Published in final edited form as:
Hypertension. 2010 May ; 55(5): 1246–1253. doi:10.1161/HYPERTENSIONAHA.110.150540.

Autoantibody-mediated angiotensin receptor activation contributes to preeclampsia through TNF-alpha signaling

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Abstract

Preeclampsia is a prevalent life-threatening hypertensive disorder of pregnancy whose pathophysiology remains largely undefined. Recently, a circulating maternal autoantibody, the angiotensin II type I receptor agonistic autoantibody (AT_1-AA), has emerged as a contributor to disease features. Increased circulating maternal tumor necrosis factor alpha (TNF-α) is also associated with the disease, however it is unknown if this factor directly contributes to preeclamptic symptoms. Here we report that this autoantibody increases the pro-inflammatory cytokine TNF-α in the circulation of AT_1-AA-injected pregnant mice, but not in non-pregnant mice. Co-injection of AT_1-AA with a TNF-α neutralizing antibody reduced cytokine availability in AT_1-AA-injected pregnant mice. Moreover, TNF-α blockade in AT_1-AA-injected pregnant mice significantly attenuated the key features of preeclampsia. Autoantibody-induced hypertension was reduced from 131±4 to 110±4 mmHg and proteinuria was reduced from 212±25 to 155±23 μg albumin/mg creatinine (both $P<0.05$). Injection of PE-IgG increased the serum levels of circulating soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) (34.1±5.1, 2.4±0.3 ng/ml, respectively), and co-injection with the TNF-α blocker significantly reduced their levels (21.7±3.4, 1.2±0.4 ng/ml, respectively). Renal damage and placental abnormalities were also decreased by TNF-alpha blockade. Lastly, the elevated circulating TNF-α in preeclamptic patients is significantly correlated to the AT_1-AA bioactivity in our patient cohort. Similarly, the autoantibody, through AT_1 receptor mediated TNF-α induction, contributed to increased sFlt-1, sEng secretion and increased apoptosis in cultured human villous explants. Overall, AT_1-AA is a novel candidate that induces TNF-α, a cytokine which may play an important pathogenic role in preeclampsia. Keywords: Basic Science; Experimental models; Pre-eclampsia/pregnancy; Angiotensin receptors; Inflammation.

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Disclosures None.

Introduction

Preeclampsia (PE) is a disorder of pregnancy characterized by maternal hypertension and renal dysfunction. It affects ~7% of first pregnancies and is a leading cause of maternal and perinatal morbidity and mortality[1, 2]. Available strategies used to manage PE are poor and currently limited to the delivery of the baby and placenta. By understanding the molecular pathways involved in the development of PE, we can expand the therapeutic strategies used to treat this disease. Recent studies report that preeclamptic women possess angiotensin II type I receptor agonistic autoantibodies (AT_1-AA) that bind to and activate AT_1 receptors in multiple cellular systems[3-8]. AT_1-AA provoke biologic responses relevant to the pathophysiology of the disorder[9-13]. Exploring beyond these *in vitro* studies, we have recently demonstrated that the injection of pregnant mice with AT_1-AA recapitulates key preeclamptic symptoms: hypertension, proteinuria, renal and placental abnormalities, and the increase of the anti-angiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng)[14, 15]. These *in vivo* studies offered direct evidence of the pathophysiologic role of AT_1-AA in PE and provided an animal model to use as an investigative tool to analyze the underlying pathogenic mechanisms associated with the disorder.

For example, increased tumor necrosis factor-alpha (TNF-α) is associated with PE and has been speculated to contribute to the disease[16-20]. However, the factors which elevate this cytokine in PE are unknown and the exact contribution of TNF-α to disease features remains largely undefined. There is considerable evidence linking angiotensin II (ANG II) to the regulation of TNF-α. TNF-α can be increased via ANG II induced AT_1 receptor activation in endothelial cells[21] and can result in end-organ damage in both the heart[22] and kidney[23-25]. In addition, both Papp *et al.* and Wang *et al.* have reported that apoptosis by TNF-α requires functional AT_1 receptor activation by ANG II in target cells[26, 27]. Taken together, these and other reports suggest that AT_1 receptor signaling and the release of TNF-α are closely related. Therefore, in the setting of PE, excessive activation of the AT_1 receptor by the autoantibody may lead to deleterious increases in TNF-α, resulting in maternal symptoms. Here we investigate the contributory role of AT_1-AA-induced elevation of TNF-α in the pathogenesis of PE using a mouse model of the disease.

Materials and Methods

For an expanded Methods section, please refer to http://hyper.ahajournals.org.

Patients

Patients admitted to Memorial Hermann Hospital were identified by the Obstetrics faculty of the University of Texas Medical School at Houston. Preeclamptic patients (n=20) were diagnosed with severe disease based on the definition set by the National High Blood Pressure Education Program Working Group Report[28]. The criteria of inclusion, including no previous history of hypertension, are previously reported[14, 15, 29]. Control pregnant women were selected on the basis of having an uncomplicated, normotensive pregnancy with a normal term delivery (n=16). The research protocol was approved of by the Institutional Committee for the Protection of Human Subjects.

Human placental explant collection and culture

Human placentas were obtained from normotensive patients who underwent an elective term cesarean section at Memorial Hermann Hospital in Houston, Texas. The explant culture system was developed from Ahmad, *et al.*[30]. Upon delivery, the placentas were placed on ice and submerged in phenol red-free DMEM containing 0.2% bovine serum albumin and

1% antibiotics. Five to seven chorionic villous explant fragments were carefully dissected from the placenta and transferred to 24-well plates for an overnight equilibration period at 37°C and 5% CO_2. All initial processing occurred within 30 minutes of delivery. The next day, the explants were incubated with either saline, ANG II (100nM), IgG from normotensive women (NT-IgG; 1:10 dilution), NT-IgG +/− losartan (5μM) or 7-aa (1μM), AT_1-AA (PE-IgG), PE-IgG +/− losartan (5μM), 7-aa (1μM) or anti-TNF-α antibody (5μg/mL). After 24h, the collection media was siphoned and stored at -80°C and the villous explants were lysed or fixed overnight in 10% formalin for embedding in paraffin wax for further analysis.

