AT$_1$ Receptor Agonistic Antibodies From Preeclamptic Patients Cause Vascular Cells to Express Tissue Factor

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Background—We recently described autoantibodies (angiotensin-1 receptor autoantibodies, AT$_1$-AA) directed at the AT$_1$ receptor in the serum of preeclamptic patients, whose placentas are commonly infarcted and express tissue factor (TF). Mechanisms of how AT$_1$-AA might contribute to preeclampsia are unknown. We tested the hypothesis that AT$_1$-AA cause vascular smooth muscle cells (VSMC) to express TF.

Methods and Results—IgG from preeclamptic patients containing AT$_1$-AA was purified with anti-human IgG columns. AT$_1$-AA were separated from the IgG by ammonium sulfate precipitation. We transfected Chinese hamster ovary cells overexpressing the AT$_1$ receptor with TF promoter constructs coupled to a luciferase reporter gene. VSMC were obtained from human coronary arteries. Extracellular signal-related kinase activation was detected by an in-gel kinase assay. AP-1 activation was determined by electromobility shift assay. TF was measured by ELISA and detected by immunohistochemistry. Placentas from preeclamptic women stained strongly for TF, whereas control placentas showed far less staining. We proved AT$_1$-AA specificity by coimmunoprecipitating the AT$_1$ receptor with AT$_1$-AA but not with nonspecific IgG. Angiotensin (Ang) II and AT$_1$-AA both activated extracellular signal-related kinase, AP-1, and the TF promoter transfected VSMC and Chinese hamster ovary cells, but only when the AP-1 binding site was present. We then demonstrated TF expression in VSMC exposed to either Ang II or AT$_1$-AA. All these effects were blocked by losartan. Nonspecific IgG or IgG from nonpreeclamptic pregnant women had a negligible effect.

Conclusions—We conclude that AT$_1$-AA and Ang II both stimulate the AT$_1$ receptor and initiate a signaling cascade resulting in TF expression. These results show an action of AT$_1$-AA on human cells that could contribute to the pathogenesis of preeclampsia. (Circulation. 2000;101:2382-2387.)

Key Words: angiotensin receptors; muscle, smooth; pregnancy

Preeclampsia occurs after the 20th week of gestation and features hypertension and an increased peripheral vascular resistance; the mechanisms are unknown. The condition occurs in 5% to 10% of pregnancies and is a major cause of maternal and fetal death. Endothelial dysfunction plays an important role in the disorder. Evidence has been presented that an as-yet unidentified circulating factor(s) is released into the maternal circulation, which leads to endothelial cell activation and subsequently to endothelial dysfunction. Studies of sera from preeclamptic patients have shown that cultured endothelial cells respond with increased expression of growth factors, fibronectin, oxygen free radical production, and inhibition of prostacyclin production. We showed earlier that serum from preeclamptic women stimulates surface adhesion molecule expression and increases endothelial cell

See page 2335

layer permeability. Recently, we observed that circulating antibodies to a vascular receptor might be responsible for the hypertension observed in preeclampsia. We found that the IgG fraction from preeclamptic women contains an angiotensin-1 receptor autoantibody (AT$_1$-AA) that stimulates the AT$_1$ receptor. Placentas from preeclamptic women exhibit relative placental insufficiency, placental infarctions, and increased tissue factor (TF) expression compared with placentas from normal pregnancies. We therefore tested the hypothesis that AT$_1$-AA might cause vascular cells to express TF.

