Preeclampsia is a pregnancy-specific disorder in humans, characterized by hypertension and proteinuria occurring after the 20th week of gestation. These symptoms resolve after delivery, suggesting that the placenta plays a central role in the pathogenesis of this disorder. It is generally agreed that poor invasion of the uterine spiral arteries by the trophoblast leads to an ischemic placenta that subsequently releases a number of circulating factors into the maternal circulation. The factors released into the maternal circulation include a number of vasoactive molecules and proinflammatory cytokines, which can potentially cause dysfunction of the maternal endothelium. Such factors can induce the endothelial cells to generate excess of oxygen-derived free radicals, resulting in the development of oxidative stress.

One of the early changes that may occur as a result of endothelial injury in the uterine spiral arteries is the accumulation of neutral lipids, a phenomenon called “acute atherosis” of pregnancy. Whether lipid accumulation occurs in the maternal systemic vasculature and, if so, the possible mechanisms involved remain unknown. Several studies have shown increased serum levels of triglycerides, low-density lipoproteins (LDLs), and lipid peroxides in women with preeclampsia when compared with normotensive pregnant women. In addition, small dense LDL is also increased in the plasma of women with preeclampsia. Small dense LDLs are more susceptible to oxidation, resulting in the generation of oxidized LDL (oxLDL). OxLDL can bind to the lectin-like oxidized LDL receptor-1 (LOX-1) on endothelial cells. LOX-1 is a type II membrane protein cell surface receptor identified on endothelial cells, vascular smooth muscle cells, and monocyte macrophages.
rotic lesions in humans and has also been shown to be elevated in hypertensive rats. LOX-1 is responsible for the binding, uptake, and degradation of oxLDL. During this process, the binding of oxLDL activates the NADPH oxidase enzyme system, resulting in the excessive generation of superoxide. Scavenging of NO by superoxide may not only reduce NO bioavailability but also generate a more potent oxidant, peroxynitrite. Peroxynitrite formation has been observed in both the placenta and the maternal vasculature of women with preeclampsia. One recent study has observed elevated LOX-1 expression in the placenta of women with preeclampsia. However, the expression, regulation, and significance of LOX-1 in the maternal systemic vasculature of preeclampsia remain unknown.

We hypothesized that LOX-1 is upregulated in small resistance-sized arteries obtained from women with preeclampsia. We further hypothesized that the circulating factors in the plasma of women with preeclampsia via the formation of peroxynitrite provide a feed-forward loop to upregulate LOX-1.

Methods

Subjects

Pregnant subjects were recruited at the time of delivery, and nonpregnant subjects were recruited at the time of abdominal surgeries at the Royal Alexandra Hospital (Edmonton, Canada). The protocols were approved by the University of Alberta Ethics Committee, and the studies were conducted according to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All of the subjects provided informed consent before inclusion in the study. Twelve subjects had preeclampsia characterized by the de novo onset of hypertension and proteinuria after the 20th week of gestation. Hypertension was defined as a blood pressure of >140/90 mm Hg on 2 occasions 6 hours apart and proteinuria of >500 mg in a 24-hour urine collection or more than 2+ on a dip stick. Normal pregnant subjects (n = 12) were normotensive throughout pregnancy. Nonpregnant subjects (n = 12) were also normotensive. None of the subjects had a past history of chronic hypertension, renal, liver, or other metabolic diseases and were not on any medications. Either blood samples at the time of admission or fat biopsies during surgeries were collected from these women as detailed below. Blood was collected by routine forearm venipuncture at the time of admission (before delivery) in tubes containing EDTA. Blood samples were immediately centrifuged at 2000g for 20 minutes and then aliquoted under sterile conditions and stored at −80°C. Omental fat biopsies were obtained at the time of cesarean section for normotensive pregnant and preeclamptic women or during abdominal surgeries for nonpregnant women who were admitted for indications such as dysfunctional uterine bleeding, ovarian cyst, menorrhagia, and dysmenorrhea and then snap frozen in liquid nitrogen and stored at −80°C. The patient characteristics are shown on Tables S1 and S2 (see online data supplement at http://hyper.ahajournals.org). In the nonpregnant group, all of the subjects were white, nonsmokers, not previously pregnant, and not on any medications.

Immunohistochemistry

Our first aim was to compare the expression of LOX-1 and to detect the presence of oxLDL in small arteries from nonpregnant, pregnant, and preeclamptic women. Omental fat biopsies were flash frozen in liquid nitrogen and stored at −80°C. Later they were cut into ~0.5-cm diameter in size and frozen in optimal cutting temperature compound, cut into 8-μm sections, mounted on glass slides at −25°C, and stored at −80°C until use. All of the arteries in the section were counted. The slides were immunostained using rabbit polyclonal antibodies for LOX-1 (1:100; Santa Cruz Biotechnologies) and oxLDL (1:100; Calbiochem). Antibody secondary antibody (1: 200; Alexa fluor 488, Invitrogen) was used to detect the primary antibody and was visualized using a fluorescein isothiocyanate filter.

