Lectin-Like Oxidized Low-Density Lipoprotein 1 Receptor in a Reduced Uteroplacental Perfusion Pressure Rat Model of Preeclampsia


Abstract—Preeclampsia is a major cause of maternal and fetal morbidity and mortality that has been associated with endothelial dysfunction attributed, in part, to dyslipidemia, an imbalance in angiogenic factors and oxidative stress. One of the many factors that have been shown to be elevated in women with preeclampsia is low-density lipoprotein (LDL) and the more oxidizable, small dense LDL, which can lead to increased vascular oxidative stress and decreased bioavailability of NO. Lectin-like oxidized LDL-1 receptor (LOX-1) is a specific receptor for oxidized LDL. We hypothesized that a reduction of placental perfusion using a rat model of reduced uteroplacental perfusion pressure would result in enhanced LOX-1 expression in the maternal vasculature causing impaired vascular endothelial function through the actions of increased superoxide production and decreased NO-mediated vasodilation. We demonstrated a significant increase in the expression of the LOX-1 receptor (4.3-fold; P=0.002), endothelial NO synthase (2.7-fold; P=0.001), and superoxide (P=0.02) in thoracic aorta of the reduced uteroplacental perfusion pressure model, whereas maximal vasodilator function was modestly decreased (P<0.05). Endothelial-dependent vasodilator function was unaffected by either oxidized LDL or an LOX-1 receptor inhibitor in controls but was modestly increased in the presence of both oxidized LDL and the LOX-1 receptor inhibitor in reduced uteroplacental perfusion pressure (P=0.03). In summary, we have shown that, in a rat model of preeclampsia, there is a dramatic increase in the expression levels of both the LOX-1 receptor and the endothelial NO synthase enzyme, along with evidence of increased superoxide production and subsequent modestly decreased endothelial function. (Hypertension. 2012;59:00.)

Key Words: preeclampsia ■ vascular function ■ LOX-1 receptor ■ oxidized LDL ■ RUPP

Preeclampsia is one of the most enduring and inadequately understood pathologies of pregnancy, which affects 5% to 8% of pregnancies and is a major cause of maternal and fetal morbidity and mortality.1,2 This almost uniquely human disorder is characterized by de novo hypertension >140/90 mm Hg after the 20th week of gestation accompanied by proteinuria more than +1. Although the diagnosis is reasonably clear, the underlying causes remain obscure, likely because of the extreme complexity of this disorder. Interestingly, the only currently known cure for preeclampsia is delivery of the placenta and, hence, delivery of the baby, suggesting a central role for this organ. It is thought that an underperfused placenta is responsible for the release of placental debris and multiple factors, which, in turn, causes the maternal syndrome.3,4 Indeed, preeclampsia has been associated with both increased systemic vascular resistance and endothelial dysfunction.

One of the many factors that have been shown to be elevated in women with preeclampsia is low-density lipoprotein (LDL) and the more oxidizable, small dense LDL.5–10 Combined with a significant volume of evidence for increased vascular oxidative stress in preeclampsia (reviewed in References 2 and 4), this suggests a greater capacity for the formation of oxidized LDL (oxLDL) in the vasculature of these women, as has actually been demonstrated in previous studies.11,12 In addition, our group has recently demonstrated both increased oxLDL and expression of its receptor, the lectin-like oxidized LDL receptor 1 (LOX-1), in the vasculature of women with preeclampsia.13 However, it is not known whether the pregnancy complication, such as reduced placental perfusion, caused an increased LOX-1 expression or whether women with preeclampsia had a predisposition for enhanced LOX-1 expression. Activation of the LOX-1 receptor, which can occur by a number of factors, such as oxLDL, activated platelets, or neutrophils, leads to the formation of a complex with the membrane-type 1 matrix metalloproteinase and subsequent
activation of RhoA and Rac1. This then causes activation of NADPH oxidase, which has several downstream effects, reaction of NADPH with oxygen to produce superoxide and reduction of endothelial NO synthase (eNOS) activity (reviewed in References 15 and 16). Superoxide is a known scavenger of NO, a reaction that produces the reactive oxygen species peroxynitrite. Combined with a reduction in the production of NO by eNOS, there is the potential for a significant impact on endothelial function attributed to a lack of bioavailable NO. However, the role of LOX-1 on vascular function in preeclampsia is not known. We hypothesized that a reduction of placental perfusion using an established rat model would result in enhanced LOX-1 expression in the maternal vasculature causing impaired vascular endothelial function through the actions of increased superoxide production and decreased NO-mediated vasodilation.

