Published in final edited form as:
Nat Med. 2008 August ; 14(8): 855–862. doi:10.1038/nm.1856.

Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice

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Abstract

Pre-eclampsia affects approximately 5% of pregnancies and remains a leading cause of maternal and neonatal mortality and morbidity in the United States and the world[1,2]. The clinical hallmarks of this maternal disorder include hypertension, proteinuria, endothelial dysfunction and placental defects. Advanced-stage clinical symptoms include cerebral hemorrhage, renal failure and the HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome. An effective treatment of pre-eclampsia is unavailable owing to the poor understanding of the pathogenesis of the disease. Numerous recent studies[3–5] have shown that women with pre-eclampsia possess autoantibodies, termed AT_1-AAs, that bind and activate the angiotensin II receptor type 1a (AT_1 receptor). We show here that key features of pre-eclampsia, including hypertension, proteinuria, glomerular endotheliosis (a classical renal lesion of pre-eclampsia), placental abnormalities and small fetus size appeared in pregnant mice after injection with either total IgG or affinity-purified AT_1-AAs from women with pre-eclampsia. These features were prevented by co-injection with losartan, an AT_1 receptor antagonist, or by an antibody neutralizing seven–amino-acid epitope peptide. Thus, our studies indicate that pre-eclampsia may be a pregnancy-induced autoimmune disease in which

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Note: Supplementary information is available on the Nature Medicine website.

AUTHOR CONTRIBUTIONS

C.C.Z. performed total IgG purification; blood pressure, urinary protein, sFlt1 and sEng measurements in antibody-injected pregnant mice; fetal kidney and placenta isolation and cell culture bioassays for AT_1-AA biological activity; and contributed to writing, editing and generation of figures. Y.Z. carried out antibody injections, affinity purification and characterization of AT_1-AAs, western blot experiments and ELISA for human IgG; and contributed to writing and generation of figures. R.A.I. procured human subjects' approvals, maintained the database of de-identified human subject information, measured blood pressure and urinary protein in antibody-injected nonpregnant mice, performed kidney and placental histology and contributed to writing and generation of figures. H.Z. provided expertise in kidney and placental histology and contributed to writing and editing of histological studies in both placenta and kidney. T.M. injected mice with antibody and helped with blood pressure and other measurements. E.J.P. provided expertise regarding the electron microscopic analysis of kidney. M.J.H. provided expertise in placental histology. S.M.R. provided expertise in pre-eclampsia, collected human subject samples amd analyzed human subject data. R.E.K. provided expertise in pre-eclampsia and the renin angiotensin system and contributed to the writing and editing of the manuscript. Y.X. was the principal investigator of the study, oversaw the overall design of experiments and interpretation of all results, organized the manuscript including text and figures, and did the final editing of the manuscript.

key features of the disease result from autoantibody-induced angiotensin receptor activation. This hypothesis has obvious implications regarding pre-eclampsia screening, diagnosis and therapy.

The pathophysiology of pre-eclampsia remains largely unknown. A widely held view is that placental ischemia, stemming from shallow trophoblast invasion and improper spiral artery remodeling, is a crucial initiating event[1,2,6]. Numerous studies have focused on circulating factors secreted by the ischemic placenta that contribute to the maternal syndrome[7–10]. The oxidative stress and vascular damage resulting from placental ischemia are believed to underlie the enhanced maternal inflammatory response associated with pre-eclampsia[11]. Immune mechanisms and the renin-angiotensin system are also implicated in pre-eclampsia[3–5,12,15]. These two concepts were united in a previous report[16], in which it was shown that sera from women with pre-eclampsia contain autoantibodies that react with AT_1 angiotensin receptors in a stimulatory fashion. Subsequent to these findings, multiple other groups, including our own, showed that many features of pre-eclampsia could be explained by the ability of these autoantibodies to activate AT_1 receptors on a variety of cells and provoke biological responses that are relevant to the pathophysiology of pre-eclampsia[17–22]. However, previous work has been restricted to the use of *in vitro* systems and has been unable to specifically address the relevance of AT_1-AAs to the defining features of pre-eclampsia, hypertension and proteinuria.

To evaluate the *in vivo* pathophysiological consequences of AT_1-AAs, we introduced IgG (approximately 800 μg) from either normotensive pregnant women or pregnant women with pre-eclampsia into pregnant mice on day 13 of gestation. We chose day 13 because this stage of mouse pregnancy is comparable to early onset pre-eclampsia in humans and is a time at which we can reliably determine whether a mouse is pregnant. We initially used western blot analysis to show that human IgG was readily detectable for at least 5d after injection (Fig. 1a). By ELISA we found that human IgG persisted in the circulation of injected mice until the time of killing on gestation day 18 and that the IgG concentrations in injected mice were similar for mice injected with IgG from normotensive pregnant women or women with pre-eclampsia (Fig. 1b). To determine whether the injected antibody retained biological activity, we killed pregnant mice 5 d after antibody injection, purified IgG from the maternal mouse sera and assayed the isolated IgG for AT_1 receptor agonistic activity with a reporter cell line in which AT_1 receptor activation results in increased expression of a 4× NFAT *cis* element–driven luciferase reporter. The results (Fig. 1c) show that IgG from women with pre-eclampsia retained the ability to activate AT_1 receptors for at least 5 d after retro-orbital injection into pregnant mice. In contrast, IgG isolated from pregnant mice injected with IgG from normotensive pregnant women did not stimulate luciferase synthesis (Fig. 1c). These results show that it is possible to introduce physiologically relevant concentrations of human IgG into pregnant mice and that the injected antibody persists in a biologically active form for many days in the maternal circulation.

Hypertension is a defining feature of pre-eclampsia. To determine whether IgG from women with pre-eclampsia has the ability to contribute to gestational hypertension, we measured systolic blood pressure at daily intervals after retro-orbital injection of IgG from pregnant women into pregnant mice on day 13 of gestation. IgG from women with pre-eclampsia but not IgG from normotensive pregnant women induced a significant increase in blood pressure that was evident 4 d after antibody injection (Fig. 1d). The increased blood pressure resulting from injection of IgG from women with pre-eclampsia was prevented by co-injection with losartan, an AT_1 receptor antagonist (Fig. 1d), suggesting that the antibody-induced increase in blood pressure required AT_1 receptor activation.