To address the mechanisms of LOX-1 upregulation, we performed a bioassay by observing the effects of plasma from the 3 groups of women on endothelial cells in culture. Human umbilical vein endothelial cells were treated with 2% plasma from nonpregnant, pregnant, and preeclamptic women for 24 hours. Individual plasma samples but not pooled plasma from each group were used in this study. In response to treatment with plasma, DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate)-labeled oxLDL uptake, intracellular superoxide levels using dihydroethidium, NADPH oxidase activity by lucigenin chemiluminescence, and nitrotyrosine staining as a marker of peroxynitrite formation were measured. In some experiments, cells treated with plasma were pretreated with 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (II), chloride (FeTPPS; 5 μmol/L), a peroxynitrite scavenger; monoclonal antibody to LOX-1 (mAbLOX-1; 10 μg/mL); or small interfering RNA (siRNA; 30 nmol/L) to LOX-1 for 6 hours. LOX-1 protein and mRNA expressions were assessed by Western blot and PCR, respectively. Please see the online data supplement for detailed methods used in this study.

Statistical Analysis

Values are expressed as means ± SEMs. Comparison of ≥3 groups was done using a 1-way ANOVA followed by a Tukey’s posthoc test. Comparison of 2 groups was conducted using a Student t test. A P value of <0.05 was deemed significant.

Results

Arterial Expression of LOX-1 and OxLDL

We detected abundant LOX-1 expression (P < 0.001) in arteries from women with preeclampsia (15.00 ± 6.50 arbitrary units [AU]), whereas there was negligible expression in arteries from nonpregnant (0.18 ± 0.09 AU) and pregnant women (0.25 ± 0.22 AU; Figure 1). In arteries from preeclamptic women, LOX-1 expression was localized to both the endothelium and the vascular smooth muscle cells. However, LOX-1 expression is greater in the endothelial layer.

Because LOX-1 is involved in the binding and uptake of oxLDL, we performed immunohistochemistry to identify the presence of oxLDL in these arteries. OxLDL was present only in arteries from women with preeclampsia (6.00 ± 0.50 AU; P < 0.001) but not in arteries from nonpregnant and pregnant women (0.07 ± 0.02 and 0.07 ± 0.06 AU; Figure 1). Also, OxLDL appears to accumulate immediately beneath the endothelial layer.

Endothelial Response to Plasma

LOX-1 Expression in Response to Plasma

LOX-1 expression was significantly increased in endothelial cells exposed to plasma from women with preeclampsia (0.419 ± 0.018 AU; P < 0.01) in comparison with cells treated with plasma from nonpregnant and pregnant women (0.145 ± 0.0039 and 0.1930 ± 0.0053 AU), respectively (Figure 2).
DiI-Labeled OxLDL Uptake
We observed significantly increased oxLDL uptake by endothelial cells in response to treatment with preeclamptic plasma when compared with treatment with nonpregnant and pregnant plasma. This uptake of oxLDL was significantly reduced by competition with unlabeled oxLDL and mAbLOX-1 (Figure 3).

NADPH Oxidase Activity
NADPH oxidase activity was significantly increased in endothelial cells treated for 24 hours with plasma from women with preeclampsia (53.92±1.40 AU/mg of protein; P<0.01) when compared with treatment with plasma from nonpregnant and pregnant women (31.37±1.10 and 29.60±1.20; AU/mg of protein), respectively (Figure 4A). LOX-1 blockade with mAbLOX-1 caused a marked reduction in NADPH oxidase activity only in the preeclamptic group (24.70±1.05 AU/mg of protein; P<0.01) but did not significantly affect NADPH oxidase activity in the nonpregnant and pregnant groups (26.58±1.50 and 26.10±1.30 AU/mg of protein, respectively). An isoimmune IgG control did not affect the NADPH oxidase activity in endothelial cells in response to preeclamptic plasma. These results were also confirmed by using siRNA to LOX-1, which reduced preeclamptic plasma-induced NADPH oxidase activity to 25.90±0.50 AU/mg of protein (Figure 4B).

In a separate set of experiments, LOX-1 expression and NADPH oxidase activity in endothelial cells in response to plasma were assessed at a time point (6 hours) before LOX-1 expression was increased. Although LOX-1 expression did not change at 6 hours (data not shown), NADPH oxidase activity was increased significantly (P<0.05) in the pre-

Figure 2. LOX-1 protein expression. A representative Western blot for LOX-1 expression from endothelial cells treated for 24 hours with 2% plasma from nonpregnant, pregnant, and pre-eclamptic women. Summary graph showing densitometric analysis of LOX-1 expression normalized to tubulin from 6 samples in each group. Bars represent means±SEs. Different letters denote significant difference (P<0.05) from each other.