Introduction of human antibody into pregnant mice & blood pressure measurement

Purified IgG were isolated from preeclamptic or normotensive patient sera (PE-IgG and NT-IgG, respectively) and their adoptive transfer into pregnant mice was carried out as previously published[14, 15, 31]. Briefly, pregnant C57Bl/6J mice (Harlan) were used. Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and concentrated IgG purified from 200μl patient serum was introduced into pregnant mice by retro-orbital sinus injection twice, on gestational day (GD) 13 and GD14 (PE, n=9; NT, n=9). For neutralization experiments, the autoantibody was simultaneously co-injected twice, with either losartan (8 mg/kg i.v.) (n=9) or the 7-aa epitope peptide (50 mg/kg i.v.; sequence AFHYESQ) (n=9). Some dams were co-injected with purified autoantibody and a polyclonal antibody against TNF-α (Abcam) (n=9). They received 0.6μg/g body weight intraperitoneal shots of antibody daily. This dosage was adapted from experiments previously described[32-34]. As a control, another group of mice was injected with the anti-TNF-α antibody in the same manner, but with no accompanying purified human IgG (n=9). The systolic blood pressure of all mice was measured at the same time daily (+/−1h) by a carotid catheter-calibrated tail-cuff system and the mice were kept warm using a warming pad (AD Instruments). Urine was collected for analysis using metabolic cages (Nalgene). All mice were sacrificed on GD18 prior to delivery when their serum and organs, including placentas, were collected. All animal protocols were reviewed and approved of by the institutional Animal Welfare Committee, University of Texas at Houston Health Science Center, Houston, TX.

ELISAs

The serum concentrations of TNF-α, sFlt-1 and sEng were determined quantitatively using commercial kits (R&D Systems). For the standard curve experiment either 0.0 (control), 0.5 or 5.0 μg/ml of anti-TNF-α (Abcam) was added to known concentrations of recombinant mouse TNF-α, and the mixtures were assessed by ELISA for its ability to detect either bound or free cytokine (R&D Systems). To determine if the ELISA kit used measured only free, unbound TNF-α, or if it was capable of detecting the TNF-α bound to the anti-TNF-α antibody, a standard curve for the cytokine was generated in the absence or presence of varying amounts of the TNF-α blocker (0.0, 0.5 and 5.0 ng/ml). The ELISA procedure was carried out according to the manufacturer's protocol and the optical density was determined at 450 nm. All assays were performed in duplicate and the TNF-α protein concentrations were derived from a standard curve generated from known amounts of the recombinant mouse protein.

Statistical analysis

All data were expressed as the mean ± SEM. Data were analyzed for statistical significance using GraphPad Prism 4 software (GraphPad Software). Student's *t* tests (paired or unpaired as appropriate) were applied in two-group analysis. Differences between the means of multiple groups were compared by the one-way analysis of variance (ANOVA), followed by post-hoc analysis. To determine a statistical correlation between AT_1-AA bioactivity and serum TNF-α, Spearman's rank correlation was applied and an "r" coefficient value was

calculated. A value of $P<0.05$ was the threshold to reject the null hypothesis and was considered statistically significant.

Results

Circulating TNF-α is increased by AT_1 receptor activation in autoantibody-injected pregnant mice but not in non-pregnant mice

To determine the role of AT_1-AA in TNF-α increase in PE, we injected NT-IgG or PE-IgG into pregnant mice at GD13 and GD14 as previously described[14, 15]. Upon sacrifice on GD18, the sera of antibody-injected pregnant mice were used to quantify TNF-α using a sensitive ELISA (Fig. 1). IgG isolated from preeclamptic women (PE-IgG) increased serum TNF-α in pregnant mice, as compared to that derived from normotensive pregnant women (NT-IgG) (24.1±2.6 and 12.1±1.7 pg/ml, respectively; PE, n=9; NT, n=9). When PE-IgG was co-injected into pregnant mice with losartan, an AT_1 receptor blocker, or 7-aa, an autoantibody-neutralizing epitope peptide, the autoantibody-mediated induction of TNF-α was specifically inhibited. These results indicate that AT_1-AA, by activating the AT_1 receptor, could be responsible for the upregulation of TNF-α in pregnant mice.

To determine if TNF-α induction by AT_1-AA *in vivo* is dependent upon pregnancy, we injected NT-IgG or PE-IgG into non-pregnant mice. PE-IgG injected non-pregnant mice had lower levels of TNF-α than PE-IgG injected pregnant mice (11.3±2.4 and 24.1±2.6 pg/ml, respectively), and the level of TNF-α was not significantly higher in non-pregnant mice injected with either PE-IgG or NT-IgG (11.3±2.4 and 9.4±3.2 pg/ml, respectively). Thus, AT_1-AA-mediated TNF-α induction is pregnancy-dependent.

Hypertension and proteinuria are reduced in AT_1-AA-injected pregnant mice through TNF-α blockade

To elucidate the role of TNF-α in the pathogenesis of PE, we co-injected pregnant mice with PE-IgG and a TNF-α neutralizing antibody (n=9). We quantitatively confirmed that the TNF-α neutralizing antibody attenuated the induction of the cytokine in the serum of PE-IgG injected pregnant mice (Fig. 1). Furthermore, to determine if the ELISA kit used measured only free, unbound TNF-α, or if it was capable of detecting the TNF-α bound to the anti-TNF-α antibody, a standard curve for the cytokine was generated in the absence or presence of varying amounts of the TNF-α blocker (0.0, 0.5 and 5.0 ng/ml) (Fig. S1). The resultant curves showed no statistically significant differences. This finding suggests that any reductions of TNF-α observed using this ELISA are physiologic, and not due to interference of the neutralizing antibody.

In addition, the key diagnostic features of PE, hypertension and proteinuria, were both partially attenuated by TNF-α blockade in comparison to pregnant mice injected with the autoantibody alone (Figs. 2A-B). By GD18, neutralization of TNF-α in AT_1-AA-injected pregnant mice reduced their hypertension from 131±4 to 110±4 mmHg and urinary protein 212±25 to 155±23 μg albumin/mg creatinine ($P<0.05$). Pregnant mice injected with NT-IgG retained their baseline blood pressure and renal function. Histological analysis by H&E and TEM of mouse kidneys revealed that TNF-α blockade prevented autoantibody-mediated renal damage (Fig. S2A-B). The glomeruli of mice injected with NT-IgG did not display any renal morphologic changes. These findings provide evidence for the role of AT_1-AA-induced TNF-α in the key maternal features of PE seen in autoantibody-injected pregnant mice. Finally, injection of PE-IgG increased the serum levels of sFlt-1 and sEng (34.1±5.1, 2.4±0.3 ng/ml, respectively), and co-injection with an anti-TNF-α antibody significantly reduced the levels of sFlt-1 and sEng (21.7±3.4, 1.2±0.4 ng/ml, respectively) (Figs. 2C-D).

