SEMINARS IN
NEPHROLOGY

AT1 Receptor Agonistic Antibodies, Hypertension, and Preeclampsia

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Immunologic mechanisms and putatively circulating mediators of preeclampsia are not a new idea, but are nevertheless compelling. Here we review studies relating to the role of agonistic antibodies that bind the second extracellular loop of the angiotensin II (AII) AT1 receptor in the pathogenesis as well as a pathologic phenotype of this disorder, focusing on observations in our laboratory. These agonistic autoantibodies (AT1-AA) appear with the development of preeclampsia and mostly are gone by 6 weeks after delivery. We have purified AT1-AA and have shown that they belong to the immunoglobulin (Ig)G3 subclass. We have shown their specificity by Western blotting, colocalization, and coimmunoprecipitation experiments. AT1-AA induce signaling in vascular cells and trophoblasts including activating protein-1 (AP-1) and nuclear factor κ B (NF-κB) activation. The signaling results in tissue factor production and reactive oxygen species generation, both of which have been implicated in preeclampsia. AT1-AA also signal via the calcineurin-nuclear factor of activated T cells and contribute to plasminogen activator inhibitor-1 (PAI-1) production and decreased trophoblast invasion. The role of AT1-AA in preeclampsia and other severe hypertensive conditions has not yet been proven with certainty. However, we believe the findings are compelling and warrant further study.
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KEYWORDS preeclampsia, immune system, angiotensin receptor, autoantibody

The term *preeclampsia* refers to the new onset of hypertension and proteinuria after 20 weeks of gestation in previously normontensive, nonproteinuric women.[1] The condition can be devastating and remains a major obstetric health problem affecting both the fetus and the mother. Moreover, a host of epidemiologic research has drawn attention to the possibility of long-term cardiovascular problems in children with low-for-gestational-age birth weight, often the result of fetal growth restriction associated with preeclampsia, and similar long-term risks have been suggested for the mothers as well.[2] Recently, exciting new insights have been gained into how preeclampsia develops that may lead to rapid progress in predicting, preventing, and managing the disease. However, the fundamental mechanisms responsible for this disease still are unclear. Recent advances (many described elsewhere in this issue) have yet to be translated into effective prevention and/or treatment.

This article focuses on the role of circulating mediators in the pathology of preeclampsia, focusing on our laboratory's contribution in this area. The notion that a circulating mediator is present in the blood of preeclamptic women that then disappears after delivery is an old one.[1] The idea that such a factor could have an immunologic basis has appeal because pregnancy is an immunologic marvel in its own right.[1]

Initial Observations

Our interest in circulating mediators and an important place for angiotensin II (AII) in the preeclampsia equation stem from early observations that platelets from preeclamptic women have increased intracellular free calcium (Ca_i^{2+}) concentrations and that these concentrations increase even further with AII stimulation, but not with thrombin.[3] The phenomenon disappears after delivery. These increments in intracellular calcium levels also have been observed by others.[4] Our initial study involved possible endothelial cell acti-

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vation by circulating components in the plasma of preeclamptic patients.[5] Endothelial cell activation is important in the pathogenesis of preeclampsia; however, the nature of the activation is unknown. We investigated 29 patients with preeclampsia, 22 normotensive pregnancies, and 18 nonpregnant women to test the hypothesis that serum from preeclamptic patients induces expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 and stimulates Ca_i^{2+} in cultured endothelial cells.[6] We then asked whether the corresponding integrin adhesive counterreceptors LFA-1 (CD11a/CD18), MAC-1 (CD11b/CD18), p 150,95 (CD11c/CD18), and VLA-4 (CD49/CD29) are increased in patients with preeclampsia. In the pregnant women, the measurements were conducted both before and after delivery. Integrin expression was measured by fluorescent antibody cell sorting analysis by using monoclonal antibodies. ICAM-1 and vascular cell adhesion molecule-1 were analyzed on endothelial cells by enzyme-linked immunosorbent assay. $CaCa_i^{2+}$ was measured with fura-2. Serum from preeclamptic patients increased endothelial cell ICAM-1 expression, but not vascular cell adhesion molecule-1 expression. Preeclamptic patients' serum levels also increased $CaCa_i^{2+}$ in endothelial cells compared with serum from normal nonpregnant or normal pregnant women. Endothelial cell $CaCa_i^{2+}$ concentrations were highly correlated with the ICAM-1 expression in preeclamptic patients before, but not after, delivery. Expression of the integrin counterreceptors on leukocytes was not increased in preeclampsia compared with normal pregnancy, but was increased compared with nonpregnant women. The expression decreased significantly after delivery in both groups. The results showed that serum from preeclamptic women induced increased ICAM-1 surface expression on endothelial cells, whereas the expression of the integrin counterreceptors was not different. We speculated that the effect on endothelial cells could be related to an increase in $CaCa_i^{2+}$. The effect on cultured endothelial cells and the rapid decrease after delivery suggested the presence of a circulating serum factor that increased endothelial cell $CaCa_i^{2+}$ and enhanced adhesion molecule expression.

