**Methods and Results**—We investigated human vascular smooth muscle cells (VSMC) and trophoblasts, as well as placenta and maternal tissues, to determine if ROS production is increased in preeclamptic women and may be involved. Lipid hydroperoxides are increased in preeclampsia, whereas antioxidant activity is reduced. Walsh et al. showed earlier that serum from preeclamptic women stimulates NADPH oxidase through the AT1 receptor, AT1-AA could act by causing the placenta to produce ROS and inflammatory responses in preeclampsia. (Circulation. 2003;107:1632-1639.)

**Conclusions**—NADPH oxidase is potentially an important source of ROS that may upregulate NF-kB in preeclampsia. We suggest that AT1-AA through activation of NADPH oxidase could contribute to ROS production and inflammatory responses in preeclampsia. (Circulation. 2003;107:1632-1639.)

**Key Words:** receptors, AT1 receptor, pregnancy, angiotensin, oxygen, cells, antibodies
Figure 1. A, Effect of Ang II, AT1-AA, ns IgG, and np IgG from normal pregnant women (np) on DCHF oxidation. Ang II and AT1-AA caused intracellular ROS release \( (P < 0.05) \). Effect of various inhibitors on ROS generation. B, Pretreatment with antisense but not sense oligonucleotides against p22 phox inhibited AT1-AA and Ang II–induced ROS generation in VSMC (left) \( (P < 0.05) \). VSMC from p47 phox \(-/-\) mice showed attenuated ROS generation after AT1-AA and Ang II (right). C, RT-PCR (mRNA) in trophoblasts day 0, 1, and 4, normal placenta, and primary human adipocytes for renin, angiotensinogen, angiotensin converting enzyme (ACE), AT1, and AT2 receptor (left). Trophoblasts expressed all genes more than adipocytes \( (P < 0.05) \). Intracellular ROS generation from trophoblasts. Ang II and AT1-AA induced time-dependent ROS production (right) \( (P < 0.05) \).
The control subjects were 15 healthy normotensive women in the third trimester of pregnancy whose newborns had an appropriate birth weight for their gestational age. Placentas were obtained only from women delivered by cesarean section without prior contractions (5 in each group). We eliminated any potential influence of uterine contraction on ROS, NADPH expression, and NF-κB activity. The nonpreeclamptic patients underwent cesarean section because of breech presentation or other obstetrical reasons. None of the control women had preeclampsia, gestational hypertension, choorioamnionitis, or chronic hypertension. Placenta tissue was matched for gestational age. From these women we also obtained blood for IgG. We had institutional review board approval, and written informed consent was obtained.

Preparation of the IgG fraction is outlined in detail elsewhere. The immunoglobulin fractions from the patients were loaded on a sephrose 4B CNBr-activated gel (Pharmacia) to which the peptide corresponding to the second extracellular loop of the human AT1 receptor was covalently linked and eluted. In preeclamptic women, the remaining IgG, after AT1-AA had been eluted, was used as a negative control and is termed "nonspecific" IgG (ns IgG). We also had control IgG from 15 healthy nonpreeclamptic pregnant women (np IgG) who had not participated in our earlier studies.

Cell Culture
We used primary human VSMC, primary human trophoblasts, and VSMC from mice lacking p47 phox. Homozygous p47phox−/− mice and control mice were a gift from Dr Steven M. Holland at NIH. Human VSMC were obtained from Clonetics and were grown in SmGM2 Medium. Trophoblasts were used in first passage. Cultures were stimulated with Ang II, (10−8 mol/L), AT1-AA (10−8 μg/mL), ns IgG (2 mg/mL), or np IgG (2 mg/mL) at a dilution 1:40. Antisense experiments against p22 phox were performed with 1 μmol/L antisense oligonucleotides (antisense p22 phox: 5′-GAT CTG CCC CAT GGT GAG GACC-3′) or sense oligonucleotides (sense p22phox: 5′-GCT CCT CAC CAT GGG GCA CTA C-3′). Anti-I-κBα, p50 NF-κB, p47 phox, and p67 phox antibodies were purchased from Santa Cruz Biotechnology. Antibodies against p65 NF-κB, smooth-muscle α-actin, and tubulin were obtained from Boehringer-Mannheim. Cytokeratin 7 was obtained from Dako.

Details on antibodies to p22 phox were described previously.