Overall, these findings provide animal evidence of the contributory role of AT_1-AA-induced TNF-α in PE.

AT_1-AA-induced placental abnormalities are reduced by TNF-α blockade in pregnant mice

In addition to abnormal kidneys, H&E staining (Fig. 3A) demonstrated that the labyrinth zones of the placentas of PE-IgG injected mice had placental calcifications, a hallmark of placental distress, and centers of fibrinoid necrosis similar to that of acute atherosis, a feature observed in human placentas from women with preeclampsia[35, 36]. The placentas of mice injected with NT-IgG had undamaged placentas free from calcifications and fibrinous centers. Co-injection of pregnant mice with PE-IgG and an anti-TNF-α antibody reduced the histopathologic changes observed in the placentas of PE-IgG injected animals. Placental weights of PE-IgG injected pregnant mice were smaller (0.09±0.02g) than placentas from NT-IgG injected mice (0.11±0.02g) (P<0.05). Co-injection of an anti-TNF-α antibody restored the autoantibody-induced placental weight reductions to 0.10±0.04g. In addition, the weight of fetuses born in litters of 6-8 pups was analyzed. Autoantibody-injected mice bore fetuses of less weight (1.06±0.19g) as compared to dams injected with NT-IgG (1.24±0.06g) (P<0.05). Co-injecting AT_1-AA with a TNF-α blocker restored fetal size to 1.11±0.43g. As compared to the NT-IgG-injected animals, injection of the anti-TNF-α antibody alone had no statistically significant effect on placental or fetal weight (0.16±0.05g and 1.27±0.10g, respectively). Fetal and placental pairs: PE, n=46; NT, n=53; PE+Anti-TNF-α, n=37; Anti-TNF-α alone, n=34. Overall, the autoantibody induced reductions in placental and fetal weights were restored by co-injection of a TNF-α blocker, implying an important role for this cytokine in the regulation of these effects.

Finally, we demonstrated that programmed cell death was increased in the labyrinth zone of placentas from mice injected with AT_1-AA as seen by quantified TUNEL staining (Figs. 3B-C). This was further confirmed by western blot analysis of Bax and Bcl-2, two apoptotic regulatory proteins (Fig. S3A-B). The degree of apoptosis was reduced in the placentas of mice co-injected with PE-IgG and the anti-TNF-α antibody. Mice injected with NT-IgG did not show increased apoptosis. This evidence confirms the fact that AT_1 receptor activation can increase mouse placental damage and TNF-α blockade can reduce these detrimental effects.

Serum TNF-α level correlates to AT_1-AA bioactivity in preeclamptic women

To determine if a relationship exists between AT_1-AA and TNF-α, we compared the serum level of TNF-α with AT_1–AA bioactivity of NT pregnant women (n=16) and women with PE (n=20). First, we confirmed that serum TNF-α was elevated in our preeclamptic cohort (Fig. S4). Next, the bioactivity level of AT_1-AA in these two patient groups was determined by an established luciferase reporter gene system[14]. The preeclamptic patients showed increased AT_1-AA-induced bioactivity as compared to their NT counterparts (5.17±1.07, n=20, and 0.14±0.04, n=16, fold induction, respectively, P<0.001) (Table S1). Intriguingly, the level of AT_1-AA bioactivity significantly correlated to serum TNF-α level when we analyzed the preeclamptic patients (Fig. 4, r=0.85, n=20, P<0.001). These data confirm earlier reports that preeclamptic patients harbor AT_1-AA and show for the first time that autoantibody bioactivity is correlated to serum TNF-α level in preeclamptic women.

AT_1 receptor-mediated TNF-α induction contributes to placental damage and sFlt-1 and sEng secretion in human villous explants

No elevation of the cytokine was observed in non-pregnant animals injected with the autoantibody, therefore the placenta may contribute to the production of autoantibody-induced TNF-α. As such, we took advantage of human placental villous explants to assess the direct role of AT_1-AA in TNF-α production in humans. Placental explants incubated

with PE-IgG showed an increase in secreted TNF-α, whereas the cytokine was not induced in explants incubated with NT-IgG (913.1±62.3 and 250.6±21.6 pg/ml, respectively, $P<0.05$) (Fig. 5A). AT_1 receptor activation was required for TNF-α secretion, as co-incubation of PE-IgG with either losartan or a 7-aa attenuated the induction of TNF-α levels (214.4±24.1 and 506.4±163.8 pg/ml, respectively, $P<0.05$ versus PE-IgG). Thus, the autoantibody is capable of inducing TNF-α secretion via AT_1 receptor activation by human placental villous explants.

Then, human placental explants and the explant culture medium were examined for pathological changes associated with PE. Explants exposed to PE-IgG demonstrated increased placental apoptosis, as determined by a TUNEL assay and index, which was blocked by the presence of a TNF-α blocking antibody (Fig. 5B-C). Placental fragments incubated with NT-IgG did not show significant apoptosis. This evidence was corroborated with western blot analysis (Fig. S5A-B). In addition, autoantibody-mediated increases in sFlt-1 and sEng by human placental explants were reduced by TNF-α blockade (Figs. 5D-E). These findings are consistent with those observed in the mouse model and suggest that AT_1-AA-induced TNF-α mediates placental damage.

Discussion

In this study, we have identified for the first time that an elevated TNF-α level is correlated to AT_1-AA bioactivity in preeclamptic women and provided both *in vitro* human studies and *in vivo* mouse evidence that AT_1-AA is a novel candidate directly inducing TNF-α production via AT_1 receptor activation. Neutralizing AT_1-AA-mediated TNF-α induction attenuates the increased placenta apoptosis and sFlt-1 and sEng secretion by cultured human villous explants. Moreover, TNF-α blockade ameliorates the key features associated with PE seen in autoantibody-injected pregnant mice *in vivo*. Both the mouse and human studies reported here provide strong evidence that AT_1 receptor activation by the autoantibody induces TNF-α and that increased TNF-α production may be an underlying mechanism contributing to the pathophysiology of the disease.

