Oxidative Stress and Preeclampsia
Rationale for Antioxidant Clinical Trials
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Abstract—Preeclampsia remains a frequent and potentially dangerous complication of pregnancy. The cause remains largely unknown, but oxidative stress and a generalized inflammatory state are features of the maternal syndrome. The placenta appears to be the principal source of free radical synthesis but maternal leukocytes and the maternal endothelium are also likely contributors. Recent reports have suggested an important role for placental trophoblast NAD(P)H oxidase in free radical generation in preeclampsia. The antioxidant vitamin E is now known to have multiple actions in addition to prevention of lipid peroxidation (ie, inhibition of NAD(P)H oxidase activation and the inflammatory response). In view of the abnormally low plasma vitamin C concentrations in preeclampsia, a combination of vitamins C and E is a promising prophylactic strategy for prevention of preeclampsia. Several multicenter randomized clinical trials are now underway. The potential use of antioxidants and the recognized, albeit modest, benefit of low-dose aspirin prophylaxis have heightened the need for a reliable predictive test for preeclampsia. A combination test involving several relevant biomarkers is likely to provide the best predictive potential. (Hypertension. 2004;44:374-380.)

Key Words: preeclampsia ■ oxidative stress ■ antioxidants ■ free radicals

Preeclampsia affects between 0.4% and 2.8% of all pregnancies in developed countries and many more in developing countries, leading to as many as 8,370,000 cases worldwide per year.1 This common disorder, which is more prevalent in first pregnancies, is associated with the highest maternal and fetal morbidity and mortality of all pregnancy complications, with >90% of the most serious outcomes occurring in developing countries.1 According to the criteria of the International Society of the Study of Hypertension in Pregnancy, the preferred definition is a diagnosis of pregnancy-induced hypertension (diastolic blood pressure >90 mm Hg) occurring after week 20 of gestation with proteinuria (either ≥300 mg protein per day or an urinary protein/creatinine ratio ≥30 mg/mmol).2 When patients have liver dysfunction, thrombocytopenia, and hemolysis, they are classified as having HELLP syndrome (ie, hemolysis, elevated liver enzymes, low platelets).3 Although the definitions focus on these simply measured clinical parameters, preeclampsia must be recognized as a multisystem disorder, which variably may affect the brain, lungs, kidney, and liver.

The risk of preeclampsia markedly increases in women with previous preeclampsia and in those with either preexisting vascular disease or conditions associated with increased cardiovascular risk, including renal disease, hypertension, diabetes, thrombophilia, and obesity (body mass index >29).4 Additionally, occurrence in first-degree relatives increases the risk. Thus preeclampsia also has a hereditary origin, but inheritance does not follow simple Mendelian characteristics and a single “preeclampsia gene” is unlikely. Despite intensive effort, associations with polymorphisms of likely candidate genes, mostly associated with cardiovascular disease, have been weak, inconsistent, or negative.5 Women who have had preeclampsia are also at greater risk for cardiovascular disease in later life,6,7 and pregnancy, itself a transient state of the metabolic syndrome, is considered to represent a “stress test” that unmasks latent cardiovascular risk.7

The cause of preeclampsia remains largely unknown, but poor placentation is an important predisposing factor. The proposed “2-stage model”8 in which reduced placental perfusion (stage 1) leads to the maternal syndrome (stage 2) is likely to provide a simplified, yet largely accurate, description of the origin of severe early-onset disease, but may be less relevant for later-onset milder disease.9 The proposed role of the placenta in the pathology of preeclampsia is also strongly supported by the rapid resolution of symptoms after delivery. Although there is clearly a focal role for placental dysfunction in preeclampsia, a number of theories are proposed to explain how this may be associated with the maternal syndrome.10–12 A pivotal role of enhanced placental superoxide generation leading to oxidative stress is increasingly recognized.10,13 Deleterious effects of free radicals include initiation of lipid peroxidation, oxidative damage of biomolecules, and cellular dysfunction, and it is proposed that these may initiate maternal vascular endothelial dysfunction.
and leukocyte activation, recognized features of this disorder. This review focuses on recent investigations into oxidative stress and its relevance to the cause and prevention of preeclampsia.

Placenta and Oxidative Stress in Preeclampsia

Is the Placenta the Origin of Free Radical Synthesis?