Figure 3. Dil-labeled oxLDL uptake. A representative image showing endothelial cells incubated with DiI-labeled oxLDL (10 μg/mL) for the last 3 hours of a 24-hour treatment period with plasma from (A) nonpregnant, (B) pregnant, and (C) pre-eclamptic women and (D) competition with excess unlabeled oxLDL (50 μg/mL) in the presence of preeclamptic plasma. Six samples of plasma from each group were used.
preeclampsia for 24 hours in the presence or absence of mAbLOX-1 (10 μg/mL) or siRNA LOX-1 (30 nmol/L) and their respective controls, nonimmune IgG (10 μg/mL) and control siRNA (50 nmol/L). Different letters denote significant difference (P<0.05) from each other.

Figure 4. NADPH oxidase activity assay. A, Summary graph showing NADPH oxidase activity from endothelial cells treated for 24 hours in the presence or absence of mAbLOX-1 (10 μg/mL) with 2% plasma from nonpregnant, pregnant, and preeclamptic women. B, Summary graph showing NADPH oxidase activity from cells treated with 2% plasma from women with preeclampsia for 24 hours in the presence or absence of mAbLOX-1 (10 μg/mL) or siRNA LOX-1 (30 nmol/L) and their respective controls, nonimmune IgG (10 μg/mL) and control siRNA (50 nmol/L). Different letters denote significant difference (P<0.05) from each other.

Eclamptic group as early as 6 hours (11.21±1.00 AU/mg of protein) in comparison with nonpregnant (8.30±1.00 AU/mg of protein) and pregnant (8.10±1.00 AU/mg of protein) groups. Furthermore, mAbLOX-1 also reduced this increase in NADPH oxidase activity (8.40±1.00 AU/mg of protein; P<0.05) in response to preeclamptic plasma. These results suggest that increased plasma levels of ligands to LOX-1 are responsible for this early increase in NADPH oxidase activity, although at a later time point (24 hours), the increased NADPH oxidase was likely attributable to increases in both LOX-1 expression and the higher levels of ligands in the preeclamptic plasma.

Superoxide Detection in Live Cells
Endothelial cells treated with plasma from women with preeclampsia generated significantly high levels of superoxide (1.309±0.032 AU; P<0.01) when compared with treatment with plasma from nonpregnant (0.574±0.027 AU) and pregnant (0.265±0.019 AU) women (Figure 5). This increase in superoxide generation in response to preeclamptic plasma was reduced by pretreatment with κ-carrageenan (0.445±0.018 AU), a nonspecific LOX-1 blocker; mAbLOX-1 (0.649±0.023 AU), a specific monoclonal blocking antibody to LOX-1; and NADPH oxidase inhibitors apocynin (0.629±0.017 AU) and diphenylene iodonium chloride (0.0676±0.027 AU). Endothelial cells treated with superoxide dismutase were used as a negative control (data not shown).

Detection of Nitrotyrosine as a Marker of Peroxynitrite
Endothelial cells treated with plasma from women with preeclampsia (18.769±4.022 AU; P<0.01) showed enhanced nitrotyrosine formation when compared with endothelial cells treated with plasma from nonpregnant and pregnant women (1.016±0.132 and 0.722±0.106 AU), respectively (Figure 6). Also, mAbLOX-1 and siRNA to LOX-1 reduced preeclamptic plasma–induced nitrotyrosine levels significantly (10.347±0.580 and 9.633±0.639 AU, respectively; P<0.05). Also, FeTPPS almost completely abolished (0.762±0.051 AU) the generation of superoxide by endothelial cells in response to plasma from women with preeclampsia.

Effect of Peroxynitrite on LOX-1 Expression
Having observed increased peroxynitrite generation in endothelial cells treated with plasma from women with preeclampsia, we sought to determine whether peroxynitrite plays a role in the upregulation of LOX-1 in response to plasma. LOX-1 expression was assessed by Western blot, in response to preeclamptic plasma in the presence of FeTPPS, a peroxynitrite scavenger. Interestingly, FeTPPS, significantly reduced LOX-1 expression by 30% (P<0.05), suggesting that peroxynitrite may play a role in inducing LOX-1 in preeclampsia (Figure 7).

In a separate series of experiments, we observed that exogenous peroxynitrite induced a modest but significant increase in LOX-1 protein expression by 40% (Figure 8A). This was also confirmed by using SIN-1, an agent that generates endogenous peroxynitrite by increasing both NO and superoxide production. SIN-1 also increased LOX-1 protein expression (Figure 8B; P<0.05). We also determined whether peroxynitrite can upregulate LOX-1 mRNA. We assessed LOX-1 mRNA expression in response to peroxynitrite or SIN-1. Both peroxynitrite and SIN-1 induced a 1.5-fold (P<0.05) increase in LOX-1 mRNA expression in 6 hours (Figure 8C and 8D).

Discussion
In women with preeclampsia, there is evidence for focal accumulation of lipid-laden macrophages in decidual vessels and accumulation of neutral lipids in uterine spiral arteries. These phenomena have been termed “acute atherosclerosis,” which is analogous to atherosclerosis. However, reports of such vascular abnormalities in the maternal systemic vasculature have not been described. In the present study, we report for the first time the accumulation of oxLDL and increased LOX-1 expression in the maternal vasculature of women with preeclampsia. Our data also indicate that per-
Oxynitrite generated secondary to LOX-1 upregulation, in turn, provides a feed-forward loop to further increase LOX-1 in preeclampsia. In women destined to develop preeclampsia, the symptoms continue to progress until delivery, suggesting a feed-forward mechanism of vascular endothelial dysfunction that could be possibly mediated by peroxynitrite through LOX-1 pathway.