Antibody-injected mice were also analyzed for proteinuria, another defining feature of pre-eclampsia, by determining the ratio of urinary albumin to creatinine on gestation day 18,

when the dams were killed. The results (Fig. 1e) show that a significant increase in urinary albumin occurred after injection of IgG from women with pre-eclampsia in contrast to IgG from normotensive pregnant women. The autoantibody-induced proteinuria was prevented by co-injection with losartan (Fig. 1e), indicating that it resulted from autoantibody-induced AT_1 receptor activation. Overall, the results (Fig. 1) indicate that IgG from women with pre-eclampsia, in contrast to IgG from normotensive pregnant women, induced hypertension and proteinuria in pregnant mice and that these antibody-induced features of pre-eclampsia required AT_1 receptor activation. Our inability to detect increased blood pressure and urinary protein in the pregnant mice injected with IgG from the controls allows us to conclude that it is unlikely that immune complexes formed between mouse antibodies raised against the injected human IgG account for the features observed in the pregnant mice injected with human IgG from subjects with pre-eclampsia.

AT_1-AAs interact with a specific seven–amino acid sequence on the second extracellular loop of the AT_1 receptor[16]. Competition experiments have shown that this seven–amino acid epitope peptide blocks autoantibody-induced AT_1 receptor activation in a variety of cell lines[16–22]. To determine whether the hypertension and proteinuria in antibody-injected pregnant mice was a result of AT_1-AAs, we injected mice on gestational day 13 with IgG from women with pre-eclampsia in the presence or absence of the seven–amino acid epitope peptide and subsequently monitored blood pressure and urinary protein. The results (Fig. 1d,e) show that antibody-induced hypertension and proteinuria were prevented by co-injection with the seven–amino acid epitope peptide. These findings indicate that the IgG-induced hypertension and proteinuria resulted from autoantibodies that interacted with a seven–amino acid epitope on the second extracellular loop of the AT_1 receptor.

The ability of the seven–amino acid epitope peptide to block AT_1-AA–mediated AT_1 receptor activation[16–22] suggests a physical association between AT_1-AAs and the second extracellular loop of the AT_1 receptor. We tested this possibility directly with a GST-peptide fusion protein containing a 27–amino acid sequence corresponding to the second extracellular loop of the AT_1 receptor (GST–27-aa). We initially tested the ability of AT_1-AAs to bind the GST–27-aa fusion protein by incubating total IgG from women with pre-eclampsia with the GST–27-aa fusion protein coupled to AminoLink Plus Coupling gel. Column chromatography was performed and the original IgG preparation was separated into a flow-through fraction that did not bind the GST–27-aa fusion protein and a fraction that initially bound and was subsequently eluted from the GST–27-aa peptide fusion protein. Antibodies in the flow-through and eluted fractions were assayed for their ability to detect AT_1 receptor protein by western blot analysis. A commercially available antibody to the AT_1 receptor was used as a positive control (Fig. 2a). The results (Fig. 2a) show that the antibodies in the flow-through fraction were unable to detect a protein at 43 kDa, corresponding to the AT_1 receptor. However, the fraction that initially bound the column and that was subsequently eluted (that is, the elution fraction, Fig. 2a) readily detected a protein of approximately 43 kDa that co-migrated with the protein detected by the commercially available anti-AT_1 receptor antibody. To determine whether the affinity-purified AT_1-AAs retained biological activity and to determine the effectiveness of the affinity purification procedure, we assayed the flow-through and elution fractions for AT_1-AA biological activity using the 4× NFAT *cis* element–driven luciferase reporter cell line mentioned above. The bioassay revealed essentially no activity in the flow-through fraction (Fig. 2b), which is consistent with the western blotting results (Fig. 2a). However, the elution fraction was highly enriched in AT_1-AA activity, approximately 40-fold over the original IgG preparation (Fig. 2b) with a recovery of greater than 30% (Fig. 2c). The ability to stimulate luciferase synthesis in this cell line was inhibited by losartan and by the seven–amino acid epitope peptide (Fig. 2d). Thus, the affinity-purified AT_1-AAs are highly enriched and retain biological activity.

The affinity-purified AT_1-AAs were tested for the ability to induce symptoms of pre-eclampsia when injected into pregnant mice. Retro-orbital injection of ~20 μg of these autoantibodies into pregnant mice resulted in a robust stimulation of blood pressure that was apparent within 3 d (Fig. 2e), as was the development of significant proteinuria (Fig. 2f). As with total IgG from women with pre-eclampsia, the hypertensive and proteinuric effects of affinity-purified AT_1-AAs injection were inhibited by losartan or the seven–amino acid epitope peptide (Fig. 2e,f). None of the mice injected with control IgG showed a significant increase in blood pressure or urinary protein. These results provide direct evidence that autoantibodies from women with pre-eclampsia that bind the second extracellular loop of the AT_1 receptor induce hypertension and proteinuria when injected into pregnant mice.

