Evidence for Increased Methylglyoxal in the Vasculature of Women With Preeclampsia
Role in Upregulation of LOX-1 and Arginase

Sowndramalingam Sankaralingam, Han Xu, Yanyan Jiang, Tatsuya Sawamura, Sandra T. Davidge

Abstract—Preeclampsia is characterized by vascular endothelial dysfunction partly attributed to oxidative stress. In the vasculature of preeclamptic women, we have shown increased lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) and arginase expression, which can contribute to vascular oxidative stress. However, the mechanisms of such upregulation are unknown. Methylglyoxal (MG) that plays a role in the vascular complications of diabetes mellitus and the development of hypertension can be one potential factor that can affect LOX-1 and arginase through its ability to induce oxidative stress in vascular cells. MG also reacts with lysine residues in proteins to generate advanced glycation end product, \( N^\epsilon \)-carboxy ethyl lysine, which also serves as a marker of MG. We hypothesized that markers of MG formation will be increased in the vasculature of preeclamptic women and that exogenous MG will induce oxidative stress by the upregulation of LOX-1 via arginase. We observed increased \( N^\epsilon \)-carboxy ethyl lysine expression in the vasculature of women with preeclampsia in comparison with normotensive pregnant women. Moreover, glyoxalase I and II, enzymes that detoxify MG, and glutathione reductase, which generates reduced glutathione, a cofactor for glyoxalase, are also reduced in preeclampsia. In cultured endothelial cells, MG increased arginase expression by 6 hours and LOX-1 expression by 24 hours. Inhibition of arginase or NO synthase significantly reduced MG-induced LOX-1 expression, superoxide levels, and nitrotyrosine staining. In conclusion, MG-induced LOX-1 expression is mediated via arginase upregulation likely because of uncoupling of NO synthase, which may have implications in preeclampsia. (Hypertension. 2009;54:00-00.)

Key Words: preeclampsia ■ methylglyoxal ■ LOX-1 ■ arginase ■ peroxynitrite ■ endothelium

Preeclampsia is a disorder of pregnancy characterized by hypertension and proteinuria occurring after the 20th week of gestation.\(^1\) A history of hypertension, diabetes mellitus, hyperlipidemia, obesity, renal disease, and extremes of age are some of the risk factors for the development of preeclampsia. Preeclampsia is also characterized by inflammatory, oxidative stress, and endothelial dysfunction.\(^2\) The maternal vascular endothelial dysfunction is thought to occur because of the actions of factors and molecules released by the placenta into the maternal circulation.\(^4\) Because diabetes mellitus is one of the risk factors for the development of preeclampsia, factors involved in vascular complications of diabetes mellitus may also influence the development and maintenance of vascular abnormalities and possibly hypertension in preeclampsia. One factor involved in the vascular complications of diabetes mellitus is methylglyoxal (MG).\(^5,6\) MG also plays a role in the development of hypertension in spontaneously hypertensive rats.\(^7,8\) Moreover, MG treatment resulted in increased blood pressure in Wistar Kyoto rats.\(^9\)

Other than increasing blood pressure, MG induces oxidative stress, resulting in the generation of reactive oxygen species and peroxynitrite in cultured vascular smooth muscle cells.\(^10,11\) MG is a highly reactive dicarbonyl compound formed by both enzymatic and nonenzymatic processes from glucose, proteins, and amino acids.\(^12\) The enzymatic sources of MG includes not only glucose but also fatty acids and proteins by the action of the enzymes, MG synthase, acetol-mono-oxygenase,\(^13\) and semicarbazide-sensitive amine oxidase (SSAO),\(^14\) respectively. MG is a highly reactive molecule that reacts with various amino acid residues in proteins to form advanced glycation end products (AGEs). Although AGEs can be formed from glyoxal as well, MG is the most reactive\(^15\) and most important precursor for AGEs.\(^6,16\) MG is degraded by the glyoxalase system.\(^17\) Glyoxalase I catalyzes the conversion of hemithioacetyl (an adduct of reduced glutathione [GSH] and MG) into S\(-\)lactoylglutathione, which is acted on by glyoxalase II to regenerate GSH. Glutathione peroxidase and glutathione reductase (GR) play roles in the formation and regeneration of GSH that serve to detoxify MG.
Whether MG is increased in the vasculature of women with preeclampsia and, if so, the mechanisms by which it affects endothelial function are unknown. We have shown previously in the vasculature of women with preeclampsia an enhanced expression of lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1).\textsuperscript{18} LOX-1 is responsible for the binding, uptake, and degradation of oxidized low-density lipoprotein (oxLDL).\textsuperscript{19} On activation via binding of oxLDL, LOX-1 generates superoxide via NADPH oxidase.\textsuperscript{20} Other than LOX-1, increased arginase expression can also result in the generation of reactive oxygen species.\textsuperscript{21} Increased arginase expression can deplete L-arginine\textsuperscript{22,23} as a substrate, which can result in the decreased synthesis of NO\textsuperscript{24} and the increased generation of superoxide by NO synthase (NOS).\textsuperscript{25,26} This phenomenon by which NOS generates superoxide because of deficiency of the substrate, L-arginine, or the cofactor tetrahydrobiopterin is known as uncoupling of NOS.\textsuperscript{25,26} Thus, overexpression of arginase in endothelial cells can lead to the uncoupling of NOS. Studies have shown increased arginase expression in both the placenta\textsuperscript{21} and the maternal vasculature of women preeclampsia\textsuperscript{27} and could contribute to decreased NO and enhanced superoxide formation. It is well known that MG can generate reactive oxygen species and peroxynitrite when added to vascular smooth muscle cells.\textsuperscript{10} However, it is not known whether MG can generate reactive oxygen species via the arginase/LOX-1 pathway and contribute to endothelial dysfunction and oxidative stress in preeclampsia.