While TNF-α is reportedly increased in the circulation of preeclamptic women[37-39], the exact cause of increased cytokine production is unknown, as is its pathogenic role. Multiple *in vitro* studies demonstrate that increased inflammatory cytokine production may lead to endothelial dysfunction, increased placenta apoptosis, decreased angiogenesis and kidney abnormalities that are relevant to the pathophysiology of PE[40-42]. There are few animal models of PE available and none of them have delineated the cause of increased TNF-α and its pathogenic role. Here, using a novel autoantibody-induced model of PE in pregnant mice, we demonstrate that autoantibody-mediated AT_1 receptor activation induces TNF-α, and that its production through this mechanism is pregnancy-dependent. Since IgG purified from normotensive pregnant women did not elicit the same increase, the effect can be attributed to the autoantibody itself and not a non-specific immunologic response.

Next, we found that TNF-α blockade attenuates AT_1-AA-induced preeclamptic features in autoantibody-injected pregnant mice, including hypertension and proteinuria. This finding indicates that anti-TNF-α antibody treatment decreases cytokine induction in autoantibody-injected pregnant mice. We believe that without interference, TNF-α-induced cell damage and inflammation create a detrimental cycle, facilitating further cell damage and inflammation. However, in the presence of an anti-TNF-α antibody which neutralizes TNF-α effects, this damage is decreased, slowing the malicious cycle. Thus, we have revealed that AT_1-AA is a key mediator in inducing the increased TNF-α in PE and blockade of this cytokine can attenuate disease features. In fact, similar to the effects of anti-TNF-α treatment in our AT_1-AA-injected pregnant mice, a soluble TNF-α receptor also attenuates

preeclamptic-like features seen in pregnant rats generated by reduced uterine placental perfusion (RUPP)[43]. Thus, both of these animal studies provide strong preclinical evidence to support the novel therapeutic possibility of targeting this deleterious cytokine associated with PE.

It is well-established that ANG II can act through the AT_1 receptor to increase TNF-α[21-27]. In this way, the autoantibody may regulate the secretion of TNF-α resulting in maternal symptoms. Although this potential role of TNF-α in preeclamptic hypertension and proteinuria has been suggested, the pathogenic mechanisms underlying its effects are not clearly identified. Earlier studies have shown that the pro-inflammatory TNF-α is associated with both vascular damage and hypertension[44]. Jovinge *et al.* have shown that TNF-α-deficient mice have reduced atherosclerotic lesions, suggesting that the cytokine plays a key role in vascular injury[45]. Similarly, in salt-sensitive rats, TNF-α blockade has been successful in alleviating both the hypertension and renal damage observed in this model[46]. In pregnant rats, TNF-α enhances contraction and inhibits endothelial nitric oxide-cGMP-mediated relaxation in systemic vessels, which could contribute to hypertension[47]. Chronic infusion of TNF-α into pregnant rats to achieve two-fold increase in concentration is sufficient to induce hypertension and increase endothelin-1 production, which the authors believe contributes to the vascular damage associated with the maternal symptoms of PE[48]. These examples illustrate that the inflammatory properties of TNF-α contribute to vascular damage and high blood pressure, which could therefore do the same in PE. In addition, Muller *et al.* report a double transgenic rat model with increased levels of circulating ANG II which exhibits hypertension, renal dysfunction as well as increased TNF-α[49]. In this model, the authors believe that increased TNF-α contributes to kidney injury via complement activation and that excess ANG II sensitizes the vasculature to the effects of the cytokine. The induction of TNF-α in the autoantibody-injection model of PE is accompanied with an autoantibody-mediated increases in sFlt-1[9, 14]. Others have also shown that sFlt-1 and sEng are induced by TNF-α[30, 50]. In conjunction with these studies, the results of the PE animal model reported here provide evidence to support the novel concept that autoantibody-mediated AT_1 receptor activation induces TNF-α production resulting in the maternal features of PE.

It should not be overlooked that AT_1-AA alone may contribute directly to certain features of PE which are independent of TNF-α. For example, the autoantibody can directly stimulate the AT_1 receptors of vascular smooth muscle cells and induce vasoconstriction[51-53]. Likewise, the autoantibody could activate AT_1 receptors on endothelial cells resulting in the synthesis of endothelin-1, a powerful vasoconstrictive agent[54, 55]. The autoantibody may also directly bind to AT_1 receptors on renal mesangial cells to induce PAI-1 secretion[13]. Therefore, it is not surprising that TNF-α blockade only partially relieves autoantibody-induced features of PE, including the partial attenuation of hypertension and proteinuria observed in the pregnant mice co-injected with the autoantibody and an anti-TNF-α antibody (Fig. 2). However, it is clear through the evidence presented here that reducing TNF-α significantly attenuates the key preeclamptic symptoms initiated by AT_1-AA in pregnant mice, indicating an important role for this cytokine which warrants further investigation.

Decreasing the amount TNF-α circulating in preeclamptic mice may both directly and indirectly alleviate many disease features. In the placenta, decreasing TNF-α production may directly reduce the amount of trophoblast apoptosis and result in a healthier organ. By limiting placental damage, reductions in TNF-α may decrease the release of key anti-angiogenic factors, sFlt-1 and sEng. With little increase in these factors, the subsequent maternal vascular and renal damage may be alleviated, thereby reducing maternal symptoms. As mentioned earlier, TNF-α is capable of inducing vascular injury through the

initiation of inflammatory cascades. Should this pathway not be instigated, then the endothelial damage associated with PE may not be as severe, and the symptoms may be lessened. Together, these scenarios indicate that TNF-α may be either directly or indirectly contributing to preeclamptic features and its blockade can reduce their severity.

Perspectives

Taken together, our studies identify AT_1-AA as a novel candidate contributing to the increased TNF-α production in PE. Both human and mouse studies demonstrate that this inflammatory cytokine plays an important role in the pathogenesis of this hypertensive condition. Of significant importance, neutralization of TNF-α reduces the maternal features of the disease, such as hypertension and proteinuria, in an adoptive transfer mouse model of PE. In addition, AT_1-AA-induced placental damage can be alleviated by preventing TNF-α action in human villous explants. These findings indicate a critical role of TNF-α in placental damage and symptom development. The work reported here could be the foundation for future studies leading to a new therapeutic strategy for PE, a life-threatening disorder of pregnancy for which the current treatment is extremely limited and the complications are especially dire.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge Dr. Edwina Popek for facilitating the TEM analysis with Texas Children's Hospital, and Dr. Carlos Carreno for aiding in the collection of human placental tissue at Memorial Herman Hospital, both in Houston, TX.