Preeclampsia is characterized by hyperlipidemia, including alterations in LDL. For instance, small dense LDLs are significantly elevated in the circulation of women with preeclampsia. These small dense LDL particles are more atherogenic and are more susceptible for oxidative modification, resulting in the formation of oxLDL. OxLDL is immunogenic and subsequently results in the formation of autoantibodies to oxLDL in the circulation. Thus, circulating autoantibodies to oxLDL have also provided evidence for the presence of oxLDL. Data from a previous study has shown increased levels of autoantibodies to oxLDL in women with preeclampsia. In contrast, however, other studies have reported no change in levels of autoantibodies to oxLDL in the plasma of women with preeclampsia. In addition, another recent study has shown decreased plasma levels of oxLDL in preeclamptic women, which the authors suggested could be attributed to

Figure 5. Live cell dihydroethidine staining for superoxide. Representative image showing superoxide production by live endothelial cells in response to treatment with 2% plasma for 24 hours from (A) nonpregnant, (B) pregnant, or (C) preeclamptic women. Additionally, (D) mAbLOX-1 (10 μg/mL), (E) κ-carrageenan (KC; 250 μmol/L), or (F) apocynin (AP; 30 μmol/L) was added in the presence of preeclamptic plasma. G, Summary graph showing live cell superoxide production in response to plasma from 6 subjects in each group. Bars represent means±SEs. Different letters denote significant difference (P<0.05) from each other.

Figure 6. Nitrotyrosine staining as a marker of peroxynitrite. Representative figure showing nitrotyrosine staining (green) in endothelial cells treated with 2% plasma for 24 hours from (A) nonpregnant, (B) pregnant, and (C) preeclamptic women. The effect of (D) mAbLOX-1 (10 μg/mL), (E) siRNA LOX-1 (30 nmol/L), and (F) FeTPPS (5 μmol/L) in the presence of plasma from women with preeclampsia is shown. Nuclei stained with Hoechst 33342 are shown in blue. G, Graph showing mean fluorescence intensity of nitrotyrosine staining from 6 subjects per group. Bars represent means±SEs. Different letters denote significant difference (P<0.05) from each other.

Figure 7. Effect of FeTPPS on LOX-1 expression. A, A representative Western blot for LOX-1 expression from endothelial cells treated with 2% plasma from nonpregnant, pregnant, and preeclamptic women for 24 hours. Some groups of cells were pretreated with FeTPPS before incubation with plasma from women with preeclampsia. B, Summary graph showing densitometric analysis of LOX-1 expression normalized to tubulin from 6 samples in each group. Different letters denote significant difference (P<0.05) from each other.
Binding of oxLDL to LOX-1 could activate the NADPH oxidase enzyme system to generate superoxide. We demonstrated increased NADPH oxidase activity in cultured endothelial cells in response only to plasma from women with preeclampsia, which was significantly reduced by blocking with mAbLOX-1 or siRNA to LOX-1. This suggests that ligands to LOX-1, possibly oxLDL, play a role in upregulating the NADPH oxidase enzyme system specifically in preeclampsia. To further address whether the increased NADPH oxidase activity and the observed increase in superoxide levels were attributable not only to increased LOX-1 expression but also to increased levels of ligands circulating in the plasma, we examined LOX-1 protein expression and NADPH oxidase activity at an earlier time point (6 hours) before LOX-1 expression was induced. We found that, despite normal LOX-1 expression in response to plasma from 3 groups of women at 6 hours, NADPH oxidase activity was increased significantly only in the preeclampsia group, suggesting that initial increases in superoxide levels might stem from increased circulating levels of ligands to LOX-1. Long-term increases in NADPH oxidase and subsequent superoxide levels could be a combined effect of both increases in oxLDL levels and increased LOX-1 expression, as seen in our 24-hour experimental protocol.

In this study, we have also shown a novel pathway for the regulation of LOX-1 by peroxynitrite. Peroxynitrite increased both LOX-1 mRNA and protein expression. Furthermore, we have demonstrated a feed-forward loop by which peroxynitrite further upregulates and maintains a higher LOX-1 expression. Thus, it appears that generation of peroxynitrite through LOX-1 further upregulates LOX-1 and may be a key player in perpetuating oxidative stress in preeclampsia. Indeed, blocking LOX-1 in endothelial cells in response to preeclamptic plasma significantly reduced superoxide and peroxynitrite levels.