Pre-eclampsia is also associated with impaired renal function and characteristic alterations in renal histology. To evaluate the potential role of AT_1-AAs in renal pathophysiology, we injected mice on gestational day 13 with IgG from normotensive pregnant women or from women with pre-eclampsia in the presence or absence of losartan or the seven–amino acid epitope peptide. Five days after antibody injection, pregnant mice were killed and their kidneys were isolated, fixed, sectioned and analyzed by H&E staining, which revealed extensive renal damage in the samples from the mice injected with the IgG from women with pre-eclampsia (Fig. 3a,b). The majority of the glomeruli seen in these mice showed normal to smaller-sized glomeruli with endothelial swelling, causing narrowing and total obliteration or occlusion of the glomerular capillary spaces (a condition termed endotheliosis; Fig. 3b). No marked cellular proliferation or increase in mesangial matrix was observed in these mice. These changes were not evident in mice after injection of IgG from normotensive pregnant women and were diminished when preeclamptic IgG was co-injected with either losartan or 7-aa (Fig. 3a–c). The glomeruli showed a characteristic ‘bloodless’ consolidated appearance, in contrast to the open glomerular tufts of the pregnant mice injected with IgG from normotensive pregnant women (Fig. 3b). Examination of kidneys from these mice by electron microscopy confirmed the glomerular changes observed by light microscopy. Narrowing and complete obliteration of the capillary loop spaces by the swollen cytoplasm of the endothelial cells are seen in the affected glomeruli, together with loss of the fenestrations of the endothelial cells (Fig. 3d). Foot process effacement is seen focally in the podocytes (Fig. 3d). Occasional electron-dense materials are also observed in the subendothelial and mesangial areas (Fig. 3d). These series of histological changes in the kidney are pathognomonic for the renal disease that often occurs in women with pre-eclampsia[24]. In contrast, no segmental glomerulosclerosis, significant mesangial cell proliferation or vessel wall changes were seen on light microscopy or electron microscopy (Fig. 3c,d). No renal histopathological changes were observed in mice injected with IgG from normotensive pregnant women (Fig. 3a–d). In mice co-injected with losartan or the seven–amino acid epitope peptide and IgG from women with pre-eclampsia, the histological changes were partially reversed (Fig. 3a–d). Consistent with these results, we found similar renal damages in pregnant mice injected with affinity-purified AT_1-AAs (data not shown). Thus, this autoantibody can induce renal histopathological changes characteristic of pre-eclampsia via AT_1 receptor activation.

Pre-eclampsia is frequently associated with placental abnormalities and intrauterine growth retardation[26]. To evaluate the potential contribution of AT_1-AAs to these features of pre-eclampsia, we injected pregnant mice (gestational day 13) with IgG from normotensive pregnant women or women with pre-eclampsia and examined placentas and fetuses on gestational day 18. The placentas were significantly smaller in mice injected with IgG from women with pre-eclampsia (0.083 ± 0.015 g) in comparison to those from mice injected with IgG from normotensive pregnant women (0.125 ± 0.013 g; Supplementary Table 1 online). The reduction in placenta size was largely prevented by co-injection with losartan or the seven–amino acid epitope peptide, indicating that the reduction placental size or fetal

weight was mediated by AT_1-AA–induced AT_1 receptor activation (Supplementary Table 1). Placentas from the mice injected with preeclamptic IgG showed increased intraparenchymal calcifications compared to those from mice injected with IgG from normotensive pregnant women (Supplementary Fig. 1 online). Significant fetal growth retardation was also observed by gestation day 18 (Supplementary Table 1) in mice injected with IgG from women with pre-eclampsia (1.28 ± 0.15 g for normotensive versus 1.01 ± 0.17 g for pre-eclamptic). The reduction in fetal weight was largely prevented when pre-eclamptic IgG was co-injected with losartan or the seven–amino acid epitope peptide (Supplementary Table 1).

Soluble fms-related tyrosine kinase-1 (sFlt1) is a soluble form of the vascular endothelial growth factor (VEGF) receptor that is significantly elevated in the plasma of women with pre-eclampsia and is believed to contribute to disease pathology by interference with VEGF signaling[8,9,24]. Soluble endoglin (sEng) is a soluble form of endoglin, a cell surface co-receptor of transforming growth factor-β receptor. It is also secreted by the placenta at elevated levels in women with pre-eclampsia and is believed to contribute to disease by interference with transforming growth factor-β signaling[25]. To evaluate the potential contribution of AT_1-AAs to increased production of sFlt1 and sEng in pre-eclampsia, we gave pregnant mice a single injection (at gestation day 13) or double injections (at gestation days 13 and 14) of IgG from normotensive pregnant women or women with pre-eclampsia and determined the serum abundance of sFlt1 and sEng of injected mice on gestation day 18. The results (Supplementary Fig. 2 online) show that sFlt1 concentrations were significantly higher in pregnant mice receiving either single or double injections of IgG from women with pre-eclampsia over those observed with pregnant mice injected with IgG from normotensive pregnant women. sEng was also induced by injection with IgG from women with pre-eclampsia, although in this case two injections were required to see a significant increase (Supplementary Fig. 2). Autoantibody-mediated induction of sFlt1 and sEng in pregnant mice was inhibited by co-injection with losartan or the seven–amino acid epitope peptide, indicating that the induction was mediated by AT_1-AAs and required AT_1 receptor activation. These results indicate that autoantibodies from women with pre-eclampsia mediate a dosage-dependent stimulation of sFlt1 and sEng production in pregnant mice via AT_1 receptor activation.

Autoantibody-induced hypertensive and proteinuric effects in pregnant mice could be mediated through the action of excessive sFlt1 production by the placenta[22]. To assess the potential contribution of AT_1-AAs independently of excess sFlt1, we introduced IgG from normotensive pregnant women or women with pre-eclampsia into nonpregnant mice and monitored sFlt1 abundance, blood pressure and proteinuria at various times after injection. The concentration of sFlt1 was very low in nonpregnant mice injected with IgG from normotensive pregnant women and was not increased significantly by injection with IgG from women with pre-eclampsia (Fig. 4a). In contrast, sFlt1 concentrations were much higher in pregnant mice and showed a significant additional increase in pregnant mice injected with IgG from women with pre-eclampsia over levels observed in mice injected with IgG from normotensive pregnant women (Fig. 4a). Thus, nonpregnant mice have very low amounts of sFlt1 in comparison to pregnant mice, and the low concentration is not significantly increased as a result of injection with IgG from women with pre-eclampsia.

Subsequently, we monitored blood pressure and proteinuria in IgG-injected nonpregnant mice. The results (Fig. 4b) show that IgG from women with pre-eclampsia, in contrast to IgG from normotensive pregnant women, stimulated an increase in blood pressure. None of ten mice injected with control IgG showed a significant increase in blood pressure. The autoantibody-mediated increase in blood pressure was inhibited by co-injection with losartan or the seven–amino acid epitope peptide (Fig. 4b). In addition, there was no

significant difference between the autoantibody-induced hypertension observed in pregnant mice (Fig. 1b and Fig. 2e) and that in nonpregnant mice (Fig. 4b), suggesting that AT_1-AA–mediated hypertension does not require antibody-induced sFlt1 produced by the placenta.