We extended these observations in endothelial cells to endothelial cell layers.[7] In these studies we were interested in the possibility that a circulating factor might influence permeability of the capillary wall. Such an alteration might account for edema and proteinuria in preeclampsia that occurred in the face of volume depletion and markedly increased peripheral vascular resistance. We tested the hypothesis that plasma from preeclamptic patients increased endothelial cell permeability and examined possible signal transduction pathways. We studied 8 patients with preeclampsia, 8 normotensive pregnant patients, and 8 nonpregnant women, as well as 5 pregnant patients with preexisting hypertension and 4 age-matched hypertensive nonpregnant women. Cultured human umbilical vein endothelial cell monolayers were used and permeability was measured by albumin flux. The role of protein kinase C (PKC) signaling was examined by down-regulation with phorbol ester and with specific PKC inhibitors. PKC isoforms were assessed by Western blot and confocal microscopy. Antisense oligodesoxynucleotides were used to test the role of specific PKC isoforms. Plasma from preeclamptic patients doubled endothelial permeability. The change in permeability decreased rapidly after delivery to control levels. In contrast, plasma from normotensive pregnant women and nonpregnant women had no effect. Permeability also was not influenced by plasma from patients with essential hypertension or pregnant patients with preexisting hypertension. Plasma from preeclamptic patients induced a translocation of PKC isoforms α and ϵ to the plasma membrane. PKC blockers inhibited the increase in permeability induced by serum from preeclamptic patients. Down-regulation of PKC α and, to a lesser extent, PKC ϵ also inhibited the preeclampsia-induced permeability increase.

Evaluating the Role of AII

In the face of earlier observations that women with preeclampsia are markedly hyperresponsive to circulating AII[7] and the findings we had observed earlier in the platelets,[3] we were encouraged to direct our attention specifically toward AII. However, AII levels had been studied in detail in preeclamptic women by other investigators. In general, circulating AII levels were normal or even below normal in these studies. Moreover, the findings indicated that in preeclampsia, the activity of the renin-angiotensin system in term human placenta and fetal membranes also essentially was normal.[9] However, these findings by no means exclude a possible role for the renin-angiotensin system in the mediation of vascular injury. Were the renin-angiotensin system activated by an external event, no increase in AII or other components would be expected. Fu et al first raised the possibility that an autoantibody might stimulate the AII AT1 receptor.[10] These investigators produced a synthetic peptide corresponding to amino acids 165 to 191 of the second extracellular loop of the human AT1 receptor in rabbits. The purified antibodies had an apparent affinity of about 1 nmol/L and were monospecific for the AT1-receptor peptide. The antibodies specifically stained Chinese hamster ovary cells expressing the rat AT1a receptor. Immunoblots on rat kidney revealed that the antibody recognized a protein band of 59 $\pm$ 3 kd in a dose-dependent manner. The band was no longer detected after preincubating the antibodies with AT1-receptor peptide. By using electron microscopic and immunofluorescence immunocytochemistry techniques, these investigators[9] detected AT1 receptors in the sarcolemma, the T-tubules and nuclei of rat cardiomyocytes, the transluminal side of endothelial cells, and fibroblasts. Functionally, the antibodies did not affect the ligand binding properties of the receptors but instead displayed agonist-like activity. They also described dose-dependent increases in the intrinsic beating rate of cultured neonatal cardiomyocytes. Their results suggested that the antibodies against the second extracellular loop of human AT1 receptors could specifically recognize AT1 receptors. In addition, they raised the possibility that the