Other than peroxynitrite, LOX-1 can be upregulated by a number of factors in the plasma, such as tumor-necrosis factor-α, transforming growth factor-β, oxLDL, angiotensin II, endothelin I, C-reactive protein, and 8-isoprostanote, to mention a few. Many of these factors have been shown to be elevated in the plasma of women with preeclampsia; thus, the upregulation of LOX-1 and activation of NADPH oxidase could be through the action of multiple factors. In our experiments, mAbLOX-1 reduced NADPH oxidase activity by >50%, suggesting that this receptor could be a major factor for inducing oxidative stress in preeclampsia. Moreover, apocynin, an NADPH oxidase inhibitor, reduced superoxide generation in response to preeclamptic plasma to the same extent as mAbLOX-1, suggesting that most of the NADPH oxidase activity in preeclampsia could be through LOX-1, as demonstrated in this bioassay. This does not exclude the role of other factors in activating the NADPH oxidase enzyme system, because mAbLOX-1 did not completely suppress NADPH oxidase activity. Importantly, apart from oxLDL, there is an array of structurally different, negatively charged molecules, such as polyanionic chemicals (polyinosinic acid and carrageenan), anionic phospholipids (phosphatidylserine and phosphatidylinositol), and cellular
ligands, such as apoptotic/aged cells, activated platelets, and bacteria, that can act as ligands for LOX-1.27,28 Although this study did not focus on the ligands, but on the receptor itself, it is possible that multiple factors could be involved in the activation of LOX-1, thus having broad implications for a common mechanism for vascular dysfunction in women with preeclampsia.

Because of the heterogeneity of preeclampsia, different circulating factors could play a role in different women or during different stages of the disease process. Nonetheless, LOX-1 pathway appears to be a predominant pathway in inducing cellular oxidative stress in response to circulating factors in the plasma of women with preeclampsia. Moreover, because the effect of a number of circulating factors converges on LOX-1 pathway, LOX-1 could be a potential target for therapeutic intervention.

Finally, preeclampsia is often considered the extreme of a pregnancy continuum, with evidence of inflammation and oxidative stress increased in pregnancy when compared with the nonpregnant state.29 However, in our study, the responses in the nonpregnant and the pregnant groups were similar in most of the outcome measures in the vasculature and in isolated endothelial cells. However, the levels of superoxide were in fact reduced in endothelial cells treated with pregnant plasma relative to nonpregnant plasma. Thus preeclampsia, in part, could be a lack of adaptation to pregnancy, in addition to circulating factors that activate the endothelium.

**Perspectives**

Preeclampsia is likely a multifactorial disorder, with inflammation, oxidative stress, immune mechanisms, and other pathways playing a role. Although a number of studies have clearly shown evidence for vascular oxidative stress in preeclampsia,14,30–32 the antioxidant trials with vitamins C and E have failed to reduce the incidence of preeclampsia33 and in some cases have even been detrimental by increasing the rate of low birth weight babies.34 These antioxidants are designed to scavenge oxidants and not to inhibit generation of such molecules. Furthermore, they would not provide the first line of defense in scavenging intracellular superoxide. In light of such evidence, identification of pathways, such as LOX-1, that could be blocked may prove to be more effective in reducing intracellular oxidative stress. Our study clearly suggests that LOX-1 pathway could be a major pathway involved in promoting and maintaining a vicious cycle of events resulting in oxidative stress and ultimately leading to endothelial cell dysfunction in preeclampsia.

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**Disclosures**

None.

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Increased Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Expression in the Maternal Vasculature of Women With Preeclampsia: Role for Peroxynitrite
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INCREASED LOX-1 RECEPTOR EXPRESSION IN THE MATERNAL VASCULATURE OF WOMEN WITH PREECLAMPSIA: ROLE FOR PEROXYNITRITE

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Materials

Medium M199, Heparin, Fetal Bovine Serum, NADPH, Lucigenin, Native LDL, SIN-1, κ-carrageenan, apocynin were purchased from Sigma (Oakville, ON, Canada). Q Media M199 (without phenol red), Hanks Balanced Salt Solution (HBSS), were purchased from GIBCO and Dihydroethidium (DHE), DiI, Hoescht 33342, fluorescent secondary antibodies were purchased from Molecular Probes (Missisauga, ON, Canada). SIN-1 and peroxynitrite were purchased from Cayman Chemicals (Ann Arbor, MI, USA). siPORT™ NeoFX and siRNA were purchased from Ambion Inc (Streetsville, ON, Canada).

HUVEC Culture

For our study, we selected human umbilical vein endothelial cells (HUVECs) because they are of human origin, easily available, and although of fetal origin, they are in a pregnant milieu. Importantly, our pilot study showed LOX-1 receptor expression in this cell type which makes these cells an appropriate tool for our study. We examined the effects of 2% plasma from the three groups of women on these endothelial cells as this concentration of plasma minimized the cytotoxic effects while maximizing differences between groups. Furthermore, we used plasma, but not serum to avoid the confounding effects of cellular products released into serum during blood coagulation.