Finally, we measured the urinary protein in antibody-injected nonpregnant mice. The results (Fig. 4c) show that neither IgG from women with pre-eclampsia nor IgG from normotensive pregnant women induced proteinuria in antibody-injected nonpregnant mice. Consistent with the lack of antibody-induced proteinuria was the absence of antibody-induced renal histopathology (Fig. 4d). Specifically, the glomeruli from nonpregnant mice injected with IgG from women with pre-eclampsia showed no evidence of hypercellularity or endotheliosis and appeared normal in both size and shape (Fig. 4d). This is in contrast to the renal histopathological changes observed in pregnant mice injected with the IgG from women with pre-eclampsia (Fig. 3). These findings indicate that whereas IgG from women with pre-eclampsia is capable of inducing hypertension in nonpregnant mice, it is unable to induce proteinuria and glomerular endotheliosis at this antibody concentration, suggesting that AT_1-AA–induced sFlt1 production from the placenta contributes to the renal damage and proteinuria observed in antibody-injected pregnant mice.

It is a widely held view that the maternal features of pre-eclampsia are secondary to placental ischemia[2,26] and vascular damage resulting in a local and systemic inflammatory response. From this perspective, researchers developed a rat model of pre-eclampsia on the basis of experimentally induced placental ischemia resulting from reduced uterine perfusion pressure (RUPP)[7,27–30] and found that tumor necrosis factor-α levels, along with those of other inflammatory markers, were elevated[31,32]. Such experimentally manipulated pregnant rats developed hypertension, proteinuria and other features of pre-eclampsia, including elevated sFlt1[33,34] and the production of AT_1-AAs[32,34]. Sera from RUPP-manipulated pregnant rats stimulated increased endothelin-1 synthesis by endothelial cells through AT_1 receptor activation[7], a finding consistent with the presence of AT_1-AAs. RUPP-induced hypertension was markedly attenuated by antagonism of the AT_1 receptor[35] or the endothelin receptor type A[32], suggesting that RUPP-induced hypertension relied on AT_1-AA–induced endothelin-1 synthesis. Collectively, these results indicate that placental ischemia and the associated vascular damage and inflammatory response may serve as key stimuli for AT_1-AA production during pregnancy and that AT_1 receptor activation has a major role in the hypertension produced by placental ischemia[34].

Angiotensin II is a well recognized hypertensive agent that mediates its physiological effects primarily through AT_1 receptor activation. Because AT_1-AAs also mediate their physiological effects through AT_1 receptor activation, we expected these autoantibodies to stimulate an increase in blood pressure in the absence of elevated sFlt1. Thus, it came as no surprise that these autoantibodies could induce hypertension in nonpregnant mice. Because both AT_1-AAs and sFlt1 can stimulate a rise in blood pressure, it is possible that each contributes to hypertension associated with pre-eclampsia.

Proteinuria and renal lesions, especially glomerular endotheliosis, are characteristic features of pre-eclampsia and occur in rat models of pre-eclampsia induced by elevated sFlt1 concentrations[24]. Because sFlt1 is induced by AT_1-AAs in pregnant mice, it is possible that proteinuria and glomerular endotheliosis in antibody-injected mice are due to AT_1-AA–mediated induction of sFlt1. Consistent with this possibility is the fact that IgG from women with pre-eclampsia readily induced hypertension in nonpregnant mice under conditions in which antibody-induced proteinuria and renal histopathological changes were lacking. In addition, AT_1-AA–mediated sFlt1 induction is absent in nonpregnant mice (Fig. 4c) because the major source of sFlt1 (that is, the placenta) is missing[15,23]. It is likely that autoantibody-

induced renal damage and proteinuria in pregnant mice are mediated through the induction of sFlt1.

It is possible that the increased occurrence of pre-eclampsia among women who are pregnant for the first time[36] may result from an increased risk of placental ischemia in first pregnancies, owing to the small size of the spiral arteries in the uterus of such women. After these women become pregnant for the first time, the spiral artery diameters increase four to five times as they become flaccid and nonmuscular in comparison to those before pregnancy[37]. After pregnancy, the uterine spiral arteries do not return to their original small size but instead remain significantly larger[37]. Permanent enlargement of the uterine spiral arteries allows for enhanced placental perfusion in subsequent pregnancies[36,37] and in this way may contribute to a reduced risk of ischemia, the resulting inflammatory response and the production of AT_1-AAs. In pre-eclampsia, the decline of the inflammatory state that occurs at childbirth may deprive autoreactive lymphocytes of key cytokines required for continued AT_1-AA production. Because AT_1-AAs are predominantly of the IgG3 subclass[38], with a relatively short 7-d half-life[16], their levels decline substantially during the first few days after childbirth.

The antibody injection model of pre-eclampsia described here provides strong experimental support for our working hypothesis that pre-eclampsia is an autoimmune disease in which angiotensin receptor–activating autoantibodies contribute to many features of the disease. The biological properties of these autoantibodies can be blocked by a seven–amino acid epitope peptide that corresponds to a specific epitope associated with the second extracellular loop of the AT1 receptor. This fact suggests a common immunological origin for these autoantibodies in different individuals and has profound therapeutic potential. Available evidence suggests that AT_1-AAs can be detected as early in pregnancy as 18 weeks, making them one of the earliest markers to identify women at risk for pre-eclampsia[39]. The fact that AT_1-AAs can be detected many weeks before the symptoms of pre-eclampsia has far reaching implications regarding presymptomatic screening, disease diagnosis and treatment. If maternal circulating AT_1-AAs contribute to pre-eclampsia, as our adoptive transfer animal model suggests, the timely identification and removal or inhibition of these autoantibodies from women with pre-eclampsia may provide considerable therapeutic benefit. If AT_1-AAs play a major part in the etiology and pathophysiology of pre-eclampsia, as we hypothesize, it may one day be possible to block autoantibody-mediated AT_1 receptor activation and thereby forestall or prevent the onset of the symptoms of pre-eclampsia.