Sources of Funding Support for this work was provided by the National Institute of Health grants HL076558 and HD34130, March of Dimes (6-FY06-323) and Texas Higher Education Coordinating Board.

Abbreviations

ANG II	angiotensin II
AT_1-AA	angiotensin receptor agonistic autoantibody
NT	normotensive
PE	preeclampsia
sEng	soluble endoglin
sFlt-1	soluble fms-like tyrosine kinase-1
TNF-α	tumor necrosis factor alpha

References

1. Roberts JM, Cooper DW. Pathogenesis and genetics of pre-eclampsia. Lancet. 2001; 357:53–56. [PubMed: 11197372]
2. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science. 2005; 308:1592–1594. [PubMed: 15947178]
3. Wallukat G, Homuth V, Fischer T, Lindschau C, Horstkamp B, Jupner A, Baur E, Nissen E, Vetter K, Neichel D, Dudenhausen JW, Haller H, Luft FC. Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor. J Clin Invest. 1999; 103:945–952. [PubMed: 10194466]

4. Dechend R, Homuth V, Wallukat G, Kreuzer J, Park JK, Theuer J, Juepner A, Gulba DC, Mackman N, Haller H, Luft FC. AT(1) receptor agonistic antibodies from preeclamptic patients cause vascular cells to express tissue factor. Circulation. 2000; 101:2382–2387. [PubMed: 10821814]
5. Li X, Shams M, Zhu J, Khalig A, Wilkes M, Whittle M, Barnes N, Ahmed A. Cellular localization of AT1 receptor mRNA and protein in normal placenta and its reduced expression in intrauterine growth restriction. Angiotensin II stimulates the release of vasorelaxants. J Clin Invest. 1998; 101:442–454. [PubMed: 9435317]
6. Li C, Ansari R, Yu Z, Shah D. Definitive molecular evidence of renin-angiotensin system in human uterine decidual cells. Hypertension. 2000; 36:159–164. [PubMed: 10948071]
7. Shibata E, Powers RW, Rajakumar A, von Versen-Hoynck F, Gallaher MJ, Lykins DL, Roberts JM, Hubel CA. Angiotensin II decreases system A amino acid transporter activity in human placental villous fragments through AT1 receptor activation. Am J Physiol Endocrinol Metab. 2006; 291:E1009–1016. [PubMed: 16787961]
8. Matsusaka T, Ichikawa I. Biological functions of angiotensin and its receptors. Annu Rev Physiol. 1997; 59:395–412. [PubMed: 9074770]
9. Zhou CC, Ahmad S, Mi T, Xia L, Abbasi S, Hewett PW, Sun C, Ahmed A, Kellems RE, Xia Y. Angiotensin II induces soluble fms-Like tyrosine kinase-1 release via calcineurin signaling pathway in pregnancy. Circ Res. 2007; 100:88–95. [PubMed: 17158338]
10. Thway TM, Shlykov SG, Day MC, Sanborn BM, Gilstrap LC 3rd, Xia Y, Kellems RE. Antibodies from preeclamptic patients stimulate increased intracellular Ca2+ mobilization through angiotensin receptor activation. Circulation. 2004; 110:1612–1619. [PubMed: 15381659]
11. Xia Y, Wen H, Bobst S, Day MC, Kellems RE. Maternal autoantibodies from preeclamptic patients activate angiotensin receptors on human trophoblast cells. J Soc Gynecol Investig. 2003; 10:82–93.
12. Dechend R, Viedt C, Muller DN, Ugele B, Brandes RP, Wallukat G, Park JK, Janke J, Barta P, Theuer J, Fiebeler A, Homuth V, Dietz R, Haller H, Kreuzer J, Luft FC. AT1 receptor agonistic antibodies from preeclamptic patients stimulate NADPH oxidase. Circulation. 2003; 107:1632–1639. [PubMed: 12668498]
13. Bobst SM, Day MC, Gilstrap LC 3rd, Xia Y, Kellems RE. Maternal autoantibodies from preeclamptic patients activate angiotensin receptors on human mesangial cells and induce interleukin-6 and plasminogen activator inhibitor-1 secretion. Am J Hypertens. 2005; 18:330–336. [PubMed: 15797649]
14. Zhou CC, Zhang Y, Irani RA, Zhang H, Mi T, Popek EJ, Hicks MJ, Ramin SM, Kellems RE, Xia Y. Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice. Nat Med. 2008; 14:855–862. [PubMed: 18660815]
15. Irani RA, Zhang Y, Blackwell SC, Zhou CC, Ramin SM, Kellems RE, Xia Y. The detrimental role of angiotensin receptor agonistic autoantibodies in intrauterine growth restriction seen in preeclampsia. J Exp Med. 2009; 206:2809–2822. [PubMed: 19887397]
16. Conrad KP, Benyo DF. Placental cytokines and the pathogenesis of preeclampsia. Am J Reprod Immunol. 1997; 37:240–249. [PubMed: 9127646]
17. Conrad KP, Miles TM, Benyo DF. Circulating levels of immunoreactive cytokines in women with preeclampsia. Am J Reprod Immunol. 1998; 40:102–111. [PubMed: 9764352]
18. LaMarca BD, Ryan MJ, Gilbert JS, Murphy SR, Granger JP. Inflammatory cytokines in the pathophysiology of hypertension during preeclampsia. Curr Hypertens Rep. 2007; 9:480–485. [PubMed: 18367011]
19. Gulati R. Raised serum TNF-alpha, blood sugar and uric acid in preeclampsia in third trimester of pregnancy. Jnma, Journal of the Nepal Medical Association. 2005; 44:36–38. [PubMed: 16554868]
20. Bertani T, Abbate M, Zoja C, Corna D, Perico N, Ghezzi P, Remuzzi G. Tumor necrosis factor induces glomerular damage in the rabbit. Am J Pathol. 1989; 134:419–430. [PubMed: 2916653]
21. Arenas IA, Xu Y, Lopez-Jaramillo P, Davidge ST. Angiotensin II-induced MMP-2 release from endothelial cells is mediated by TNF-alpha. Am J Physiol Cell Physiol. 2004; 286:C779–784. [PubMed: 14644777]