Umbilical cords were obtained from normotensive pregnancies and endothelial cells were isolated as previously described. Cells were grown on 0.1% gelatin-coated dishes in M199 with 1% endothelial cell growth supplement, heparin, and 20% fetal bovine serum (FBS) and used at passages 2–4. Before stimulation human umbilical vein endothelial cells were plated in six-well plates and incubated in M199 medium (without phenol red) containing 1% FBS and no endothelial cell growth supplement. At the end of each experiment, cellular protein extracts were prepared or total cellular RNA was extracted.

Experimental Protocol

To compare the effect of plasma from three groups of women on LOX-1 receptor protein expression, HUVECs grown in 6 well plates were treated with 2% plasma from non-pregnant, pregnant and preeclamptic women for 24h and LOX-1 receptor protein expression was determined by Western blot. Individual plasma samples from each of these women were assessed and not the pooled plasma from each group was used. To determine the contribution of peroxynitrite generated in response to plasma on LOX-1 receptor expression, FeTPPS (5 µmol/L) a specific peroxynitrite scavenger was added 30 min prior to plasma treatment. To further confirm the effects of peroxynitrite, we
conducted experiments using exogenous peroxynitrite. To determine the optimal dose of peroxynitrite, preliminary studies were conducted using 25, 50, 100 µmol/L peroxynitrite (Cayman Chemicals, Ann Arbor, MI) for 6, 12 and 24h and cells were stimulated with a dose of 25 µmol/L for 6h. Incubation with higher doses or for longer periods of time resulted in cell death. Exogenous peroxynitrite, used in these experiments has a very short half life at physiological pH. We therefore, have also used SIN-1 (Cayman Chemicals, Ann Arbor, MI), a peroxynitrite donor. SIN-1 at physiological pH releases both nitric oxide and superoxide within the cell, which combine together to form peroxynitrite. Similarly, after conducting preliminary studies with 0.25, 0.50 and 1 mmol/L SIN-1 for 6, 12 and 24 hours, a dose of 0.25 mmol/L for 6h was chosen. To determine if the LOX-1 receptor expression can be regulated at the transcriptional level, LOX-1 receptor mRNA expression was also examined in response to treatment with peroxynitrite and SIN-1 for 6h using RT-PCR.

Since the LOX-1 receptor is responsible for the binding and uptake of oxLDL, we next compared the uptake of oxLDL in cells treated with plasma. Cells grown in 48 well plates were quiesced using phenol-red free media (Q-media) with 5% v/v lipoprotein deficient serum. The cells were then treated with plasma for 24h. During the last three hours of treatment, Dil-oxLDL (10 µg/ml) was added in the presence or absence of 50 µg/ml unlabelled oxLDL (for competition) or mAbLOX-1 (10 µg/ml) as appropriate. The mAbLOX-1 was a kind gift from Dr. Sawamura, Osaka, Japan.

Since the binding of oxLDL can activate the NADPH oxidase enzyme system, we compared the NADPH oxidase activity in HUVECs treated with plasma from three groups of women for 24h in the presence or absence of monoclonal antibody to the LOX-1 receptor (mAbLOX-1, 10 µg/ml) or siRNA to the LOX-1 receptor. In some experiments, a non-immune IgG was used to confirm the specificity of the mAbLOX-1.

The activation of NADPH oxidase leads to the generation of superoxide. We therefore, measured superoxide generation in HUVECs grown in 48 well plates in response to plasma treatment from three groups of women for 24h. Cells exposed to plasma from preeclamptic women were also pre-treated with k-carrageenan (250 µmol/L) a non-specific LOX-1 blocker, mAbLOX-1 (10 µg/ml), apocynin (30 µmol/L) or diphenylene iodonium chloride (DPI, 10 µmol/L).

Excess superoxide generated can combine with nitric oxide to generate peroxynitrite. Peroxynitrite further nitrates tyrosine residues in several proteins resulting in the formation of nitrotyrosine. We therefore, treated cells with plasma from three groups of women for 24h in the presence or absence of mAbLOX-1(10 µg/ml) or siRNA to the LOX-1 receptor and performed immunocytochemistry to detect nitrotyrosine.

**Western Blot analysis for LOX-1**

After HUVECs were treated with plasma, peroxynitrite or SIN-1, cells were washed in ice-cold phosphate buffered saline, and lysed using lysis buffer (25 mmol/L Tris-HCl pH 7.5 with 0.5% Triton X-100) and sonicated for ~5 s. Protein content was determined using bicinchoninic acid reagent. 10 µg of the protein was loaded on to a 10% gel for LOX-1. The gel was run at 120 V and the protein was transferred onto a nitrocellulose membrane, blocked with 5% fat free milk and the membranes were probed with primary antibodies for LOX-1 (rabbit anti-human) (1:200 Santa Cruz...
Biotechnology), overnight at 4°C. The primary antibody was then detected with a peroxidase-conjugated anti-rabbit secondary antibody (1:5,000). Membranes were scanned with Fluor S Max Multimager (Bio-Rad) and the densitometric analysis was conducted using the software.

