controls (2.5±0.4 milliunit/ml), a higher level of plasma TG activity is identified in either mild PE (6.3±0.5 milliunit/ml) or severe PE (9.7±0.7 milliunit/ml) with *in vitro* TG activity assay (Sigma-Aldrich, MO) (***P*<0.01 NT

(n=25) versus mild PE (n=25) or severe PE (n=25); ++P<0.01 mild PE versus severe PE). Data in Figure 1 represent mean±S.E.M. from indicated experiments.

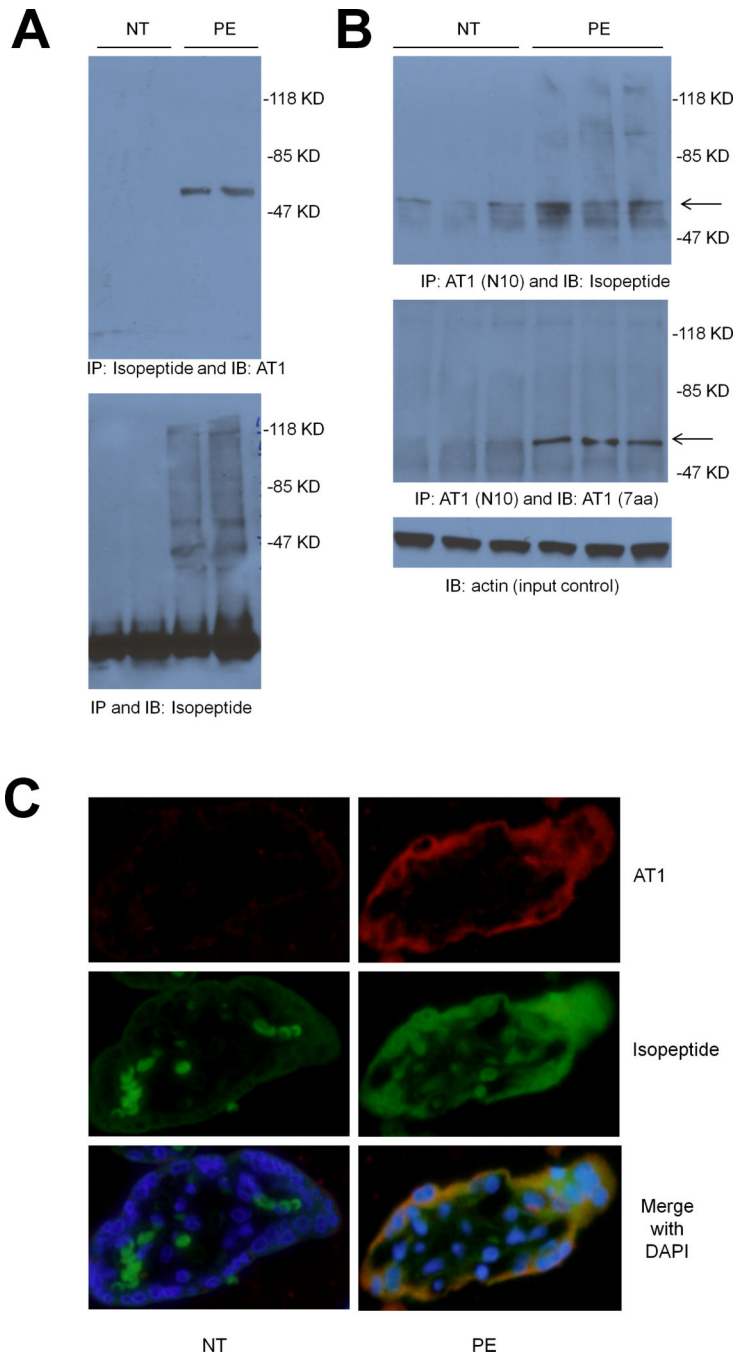

Figure 2. AT1 receptor is modified with ε-(γ-glutamyl)-lysine isopeptide isopeptide in PE placentas

(A) Following 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 NT placental lysates (n=2 randomly chosen patient samples in each category). **(B)** Following immunoprecipition, 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) co-localize (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.