Methods

Preparation of Immunoglobulin Fraction

We used IgG fractions from preeclamptic women containing AT$_1$-AA. Preparation of the IgG fraction is outlined in detail elsewhere.
Briefly, affinity purification was performed. The immunoglobulin fractions from the patients were loaded on a Sepharose 4B CNBr-activated gel (Pharmacia), to which the peptide corresponding to the second extracellular loop of the human AT₁ receptor was covalently linked. The antibodies were eluted with 3 mol/L potassium thiocyanate (pH 7.4) followed by immediate extensive dialysis against PBS. The AT₁-AA were prepared from the sera of 12 preeclamptic women, diagnosed according to criteria outlined elsewhere. These criteria rely on documenting no hypertension before pregnancy, no proteinuria before the second trimester, and proteinuria after the 20th week of pregnancy with increased blood pressure. IgG from pre-eclamptic women after AT₁-AA had been eluted, was used as a negative control, and is termed “nonspecific” IgG. We also had control IgG from 12 healthy nonpreeclamptic pregnant women who had not participated in our earlier studies. Written informed consent was obtained.

Cell Culture
We used human coronary artery vascular smooth muscle cells (VSMC) in this study because of their reliable expression of the AT₁ receptor. VSMC were obtained from Clonetics and were grown in SmGM2 medium (Clonetics). Chinese hamster ovary (CHO) cells overexpressing the AT₁ receptor and CHO wild-type cells in DMEM/HAMs F-12 containing geneticine (63 mg/L), 10% FCS, 0.1% penicillin/streptomycin, and glucose. Cells were grown to 75% confluence.

Immunohistochemistry
Immunohistochemistry was done on cryosections with the use of standard peroxidase-antiperoxidase techniques as described earlier. Endogenous peroxidase was inactivated by immersing the sections in methanol containing 0.6% H₂O₂ for 10 minutes. After the standard steps, the sections were developed in 3-amo-ethylcarbazole until a red/brown reaction product could be seen. Finally, counterstaining steps, the sections were developed in 3-amino-ethylcarbazole until a red/brown reaction product could be seen. Finally, counterstaining was performed with Mayer’s hemalaum (Merck). Preparations were examined under a Zeiss Axiosplan-2 microscope. Fifteen different areas of each placenta sample were analyzed. The placenta samples were examined without knowledge of the disease identity. The antibody against human TF was a kind gift of Dr Luther, University of Dresden (Germany).

Extracellular Signal-Related Kinase Assay
VSMC were set serum free for 24 hours and stimulated with Angiotensin (Ang) II, AT₁-AA, or nonspecific IgG for 15 minutes. After stimulation, cells were harvested by aspirating the medium and washing twice with PBS (4°C). Cells were lysed by addition of lysis buffer (4°C: 20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP-40, 1% Na-deoxycholate, 0.2% SDS, and protease inhibitors. Cell lysates, 20 μL staphylococcal protein A–sepharose beads (50% slurry, Promega), and the indicated antisera were incubated at 4°C for 2 hours. Precipitates were washed with RIPA buffer 6 times, subjected to SDS-PAGE, blotted to the PVDF (polyvinylidene fluoride resin) membrane, and probed with the indicated antisera. Antibody binding was revealed with the use of enhanced chemiluminescence detection (Amersham). Anti-AT₁, anti–interleukin-1 receptor, and anti-human antibodies were obtained from Santa Cruz Biotechnology Inc.

Electromobility Shift Assay
Tissue extracts and electromobility shift assay (EMSA) were performed as described earlier. Double-stranded oligonucleotides containing the consensus sequence for AP-1 (Santa Cruz Biotechnology Inc, 5’-GAT CCA GGG CTT GGG ATT CCC CAT CTC CAC AGG) were radiolabeled with gamma-³²P with the use of T4 polynucleotide kinase by standard methods and purified over a column. Antibodies directed against c-Jun, c-Myb, and nuclear factor (NF)-κB (P65) were obtained from Santa Cruz Biotechnology Inc.

Transfection Experiments
The human TF luciferase promoters have been described previously and included the promoters of TF (−244 to +1) and TF (−111 to +1). For transient transfection, 2 μg of the appropriate luciferase promoter construct per milliliter of medium was transfected with Fugene6 (Roche Boehringer) according to the manufacturer’s description. Transfected cells were stimulated for 15 minutes with no or 10−7 mol/L Ang II. AT₁ receptor was blocked by a 30-minute preincubation with 10−5 mol/L losartan. Cells were harvested and lysed as described earlier. Relative luciferase units were calculated as percentage of basal luciferase activity of the nonstimulated cell line. The measurements were performed in duplicate. The data were confirmed in 3 to 5 independent transfections. For these studies, separate transfections were performed from 7 preeclamptic women and 7 control women.

