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 receptor muscle, smooth pregnancy
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 glutamine. 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 standard peroxidase-antiperoxidase techniques as described earlier. Immunohistochemistry was done on cryosections with the use of 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 MgCl2, 0.1 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-A T1, anti– interleukin-1 receptor, and anti-human antibodies were obtained from Santa Cruz Biotechnology Inc.

**TF Activity**

Protein concentrations of the cellular extracts were quantified by use of the Bradford method. TF activity was determined in cell extracts with a clotting-based assay according to the manufacturer’s instructions (ACTICLOT, Diagnostics International). Data and standard deviations represent means of triplicates.

**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 vessels from a normal pregnancy than from a preeclamptic 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-AA. A band results at the same position as lane 1 at 48 kDa. Lane 4 shows that the
communoprecipitations 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 commune 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 7 shows VSMC exposed to IgG from an 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 (mean ± SD). 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 binding sites (TF promoter ΔNF-κB ΔAP1), no stimulation (no stim) occurred. B, Same experiment performed in CHO cells expressing AT1 receptor. Similar responses were observed. Wild-type CHO cell lines with minimal AT1 receptor expression showed no effect.

AT1-AA from a preeclamptic patient gave a response similar to Ang II. This effect was also blocked with losartan. Nonspecific IgG from the same patient had no effect. IgG from nonpreeclamptic pregnant patient had no effect. When the experiments were repeated with TF promoter lacking both AP-1 and NF-κB binding sites, no stimulation was observed with either Ang II or AT1-AA treatment. The lower panel shows the same experiment performed in CHO cells expressing AT1 receptor. Similar responses were observed. A wild-type CHO cell line with minimal AT1 receptor expression but transfected with the full-length TF promoter showed no effect when exposed to Ang II or AT1-AA treatment. This experiment documents that the TF promoter activation resulted from AT1 receptor stimulation.

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 activity. 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 (ns) 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.

Figure 5. TF activity from VSMC extracts exposed to treatments (mean±SD). 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 (ns) 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 activity than the normal placenta.

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 PPs/TFP12, a Kunitz-type proteinase 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 compared with placentas from women with normal pregnancies. Brar et al. observed increased choriocytic 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, and a mutation leading to the replacement of leucine by phenylalanine at position 10 of mature angiotensinogen, the site of renin cleavage. We have not yet shown precisely how AT1-AA activate the AT1 receptor, although we have demonstrated the binding site of the antibody. Possibly, the AT1-AA do not activate the receptor directly. An alternative mechanism could involve an alteration in the receptor’s configuration, permitting greater accessibility to available Ang II.

In summary, we showed that AT1-AA from IgG of preeclamptic patients specifically communoprecipitated with a commercially available AT1 receptor antibody. AT1-AA induced a signal transduction pathway through the AT1 receptor involving ERK1/2 and AP-1 activation. This cascade of events resulted in TF expression, which was inhibited by AT1 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 placentas of preeclamptic women, raising the possibility that AT1-AA contribute to the pathogenesis of preeclampsia.

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References
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