METHODS

Reagents

We purchased cell culture medium RPMI 1640, antimycotics, antibiotics and FBS from Invitrogen. Losartan was a generous gift from Merck.

Human subjects

Pregnant human subjects with pre-eclampsia who were admitted to Memorial Hermann Hospital were identified by the obstetrics faculty of the University of Texas Medical School at Houston. The subjects were diagnosed with severe pre-eclampsia on the basis of the definition set by the National High Blood Pressure Education Program Working Group report. These criteria included the presence of a high blood pressure of ≥160/110 mm Hg and the presence of protein in urine of ≥3.5 g in a 24-h period or a random urinary protein/creatinine ratio of >0.3. These women had no previous history of hypertension. Other criteria included the presence of persistent headache, visual disturbances, epigastric pain or

the HELLP syndrome in women with a blood pressure of ≥140/90 mm Hg. Normotensive pregnant individuals were characterized by uncomplicated pregnancies with normal-term deliveries. The clinical characteristics of the study subjects are summarized in Supplementary Table 2 online. We centrifuged blood samples at 18,000*g* for 10 min and stored the serum samples at −80 °C. The research protocol, which included informed consent from the subjects, was approved by the University of Texas Health Science Center at Houston Committee for the Protection of Human Subjects.

Preparation of the immunoglobulin fraction

We isolated the IgG fraction from human or mouse sera as previously described[20] with GammaBind G Sepharose (Amersham Biosciences).

Construction and expression of glutathione-*S*-transferase–27–amino acid fusion protein encoding the second extracellular loop of the angiotensin II receptor

We used a pGEX-4T-1 GST expression vector (Promega) expressing GST and an in-frame DNA insertion encoding the 27 amino acids corresponding to the second extracellular loop of human AT_1 receptor (accession code: NM_009585.2). Specifically, we mixed equal amounts of two complementary oligonucleotides corresponding to the second extracellular loop of human AT_1 receptor flanked by *Eco*RI restriction enzyme sites, boiled them for 5 min and then cooled to 23 °C. We cut the annealed double-stranded DNA with *Eco*RI and subcloned it into the *Eco*RI polycloning site of the pGEX-4T-1 vector. We confirmed the correct sequence by DNA sequencing. We performed the expression and purification of the GST–27-aa fusion protein following the protocol provided by the vendor (Amersham Bioscience). The purified GST-peptide fusion proteins were either immediately used or stored at −80 °C.

Affinity purification

We performed affinity purification of AT_1-AAs with the MicroLink Protein Coupling Kit (PIERCE). Briefly, we coupled the GST-peptide fusion protein (200 μg) purified as above to an AminoLink Plus Coupling Gel column (PIERCE) for 4 h or overnight at 4 °C according to the protocol provided by the vendor. We loaded IgG fractions (400 μl) that had been purified by GammaBind G Sepharose chromatography from sera of donors with pre-eclampsia or normotensive donors on the columns and incubated for 3 h. We collected the flow-through by centrifugation at 1,000*g* for 1 min. We eluted bound IgG by centrifugation at 1,000*g* for 1 min after incubation with 100 μl 100 mM glycine (pH 2.7) for 10 min. We neutralized the low pH of the eluted fraction by adding 5 μl of 1 M Tris, pH 9.0. We performed all steps at 4 °C. The elution fraction (affinity-purified AT_1-AAs) was either used immediately or stored at −80 °C.

Synthetic seven–amino acid epitope peptide

For the neutralization experiments, we used a synthetic peptide corresponding to a sequence on the second extracellular loop of the human AT_1 receptor, AFHYESQ. We purchased this peptide from Baylor College of Medicine.

Introduction of antibody into mice

We used C57BL/6J pregnant or non-pregnant mice (18–22 g; Harlan) in our study. All animal studies were reviewed and approved by the Animal Welfare Committee, University of Texas Houston Health Science Center. Mice were anesthetized with sodium pentobarbital (50 mg kg^{-1} intraperitoneally). We concentrated total IgG (~800 μg) or affinity-purified AT1-AAs (~20 μg) from 200 μl donor sera by lyophilization and resuspended them with ~200 μl saline and then introduced them into either 13-d pregnant mice or nonpregnant mice

by retro-orbital injection. Some mice also received losartan (0.24 mg) or a seven–amino acid peptide corresponding to an epitope on the second extracellular loop of the AT_1 receptor (1.5 mg). We collected plasma on gestation days 13 through 18 for measurement of sFlt1 concentration. We measured mouse systolic blood pressure by tail-cuff (AD Instruments) at each of these time points[17,40]. We collected mouse urine at gestation day 18 at the time the dams were killed. We quantified urinary albumin by ELISA (Exocell) and measured urinary creatinine by a picric acid colorimetric assay kit (Exocell). We used the ratio of urinary albumin to urinary creatinine as an index of urinary protein[24].

Western blot analysis

We analyzed human IgG in mouse circulation by western blotting as previously described[20]. Briefly, we ran mouse plasma on 10% SDS-PAGE gels and transferred the gels to a nitrocellulose membrane. We probed the membrane with horseradish peroxidase–conjugated mouse antibody to human IgG (1:5,000 dilution, Jackson ImmunoResearch Laboratories). We detected the signal with an ECL Kit (Amersham Bioscience). In addition, we evaluated affinity-purified AT_1-AA binding to the AT_1 receptor by western blotting analysis. Specifically, we ran cellular extracts from the 4× NFAT *cis* element–driven luciferase reporter cells (prepared as described below) on 10% SDS-PAGE gels and transferred the gels to a nitrocellulose membrane. We then incubated membranes with either commercially available antibody to the AT_1 receptor (1:1,000 dilution, Santa Cruz), flow-through fraction antibody (1:100 dilution) or the elution fraction antibody (1:100 dilution) as the primary antibody. We used horseradish peroxidase–conjugated antibody to rabbit IgG to detect the commercial antibody to AT_1 receptor or antibody to human IgG for the flow-through or elution fraction antibodies as the secondary antibody. We detected the signal with an ECL Kit (Amersham Bioscience).