Comununoprecipitation
VSMC were lysed in modified RIPA lysis buffer containing 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 20% glycerol, 1 mmol/L MgCl₂, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 1% NP-40, 1% Na-deoxycholate, 0.2% SDS, and protease inhibitors. Cell lysates, 20 μL staphylococcal protein A–sepharose beads (50% slurry, Promega), and the indicated antisera were incubated at 4°C for 2 hours. Precipitates were washed with RIPA buffer 6 times, subjected to SDS-PAGE, blotted to the PVDF (polyvinylidene fluoride resin) membrane, and probed with the indicated antisera. Antibody binding was revealed with the use of enhanced chemiluminescence detection (Amersham). Anti-AT₁, anti–interleukin-1 receptor, and anti-human antibodies were obtained from Santa Cruz Biotechnology Inc.

Results
Figure 1 shows immunohistochemistry of 2 placentas, one from a normal pregnancy and one from a patient with preeclampsia. An antibody against TF, followed by a second antibody coupled with peroxidase, was applied. Strongly positive staining is seen in the preeclamptic placenta. The upper panel shows cytotrophoblasts; lower panels show blood vessels at 2 levels of magnification. Far more TF is seen in the placental vessel from the preeclamptic placenta compared with the control placenta.

Figure 2 shows a series of coimmunoprecipitations. From the left in lane 1 is the positive control. Immunoprecipitation with a commercial AT₁ receptor antibody and subsequent immunoblotting with a different commercial AT₁ receptor antibody directed against a different AT₁ receptor epitope results in the band as shown at 48 kDa. Lane 2 shows a negative control, in which the VSMC extracts were exposed to an IL-1 receptor antibody and immunoblotted with an AT₁ receptor antibody. No band resulted. Lane 3 shows the coimmunoprecipitation experiment in which VSMC extracts were immunoprecipitated with a commercial AT₁ receptor antibody and immunoblotted with AT₁-AAA. A band results at the same position as lane 1 at 48 kDa. Lane 4 shows that the
coimmunoprecipitations in the earlier lane were specific, since nonspecific IgG from the same patient failed to react with the immunoprecipitated AT1 receptor and no band resulted. Lane 5 shows reciprocal coimmune precipitation, in which the AT1-AA were used as the immunoprecipitating antibody and the commercial AT1 antibody as the detection antibody. To demonstrate specificity, we used nonspecific IgG from the same patient as the immunoprecipitating antibody in lane 6, and no band resulted. These experiments show specific binding of the AT1-AA to the AT1 receptor.

We next tested whether or not AT1-AA would initiate the ERK1/2 pathway involved in AP-1 activation. These results are shown in Figure 3A. Ang II induced ERK1/2 (p44 and p42), which was diminished by losartan. AT1-AA also induced ERK1/2, which was inhibited by losartan. Nonspecific IgG or IgG from nonpreeclamptic women had a lesser effect. We then tested whether or not AT1-AA would activate AP-1.

Figure 3B shows the EMSA for the AP-1 transcription factor. Lane 1 is the control lane (left). Lane 2 shows VSMC exposed to Ang II (1×10^{-7} mol/L) for 15 minutes. A strong AP-1 band is visible, indicating strong DNA binding activity of AP-1 complexes. Lane 3 shows the same Ang II exposure in the presence of 30 minutes of losartan (1×10^{-5} mol/L) preexposure. No AP-1 induction, compared with control, was observed. Lane 4 shows VSMC exposed to AT1-AA. A band similar to Ang II exposure was observed. Lane 5 shows the same preparation with AT1-AA–treated VSMC previously exposed to losartan. No band induction was observed. Lane 6 shows VSMC exposed to nonspecific IgG from the same patient. No band induction was observed. Lane 6 shows VSMC exposed to IgG from a nonpreeclamptic pregnant patient. Again, no band induction was observed. Figure 3C shows a supershift in lane 3 with antibody directed against c-Jun. No supershifts were observed with anti-c-Myb or anti-p65 NF-κB antibodies, used as controls. In lane 6 is shown a
competition with unlabeled AP-1, suggesting specificity of
the AP-1 activation.