Uptake of Dil-labeled oxLDL

Oxidation of LDL

LDL was purchased from Sigma Inc., as lyophilized protein 5mg in 150 mM NaCl and 0.01% EDTA. It was reconstituted using 5ml of sterile distilled water (to get a conc of 1mg/ml). After reconstitution, the LDL was dialyzed using 1 L autoclaved PBS for 24 hours at 4 °C changing the dialysis buffer (PBS) every 6 hours. The LDL was oxidized under aerobic conditions with 5µmol/L CuSO4 for 24h at 37 °C.10 The reaction was stopped with 1mmol/L EDTA and the oxidized LDL was kept at 4 °C in dark. The oxidized LDL thus prepared was dialysed to remove CuSO4 and EDTA.11 The dialysis solution consisted of (in mmol/L) NaCl – 140, NaH2PO4 – 1.9, Na2HPO4 – 8.1, EDTA – 0.1.

Labeling of oxLDL with Dil

Labeling of oxLDL with Dil was carried out according to the method of Reynolds and St.Clair.12 Both oxLDL and lipoprotein deficient serum were dialyzed against 1L of autoclaved 0.9%NaCl for 24h at 4 °C in dark, changing the dialysis solution every 6h, before labeling with Dil.13 Ox LDL (1 mg/ml) lipoprotein deficient serum (2.5 mg/ml) were mixed in a ratio of 1:2 (vol:vol) to give a protein concentration ratio of 1:5 (wt:wt). 150µg Dil/mg oxLDL protein was gently mixed and incubated at 37 °C for 18h in dark under anaerobic conditions. The density of the mixture was adjusted to 1.080 g/ml with solid KBr and overlayed with 4ml of 1.063 g/ml KBr solution. The labeled oxLDL was isolated by centrifugation at 41500 rpm (160831*gav) for 24h at 4 °C using a SW 50 rotor in Beckman L8-M ultracentrifuge. The top 3ml containing Dil-labeled oxLDL was pipetted out and dialysed extensively against autoclaved 0.9% NaCl + 0.01% EDTA for 24h at 4 °C in dark and stored in dark at 4 °C until use. Before use in cell culture experiments the Dil-oxLDL was dialyze in autoclaved normal saline at 4 °C for 24h in dark.

NADPH Oxidase Activity

The NADPH oxidase activity assay was performed by modification of a previously published method.14 After treatment with plasma, cells were washed in ice-cold Dulbecco’s phosphate-buffered saline, scraped off in lysis buffer containing 20 mmol/L KH2PO4, 1 mmol/L EGTA, and protease inhibitors, pH 7.4. The cells were sonicated briefly, and the lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in cell homogenates. The reaction was started by the addition of NAD(P)H (0.1 mmol/L) to the suspension (250 µL final volume) containing sample (50 µL), lucigenin (5 µmol/L), and phosphate buffer containing (in mmol/L) KH2PO4 -50, EGTA -1, sucrose -150, pH 7.4. Luminescence was measured
every 4 seconds for 3 minutes in a luminometer. Buffer blank was subtracted from each reading. Activity was expressed as arbitrary units/mg protein.

**Superoxide Imaging**

Superoxide generated in response to plasma treatment was detected using dihydroethidium (DHE). DHE is a cell permeable dye which exhibits blue fluorescence in the cytosol. However, once this probe is oxidized to ethidium, it intercalates within the cell's DNA, staining its nucleus and emits a bright red fluorescence. During the last 40 minutes of plasma treatment, DHE (20 µmol/L final concentration) was added and incubated in the dark at room temperature. This time point and concentration of DHE was chosen based on preliminary studies. The cells were washed off thrice with Hank’s Balanced Salt Solution containing calcium and magnesium. Imaging for superoxide was performed in live cells using a Olympus IX81 fluorescence microscope using a CY3 filter. Image analysis was conducted using Adobe Photoshop. The total intensity due to DHE in a field was divided by the number of cells in that field to obtain the mean fluorescence intensity and reported as arbitrary units.

**Immunocytochemistry**

After treatment with plasma for 24h cells were washed with ice-cold PBS and fixed with 10% formalin phosphate. After washing, the cells were blocked with 5% BSA in PBS for 3 h and incubated with primary antibody (rabbit polyclonal anti-nitrotyrosine antibody 1:200) overnight at 4°C. After washing, a FITC conjugated anti rabbit secondary antibody (Alexa Fluor 488 1: 200) was added and washed off. Nuclear counter staining using Hoechst 33342 (0.5 µg/ml) (Molecular Probes) was performed. Images were taken using a fluorescence microscope (Olympus IX 81) using the FITC and DAPI filters.

**Total RNA Isolation**

After treatment with peroxynitrite or SIN-1, cells were washed twice with ice-cold PBS. RNA was isolated using Trizol reagent. The RNA concentration and the quality were determined by measuring the absorbency at 260nm and 260/280nm ratio using a NanoSspek.