Arguably, the most important event during normal placental development is establishment of an effective maternal circulation, a process that is inextricably linked with the physiological conversion of the spiral arteries from highly tortuous and thick-walled vessels to flaccid sinusoidal conduits of low resistance. Failure of spiral artery remodeling in the placental bed of pregnancies affected by preeclampsia was first demonstrated by Brosens et al and later associated with a partial failure of placental trophoblast invasion. These observations are fundamental to the current theory of preeclampsia. More recently, it was shown in normal pregnancy that at 10 to 12 weeks’ gestation, the onset of maternal blood flow in the placenta results in a local increase in oxygen tension and parallel elevation in expression and activity of several antioxidant enzymes. The authors hypothesized that a putative diminution of the antioxidant response to this oxygenation stimulus could result in oxidative stress that may lead to trophoblast degeneration and possibly contribute to impairment of trophoblast invasion and diminished remodeling of the spiral arteries. A defective response to an oxidant stimulus could therefore be one of the earliest events in preeclampsia.

Whatever the cause of impaired trophoblast invasion, the resultant inadequacy of placental perfusion is likely to result in oxidative stress by the following potential mechanisms. Maintenance of the muscular coat of the spiral artery may lead to intermittent placental perfusion, because the spiral arteries would retain susceptibility to maternal humoral and neuronal constrictor influences. Together with frequent thrombotic occlusion followed by clot dissolution, this may lead to a repeated hypoxia/reoxygenation insult in the affected placenta throughout pregnancy. Hypoxia/reoxygenation is a potent stimulus to the activation of xanthine oxidase, an important source of superoxide generation, which is abundantly expressed in cytotrophoblast, syncytiotrophoblast, and villous stromal cells. As might be anticipated, placental tissue from women with preeclampsia demonstrates enhanced expression and activity of this enzyme. Thus xanthine oxidase is likely to play a fundamental role in free radical-induced tissue damage in the human placenta. In support of this, Hung et al have shown that in vitro hypoxia/reoxygenation in normal third trimester placenta leads to free radical-induced tissue damage as evidenced by nitrotyrosine staining in trophoblast and activation of apoptotic pathways, both of which were preventable by addition of a free radical scavenger. Their study also supports the suggestion that as a result of underperfusion, aponecrotic processes could lead to deportation of syncytiotrophoblast microvesicles into the maternal circulation. These microvesicles, normally present in the circulation in pregnancy, have been shown to increase in preeclampsia and have been directly linked to activation of maternal neutrophils, which in turn may contribute to activation of the vascular endothelium (Figure 1).

Role for NAD(P)H Oxidase

NAD(P)H oxidases are a major source of superoxide in neutrophils and vascular endothelial cells (for review see Reference 21) and have also been reported in human trophoblast. The neutrophil and nonphagocytic NAD(P)H oxidase isoforms are closely related, because many of the subunits of the enzyme complexes are homologous and share common mechanisms of activation. In contrast to an absence of basal synthesis and the extracellular release of superoxide genera-
tion in the neutrophil respiratory burst, NAD(P)H oxidase isoforms found in nonphagocytic cells show a low basal intracellular synthesis of superoxide that is increased on activation.\textsuperscript{21} Potential stimuli for activation of NAD(P)H oxidase in preeclampsia include raised feto-placental vascular shear stress,\textsuperscript{23} elevation of maternal plasma cytokine concentrations,\textsuperscript{11} and enhanced angiotensin II (Ang II) sensitivity.\textsuperscript{24} Sustained activation of NAD(P)H oxidase may, therefore, impact on the pathogenesis of preeclampsia. Studies from our laboratory (R.D.) have recently demonstrated increased expression of NAD(P)H oxidase subunits (ie, p22phox, p47phox, and p67phox) in both trophoblast and placental vascular smooth muscle cells in plasental tissue of women with preeclampsia.\textsuperscript{25} In another study, we (M.R., L.P.) have also reported higher placent al NAD(P)H oxidase activity in women with early-onset preeclampsia as compared with those with late-onset of disease,\textsuperscript{26} which accords with the suggestion that early-onset preeclampsia is more dependent on placent al dysfunction than the later-onset disease.\textsuperscript{9} In addition, a novel mechanism for activation of NAD(P)H oxidase has been proposed because the serum from preeclamptic women has recently been shown to have a high concentration of an IgG autoantibody that binds to the AT\textsubscript{1} receptor (AT\textsubscript{1}R-AA) in a stimulatory fashion.\textsuperscript{27,28} This may lead to shallow trophoblast invasion\textsuperscript{28} or to sustained activation of the epidermal growth factor receptor or by the presence of a positive feedback loop, whereby Ang II increases reactive oxygen species formation that subsequently activates the epidermal growth factor receptor.\textsuperscript{27} The addition of either Ang II or AT\textsubscript{1}R-AA to a primary culture of trophoblast or vascular smooth muscle led to induction of NAD(P)H oxidase mediated superoxide generation,\textsuperscript{25} and this response was inhibited after pretreatment with antisense, but not sense, oligonucleotides against p22phox. In response to both agonists, the expression of several subunits of the NAD(P)H oxidase (p22phox, p47phox, and p67phox) was enhanced. These experiments have clearly shown that NAD(P)H oxidase is principally involved in Ang II and AT\textsubscript{1}R-AA-induced reactive oxygen species generation. Thus there is considerable evidence to implicate activation of NAD(P)H oxidase in plasental oxidative stress associated with preeclampsia.