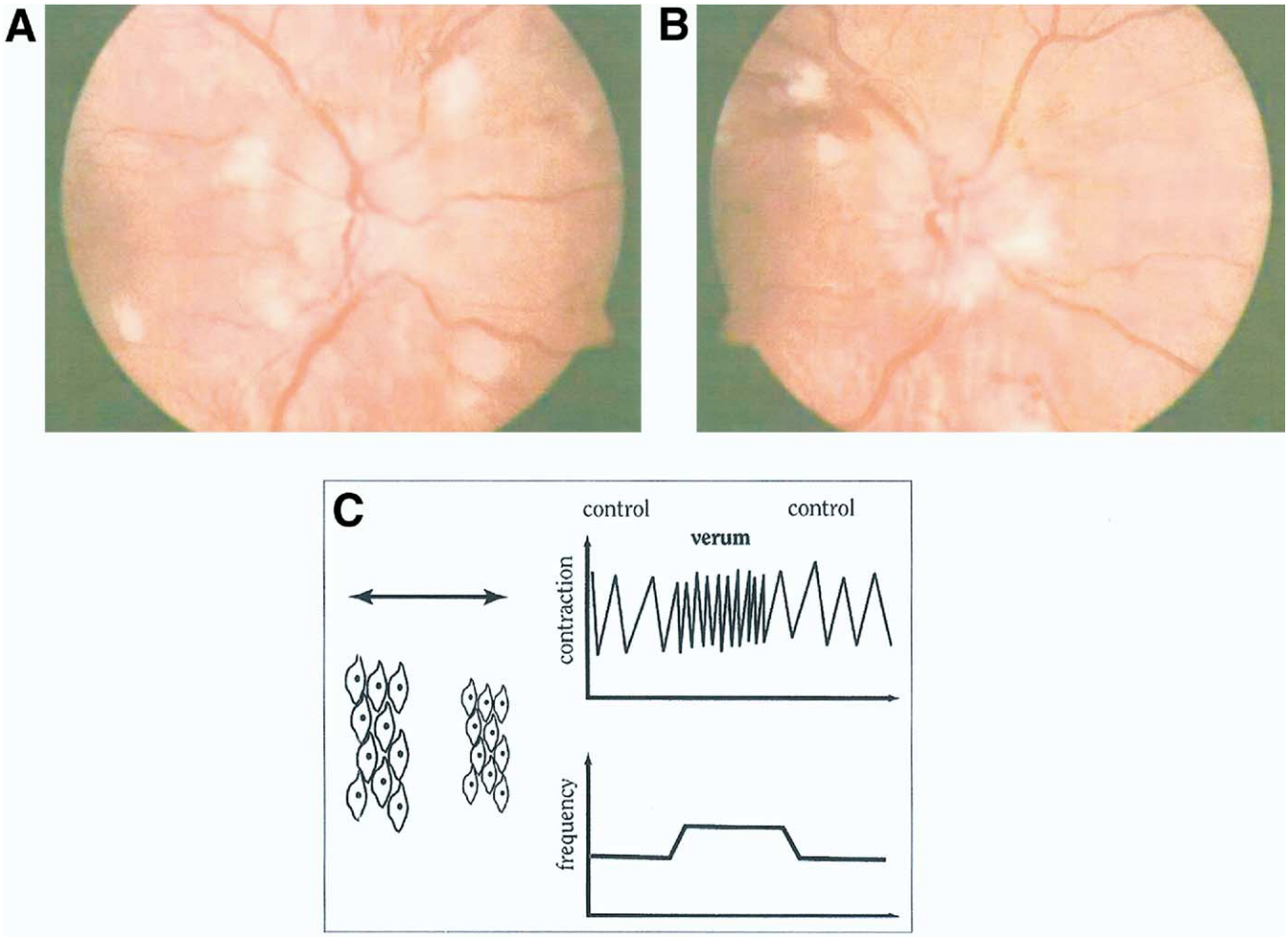

Figure 1 (A, B) Fundus photographs from a 36-year-old woman who had preeclampsia 17 years earlier. She had papilledema, cotton wool spots, and flame-shaped hemorrhages bilaterally. (C) Her serum level (dilution, 1:40) increased the spontaneous beating rate of neonatal rat cardiomyocytes. The response was blocked by AT1-receptor blocker or washout removal.

second extracellular loop of the G-protein–coupled membrane receptors is a specific target for antibodies with agonist-like activity.

Fu et al[9] produced a synthetic peptide. They made an agonistic AT1 receptor autoantibody (AT1-AA). The question remained whether or not humans might produce such AT1-AA simultaneously. There are highly suggestive precedents. Wallukat et al had gathered compelling evidence that patients with cardiomyopathy commonly have activating autoantibodies directed against β-adrenergic receptors.[11] Moreover, removing these antibodies appeared to greatly facilitate remission of this devastating condition.[11] Compelling was the observation that molecular mimicry might facilitate the development of such autoantibodies, as the investigators showed for *Typanosoma cruzi*–induced cardiomyopathy.[12]

Occasionally, clinicians stumble on findings that can contribute to clinical research. We encountered a 36-year-old nurse who had presented originally because of decreased visual acuity. Her blood pressure of 190/136 mm Hg was associated with marked hypertensive retinopathy (Fig. 1) and she had moderate renal insufficiency (serum creatinine, 230 μmol/l [2.6 mg/dL]). Of interest was a past history of preeclampsia at age 19 years, and no medical problems thereafter. When her serum was bioassayed, by using spontaneously beating neonatal cardiomyocytes, provoked an increase in the preparation's spontaneous beating rate. This increase could be reversed by removal of her serum from the assay media or by administering an AT1-receptor blocker. Indeed, the patient's clinical condition improved markedly with maximal AT1-receptor blockade, combined with other antihypertensive medications, fundoscopic changes reverting, and renal function stabilizing.

Stimulated by this clinical observation, we investigated 25 preeclamptic patients and compared them with 12 normotensive pregnant women and 10 pregnant patients with essential hypertension.[13] Antibodies were detected by the chronotropic responses to AT1 receptor–mediated stimulation of cultured neonatal rat cardiomyocytes coupled with receptor-