Methods

Ethical Approval
All of the protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee, in accordance with the Canadian Council on Animal Care guidelines.

Reduced Uteroplacental Perfusion Pressure Model of Preeclampsia
A major difficulty in studying a disorder such as preeclampsia lies in the specificity of the condition to the human and higher ape species. Many animal models have been created to reproduce particular aspects of preeclampsia, for instance, genetic manipulation of mice (NO synthase knock outs, transgenic mice, catechol-O-methyltransferase knockouts, or the BPH/5 mouse), pharmacologic induction (NO synthase blockade, angiogenic factors, tumor necrosis factor-α, Adria mycin, angiotensin I antagonists, insulin, deoxycorticosterone acetate, or endothxin), or mechanical manipulation through reduced uteroplacental perfusion. Of the last category, one of the most well characterized is the rat model of reduced uteroplacental perfusion pressure (RUPP). This model has the advantage of mimicking the central role of reduced placental perfusion and of producing a systemic, not pathway-specific, response. This model expresses many of the phenotypic characteristics of preeclampsia in humans, such as hypertension, proteinuria, fetal growth restriction, increased total peripheral resistance, decreased cardiac output, increased renal pressure natriuresis, and renal plasma flow, and, therefore, was chosen for our studies.

Three-month-old female Sprague-Dawley rats (Charles River, Wilmington, MA) were maintained on ad libitum standard rat chow and tap water in a 12:12-hour light:dark cycle. Females were acclimatized in house before breeding. Day 0 of pregnancy was determined by the presence of sperm in a vaginal smear after an overnight introduction of a male. On day 14 of pregnancy, rats were anesthetized by inhaled isoflurane (4% induction, 1% to 3% maintenance) and the abdominal cavity opened by a midline incision. The animal was then anesthetized by isoflurane and a PTFE #30 catheter inserted into the right carotid artery (n = 9 per group). Blood pressure was monitored and recorded via an attached pressure transducer (type 379, Hugo Sachs Elektronik, Harvard Apparatus). Heart rate was simultaneously monitored by ECG. The catheter was kept patent via a flush of heparinized saline. After a stable recording of blood pressure and heart rate for ≥ 10 minutes, animals were euthanized by exsanguination via excision of the superior vena cava.

Proteinuria
On day 13 before surgery and day 19 before experimental day), animals were placed into metabolic cages for the collection of 24-hour urine samples (n = 4–5). The volume of urine samples was recorded and aliquots were snap frozen in liquid nitrogen and stored at −80°C until analysis. Total urine albumin concentrations were measured using the AssayMax Rat Albumin ELISA kit (AssayPro). Urine creatinine was measured using the Creatinine (Urinary) Assay kit (Cayman Chemical Company). Proteinuria was quantified and expressed as the albumin:creatinine ratio.

Offspring Biometrics
After euthanasia, the uterine horns were externalized and the number of viable and reabsorbed fetuses counted. Fetal body weight, crown-rump length, abdominal girth, and placental weight of all of the viable offspring were measured (n = 12–20 litters).

Assessment of Kidney Endotheliosis
Kidneys were excised, bisected longitudinally, and fixed in Z-fix (buffered zinc formalin). Hematoxylin and eosin– and periodic acid-Schiff–stained slides of kidney tissues were prepared by the Alberta Diabetes Institute Histology Core (Edmonton, Alberta, Canada). All of the histological sections from kidneys were evaluated by a veterinary pathologist (R.R.E.U.) who was blinded to the study groups.

LOX-1 Receptor and eNOS Expression
The rat thoracic aorta was chosen as a model system in which to test our hypothesis because of previously published results demonstrating increased LOX-1 expression in this vascular bed in other cardiovascular conditions, such as atherosclerosis, diabetes mellitus, hypertension, and stress. In addition, we used this as a model system to specifically test the impact of LOX-1 on the NO pathway, which predominate in this vascular bed.