Enzyme-linked immunosorbent assays

We determined the concentrations of sFlt1 and sEng in mouse plasma with commercial kits (R&D Systems). We evaluated the concentration of human IgG in mouse plasma by collecting mouse plasma at various time points as indicated in Figure 1b, diluting the plasma 300-fold and quantifying human IgG abundance with a commercial ELISA kit (PIERCE).

Histological analysis

We harvested placentas and kidneys of mice and fixed them in 4% formaldehyde overnight at 23 °C. Tissues were infiltrated and embedded in paraffin. We cut 4-μm serial sections and stained them with H&E by standard techniques[24,40].

Transmission electron microscopy

After killing the mice, we isolated kidneys[24]. We fixed tissue samples (1-mm^3 in size) in 3% glutaraldehyde overnight. We then rinsed the samples in buffer, exposed them to 1% osmium tetroxide, dehydrated them and embedded them in araldite-epon mixture. We prepared semi-thin tissue sections (0.6-μm) and stained them with uranyl acetate and lead citrate and then examined them with a JEOL 1210 transmission electron microscope (JEOL Corporation).

Luciferase activity assay

We measured the biological activity of affinity-purified IgG (AT_1-AAs) and IgG isolated from donor sera or mouse plasma (100 μl) with GammaBind G Sepharose as previously described[21]. Briefly, we plated Chinese hamster ovary cells (1×10^5 cells) containing stably integrated copies of a AT_1 receptor minigene and a 4× NFAT-driven luciferase minigene on 24-well plates and grew them overnight. The next day, we changed the cells to serum-free

medium and treated them with IgG (1:10 dilution) or affinity-purified AT_1-AAs (1 μg) for 24 h. We measured luciferase activity in cell lysates with the luciferase assay kit (Promega)[21].

Statistical analyses

All values are expressed as the mean ± s.e.m. We analyzed data for statistical significance with GraphPad Prism software (Graphpad). A value of $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Merck for the gift of losartan. This work was supported by US National Institutes of Health grants HL076558 (to Y.X.) and HD34130, March of Dimes grant 6-FY06-323 and the Texas Higher Education Coordinating Board (to R.E.K.).

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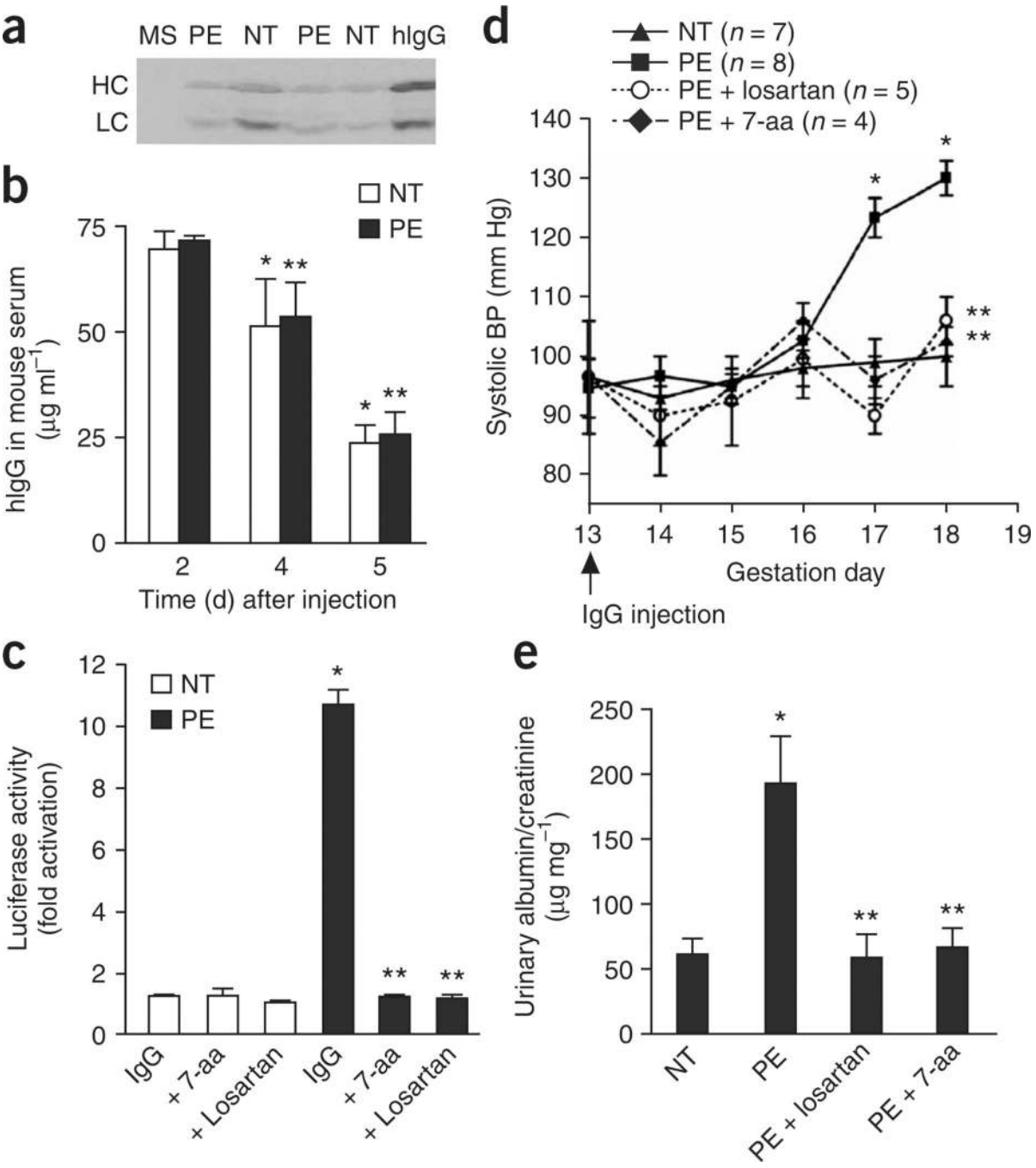

Figure 1.