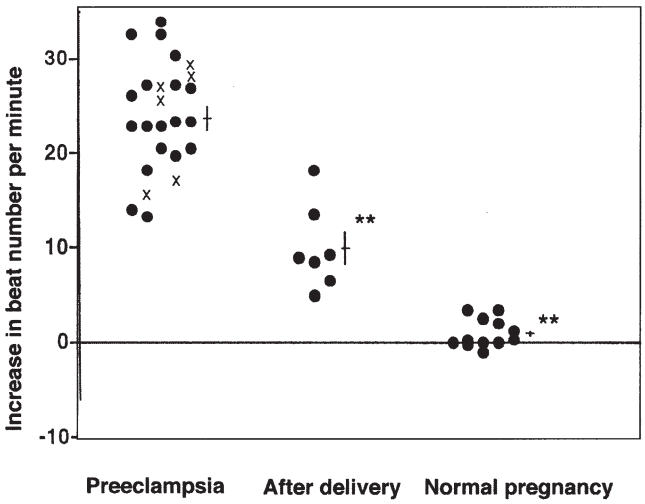

Figure 2 Increase in beat number of spontaneously beating neonatal rat cardiomyocytes when exposed to immunoglobulin from 25 patients with preeclampsia, 7 patients postdelivery, and 12 patients with normal pregnancy. Nulliparous patients are shown (●), multiparous patients are designated with an x. **Different from preceding group ($P < .01$).

specific antagonists. Immunoglobulin from all preeclamptic patients stimulated the AT1 receptor while immunoglobulin from controls had no effect (Fig. 2). The increased autoimmune activity decreased after delivery. Figure 2 shows the increase in beats per minute of spontaneously beating neonatal rat cardiomyocytes when exposed to immunoglobulin from the 25 patients with preeclampsia, the 7 patients after delivery, the 12 patients with normal pregnancies, and the hypertensive patients who became pregnant. Immunoglobulin from preeclamptic patients increased the beat number by 23 beats per minute, which was different from immunoglobulin from women with normal pregnancies and similar to the effect of 0.1 μmol/L of AII. The immunoglobulin samples from 5 of 10 nonpreeclamptic hypertensive patients appeared to contain a fraction that interacted with the 1-adrenoceptor. These 5 women had immunoglobulin that increased the beats per minute by 10; however, the addition of prazosin abolished this increase.

Affinity column purification and anti-human immunoglobulin (Ig)G and IgM antibody exposure implicated an IgG antibody directed at the AT 1 receptor. Peptides corresponding to sites on the AT 1 receptor's second extracellular loop abolished the stimulatory effect. We constructed short, overlapping peptides corresponding to the second extracellular loop of the AT 1 receptor (Fig. 3). These peptides were exposed to affinity-purified antibody preparations. We tested the effect on the spontaneous beating rate of neonatal rat cardiomyocytes. The sequence AFHYESQ abolished the response. We next used Western blotting with purified patient IgG and a commercially obtained AT 1-receptor antibody. The antibodies produced bands of identical molecular weight. Further, confocal microscopy of vascular smooth muscle cells showed colocalization of purified patient IgG and AT1-receptor antibody (Fig. 4). The PKC inhibitor calphostin C prevented the stimulatory effect. These results suggested that preeclamptic patients develop stimulatory autoantibodies against the second extracellular AT 1-receptor loop. The effect appeared to be PKC mediated. We suggested that these novel autoantibodies may participate in the AII–induced vascular lesions in these patients.

Exploring the Roles of AT1-AA

We were aware of the fact that the notion of circulating AT1-AA would evoke much skepticism. We had not shown that AT1-AA might induce changes that contributed to preeclampsia. We next embarked on cellular observations to test the notion that AT1-AA can evoke signaling in vascular cells that could contribute to preeclampsia. Women with preeclampsia have an accelerated thrombotic tendency particularly in placenta, and tissue factor has been implicated in this process. We decided to focus on tissue factor production.[14] We next purified AT1-AA further and found that they belong to the IgG3 fraction. We separated AT_1-AA from the total IgG fraction by ammonium sulfate precipitation. We focused on proving AT1-AA specificity by coimmunoprecipitating the AT1 receptor with AT1-AA. AT1-AA were successful in this experiment whereas nonspecific IgG was not (Fig. 4). We then transfected Chinese hamster ovary cells overexpressing the AT1 receptor with tissue factor promoter constructs coupled to a luciferase reporter gene. Vascular smooth muscle cells (VSMCs) were obtained from human coronaries. Extracellular signal-related kinase (ERK) activation was detected by an in-gel kinase assay. Activating protein-1 activation was determined by electromobility shift assay. Tissue Factor (TF) was measured by enzyme-linked immunosorbent assay and detected by immunohistochemistry. We found that AII and AT_1-AA both activated extracellular signal-related kinase, activating protein-1, and the tissue factor promoter–transfected VSMCs and Chinese hamster ovary cells, but only when the activating protein-1 binding site was present. We then showed tissue factor expression in VSMCs exposed to either AII or AT1-AA. All these effects were blocked by losartan. Nonspecific IgG or IgG from nonpreeclamptic pregnant women had a negligible effect. These observations gave us confidence that AT-AA can indeed induce signaling phenomena that could contribute to preeclampsia.