Sections of thoracic aortas were snap frozen in liquid nitrogen for subsequent analysis. Western blotting was performed on tissue homogenates using primary antibodies for eNOS (mouse monoclonal antibody: 1:250, BD Biosciences; n = 8–9) and LOX-1 (rabbit antibody: 1:250; Abcam). Antirabbit (IRDye 800 and IRDye 680: 1:20000; LI-COR), and antigoat (Alexa Fluor 700: 1:20000; Invitrogen) secondary antibodies were used. The protein bands were detected and quantified by a Li-Cor Odyssey version 3.0 imager system, and all of the data were expressed as a percentage increase over the corresponding untreated control.

Superoxide and Peroxynitrite Detection
Sections of thoracic aortas were embedded in optimal cutting medium (OCT) and snap-frozen in liquid nitrogen for subsequent analysis. Sections were cut at 20 μm, mounted on glass slides at −20°C, and stored at −80°C until use.

Superoxide generation was measured by staining with dihydroethidium (DHE; 20 μmol/L; n = 8–9). DHE is cell permeable and reacts with intracellular and extracellular superoxide to yield analyses. Morphine (2 mg/kg) was administered as a postsurgical analgesic.
ethidium, which binds to nDNA and generates nuclear fluorescence. Briefly, slides were thawed out, washed thrice with Hank balanced salt solution (containing calcium and magnesium), and incubated with Hank balanced salt solution for 10 minutes at 37°C. Subsequently, the respective dyes were added, incubated for 30 minutes at 37°C, washed to remove excess dye on the surface of the section, cover slipped, and imaged using an IX81 Olympus fluorescence microscope. Superoxide generation and DHE staining were measured as percentage increase over the control.

The footprint of peroxynitrite generation is the formation of nitrotyrosine residues. To detect nitrotyrosine, slides were prepared as above. Briefly, slides were then thawed, fixed in ice-cold acetone for 20 minutes, washed in PBS, and blocked for 1 hour in 1% BSA. Slides were then incubated overnight at 4°C with a primary antibody for nitrotyrosine (mouse anti-nitrotyrosine antibody: 1:150, Abcam; n=8 per group) and subsequently incubated for 1 hour with a goat antimouse secondary (Alexa Fluor 488: 1:250, Invitrogen). Slides were then mounted using a 4',6-diamidino-2-phenylindole containing mounting medium to stain for cell nuclei and cover slipped. Slides were imaged using an IX81 Olympus fluorescence microscope and peroxynitrite generation, nitrotyrosine staining, was measured as the percentage increase over the control.

### Wire Myography

A total of 13 sham and 10 RUPP animals were used for vascular function experiments. Thoracic aortas were isolated, cleaned of all surrounding adipose and connective tissues, and mounted on two 40-μm wires attached to a wire myograph (DMT, Copenhagen, Denmark) to allow isometric tension recordings. Vessels were stretched to 2-g tension over a series of adjustments in tension taking 10 minutes.

After a 30-minute equilibration period, vessels were twice exposed to a single dose of phenylephrine (10 μmol/L), followed by a single dose of methylcholine (MCh; 3 μmol/L) to check functional endothelial and smooth muscle integrity. A cumulative concentration response curve to phenylephrine was performed to determine the effective concentration producing 80% of the maximum response. Endothelial-dependent relaxation was assessed by determining a cumulative concentration response curve to MCh. To investigate the involvement of the LOX-1 receptor in vascular function, responses to MCh (0.003-3.000 μmol/L) were investigated after a 30-minute incubation with the anti-LOX-1 receptor antibody (TS20; 10 μg/mL) or a mouse IgG control (Sigma I5381; 10 μg/mL). The placlenta weight from RUPP pregnancies was unchanged (0.44±0.01 g RUPP versus 0.48±0.02 g sham).

### Statistical Analyses

Normality was tested using the Kolmogorov-Smirnov test for distributed data. Nonnormally distributed data included LOX-1 and eNOS expression, distributed data were presented as median (interquartile range), and P<0.05 was considered statistically significant.

### Results

#### Model Characteristics

RUPP surgery resulted in increased systolic (116±3 mmHg RUPP versus 104±4 mmHg sham; P=0.01) but not diastolic blood pressure (93±4 mmHg RUPP versus 84±5 mmHg sham). Although blood pressure was measured under anesthesia, the heart rate was not different (314±27 bpm RUPP versus 333±25 bpm sham), indicating a similar level of anesthesia in both groups.