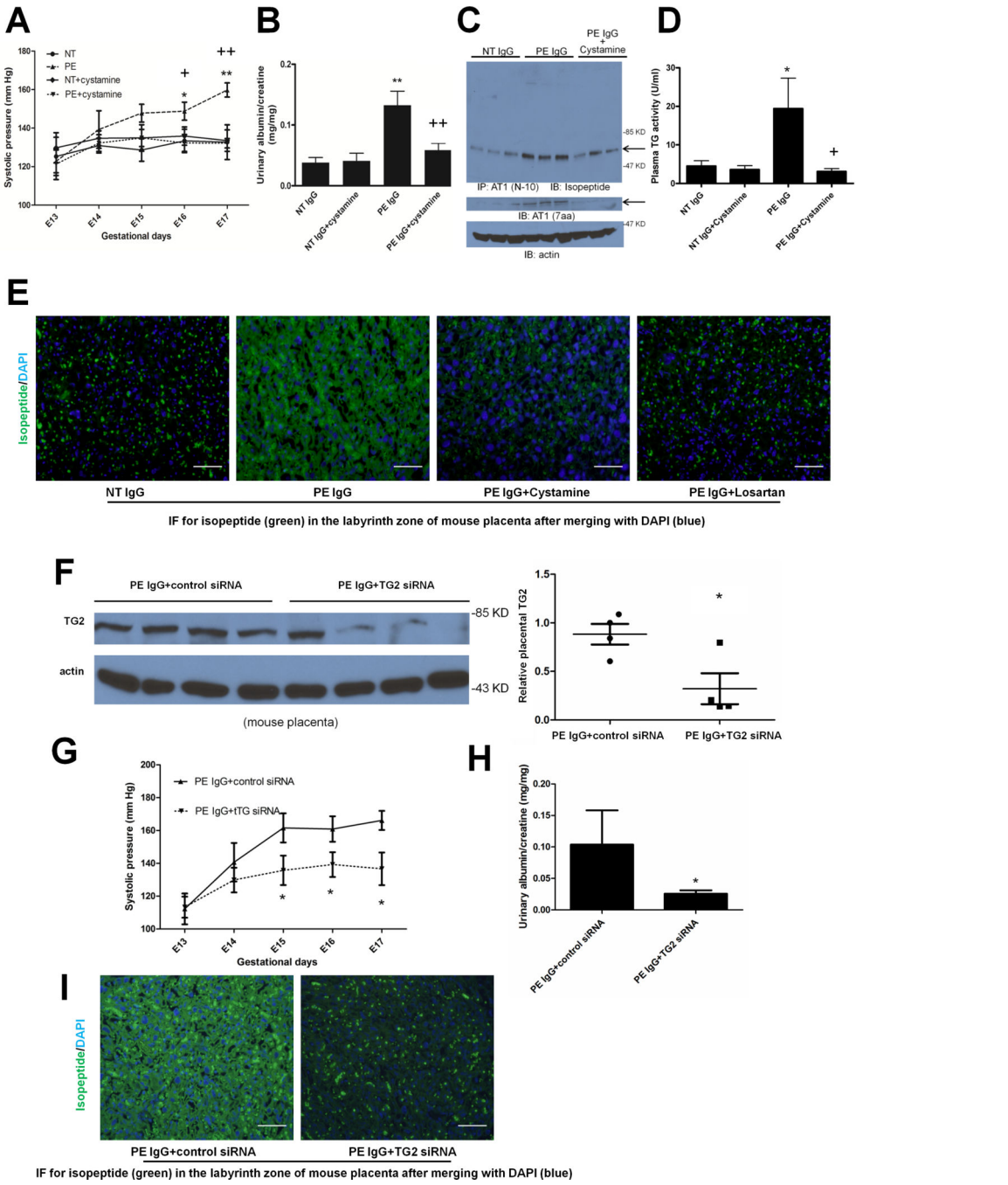

Figure 3. Cystamine and siRNA-mediated TG2 knockdown alleviate placental isopeptide modifications and clinical features of PE in autoantibody-induced mouse model

(A) The increase in systolic blood pressure is prevented by TG inhibitor cystamine in PE mice (**P*<0.05, ***P*<0.01 NT IgG versus PE IgG; +*P*<0.05, ++*P*<0.01 PE IgG versus 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 versus PE IgG; ++*P*<0.01 PE IgG versus PE IgG +cystamine; n=6 mice in each group). **(C)** Accumulation and isopeptide modification of placental AT1 receptors are inhibited by cystamine treatment. After the mouse placental lysates were pre-cleared 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 in Western blot directly with goat anti-AT1 receptor antibody (7aa, Bethyl labs). **(D)** Cystamine treatment represses PE IgG-induced increase in plasma TG activity in pregnant mice. Murine plasma collected with EDTA as anti-coagulant on E18 were assayed in *in vitro* TG activity assay (Covalab, France). (**P*<0.05, NT IgG versus PE IgG; +*P*<0.05, PE IgG versus 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 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 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 versus PE IgG+TG2 siRNA; n=4 mice in each group). **(G)** Tissue transglutaminase (tTG) siRNA thwarted blood pressure increase in PE mice from E15 to E17 (*P<0.05 PE IgG+control siRNA (n=6 mice) versus 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 versus 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).