Exploring the Roles of AT 1-AA

Much remains unknown about the nature of AT1-AA, its receptor binding properties, and subsequent signaling. We focused on the ability of the AT1-AA to recognize a specific conformation of the AT1 receptor.[14] Cleavage of the external disulfide bond with dithiothreitol caused an inactivation of the receptor when stimulated either with AII or the autoantibodies in cultured neonatal rat cardiomyocytes. Long-term stimulation of the AT1 receptor with either agonist downregulated the AT1 receptor-mediated response to a second

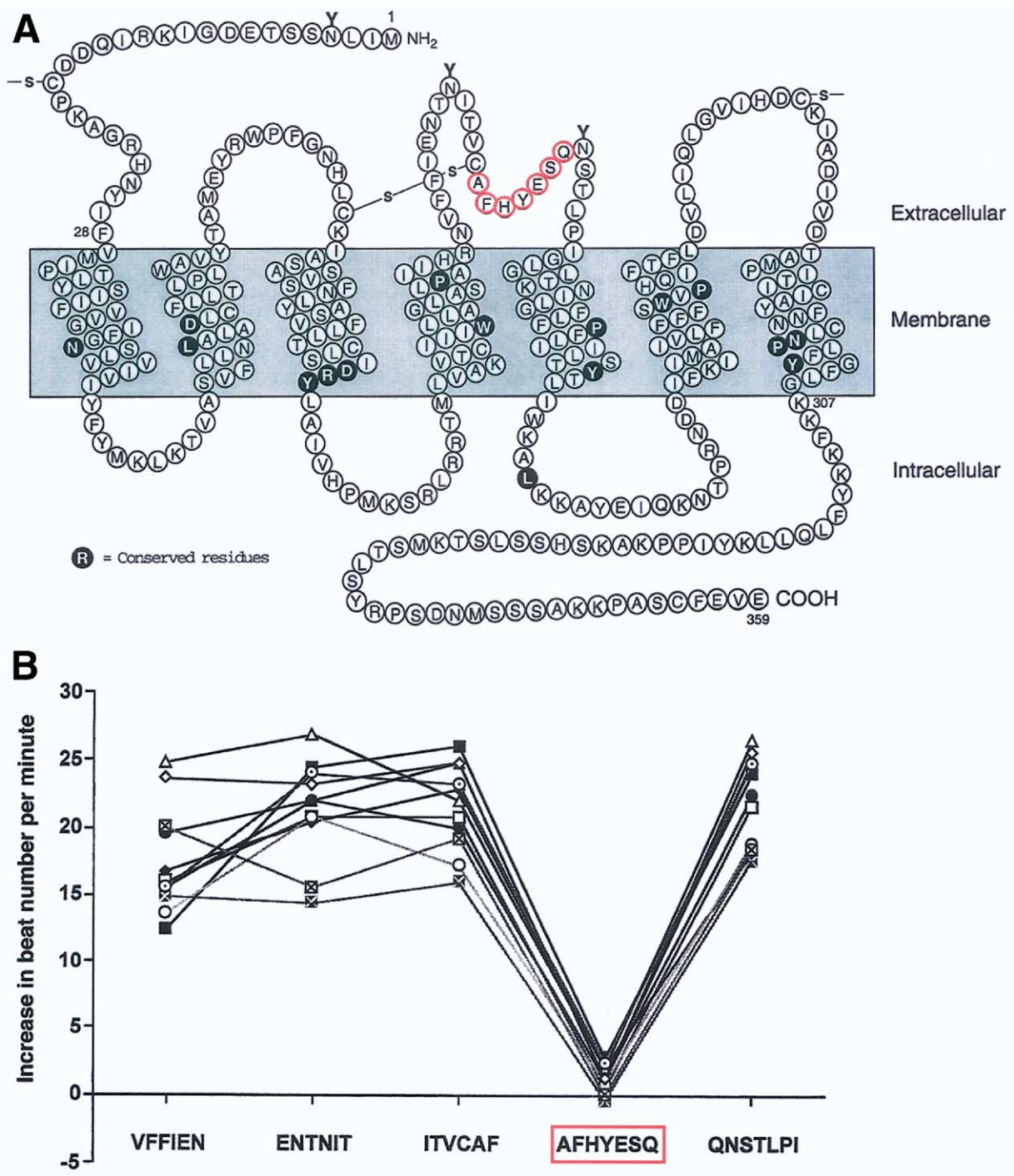

Figure 3 Short overlapping peptides corresponding to the second extracellular loop of the AT_1 receptor were prepared and exposed to affinity-purified antibody preparations. We tested the effect on the spontaneous beating rate of neonatal rat cardiomyocytes. The sequence AFHYESQ abolished the response. (A) AT1 receptor and where the agonistic AT1 receptor autoantibodies (AT1-AA) bind. (B) The sequence that completely inhibited the increased beating rate of neonatal rat cardiomyocytes.