22. Kalra D, Sivasubramanian N, Mann DL. Angiotensin II induces tumor necrosis factor biosynthesis in the adult mammalian heart through a protein kinase C-dependent pathway. Circulation. 2002; 105:2198–2205. [PubMed: 11994255]

23. Ruiz-Ortega M, Bustos C, Hernandez-Presa MA, Lorenzo O, Plaza JJ, Egido J. Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor-kappa B activation and monocyte chemoattractant protein-1 synthesis. J Immunol. 1998; 161:430–439. [PubMed: 9647253]

24. Ruiz-Ortega M, Ruperez M, Lorenzo O, Esteban V, Blanco J, Mezzano S, Egido J. Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney. Kidney Int Suppl. 2002:S12–22. [PubMed: 12410849]

25. Ferreri NR, Escalante BA, Zhao Y, An SJ, McGiff JC. Angiotensin II induces TNF production by the thick ascending limb: functional implications. Am J Physiol. 1998; 274:F148–155. [PubMed: 9458834]

26. Papp M, Li X, Zhuang J, Wang R, Uhal BD. Angiotensin receptor subtype AT(1) mediates alveolar epithelial cell apoptosis in response to ANG II. Am J Physiol Lung Cell Mol Physiol. 2002; 282:L713–718. [PubMed: 11880296]

27. Wang R, Zagariya A, Ibarra-Sunga O, Gidea C, Ang E, Deshmukh S, Chaudhary G, Baraboutis J, Filippatos G, Uhal BD. Angiotensin II induces apoptosis in human and rat alveolar epithelial cells. Am J Physiol. 1999; 276:L885–889. [PubMed: 10330045]

28. Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. Am J Obstet Gynecol. 2000; 183:S1–S22.

29. Siddiqui AH, Irani RA, Blackwell SC, Ramin SM, Kellems RE, Xia Y. Angiotensin receptor agonistic autoantibody is highly prevalent in preeclampsia: correlation with disease severity. Hypertension. 2010; 55:386–393. [PubMed: 19996068]

30. Ahmad S, Ahmed A. Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. Circ Res. 2004; 95:884–891. [PubMed: 15472115]

31. Siddiqui AH, Irani RA, Blackwell SC, Ramin SM, Kellems RE, Xia Y. Angiotensin Receptor Agonistic Autoantibody Is Highly Prevalent in Preeclampsia. Correlation With Disease Severity. Hypertension. 2009

32. Tsuji F, Oki K, Okahara A, Suhara H, Yamanouchi T, Sasano M, Mita S, Horiuchi M. Differential effects between marimastat, a TNF-alpha converting enzyme inhibitor, and anti-TNF-alpha antibody on murine models for sepsis and arthritis. Cytokine. 2002; 17:294–300. [PubMed: 12061836]

33. Franks AK, Kujawa KI, Yaffe LJ. Experimental elimination of tumor necrosis factor in low-dose endotoxin models has variable effects on survival. Infect Immun. 1991; 59:2609–2614. [PubMed: 1855980]

34. Scallon BJ, Moore MA, Trinh H, Knight DM, Ghrayeb J. Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. Cytokine. 1995; 7:251–259. [PubMed: 7640345]

35. Kraus, FT.; Redline, RW.; Gersell, DJ.; Nelson, DM.; Dicke, JM. Placental Pathology. American Registry of Pathology; Washington, DC: 2004.

36. Benirschke, K.; Kaufmann, P. Pathology of the Human Placenta. Fourth ed. Springer-Verlag; New York, NY: 2000.

37. Saito S, Shiozaki A, Nakashima A, Sakai M, Sasaki Y. The role of the immune system in preeclampsia. Mol Aspects Med. 2007; 28:192–209. [PubMed: 17433431]

38. Hamai Y, Fujii T, Yamashita T, Nishina H, Kozuma S, Mikami Y, Taketani Y. Evidence for an elevation in serum interleukin-2 and tumor necrosis factor-alpha levels before the clinical manifestations of preeclampsia. Am J Reprod Immunol. 1997; 38:89–93. [PubMed: 9272206]

39. Abbasi S, Lee JD, Su B, Chen X, Alcon JL, Yang J, Kellems RE, Xia Y. Protein kinase-mediated regulation of calcineurin through the phosphorylation of modulatory calcineurin-interacting protein 1. J Biol Chem. 2006; 281:7717–7726. [PubMed: 16415348]

40. Hayakawa S, Nagai N, Kanaeda T, Karasaki-Suzuki M, Ishii M, Chishima F, Satoh K. Interleukin-12 augments cytolytic activity of peripheral and decidual lymphocytes against

choriocarcinoma cell lines and primary culture human placental trophoblasts. Am J Reprod Immunol. 1999; 41:320–329. [PubMed: 10378027]