**Demonstration of Oxidative Stress in Preeclampsia**

**Placenta**

Given the suggested involvement of xanthine oxidase and NAD(P)H oxidase, the demonstration that placent al tissue of women with preeclampsia synthesizes abnormal quantities of superoxide is not unexpected.\textsuperscript{25,29,30} Numerous independent studies assessing oxidative damage biomarkers have also indicated placent al oxidative stress. Several report higher placent al levels of markers for lipid peroxidation, including the F\textsubscript{2}-isoprostanes,\textsuperscript{31–33} nitrotyrosine and 4-hydroxynonenal staining,\textsuperscript{34} oxidant protein damage,\textsuperscript{35} or oxidizing potential,\textsuperscript{36} and others have demonstrated higher production and secretion rates of F\textsubscript{2}-isoprostanes in vitro when compared with placentas from normal pregnancies.\textsuperscript{33} Placent al antioxidan t capacity, shown by either vitamin E concentrations or the expression/activity of antioxidant enzymes, has been reported to be decreased in preeclampsia.\textsuperscript{37,38} In contrast, some studies have described higher glutathione levels,\textsuperscript{31,39} glutathione peroxidase enzyme activity,\textsuperscript{39} or catalase enzyme activity\textsuperscript{38} in placentas from women with severe preeclampsia, which could be explicable by free radical-mediated gene transcription of the relevant genes.\textsuperscript{40}

**The Maternal Circulation**

Placental oxidative stress may directly or indirectly lead to oxidative stress in the maternal circulation. As described, free radical generation may induce shedding of placent al “debris,” and in vitro evidence suggests that syncytiotrophoblast microvesicles may lead to activation of maternal neutrophils.\textsuperscript{11} Maternal neutrophils may also be locally activated during passage of maternal blood through the placenta. Release of lipid peroxides into the maternal circulation and cytokine synthesis from activated neutrophils could contribute to maternal endothelial cell activation, to subsequent leukocyte adhesion, and hence to further neutrophil activation (Figure 1). Isolated neutrophils from women with preeclampsia synthesize more superoxide on activation with either receptor-mediated (N-formyl-methionyl-leucyl-phenylalanine) or nonreceptor-mediated (phorbol 12-myristate 13-acetate) stimuli than those of normotensive pregnant women,\textsuperscript{41–43} and this is mediated by NAD(P)H oxidase.\textsuperscript{44}

The fatty acid profile in women with preeclampsia may also predispose to oxidative stress. Serum free fatty acids, triglycerides, and very-low-density lipoprotein concentrations are elevated, whereas concentrations of cholesterol, lipoprotein(a), and the other lipoproteins\textsuperscript{45,46} are unchanged. The low-density lipoprotein particles are smaller than those of normotensive controls, which may facilitate their oxidation.\textsuperscript{47,48} One of the first biomarkers of lipid peroxidation found to be elevated in the plasma of women with preeclampsia was malondialdehyde (MDA), a major metabolite of lipid peroxide breakdown.\textsuperscript{49} Numerous subsequent studies have strengthened the evidence for lipid peroxidation, using a variety of relevant assays including thiobarbituric reactive substance, conjugated dienes, F\textsubscript{2}-isoprostanes (usually 8-epi-prostaglandin F\textsubscript{2α}), and antibodies against oxidatively modified low-density lipoprotein, as extensively reviewed.\textsuperscript{10,13} Amino acid side-chains of proteins may also be modified by direct oxidative attack or by lipid peroxidation products, resulting in the formation of additional carbonyl groups. These, too, are elevated in plasma from women with preeclampsia.\textsuperscript{50} A few isolated studies, however, are not supportive of a role for oxidative stress in the maternal circulation. Two found no differences in markers of lipid peroxidation in either urine (the isoprostane 8,12-epi-IPF\textsubscript{2α}) or plasma (8-epi-prostaglandin F\textsubscript{2α}) and MDA\textsuperscript{52}. Another, although describing similar serum MDA concentrations in women with preeclampsia to controls, has nonetheless reported a higher MDA/total antioxidant capacity ratio in women with preeclampsia, which, as the authors suggested, is indicative of oxidative stress.\textsuperscript{53}