AII stimulation. These observations show that the agonistic autoantibodies behave pharmacologically in a similar fashion to AII. The possibility remains that AT1-AA alter the conformation of the receptor and that the receptor's ability to bind circulating AII is thereby enhanced. The receptor is known to dimerize. We were extremely interested to learn that in preeclamptic women, a significant increase in heterodimerization occurs between the AT1 receptor for the vasopressor AII and the B(2)-receptor for the vasodepressor bradykinin. AbdAlla et al[17] showed that AT1-B(2)-receptor heterodimeriza-

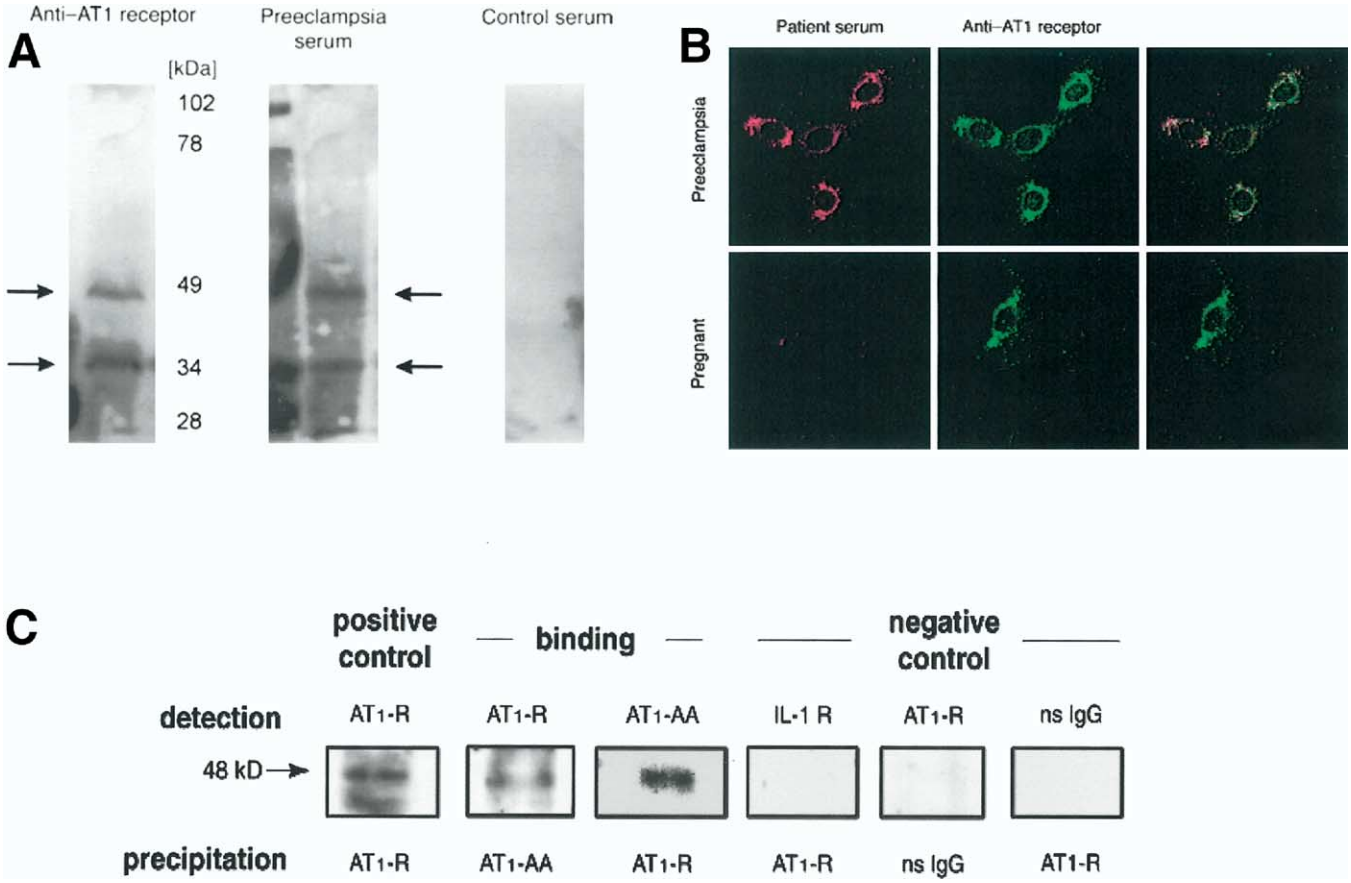

Figure 4 (A) Western blot of VSMC extract probed with column-purified IgG from preeclamptic patients' serum showed 2 bands that were identified as AT1 receptor by stripping and reprobing with commercially available anti–AT1-receptor antibody. Identically handled serum from nonpreeclamptic patients produced no bands. (B) Confocal photomicrograph of vascular smooth muscle cells exposed to affinity column–purified IgG from preeclamptic patient serum and commercially available AT1-receptor antibody. A Cy3-labeled anti-human IgG antibody produced the red staining. The same cells were exposed simultaneously to commercially available AT 1-receptor antibody with a Cy3-labeled secondary anti-human IgG antibody. Superimposition revealed that these antibodies colocalize (yellow). (G-I) The same procedure with IgG fraction from a pregnant, nonpreeclamptic patient. Only staining from the commercially available AT1-receptor antibody is seen. (C) VSMC extracts were subjected to immunoprecipitation using the indicated antibodies. Proteins were revealed by immunoblotting using the indicated antibodies. Lane 1 shows VSMC extract immunoprecipitated and immunoblotted with commercial AT 1-receptor antibody. Lane 2 shows an interleukin-1–receptor antibody coimmunoprecipitation as a negative control. Lane 3 shows the VSMC extract immunoprecipitated with commercial AT 1 antibody and the AT 1-receptor autoantibody (AT 1-AA) as the detection antibody. The same band as in lane 1 is visible. Lane 4 shows immunoprecipitation with nonspecific IgG (ns IgG) from the same patient. No band resulted. Lane 5 shows the AT 1-AA as the immunoprecipitating antibody and the commercial AT 1 antibody as the detection antibody. The same band is visible. Lane 6 shows nonspecific IgG from the same patient used as the immunoprecipitating antibody.