**Analysis of mRNA expression**

cDNA was synthesized by incubating total cellular RNA with random primers and dNTP at 65°C for 5 min and then by incubating the mixture with reverse transcription buffer. The cDNA obtained was amplified using LOX-1 primers (10 uM) (5'-TTACTCTCCATGGTGC-3') (5'-AGCTTCTTCTDCTTGTG-3') and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-CCCTCATTGACCTCAACTACATGG-3') (5'-AGTCTTCTGGGTGCGATGG-3'), was used as internal standard in the PCR reaction mixture. 15 193-base pair human LOX-1 cDNA fragment and 456-base pair human GAPDH cDNA fragments were amplified for 40 and 30 cycles, respectively. The PCR product was then subjected to
electrophoresis on a 1% agarose gel containing ethidium bromide. The bands were imaged and analyzed using Fluor Multimager (Bio-Rad).

**siRNA for LOX-1**

We used siPORT NeoFX® (Ambion) Transfection agent to transfect siRNA against LOX-1 into HUVECs. HUVECs were transfected with siRNA to LOX-1 by a process called Reverse Transfection that involves simultaneously transfecting and plating cells. Cells grown in T-75 flask were trypsinized and diluted in normal growth medium. siPORT NeoFX transfection agent was diluted in OPTI-MEM I medium (1:20) and incubated at room temperature for 10 min. siRNA was diluted in OPTI-MEM I medium. Preliminary studies were conducted using varying concentration of siRNA and a concentration of 30nM was chosen. The diluted siPORT NeoFX transfection agent and the diluted siRNA were mixed and incubated at room temperature for 10 min. Then, the mixture was dispensed into the cell culture plates and the cell suspensions were overlayed onto the transfection complexes and grown as usual.

**References**


Table S1.

Characteristics of subjects from whom fat biopsies were obtained

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-Pregnant</th>
<th>Pregnant</th>
<th>Preeclamptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>33.3 ± 2.5</td>
<td>29.6 ± 1.5</td>
<td>28.4 ± 2.0</td>
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<tr>
<td>Prepregnant weight (kg)</td>
<td>71.6 ± 3.2</td>
<td>67.69 ± 5.77</td>
<td>76.34 ± 8.0</td>
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<tr>
<td>Body Mass Index (kg/m²)</td>
<td>26.69 ± 1.7</td>
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<td>29.88 ± 1.72</td>
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<td>Term BP</td>
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<td>Systolic BP</td>
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<td>112.1 ± 1.0</td>
<td>155.7 ± 6.7†,*</td>
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<td>Diastolic BP</td>
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<td>72.1 ± 1.5</td>
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<tr>
<td>Proteinuria</td>
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<td>NA</td>
<td>2.9 ± 0.3</td>
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<tr>
<td>Gestational age at delivery (wk)</td>
<td>NA</td>
<td>38.9 ± 0.24</td>
<td>33.2 ± 2.0*</td>
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<tr>
<td>Infant birth weight (g)</td>
<td>NA</td>
<td>3356 ± 148.3</td>
<td>1986 ± 270.8*</td>
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The characteristics of subjects, 6 from each group of women are shown in the table. Data are shown as mean ± SEM. † denotes P<0.01 vs. non-pregnant women; * denotes P<0.01 vs. pregnant women.
Table S2.

Characteristics of subjects from whom plasma samples were obtained

<table>
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<tr>
<th>Characterisitics</th>
<th>Non-Pregnant</th>
<th>Pregnant</th>
<th>Preeclamptic</th>
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</thead>
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<tr>
<td>Maternal age (y)</td>
<td>28.4 ± 2.0</td>
<td>27.6 ± 1.5</td>
<td>30.6 ± 4.3</td>
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<td>Prepregnant weight (kg)</td>
<td>59.4 ± 2.8</td>
<td>60.6 ± 4.6</td>
<td>62.6 ± 4.3</td>
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<tr>
<td>Body Mass Index (kg/m²)</td>
<td>27.42 ± 2.37</td>
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<tr>
<td>Term BP</td>
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<tr>
<td>Systolic BP</td>
<td>115.6 ± 3.6</td>
<td>106.2 ± 5.4</td>
<td>148 ± 3.5†,*</td>
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<tr>
<td>Diastolic BP</td>
<td>78.8 ± 0.8</td>
<td>64.2 ± 1.8</td>
<td>101.0 ± 4.0†,*</td>
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<tr>
<td>Parity</td>
<td>1.0 ± 0.0</td>
<td>1.8 ± 0.2</td>
<td>1.4 ± 0.4</td>
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<tr>
<td>Proteinuria</td>
<td>NA</td>
<td>NA</td>
<td>3+</td>
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<tr>
<td>Gestational age at delivery (wk)</td>
<td>NA</td>
<td>38.4 ± 0.2</td>
<td>32.0 ± 2.2*</td>
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<tr>
<td>Infant birth weight (g)</td>
<td>NA</td>
<td>3196 ± 119.7</td>
<td>2017 ± 210.6*</td>
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</tbody>
</table>

The characteristics of subjects, 6 from each group of women are shown in the table. Data are shown as mean ± SEM. † denotes $P<0.01$ vs. non-pregnant women; * denotes $P<0.01$ vs. pregnant women.