Intracellular Redox State Assay
Intracellular oxidant production in living cells was based on the oxidation of 2,7′-dichlorodihydrofluorescein. Briefly, 2,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA), (Molecular Probes) is a nonpolar compound that is converted into a nonfluorescent polar derivative (H2 DCFH) by cellular esterases after incorporation into cells. H2DCFH was oxidized to the fluorescent 2,7′-dichlorofluorescein in the presence of intracellular hydrogen peroxide and peroxidases. Cells were treated for 1 hour with diphenylene iodonium (DPI, 10 μmol/L, Sigma), the xanthine oxidase inhibitor allopurinol (10 μmol/L, Sigma), the cyclooxygenase 1 and 2 inhibitor indomethacin (10 μmol/L, Sigma), the competitive inhibitor of NO synthase (all three isoforms), L-NMMA (300 μmol/L, Calbiochem), the mitochondrial chain complex I inhibitor rotenone (10 μmol/L), or catalase (1000 U/mL Sigma).

Briefly, human VSMC and trophoblasts were incubated for 30 minutes at 37°C with 5 μmol/L H2DCF-DA in HBSS-HEPES. VSMC were stimulated, and the fluorescence intensity over time for 5 groups of 15 to 20 cells was measured by fluorescence microscopy (Carl Zeiss; excitation, 488 nm; emission, 513 nm). The fluorescence intensity was measured for each group, and the relative fluorescence intensity was taken as the average of 5 values.

Cytochrome C Assay
Superoxide anion release was determined by measuring superoxide dismutase (SOD)-inhibitable reduction of ferri-cytochrome C in VSMC, grown in 12 well plates. Reduction of ferri-cytochrome C was calculated from the differences in absorbance at 550 nm normalized to the absorbance at the isosbestic point at 525 nm. Superoxide-specific cytochrome C reduction was calculated between cells incubated with and without SOD by use of an extinction coefficient of 21 100 l · mol−1 · cm−1. The rates of superoxide generation were determined as mol · min−1 · 1 000 000 cells.

Western Blots, Electrophoretic Mobility Shift Assay, Immunohistochemistry, and Immunofluorescence
After stimulation, cells were harvested and nuclear and cytoplasmic extracts were performed. Western blots and electrophoretic mobility shift assay (EMSA) were done as described earlier. Each Western blot experiment was done 5 or more times. Representative examples are shown. Immunohistochemistry was done on cryosections with standard PAP (peroxidase-antiperoxidase) techniques. For immunofluorescence, sections were stained for cytokeratin 7 and p22 phox. After a final washing with TBS, slides were mounted in Vectashield mounting medium (Vector Laboratories). Preparations were analyzed under a Zeiss Axioplan-2 microscope (Carl Zeiss) and digitally photographed with Axiovision 2 multichannel image processing system (Carl Zeiss).

TaqMan Reverse Transcription–Polymerase Chain Reaction
For reverse transcription–polymerase chain reaction (RT-PCR), RNA was isolated after the TRIZOL protocol (Gibco Life Technology). Primers were synthesized by Biotez with the following sequences or have been published:


Real-time quantitative RT-PCR was performed using the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems). Forty cycles of PCR were performed following the instructions of EZ-RT-PCR TaqMan kit protocol with manganese concentrations of 3 mmol/L for GAPDH and 4 mmol/L for p22 phox, p47 phox, and p67 phox. Each sample was tested in duplicate. Each experiment was done 5 times. For quantification of gene expression, the target sequence was normalized in relation to the expressed housekeeping gene GAPDH.

Oxidative Fluorescent Microtopography
The redox-sensitive hydroethidine (HE)/ethidium (ET) fluorescent probe is used to detect intracellular ROS production in placenta in situ. In response to ROS, HE is dehydrogenated, resulting in the formation of ET. ET is positively charged and has better cellular retention and stability compared with HE. Therefore, ET formation was chosen as a ROS production indicator. The method of Fleming was modified slightly. Briefly, frozen specimens were cryosectioned at 10-μm thickness and placed on a glass slide. The specimens were incubated with 0.1 mmol/L hydroethidine (Mobitec) and dissolved in Hepes-Tyrode solution (132 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L CaCl2, 0.5 mmol/L MgCl2, 9.5 mmol/L HEPES, 5 mmol/L Glucose) for 30 minutes at room temperature. After incubation, images were obtained with the use of an imaging system (l Ex: 520, Em: 605 nm; Attofluor).