Besides the determination of oxidative damage, many investigations have evaluated antioxidant capacity in the maternal circulation by the assessment of the total antioxidant...
capacity, the concentration of specific antioxidants, or the activity of antioxidant enzymes. The different methods used are not always comparable and do not necessarily provide a reliable overview of antioxidant status. This has led to some ambiguity in results but the consensus suggests that antioxidant capacity is decreased in the maternal circulation.\textsuperscript{13} Glutathione is widely recognized as a major intracellular water-soluble antioxidant, which has been analyzed in maternal blood using different methodologies.\textsuperscript{54} Women with preeclampsia have lower plasma glutathione concentrations\textsuperscript{55,56} and a lower erythrocyte glutathione:hemoglobin ratio when compared with normotensive control women.\textsuperscript{57} The altered lipid profile in preeclampsia is most likely to be responsible for the variable reports on plasma vitamin E concentrations, because vitamin E is transported by lipoproteins.\textsuperscript{58} Vitamin E concentrations, not adjusted for lipid profile, have been reported to be lower,\textsuperscript{59–61} similar,\textsuperscript{62,63} or increased.\textsuperscript{64,65} However, when corrected for the lipid profile, no differences have been found between women with preeclampsia and normotensive controls.\textsuperscript{52,66–68} In contrast to vitamin E, lower plasma vitamin C concentrations are consistently reported in women with preeclampsia.\textsuperscript{59–61,68}

In general, the combination of elevated lipid peroxidation markers and decreased antioxidant capacity provides a clear indication of the presence of oxidative stress. Because of the difficulties and variability in the methods of measurement, these different facets of oxidative stress should preferably be determined in the same patient population using multiple techniques of assessment.\textsuperscript{13} A recent study in which oxidative stress was investigated in maternal blood by a wide variety of methods showed evidence for oxidative stress by some (notably reduced vitamin C concentrations), but not all, methods used, leading to the conclusion of a mild state of oxidative stress, although the authors concur that locally high oxidant stress in the placenta could play an important role.\textsuperscript{69} Placental oxidative stress has recently convincingly been determined by a methodologically comprehensive study.\textsuperscript{70}

**Influence of Diet**

As soluble antioxidant levels are highly influenced by maternal dietary intake, dietary factors could play a role in preeclampsia. To date, only a few studies have addressed micronutrient intake in preeclampsia. Validated food questionnaires have been used only to assess dietary intake of vitamin C\textsuperscript{11} or vitamin E.\textsuperscript{64} One study has suggested that an intake of vitamin C below the recommended dietary allowance may double the preeclampsia risk, but there is no evidence for a similar effect for vitamin E. In view of the known synergy between vitamin C and E,\textsuperscript{72} impairment of vitamin E regeneration because of the low plasma vitamin C concentrations could compromise the free radical scavenging capacity of vitamin E in women with preeclampsia.

**Prophylaxis and Prediction of Preeclampsia**

Despite increased understanding of the cause of the syndrome, there is currently no accepted method of prevention of preeclampsia.\textsuperscript{73} A recent meta-analysis of low-dose aspirin indicates that a 19% reduction may be achievable, but some individual large trials have indicated a more modest effect.\textsuperscript{74} The evidence for oxidative stress, the newly recognized role for NAD(P)H oxidase, and the demonstration of an inflammatory state in preeclampsia offers a potential avenue of hope for development of new strategies. Some antioxidants not only detoxify free radicals but also are involved in redox-sensitive gene expression,\textsuperscript{78} inhibition of apoptosis,\textsuperscript{79} and may have anti-inflammatory properties.\textsuperscript{76,77} Importantly, vitamin E directly inhibits monocyte NAD(P)H oxidase activity by the inhibition of PKC, thereby preventing phosphorylation and translocation of p47phox.\textsuperscript{76} Because pathways of activation, including the PKC pathway, of the different isoforms are similar,\textsuperscript{21} vitamin E might be expected to also inhibit the vascular and placental isoforms. This multimode of action may be fortuitous in relation to preeclampsia prevention, because vitamin E may not only inhibit the lipid peroxidation chain reaction but also could minimize the excessive generation of free radicals by inhibition of NAD(P)H oxidase in both placental tissue and maternal neutrophils (Figure 1), reduce placental apoptosis, and inhibit leukocyte and endothelial cell activation. The potential benefit of this antioxidant through an anti-inflammatory mode of action is supported by the studies of Takacs et al, which have shown that the activation of NF-κB and the upregulation of IL-6 and intercellular adhesion molecule-1 in cultured human umbilical venous endothelial cells by preeclamptic plasma are inhibitible by addition of vitamin E.\textsuperscript{77,78}