We next verified the specificity of our observations by
showing that the AP-1 binding site must be present for Ang
II or AT1-AA–related TF promoter activation. Figure 4 shows
luciferase activity on the ordinate and treatments on
the abscissa in VSMC, transfected with 2 TF promoter
constructs. Ang II treatment resulted in full expression of intact
TF promoter. Effect was fully blocked with losartan (Los).
AT1-AA from preeclamptic patient gave response similar to Ang
II. Effect was also blocked with losartan. Nonspecific IgG (ns)
from same patient had no effect. IgG from nonpreeclamptic
pregnant patient (np) had no effect. When experiments were
repeated with TF promoter missing both AP-1 and NF-κB bind-
ing sites (TF promoter DNF-κBΔAP1), no stimulation (no stim)
ocurred. B, Same experiment performed in CHO cells express-
ing AT1 receptor. Similar responses were observed. Wild-type
CHO cell lines with minimal AT1 receptor expression showed no
effect.

losartan. AT1-AA from a preeclamptic patient gave a re-
response similar to Ang II. This effect was also blocked with
losartan. Nonspecific IgG from the same patient had no effect. IgG from nonpreeclamptic
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repeated with TF promoter lacking both AP-1 and NF-κB bind-
ing sites (TF promoter DNF-κBΔAP1), no stimulation (no stim)
ocurred. B, Same experiment performed in CHO cells express-
ing AT1 receptor. Similar responses were observed. Wild-type
CHO cell lines with minimal AT1 receptor expression showed no
effect.

After demonstrating that TF expression is increased in
preeclamptic placentas and can be induced in VSMC, we
investigated whether or not TF is functionally active. Figure 5A shows TF activity from VSMC extracts exposed to various treatments. Nonstimulated cells (no stim) showed minimal expression. Ang II exposure increased TF expression to high levels. Effect was blocked by losartan (Los). AT₁-AA from preeclamptic patient caused effect similar to Ang II exposure. Effect was blocked by losartan. Nonspecific IgG from same patient and IgG from nonpreeclamptic pregnant patient (np) generated no TF expression. B, TF expression measured from normal and preeclamptic placentas. Preeclamptic placenta expressed 6-fold more TF. Prot indicates protein.

**Discussion**

We demonstrated by reciprocal coimmunoprecipitation that AT₁-AA specifically binds to the AT₁ receptor in VSMC. We next showed ERK1/2 phosphorylation and then performed EMSA to show that AP-1 was activated by AT₁-AA in a similar fashion as with Ang II. By competition and supershift experiments, we showed that the induced DNA binding activity of AP-1 was specific. The transfection experiments with the TF promoter in VSMC and CHO cells showed that the TF promoter activation was induced by AT₁-AA and that the AP-1 binding site must be intact for the activation to occur. We further verified that the induced signal cascade is dependent on the presence of the AT₁ receptor, since a cell line not expressing the AT₁ receptor showed no induction of the TF promoter in response to either Ang II or AT₁-AA. Finally, we showed that AT₁-AA–induced TF expression is functionally active in VSMC. Similarly, placentas from preeclamptic patients also expressed TF in increased amounts, compared with that in control subjects. These findings support the notion that increased TF may be functionally relevant.