Statistics
Data are presented as mean ± SEM. We tested for differences (P < 0.05) in mean values by ANOVA and corrected t tests as appropriate.
Results

Acute application of Ang II and AT1-AA caused a time-dependent release of ROS, whereas nonspecific IgG and IgG from normal pregnant women had no effect. ROS generation in response to AT1-AA as well as Ang II was blocked by losartan, catalase, the antioxidant tiron, and to the nonspecific NADPH oxidase inhibitor DPI. In contrast, inhibition of xanthine oxidase with allopurinol, cycloxygenase with indomethacin, the respiratory chain with rotenone, and NO synthase with L-NMMA had no effect on either AT1-AA–induced or Ang II–induced DCF fluorescence in VSMC (data not shown). VSMC incubation with AT1-AA increased superoxide anion generation, as determined by SOD-inhibitable cytochrome C reduction (data not shown). Antisense oligonucleotides against p22 phox attenuated both the AT1-AA and Ang II-induced DCF fluorescence to the same extent as DPI. Sense oligonucleotides had no effect. Accordingly, VSMC from NADPH oxidase knockout mice (p47phox−/−) exhibited only a very weak, antioxidant-insensitive AT1-AA and Ang II–induced ROS generation,
compared with VSMC from p47phox+/- animals. These experiments show that NADPH oxidase is a source for ROS (O2−, H2O2) in VSMC in response to AT1-AA through the AT1 receptor (Figure 1A and 1B).

We tested whether or not trophoblasts and placenta express the products of the renin-angiotensin system genes by RT-PCR before determining their response to AT1-AA (Figure 1C, left). Adipocytes were included as a positive control.13 All the genes were present and increased as the cells differentiated from day 0 to 4. We next tested the effects of Ang II and AT1-AA on ROS production in trophoblasts. AT1-AA and Ang II induced a significant intracellular ROS generation (Figure 1C, right). Nonspecific IgG and IgG from normal pregnant women did not induce intracellular ROS production. However, the ROS production in trophoblasts was lower than in VSMC (data not shown). Losartan blocked intracellular ROS production. DPI and tiron blocked the effects of Ang II and AT1-AA (data not shown). Thus, trophoblasts can generate intracellular ROS in response to AT1-AA, and NADPH oxidase is a major source. We next tested whether or not components of NADPH oxidase are induced by AT1-AA by Western blotting for p22, p47, and p67 phox in VSMC and trophoblasts. Exposing both cell types (Figure 2A, left and right) to Ang II and AT1-AA for 24 hours increased p47 and p67 phox that was reduced by losartan (P<0.05). However, p22 phox was induced only in trophoblasts. The lack of p22 expression by Ang II and AT1-AA in VSMC was confirmed on the RNA level (data not shown). However, p67 phox was induced by both stimuli in VSMC on the protein and the RNA level. RNA in trophoblasts confirmed the protein expression data (data not shown).

We next tested whether or not AT1-AA cause increased NF-κB expression by EMSA and Western blotting in both cell types (Figure 2B). NF-κB was activated by Ang II and AT1-AA (left, VSMC and middle trophoblasts). I-κBα expression was reduced after Ang II and AT1-AA, indicating degradation of I-κBα that was prevented by losartan. Competition experiments with unlabeled oligonucleotides eliminated DNA binding (right). Antibodies to p50 and p65 resulted in supershifts. Preincubation with losartan, DPI, and the intracellular ROS scavenger tiron inhibited AT1-AA–induced NF-κB activation. In contrast, scavenging extracellular H2O2 with catalase was less potent than tiron (Figure 2C, left, VSMC, right trophoblasts). The studies were repeated in p47 phox−/− and control cells (Figure 2D). NF-κB activation in response to Ang II and AT1-AA was markedly reduced in p47 phox−/− cells, compared with wild-type controls. Calcium signaling in response to Ang II measured by Fura 3 and confocal microscopy, was preserved (data not shown).

We next examined the production of ROS in situ. Normal (left) and preeclamptic placenta (right) sections were stained with hydroethidine (Figure 3A). A signal was detected in preeclamptic placentas, especially around the vessels, in the perivascular region, and in the trophoblasts. Expression of p22, p47, and p67 phox was low in normal placenta. In preeclamptic placenta, the expression of all three components was increased. Expression was induced in the cytrophoblasts and in the placental vessels (Figure 3A). Western blotting and quantitative mRNA expression confirmed that p22, p47, and p67 phox were expressed in normal placenta in low amounts and were upregulated in placentas from preeclamptic patients (Figure 3B).

NF-κB activity in placentas from preeclamptic women was increased compared with control subjects (Figure 4A, left). Western blotting for 1κBα showed decreased 1κBα expression in preeclamptic placentas compared with controls. We performed immunohistochemistry for activated p65, as shown in Figure 4A. Intense nuclear staining can be seen along the vessels and cytrophoblasts compared with normal placenta. To further underscore the relevance of trophoblasts in ROS generation by NADPH oxidase (Figure 4B), we colocalized p22 phox (green) with trophoblast cytokeratin 7 (red) in placentas from preeclamptic patients. The yellow staining indicates colocalization. We then stained for smooth muscle α-actin and ROS by hydroethidine in two consecutive sections (Figure 4C). The results indicate that VSMC are involved in ROS formation in preeclampsia.