tion in preeclampsia correlated with a 4- to 5-fold increase in B(2)-receptor protein levels. Expression of the AT1-B(2) heterodimer increased the responsiveness to AII and conferred resistance in AT1 receptors to inactivation by reactive oxygen species (ROS) raised in normotensive and preeclamptic pregnancies. The investigators suggested that AT1-B(2) heterodimers contributed to AII hypersensitivity in preeclampsia. Moreover, they identified preeclampsia as the first disorder associated with altered G-protein–coupled receptor heterodimerization.

The work described earlier has focused on the role of agonistic AT1-AA in the vasoconstrictive phenotype of preeclampsia. More recently, studies by Xia et al[18] suggested these antibodies may be instrumental in the pathogenesis of the diseases, specifically the hypothesis that the disorder is a result of abnormal placentation. These investigators focused on 2 major features of preeclampsia: increased plasminogen activator inhibitor-1 (PAI-1) production and shallow trophoblast invasion. They studied 38 pregnant patients, 20 of whom had severe preeclampsia

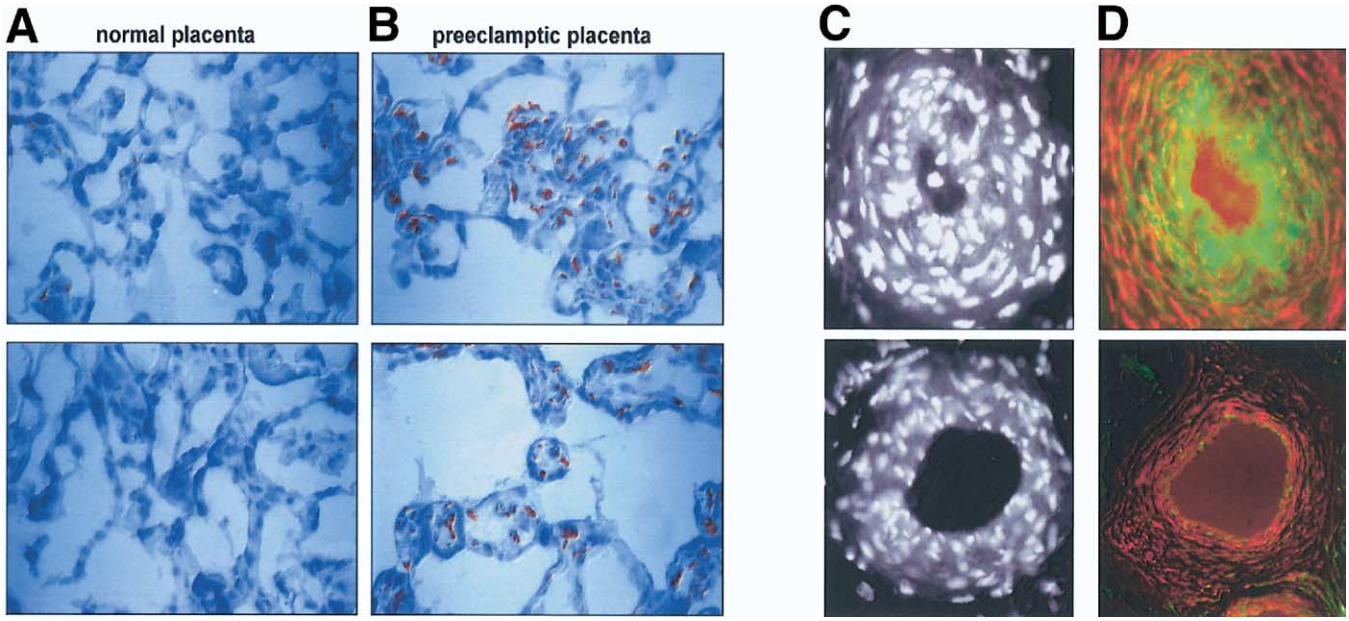

Figure 5 (A) Normal and (B) preeclamptic human placenta stained for the NF-κB p65 protein. NF-κB is activated in preeclamptic placenta. AT 1-AA can activate NF-κB. (C, D) Vessels from preeclamptic placenta. (C) Ethidium bromide staining for ROS. (D) α-smooth muscle actin staining for VSMCs in an adjacent section.