RUPP surgery resulted in a decreased number of live births (4.20±0.71 RUPP versus 13.69±1.07 sham; P<0.0001). In addition, offspring tended to be lighter (3.38±0.08 g RUPP versus 3.59±0.07 g sham; P=0.058) and were asymmetrically growth restricted (crown-rump length:abdominal girth ratio: 1.05±0.02 RUPP versus 1.12±0.02 sham; P=0.022). Proteinuria, as determined by the urinary albumin:creatinine ratio in 24-hour urine samples, was unchanged by the sham surgery. In our hands, the RUPP surgery also did not cause an increase in proteinuria from presurgery levels, nor were proteinuria levels in RUPP animals significantly different from those in shams. However, RUPP pregnancies did exhibit modest changes in kidney glomerular morphology. In these rats, multifocal to segmental sections of the renal cortex had glomeruli that were slightly enlarged compared with sham and displayed mild-to-moderate swelling of endothelia cells that appeared to reduce capillary space (glomerular endotheliosis).

### LOX-1 Receptor and eNOS Expression in Thoracic Aortas

Expression of the LOX-1 receptor in the thoracic aorta was significantly increased in RUPP animals (~4.3-fold (P=0.002; Figure 2A). Expression levels of eNOS were also significantly increased 2.7-fold in RUPP thoracic aortas (P=0.001; Figure 1B).

### Superoxide and Peroxynitrite Levels in Thoracic Aortas

DHE staining revealed increased levels of the reactive oxygen species superoxide in the thoracic aortas from RUPP pregnancies (P=0.02; Figure 2A). Although levels of nitrotyrosine, the footprint of peroxynitrite generation, were not significantly increased in aortas from RUPP animals, a trend was observed (P=0.065; Figure 2B).

### Vascular Function

Responses to phenylephrine in the thoracic aortas were unaffected by RUPP surgeries (Emax: 1.74±0.11 g RUPP versus 1.58±0.17 g sham). Vasodilatation to MCh was modestly but significantly decreased (P<0.05) in RUPP animals (pEC50: 6.60±0.05 RUPP versus 6.73±0.05 sham; Emax: 95.34±2.92% RUPP versus 102.78±2.82% sham).

In sham animals, incubation of the thoracic aorta with the LOX-1 receptor ligand (oxLDL), the LOX-1 receptor inhibitor (anti-LOX-1 antibody), or a combination of the 2 did not affect MCh-induced vasodilatation (Table). In animals exposed to the RUPP surgery, neither oxLDL nor the anti–LOX-1 antibody alone affected MCh-induced vasodilatation (Figure 3A and 3B). However, the combination of oxLDL and the anti–LOX-1 antibody significantly enhanced MCh-induced vasodilatation (P=0.028; Figure 3C).

### Discussion

In our study, the RUPP model displayed many of the characteristics of the preeclamptic condition in humans, as...
has been demonstrated by other investigators.\textsuperscript{17,19–26,28,37,38} For instance, we verified increased systolic blood pressure, altered fetal biometrics, and glomerular endotheliosis in RUPP compared with sham-operated rats.

The use of this pregnant rat model with preeclamptic-like features led to a dramatic increase in the expression of the LOX-1 receptor in the thoracic aortas from these animals, indicating a causative role for reduced placental perfusion in

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1}
\caption{Expression levels of the lectin-like oxidized low-density lipoprotein 1 receptor (LOX-1) in the thoracic aorta from sham and reduced uteroplacental perfusion pressure (RUPP)--operated groups. \textbf{A}, LOX-1 receptor expression was increased 4.3-fold in RUPP vs sham. \textbf{B}, eNOS expression was increased 2.7-fold in RUPP vs sham. All of the data were normalized to actin and expressed as a percentage of sham levels. Representative blots from a single gel are shown. \textsuperscript{**}P<0.01 by t test.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2}
\caption{Dihydroethidium (DHE) and nitrotyrosine staining in the thoracic aorta from sham and reduced uteroplacental perfusion pressure (RUPP)--operated groups. \textbf{A}, DHE staining, as a marker of superoxide levels, was increased in RUPP vs sham. Representative images are shown; red fluorescence indicates nuclear fluorescence generated by the reaction of DHE with superoxide to yield ethidium. \textbf{B}, Nitrotyrosine, a footprint of peroxynitrite generation, was unchanged in RUPP vs sham (P=0.065, t test). Representative images are shown; green fluorescence indicates the presence of nitrotyrosine, blue fluorescence indicates 4',6-diamidino-2-phenylindole--stained nuclei. All of the data were given as a mean fluorescent intensity (MFI) and expressed as a percentage of sham levels. \textsuperscript{*}P<0.05 by t test.}
\end{figure}
the upregulation of this scavenger receptor. Because activation of the LOX-1 receptor is thought to lead to increased production of superoxide and a reduction in NO-mediated vasodilation, we measured both of these in RUPP and sham animals. Correspondingly, superoxide levels, as measured by DHE fluorescence, were significantly increased in RUPP versus sham animals. Interestingly, eNOS expression was also dramatically increased. We have shown previously both increased vascular nitrotyrosine and eNOS in women with preeclampsia compared with normal-pregnant or nonpregnant controls.39 These findings are consistent with a condition of increased oxidative stress in preeclampsia that may be triggered by increased LOX-1 receptor expression.