Injection of IgG from women with pre-eclampsia into pregnant mice leads to hypertension and proteinuria. IgG (~800 μg) from normotensive (NT) or pre-eclamptic (PE) pregnant women was introduced into pregnant mice at gestation day 13. (**a**) Western blot analysis of human IgG (hIgG) abundance in maternal circulation 5 d after injection. HC, IgG heavy chain; LC, IgG light chain; MS, mouse serum without injection. (**b**) Human IgG detection by ELISA. *$P < 0.05$ versus day 2 normotensive IgG; **$P < 0.05$ versus day 2 pre-eclampsia IgG. (**c**) Luciferase activity reflecting activation of AT_1 receptors by IgG (isolated on gestation day 18 from antibody-injected mice) in the presence or absence of losartan or a seven–amino acid epitope peptide (7-aa) in an NFAT-luciferase reporter cell line. Data are

expressed as means ± s.e.m. ($n = 3$). *$P < 0.01$ versus normotensive IgG treatment; **$P < 0.05$ versus pre-eclampsia IgG treatment. (**d**) Systolic blood pressure (BP), as measured at daily intervals after IgG injection in the presence or absence of losartan or 7-aa. (**e**) Ratio of urinary albumin to creatinine in urine collected on gestation day 18 when the dams were killed. Data represent the average values from eight of fourteen mice injected with pre-eclamptic IgG that showed a significant increase in blood pressure and urinary protein. Data are expressed as means ± s.e.m. *$P < 0.01$ versus the normotensive IgG. **$P < 0.05$ versus pregnant mice injected with pre-eclampsia IgG alone.

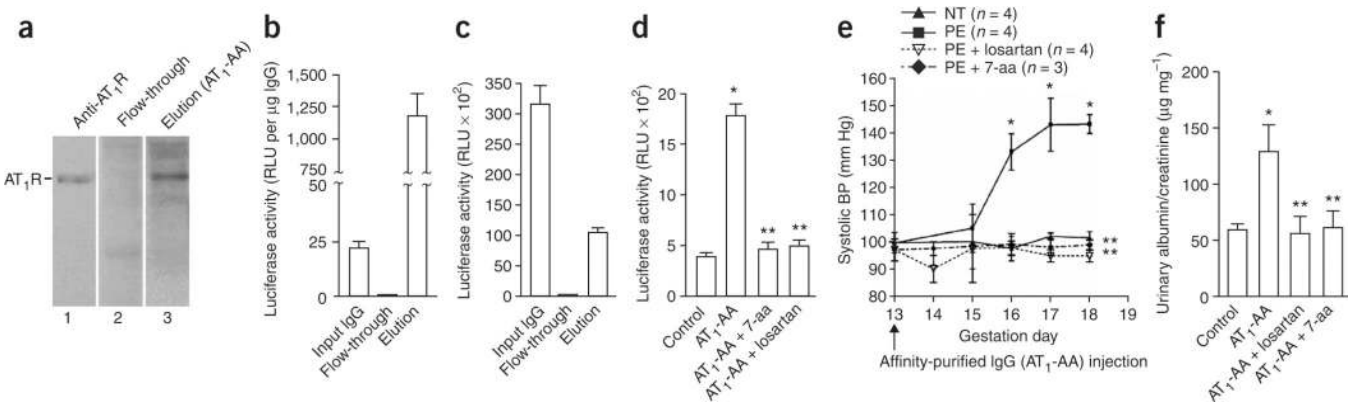

Figure 2.

Affinity-purified AT_1-AAs induced an increase in blood pressure and proteinuria in pregnant mice. (**a**) Western blot showing affinity-purified AT_1-AA detection of AT_1 receptor protein (AT_1R). Cell lysates from AT_1 receptor–positive reporter cells were analyzed with commercial antibody to the AT_1 receptor (lane 1), the flow-through fraction of IgG that did not bind the GST-peptide affinity matrix (lane 2) and the eluted fraction containing affinity-purified AT_1-AAs (lane 3). (**b**) The biological activity of AT_1-AAs in various fractions as measured by the luciferase reporter cell assay. RLU, relative light units. (**c**) The total recovered biological activity of AT_1-AAs in the affinity-purified fractions as analyzed by luciferase reporter cell assay. (**d**) Luciferase activity of cell lysates of AT_1-luciferase reporter cells incubated with or without affinity-purified AT_1-AAs in the presence or absence of losartan or the seven-amino acid epitope peptide (7-aa) for 24 h. (**e**) Systolic blood pressure in pregnant mice after injection of AT_1-AAs (~20 µg) on gestation day 13 in the presence or absence of losartan or 7-aa. IgG from normotensive pregnant women was used as a control. (**f**) The ratio of urinary albumin and creatinine on gestation day 18. Data represent the average results from four of five AT_1-AA–injected mice that showed a significant increase in blood pressure and urinary protein. Data are expressed as means ± s.e.m. $^*P < 0.05$ versus affinity-purified normotensive IgG control. $^{**}P < 0.05$ versus pregnant mice injected with pre-eclampsia IgG alone.