and 18 normotensive individuals. They purified IgG from these individuals and determined the presence of AT1-AA by the ability to stimulate an increase in the contraction rate of cultured rat neonatal cardiac myocytes. Immortalized human trophoblasts were chosen to study PAI-1 production and secretion after treatment with IgG from normotensive and preeclamptic women. Xia et al[18] used in vitro matrigel invasion assay to test the effect of AT1-AA on the invasive properties of human trophoblasts. Losartan and cyclosporin A were used to determine whether the AT1-AA–induced stimulation of PAI-1 secretion was through the AT1 receptor and the calcineurin-nuclear factor of activated T cells–dependent pathway. The investigators observed that IgG from 18 of 20 severely preeclamptic women stimulated increased cardiomyocyte contraction rates of 20 to 40 beats per minute, and also stimulated PAI-1 secretion from human trophoblasts. Activation of AT1 receptors by AT1-AA was blocked by losartan (an AT1 receptor antagonist) and by the same 7 amino acid peptides corresponding to a sequence present on the second extracellular loop of the AT1 receptor that we used. Activation of AT1 receptors by AT1-AA resulted in decreased trophoblast invasiveness as determined by the in vitro matrigel invasion assay.

The investigators showed additional data indicating that AT1-receptor activation by AT1-AA is followed by the downstream activation of the calcium-dependent calcineurin-calcineurin-nuclear factor of the activated T-cell signaling pathway, leading to increased PAI-1 gene expression. Xia et al[18] suggested that a maternal autoantibody with the ability to activate AT1 receptors may account for 2 features of preeclampsia: increased PAI-1 production and shallow trophoblast invasion. Shallow trophoblast invasion also has been attributed to a failure in terminating the expression of transforming growth factor-b3 shortly after implantation. AT1-AA also possibly could interfere with this process, although this hypothesis has not been tested.

More recently, we have described the effects of AT1-AA on ROS, nicotinamide adenine dinucleotide phosphate oxidase expression, and nuclear factor-κB (NF-κB) activation to elucidate actions of AT1-AA further.[19] We showed that AT-1AA can cause human trophoblasts or vascular smooth muscle cells to produce ROS by activating nicotinamide adenine dinucleotide phosphate oxidase. We subjected VSMCs from p47 phox gene–disrupted −/− and control +/+ mice to AT1-AA. By means of intracellular ROS generation, we found that both AII and AT1-AA produced a strong response. This ROS production was ameliorated greatly by the antioxidant tiron or by using VSMCs lacking p47 phox. In these cells, the nicotinamide adenine dinucleotide phosphate oxidase was not operative. In other studies, we used human trophoblasts and electromobility shift assays with supershifts and showed that either AII or AT-AA activated the NF-κB units p50 and p65. Human placenta expressing ROS (ethidium bromide staining of a small artery) and placental tissue showing activated NF-κB p65 by immunohistochemistry is shown (Fig. 5).

Similar to our clinical observation, AT1-AA also have been found in other patients with malignant hyperten-

sion[20] and in patients with humoral renal transplant rejection (unpublished observations). Thus, they are not a specific preeclampsia phenomenon. The existence of these antibodies is exciting. However, the field is hampered by the fact that detection still relies on a bioassay. Attempts at establishing an enzyme-linked immunosorbent assay have not yet been successful. Thus, confirmatory studies in large populations of women with preeclampsia have not yet been conducted. The pathogenic significance of AT1-AA has not yet been convincingly documented. A promising transgenic rat model relying on human renin and angiotensinogen transgenes has been developed.[21] This model features the development of AT1-AA (unpublished observations). AT1-AA could be an epiphenomenon; the possibility remains that the tree under which we are barking could be the wrong one.

AII plays some role in angiogenesis. Angiotensin-converting enzyme inhibitors and AT1-receptor blockers appear antiangiogenic and decrease microvessel formation.[22] Vascular endothelial growth factor–mediated angiogenesis can be decreased with AT1-receptor blockers.[23] Could AII or possibly AT1-AA possibly play a role in up-regulating the recently implicated soluble Fms-like receptor-1?[24] (See article by Bdolah, Sukhatme, and Kuramanchi in this issue for more detail.) The signal presumably has to do with hypoxia and therefore may involve the hypoxia-inducible factor (HIF).[25] Interestingly, AII may increase HIF-1α induction. According to Page et al,[27] AII relies on ongoing translation to maintain increased HIF-1α protein levels. AII increases HIF-1α translation by a ROS-dependent activation of the phosphatidylinositol 3-kinase pathway, which acts on the 5′-untranslated region of HIF-1α messenger RNA. Their results suggest that the nonhypoxic induction of the HIF-1α transcription factor via vasoactive hormones such as AII might be important to vascular biology. Finally, evidence recently has been presented that HIF transcription factors are overexpressed in preeclamptic placentae.[27] In vitro DNA binding activity for HIF-1α was shown in these studies. Flt1 and tyrosine hydroxylase, both of which are equipped with hypoxia response elements, were expressed to a greater degree in preeclamptic compared with normal placentae. Mechanisms that contribute to alternative Flt1 splicing would be elucidative.

In conclusion, this article focused on a circulating factor that may be responsible for the excessive vasoconstriction characteristic of preeclampsia, and even the abnormal placentation, thought by many to initiate the disease. A role for the renin-angiotensin system, once discounted in the pathophysiology of this disease because plasma renin activity and AII levels frequently are suppressed in this disorder, now has been resurrected.

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