**Discussion**

A role for oxidative stress in the pathogenesis of preeclampsia is compelling.15 Preeclampsia is associated with a distinct pathological placental lesion of the decidual arterioles known as acute “atherosclerosis” that has a striking resemblance to atherosclerotic lesions in coronary arteries and elsewhere. The endothelium is disrupted, platelet aggregates are formed, and lipid-laden macrophages accumulate.16 Interestingly, increased NADH-oxidase–mediated superoxide production has been identified in the early stages of atherosclerosis and endotoxemia. APO-E−/− mice that were also missing the p47phox gene had less total area atherosclerotic lesions than APO-E−/− control mice.17 Preeclamptic women exhibit insulin resistance and dyslipoproteinemia. Indeed, dyslipoproteinemia appears to persist in women with a history of preeclampsia. Such women have an increased long-term cardiovascular risk. However, whether or not dyslipidemia antedates the preeclampsia is not established for certain.18 Dyslipidemia could contribute to mechanisms resulting in preeclampsia through AT1 receptor–mediated NADH-oxidase stimulation.

Increased production of vascular superoxide can result from increased NADPH oxidase activity. Both endothelial and VSMC manifest extramitochondrial or membrane-bound oxidases that use NADH and NADPH as substrates for electron transfer to molecular oxygen.19 As we confirmed here, Ang II can activate NADPH oxidase in cultured VSMC. There are multiple molecular targets of ROS, including Ras, p38 mitogen-activated protein kinase, phospholipase D, and protein tyrosine phosphatases.20 In our earlier study, we demonstrated that the extracellular signal-related kinase system (ERK1/2) can be activated by AT1-AA and that the activator-protein transcription factor system AP-1 are expressed in increased amounts. This activity leads to increased tissue factor expression and activity.3 ERK1/2, AP-1, and tissue factor are activated by reactive oxygen intermediates.19 ROS generation appears to be important for the Ang II–related signaling. Viedt et al2 showed that ROS generated by
Endothelial dysfunction is a hallmark of preeclampsia, leading to decreased vasodilation, VSMC proliferation, and increased interaction between leukocytes and the vessel wall. Preeclampsia involves endothelial dysfunction, maternal inflammatory responses, complement system activation, leukocyte stimulation, and thrombosis. NF-κB is the transcription mediator for all these entities. NF-κB has not been studied in preeclampsia; however, initial data indicate that NF-κB may actually be downregulated in placentas from normal pregnancies. The results of our present study show that NF-κB is activated in AT1-AA–exposed VSMC and that NF-κB expression is also increased in the placentas from patients with preeclampsia. Thus, a chain of events can be postulated that ranges from AT1-AA production, AT1 receptor stimulation, NADPH oxidase activation, ROS production, transcription factor activation, and inflammatory mediator production in preeclampsia.

Increased nitrotyrosine immunoreactivity has been identified in placentas from preeclamptic women as well as increased xanthine oxidase activity and decreased superoxide dismutase function. Furthermore, evidence for oxidative stress in preeclampsia has accrued from studies of ascorbate radical. Manes et al recently showed that NADPH oxidase is constitutively active in normal trophoblasts and thus is a potential source for ROS.

Renin-angiotensin system gene variants have been implicated in preeclampsia. We found that all components of the renin-angiotensin system were detectable in cultured trophoblasts and placentas. We included adipocytes as control cells, since the components of the renin-angiotensin system were found to be expressed in adipocytes. A physiological role for the renin-angiotensin system in human placenta was
suggested by the observation that human trophoblasts in suspension exposed to Ang II stimulates human placental lactogen by the activation of phospholipase C.27 The local renin-angiotensin system in the placenta appears to be deregulated in preeclampsia.

We have yet to show the origin of AT1-AA. We do not understand the immunology of the AT1-AA phenomenon and we do not know for certain that AT1-AA are of pathogenic significance.28 Nevertheless, four new aspects about preeclampsia emanated from our study. First, AT1-AA from preeclamptic women induce intracellular ROS in VSMC and trophoblasts, an induction that is mediated by NADPH oxidase. Second, AT1-AA activate NF-κB as a downstream target. The experiments in p47 phox−/− VSMC render reasonable support to the notion that NADPH oxidase is involved. The identity of the associated NOX protein is unclear. Third, ROS production is increased in preeclamptic placentas; we provide some evidence that this production occurs in and around the blood vessels. NADPH oxidases are present in the placenta and are massively stimulated in preeclampsia. Finally, NF-κB is markedly upregulated.

Whether or not AT1-AA are the cause or the result of vascular damage in preeclampsia will require additional investigations.

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AT₁ Receptor Agonistic Antibodies From Preeclamptic Patients Stimulate NADPH Oxidase

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