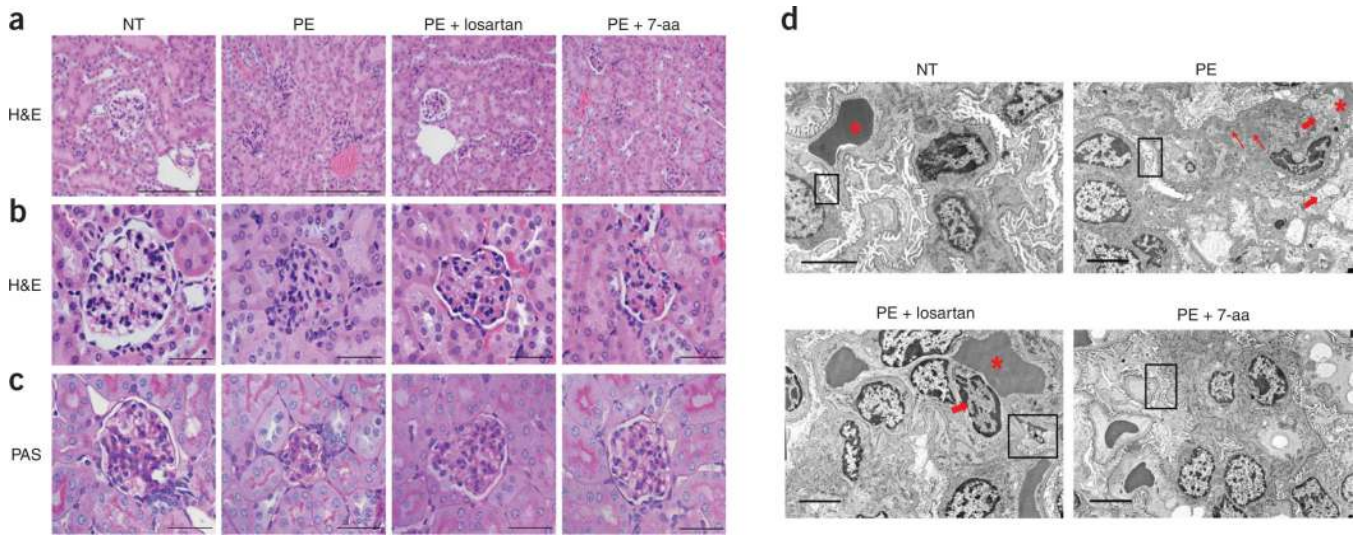

Figure 3.

Kidney damage is induced in pregnant mice by injection of IgG from women with pre-eclampsia. Kidneys were isolated from pregnant mice 5 d after IgG injection and prepared for routine histological analysis. (**a,b**) H&E staining of kidneys from pregnant mice injected with IgG from normotensive women, or IgG from women with pre-eclampsia in the presence or absence of losartan or the seven-amino acid epitope peptide (7-aa). Kidneys treated with pre-eclampsia IgG have normal to smaller sized glomeruli with a consolidated appearance and extensive endothelial swelling, narrowing and occlusion of capillary lumens compared to those from mice injected with IgG from normotensive pregnant women. Scale bars, 200 μm (**a**) and 50 μm (**b**). (**c**) Periodic acid–Schiff (PAS)-stained sections from mice injected with IgG from normotensive women or with IgG from women with pre-eclampsia in the presence or absence of losartan or 7-aa. Kidneys treated with pre-eclampsia IgG have swollen endothelial cells without PAS-positive materials in the cytoplasm. Scale bar, 50 μm. (**d**) Electron microscopic examination of kidney sections from mice injected with IgG from normotensive women or with IgG from women with pre-eclampsia in the presence or absence of losartan or 7-aa. Thick arrow shows endothelial cell swelling causing marked narrowing or total occlusion of the capillary loop spaces (*). Thin arrows indicate focal subendothelial deposits. Boxes highlight the state of the fenestrations of the endothelial cells and the podocyte foot processes in the various kidney samples.

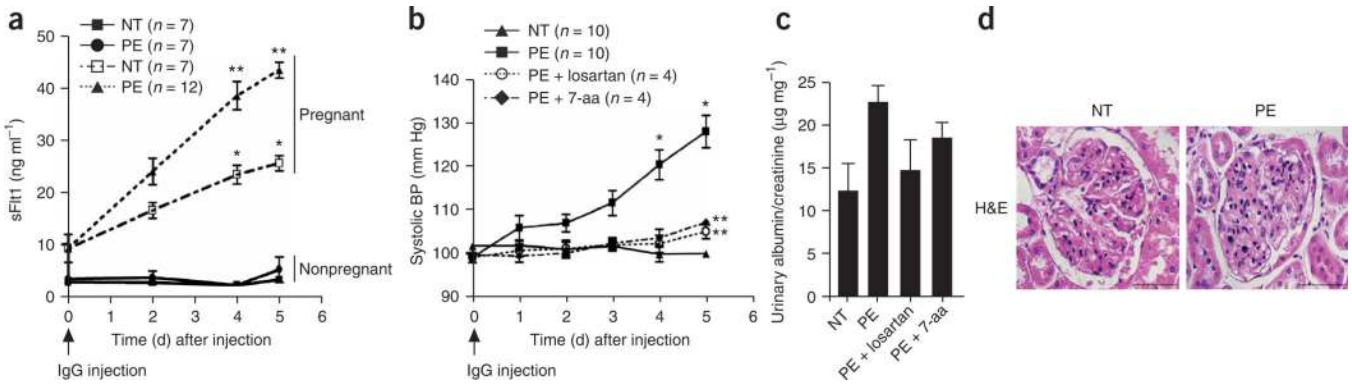

Figure 4.

Effects of injection of IgG from women with pre-eclampsia into nonpregnant mice. (**a**) sFlt1 abundance in nonpregnant and pregnant mice injected on gestation day 13 with IgG from normotensive donors or pre-eclamptic donors, as determined by ELISA. The pregnant mice were used as positive controls. *$P < 0.01$ versus nonpregnant mice injected with normotensive IgG. **$P < 0.05$ versus pregnant mice injected with normotensive IgG. (**b**) Systolic blood pressure of nonpregnant female mice injected with IgG from normotensive pregnant women or IgG from pregnant women with pre-eclampsia in the presence or absence of losartan or the seven-amino acid epitope peptide (7-aa). Data are the average results from 10 of 11 nonpregnant mice injected with pre-eclamptic IgG that showed a significant increase in blood pressure. Data are expressed as means ± s.e.m. *$P < 0.05$ versus nonpregnant mice injected with normotensive IgG. **$P < 0.05$ versus nonpregnant mice injected with pre-eclamptic IgG. (**c**) Urinary protein, as determined by the ratio of albumin to creatinine at day 6 after injection, in nonpregnant female mice injected with IgG from normotensive pregnant women or IgG from pregnant women with pre-eclampsia in the presence or absence of losartan or 7-aa. Data are expressed as means ± s.e.m. (**d**) H&E staining of kidneys from nonpregnant mice injected with normotensive IgG or IgG from women with pre-eclampsia showing no substantial renal histopathological changes. Scale bar, 50 µm.