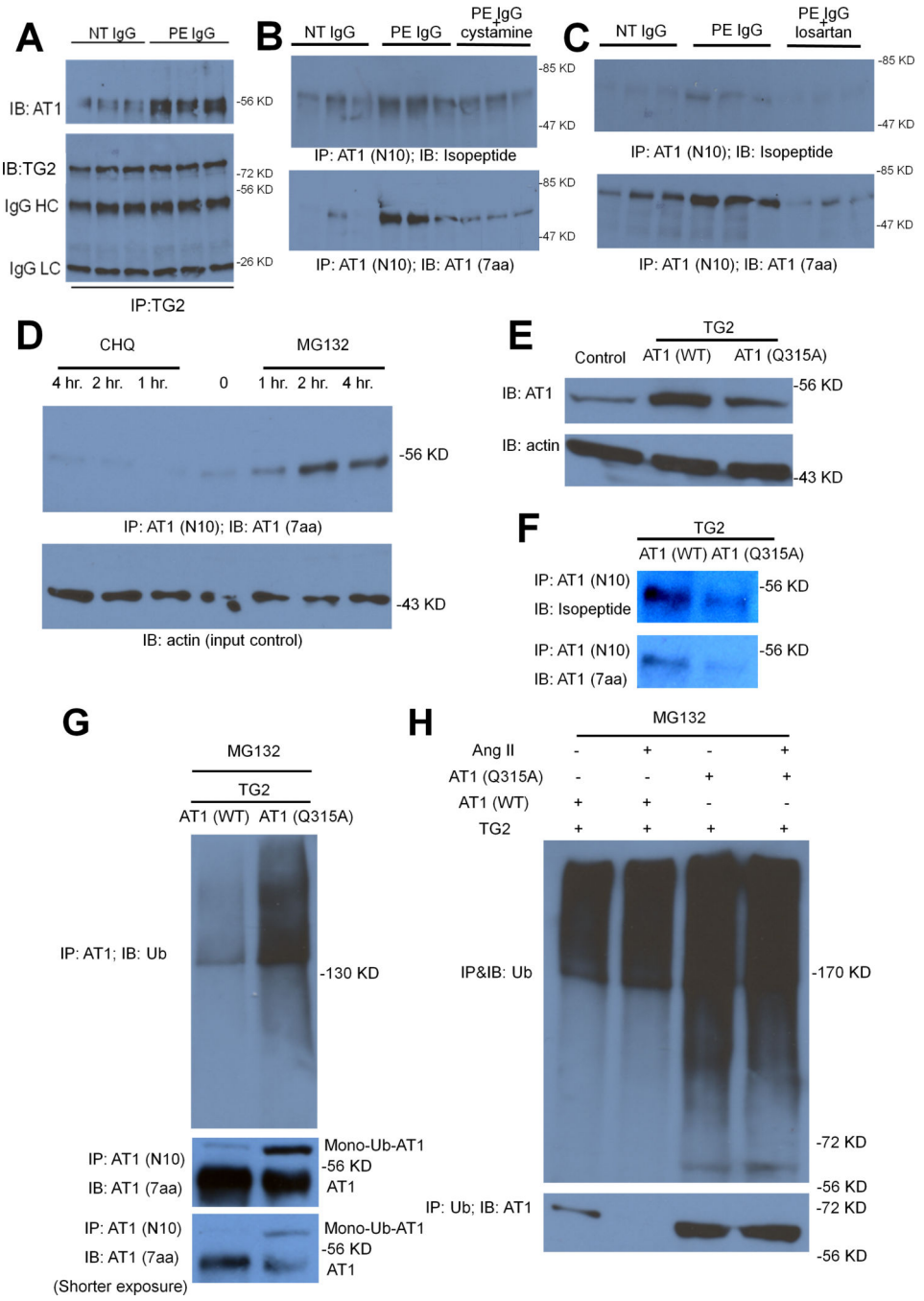

Figure 4. TG2-mediated isopeptide modification on Q315 of AT1 receptor stabilizes the receptor via preventing ubiquitination

(A) PE IgG promotes the interaction between AT1 receptors and tissue transglutaminase (TG2) in human trophoblast cell line *HTR*-8/SVneo cells. Compared with 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 pulldown products was assessed by Western blot. (n=3 independent experiments) **(B)** TG inhibitor cystamine (500 μM) represses PE IgG (100 μg)-induced accumulation and isopeptide modification of AT1 receptors. After 4-hr

treatment at 37 °C, AT1 receptors in the treated cells were immunprecipitated 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 Labs), respectively. (n=3 independent experiments) **(C)** AT1 receptor antagonist losartan (5 μM) 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 μM, proteasome inhibitor)-treated, but not chloroquine (CHQ, 200 μM, a lysosome inhibitor)-treated, trophoblasts. AT1 receptor in the treated cells was concentrated by immunprecipitation 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 Labs). 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)** Poly-ubiquitination and mono-ubiquitination levels of immunoprecipitated AT1 receptor are elevated in Q315A mutant cells treated with proteasome inhibitor MG132 (20 μM) (n=3 independent experiments). **(H)** Reciprocally, increased mono-ubiquitinated AT1 receptor is identified in the ubiquitinated proteins of Q315A mutant cells. The increase in mono-ubiquitinated AT1 receptor Q315A mutant was not changed by Ang II (100 nM) treatment that decreased the level of mono-ubiquitinated wild type AT1 receptor.