41. Yui J, Garcia-Lloret M, Wegmann TG, Guilbert LJ. Cytotoxicity of tumour necrosis factor-alpha and gamma-interferon against primary human placental trophoblasts. Placenta. 1994; 15:819–835. [PubMed: 7886023]
42. Sargent IL, Borzychowski AM, Redman CW. NK cells and pre-eclampsia. J Reprod Immunol. 2007; 76:40–44. [PubMed: 17482272]
43. LaMarca B, Speed J, Fournier L, Babcock SA, Berry H, Cockrell K, Granger JP. Hypertension in response to chronic reductions in uterine perfusion in pregnant rats: effect of tumor necrosis factor-alpha blockade. Hypertension. 2008; 52:1161–1167. [PubMed: 18981324]
44. Tang P, Hung MC, Klostergaard J. Human pro-tumor necrosis factor is a homotrimer. Biochemistry. 1996; 35:8216–8225. [PubMed: 8679576]
45. Jovinge S, Hamsten A, Tornvall P, Proudler A, Bavenholm P, Ericsson CG, Godsland I, de Faire U, Nilsson J. Evidence for a role of tumor necrosis factor alpha in disturbances of triglyceride and glucose metabolism predisposing to coronary heart disease. Metabolism. 1998; 47:113–118. [PubMed: 9440488]
46. Elmarakby AA, Quigley JE, Imig JD, Pollock JS, Pollock DM. TNF-alpha inhibition reduces renal injury in DOCA-salt hypertensive rats. Am J Physiol Regul Integr Comp Physiol. 2008; 294:R76–83. [PubMed: 17989143]
47. Giardina JB, Green GM, Cockrell KL, Granger JP, Khalil RA. TNF-alpha enhances contraction and inhibits endothelial NO-cGMP relaxation in systemic vessels of pregnant rats. Am J Physiol Regul Integr Comp Physiol. 2002; 283:R130–143. [PubMed: 12069938]
48. LaMarca BB, Cockrell K, Sullivan E, Bennett W, Granger JP. Role of endothelin in mediating tumor necrosis factor-induced hypertension in pregnant rats. Hypertension. 2005; 46:82–86. [PubMed: 15928030]
49. Muller DN, Shagdarsuren E, Park JK, Dechend R, Mervaala E, Hampich F, Fiebeler A, Ju X, Finckenberg P, Theuer J, Viedt C, Kreuzer J, Heidecke H, Haller H, Zenke M, Luft FC. Immunosuppressive treatment protects against angiotensin II-induced renal damage. Am J Pathol. 2002; 161:1679–1693. [PubMed: 12414515]
50. Cudmore M, Ahmad S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, Devey LR, Wigmore SJ, Abbas A, Hewett PW, Ahmed A. Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. Circulation. 2007; 115:1789–1797. [PubMed: 17389265]
51. Berk BC, Corson MA. Angiotensin II signal transduction in vascular smooth muscle: role of tyrosine kinases. Circ Res. 1997; 80:607–616. [PubMed: 9130441]
52. Stjernquist M, Bodelsson G, Poulsen H. Vasoactive peptides and uterine vessels. Gynecol Endocrinol. 1995; 9:165–176. [PubMed: 7502694]
53. Yang X, Wang F, Chang H, Zhang S, Yang L, Wang X, Cheng X, Zhang M, Ma XL, Liu H. Autoantibody against AT1 receptor from preeclamptic patients induces vasoconstriction through angiotensin receptor activation. J Hypertens. 2008; 26:1629–1635. [PubMed: 18622242]
54. Gilbert JS, Ryan MJ, LaMarca BB, Sedeek M, Murphy SR, Granger JP. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. Am J Physiol Heart Circ Physiol. 2008; 294:H541–550. [PubMed: 18055511]
55. Granger JP, Abram S, Stec D, Chandler D, LaMarca B. Endothelin, the kidney, and hypertension. Curr Hypertens Rep. 2006; 8:298–303. [PubMed: 16884660]

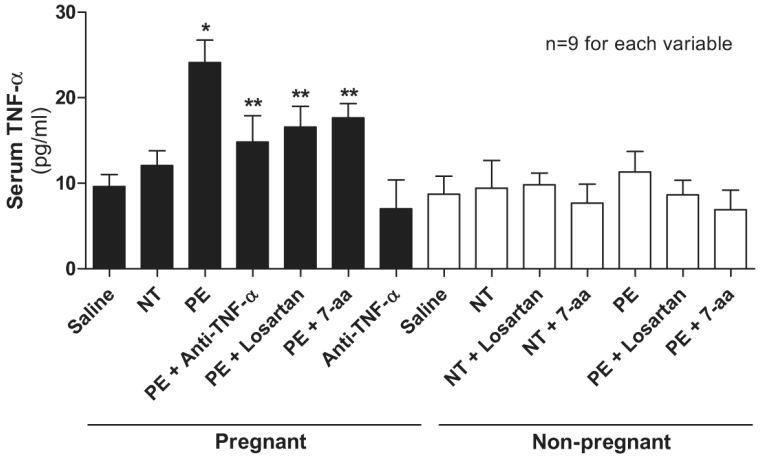

Figure 1. TNF-α is increased in AT_1-AA-injected pregnant mice

Serum TNF-α was elevated in PE-IgG injected pregnant mice but not in NT-IgG injected pregnant mice. Co-injection of losartan or 7-aa resulted in decreased serum TNF-α levels in PE-IgG injected pregnant mice. Non-pregnant animals injected with similarly purified human IgG fractions (white bars) did not demonstrate increased cytokine levels. n=9 for each variable. *P<0.05 versus NT-IgG. **P<0.05 versus PE-IgG.

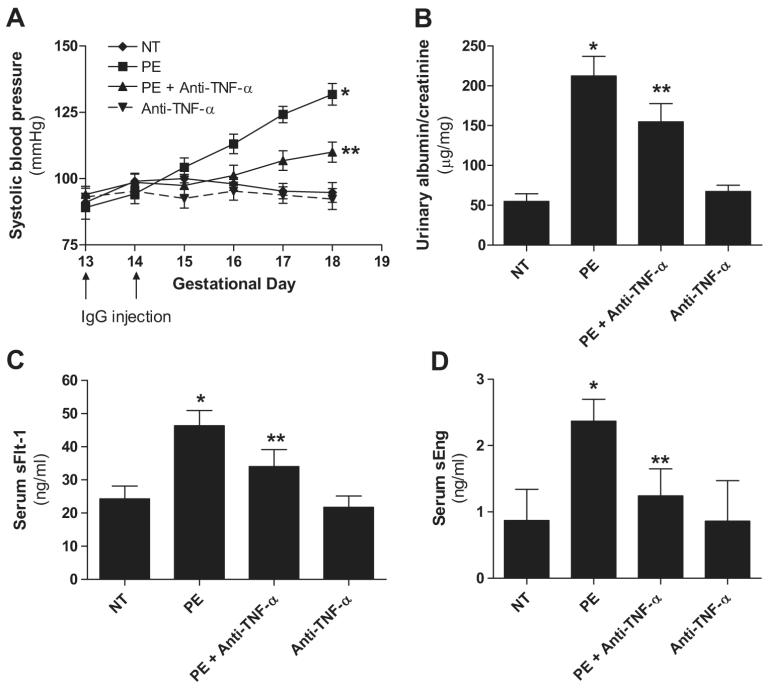

Figure 2. TNF-α blockade reduces AT_1-AA-induced preeclamptic-like features

The key features of PE, hypertension (A) and proteinuria (B) present in the PE-IgG-injected pregnant mice were reduced by co-injection with a TNF-α blocker. In addition, sFlt-1 (C) and sEng (D) were also reduced with the co-injection of the autoantibody and the TNF-α blocker. n=9 for each variable. **P*<0.05 versus NT-IgG. ***P*<0.05 versus PE-IgG.