Given the consistent demonstration of an increased involvement of components of the LOX-1 receptor pathway, we further investigated the effects of this pathway on vascular function in RUPP animals. In contrast to previous studies demonstrating increased vasoconstrictor responses in uterine,29 thoracic,19,25 and mesenteric40 arteries, we observed no change in the thoracic aorta responses to phenylephrine. Increased levels of superoxide would be expected to lead to scavenging of the endothelium-dependent vasodilator NO to produce peroxynitrite. In our study, we did not demonstrate a significant increase in peroxynitrite levels in RUPP compared with sham-operated animals, although there was a trend in this direction. However, we did see a decreased endothelial function in the thoracic aorta, as observed by other investigators,19,20,23,24,29 although it was modest similar to the report by Crews et al.19 When the effect of stimulation of the LOX-1 receptor in the presence of the anti-LOX-1 antibody, there was a significant increase in vasodilator function. However, when this was combined with blockade of the LOX-1 receptor using the anti–LOX-1 receptor antibody, there was a significant increase in vasodilator function. This suggests that the LOX-1 receptor may be active endogenously but cannot be significantly activated by exogenous oxLDL. To verify that oxLDL was used at a sufficient level, we increased the concentration 5-fold and obtained the same results (data not shown).

Considering that the LOX-1 receptor is a scavenger receptor that may be activated by multiple ligands, it may be that the changes seen in preeclampsia require many of these ligands to work in a synergistic manner. Because the LOX-1 receptor was active after the RUPP surgery, as evidenced by the increase in vasodilation observed in the presence of LOX-1 receptor inhibition, this model appears to produce the correct

Table. Maximal Percentage of Vasodilator Responses of the Thoracic Aorta From the Sham-Operated Group to the Endothelium-Dependent Agonist MCh in the Absence or Presence of the LOX-1 Receptor Agonist (OxLDL) or Inhibitor (Anti–LOX-1 Receptor Antibody)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; ± SE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCh</td>
<td>96.34 ± 2.16% (9)</td>
</tr>
<tr>
<td>MCh + oxLDL</td>
<td>98.50 ± 1.59% (6)</td>
</tr>
<tr>
<td>MCh + antiLOX-1 ab</td>
<td>94.71 ± 2.36% (7)</td>
</tr>
<tr>
<td>MCh + oxLDL + antiLOX-1 ab</td>
<td>96.24 ± 3.08% (7)</td>
</tr>
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EMax indicates methylcholine; oxLDL, oxidized low-density lipoprotein; LOX, lectin-like oxLDL-1 receptor; ab, antibody.
in vivo environment for this activation to occur. Given the very low LOX-1 receptor expression and complete lack of an effect of either oxLDL or the anti–LOX-1 antibody in sham animals, it also appears that the LOX-1 receptor has a very low level of involvement in normal pregnancy situations and is only “switched on” in the pathological environment. Thus, the LOX-1 receptor is a potential downstream target for the heterogeneous pathophysiology of preeclampsia.

**Perspectives**

In summary, we have demonstrated that, in a rat model of preeclampsia, there is a dramatic increase in the expression levels of both the LOX-1 receptor and the eNOS enzyme. In addition, there was evidence of increased superoxide production and subsequent modestly decreased endothelial function. Whether the LOX-1 receptor would have a greater role to play in models that demonstrate more extensive endothelial dysfunction remains to be seen. In addition, determination of whether the LOX-1 receptor pathway holds any therapeutic potential will require additional studies into the role of this receptor in the progression of the disease.

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