TF is a 47-kDa transmembrane protein that initiates the extrinsic pathway of coagulation through formation of an enzymatic complex with factor VII/factor VIIa. However, TF also has biological functions independent of the clotting cascade in embryogenesis, blood vessel development, cell adhesion, and migration. The TF promoter is complex and contains consensus sequences for NF-κB and AP-1. We focused on AP-1 activation in this study; however, NF-κB can also be activated by Ang II. We performed a supershift analysis, which demonstrated participation of c-Jun but not c-Myb or the p65 component of NF-κB, supporting the conclusion that AP-1 is indeed specific. A role for the coagulation system in the pathogenesis of preeclampsia has been proposed. Oian et al observed increased sensitivity to thromboplastin synthesis in monocytes from preeclamptic women. Increased antifibrinolytic activity in placentas from preeclamptic women has been attributed to plasminogen activator inhibitor-2. Multiple variables of the hemostatic system from 200 preeclamptic women and 97 control women were entered into a multivariate regression model and produced results consistent with activated coagulation in the placental vessels.

We believe that AT₁-AA from preeclamptic patients may be responsible for TF activation in the placenta and perhaps on endothelial surfaces. Nishimura et al have shown that Ang II can stimulate endothelial cells to express TF and plasminogen activator inhibitor-1. How TF expression might participate in the pathogenesis of preeclampsia, other than by promoting local coagulation and perhaps causing ischemia, is unclear. Zhou et al recently reported that human cytotrophoblasts adopt a vascular phenotype that appears to be necessary for successful endovascular invasion. In preeclampsia, human cytotrophoblasts fail to express this vascular phenotype. Consequently, integrins, cadherins, immunoglobulin superfamily members, and perhaps other structures including surface receptors are not produced appropriately. TF may be important to cell differentiation. Cytotrophoblast differentiation and the maintenance of intervillous flow has been shown to depend on PP5/TPP12, a Kunitz-type protease inhibitor, identical to TF inhibitor-2. Thus, an influence of TF expression in placental tissue could conceivably influence cytotrophoblast differentiation.

The renin-angiotensin system is implicated in preeclampsia. Gant et al identified hypersensitivity to infused Ang II in preeclamptic patients, although the Ang sensitivity test in preeclampsia is not invariably positive. Sowers et al found elevated active tissue renin concentrations and increased renin mRNA expression in placentas from preeclamptic patients.
patients compared with placentas from women with normal pregnancies. Brar et al.\(^4\) observed increased chorionic tissue active renin levels in patients with preeclampsia compared with that in control subjects. Another line of evidence implicating the renin-angiotensin system in preeclampsia stems from genetic observations, including an association between preeclampsia and the angiotensinogen variant T235,\(^5,6\) and a mutation leading to the replacement of leucine by phenylalanine at position 10 of mature angiotensinogen, the site of renin cleavage.\(^6\) We have not yet shown precisely how AT\(_1\)-AA activate the AT\(_1\) receptor, although we have demonstrated the binding site of the antibody.\(^4\) Possibly, the AT\(_1\)-AA do not activate the receptor directly. An alternative mechanism could involve an alteration in the receptor's configuration, permitting greater accessibility to antibody Ang II.

In summary, we showed that AT\(_1\)-AA from IgG of preeclamptic patients specifically communoprecipitated with a commercially available AT\(_1\) receptor antibody. AT\(_1\)-AA induced a signal transduction pathway through the AT\(_1\) receptor involving ERK1/2 and AP-1 activation. This cascade of events resulted in TF expression, which was inhibited by AT\(_1\) receptor blockade and was not elicited by nonspecific IgG from preeclamptic patients or IgG from healthy pregnant women. Increased TF expression was detected in the placenta of preeclamptic women, raising the possibility that AT\(_1\)-AA contribute to the pathogenesis of preeclampsia.

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References

5. Baker PN, Davidge ST, Roberts JM. Plasma of preeclamptic women. Increased TF expression was detected in the placenta of preeclamptic women, raising the possibility that AT\(_1\)-AA contribute to the pathogenesis of preeclampsia.

References

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