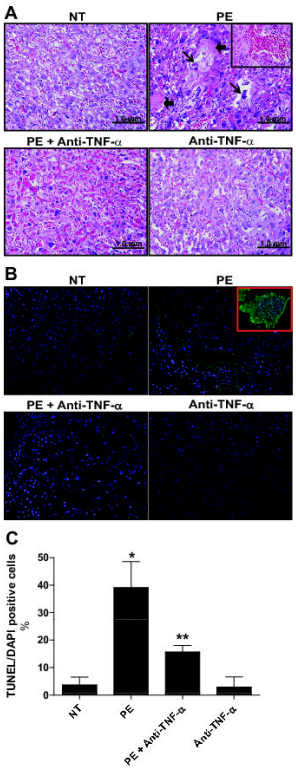

Figure 3. Autoantibody-induced placental damage can be prevented by TNF-α neutralization

Placentas assessed by H&E staining (Panel A, 40X) indicate that PE-IgG injected mice had damaged placentas: calcifications (thin arrow) and fibrotic areas (thick arrow). Their labyrinth zones appear heterogeneous and have abnormal pools of blood (inset box). Placental apoptosis was assessed by TUNEL staining (Panel B, 10X, scale bar=1 mm). PE-IgG injected mice had increased apoptosis in their labyrinth zones as compared to NT-IgG injected animals. Quantification of the TUNEL assay (C) indicates a reduction in the TUNEL-positive cells in mice co-injected with PE-IgG and a TNF-α blocker as compared to the PE-IgG injected animals. n=18 placentas per variable, from 9 different mice in each group. Green; TUNEL-positive cells. Blue; DAPI-positive nuclei. Mice injected with NT-IgG or the anti-TNF-α antibody alone had unremarkable placentas. *P<0.05 versus NT-IgG. **P<0.05 versus PE-IgG.

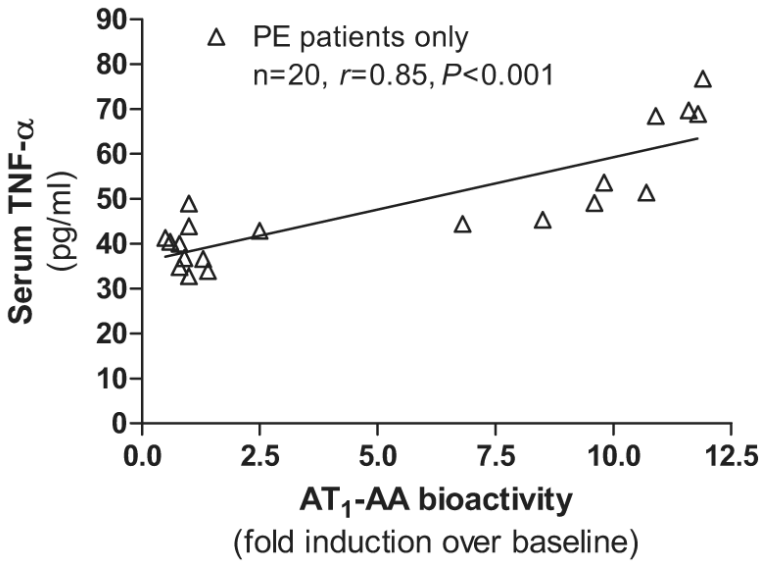

Figure 4. Serum TNF-α positively correlates to AT_1-AA bioactivity

A positive correlation between the level of AT_1-AA bioactivity and serum TNF-α level was identified in preeclamptic women. Spearman's rank correlation was used to determine an r-value (r=0.85, n=20, $P<0.001$).

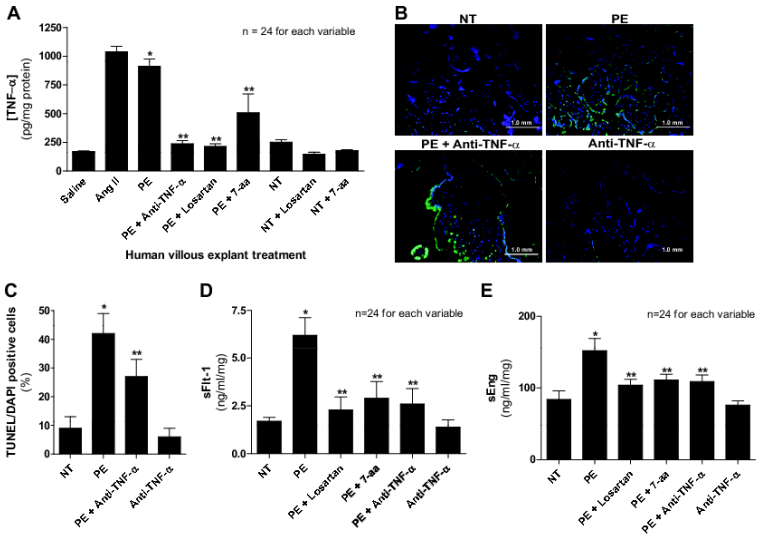

Figure 5. TNF-α blockade prevents AT_1 receptor-mediated damage in human placental villous explants

Culturing human villous explants with PE-IgG resulted in TNF-α secretion (A). Co-culturing the explants with PE-IgG and losartan (5μM) or 7-aa (1μM) reduced the cytokine level. Apoptosis was increased in explants incubated with AT_1-AA and was partially diminished by blocking TNF-α activity as demonstrated by a TUNEL assay (B). Green; TUNEL-positive cells. Blue; DAPI-positive nuclei. 10X. Quantification of TUNEL staining (C) indicates that co-incubation with PE-IgG and an anti-TNF-α agent (5μg/ml) reduces the amount of apoptosis. Secretion of sFlt-1 (D) and sEng (E) were reduced by co-incubation of the autoantibody with an anti-TNF-α antibody. Six different placentas were collected, and from each, n=4 for every variable, total n=24 per variable. **P*<0.05 versus NT-IgG. ***P*<0.05 versus PE-IgG.