To date, 3 placebo-controlled studies have investigated the potential value of antioxidant supplementation in preeclampsia. When antioxidants were administered to women with established preeclampsia, no benefit was apparent.\textsuperscript{79,80} In contrast, in a study from our unit (L.P.), treatment from early pregnancy (16 to 22 weeks) until delivery with a combination of 1000 mg vitamin C and 400 IU vitamin E daily in a high-risk population reduced the incidence of preeclampsia by >50%.\textsuperscript{81} Risk was assessed on the basis of an abnormal uterine artery waveform or by preeclampsia in the previous pregnancy. In the same study, we showed that antioxidant treatment led to prevention of lipid peroxidation, as assessed by measurement of the isoprostane, 8-epi-prostaglandin-F\textsubscript{2α}, as well as reducing concentrations of biomarkers of placental and endothelial dysfunction.\textsuperscript{68,82} Because lipid peroxidation progressively increases with gestational age, even in pregnancies with normal outcome,\textsuperscript{83} early intervention may have a positive effect on pregnancy outcome (Figure 2).

The use of α-tocopherol and vitamin C, either alone or in combination, has been reported in several clinical studies evaluating potential benefit in cardiovascular disease, and these have been disappointingly negative. Although supplementation enhanced plasma vitamin status, meta-analysis has failed to show a beneficial effect of the use of vitamin C and/or vitamin E in the amelioration of pre-existing cardiovascular disease.\textsuperscript{84} However, most of these studies have been performed in subjects with established disease and undetermined or normal vitamin baseline values. In contrast, an investigation in which subjects with low baseline levels of vitamin C were treated with vitamins C and E has shown benefit in the protection against arteriosclerosis progression.\textsuperscript{85} Primary antioxidant intervention has been proven to be effective in the prevention of transplant-associated arterio-
sclerosis and endothelial dysfunction in hyperlipidemic children. Because preeclampsia is recognized as an inflammatory state in which women at risk have low plasma vitamin C and raised 8-epi-prostaglandin F2α concentrations, affected women may similarly benefit from a combination of vitamin C and E. Multicenter trials in the United Kingdom, the United States, Canada, and 3 developing countries are now underway and will determine whether antioxidant prophylaxis may be used routinely in prevention of preeclampsia.

If early prophylaxis with antioxidants proves to be beneficial, then reliable prediction of preeclampsia in low-risk and higher-risk populations will be essential to target the intervention to those most likely to benefit. At present, there is no test that provides clinically useful sensitivity and specificity. The determination of a single biochemical marker or hemodynamic variable is unlikely to provide adequate predictive power as preeclampsia is undoubtedly of multifactorial origin. The best predictive test at present involves assessment of the uterine artery Doppler waveform but is of inadequate accuracy for general clinical use. Future approaches could include the use of a combination of relevant biochemical markers, as previously suggested in a study from our group (L.P.), in which we reported that a combination of markers for endothelial and placental function (PAI-1/PAI-2 ratio, leptin, placental growth factor) could be used to develop predictive algorithms with sensitivities ranging from 53% to 80% and specificities of 88% to 100%. The advent of new technology, such as fast throughput proteomics and advances in bioinformatics, also pave the way for improvement of preeclampsia prediction.

Perspectives
Preeclampsia is an inflammatory state characterized by maternal endothelial and leukocyte activation. Placental oxidative stress is likely to play a pivotal role in the maternal disorder. The newly recognized role for the involvement of NAD(P)H oxidase in the placenta represents a significant advance in our understanding of the disease. A combination of vitamins C and E, which act in synergy to prevent lipid peroxidation, may be effective in the prevention of preeclampsia. Moreover, the newly recognized anti-inflammatory properties of vitamin E may be particularly efficacious, because the multimode of action includes pathophysiological pathways associated with activation of NAD(P)H oxidase.

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