We hypothesize that markers of MG formation will be elevated in the maternal vasculature of women with pre-eclampsia. Also, MG will upregulate LOX-1 expression and oxidative stress markers via upregulation of arginase in endothelial cells in culture.

### Methods

#### Subjects

Nonpregnant, pregnant, and preeclamptic subjects (6 per group) were recruited for this study at the Royal Alexandra Hospital (Edmonton, Alberta, Canada). Omental fat biopsies were obtained from these subjects during abdominal surgeries or cesarean section and stored at \(-80^\circ\text{C}\). For details of the inclusion and exclusion criteria and the subject characteristics (Table S1), please see the online Data Supplement, available at http://hyper.ahajournals.org.

#### Immunohistochemistry

**Experimental Protocol**

**Immunohistochemistry**

Our first aim was to assess the expression of AGEs, N\textsuperscript{\textasteriskcentered}carboxy methyl lysine (CML) and N\textsuperscript{\textasteriskcentered}carboxy ethyl lysine (CEL), that also serve as markers of MG in small arteries from nonpregnant, pregnant, and preeclamptic women. Although CML can be formed via a number of reactions, such as oxidative cleavage of Amadori products, Schiff base, auto-oxidation of glucose, and from MG, CEL is formed primarily from MG as its precursor.\textsuperscript{28} CEL levels also serve as an index of MG in tissues.\textsuperscript{29} We have, therefore, assessed both CML and CEL, which are considered markers of MG formation. The circulating concentrations of MG have been reported to vary from nanomoles per liter\textsuperscript{10} to micromoles per liter,\textsuperscript{32} the variations being attributed to analytic techniques.\textsuperscript{17} However, the tissue levels of MG may be higher and also more important, because MG tends to react rapidly and readily with proteins in tissues.\textsuperscript{32} We have, therefore, assessed markers of MG in tissues. The arteries in omental fat biopsies were immunostained using rabbit monoclonal antibodies for CML (1:100) and CEL (1:100). Antirabbit secondary antibody (1:200 Alexa Fluor 488, Invitrogen Canada, Inc) was used to detect the primary antibody and was visualized using a fluorescein isothiocyanate filter.

We further assessed the expression of enzymes that are involved in the synthesis and degradation/detoxification of MG. We chose to measure the expression of SSAO, because it is widely studied in relation to MG formation.\textsuperscript{14} We also assessed the expressions of glyoxalase I and II, enzymes that detoxify MG. In addition, we compared the expressions of glutathione peroxidase and GR, enzymes that generate GSH, a cofactor necessary for the detoxification of MG. Rabbit antibodies against human SSAO, glyoxalase I and II, as well as glutathione peroxidase and GR, were used at 1:100 dilution. Antirabbit secondary antibody (1:200 Alexa Fluor 488, Invitrogen Canada, Inc) was used to detect the primary antibody and was visualized using a fluorescein isothiocyanate filter.

#### Statistical Analysis

Values are expressed as mean±SEM. Comparison of \(\geq 3\) groups was done using a 1-way ANOVA followed by a Tukey post hoc test. A \(P<0.05\) was deemed significant.

#### Results

**Arterial Expression of CEL, CML, and SSAO**

We assessed the arterial expression of CEL and CML. Arteries from women with preeclampsia show significantly increased fluorescent staining for CEL and CML (26.7±3.5 and 22.6±3.1 arbitrary units; \(P<0.01\)), respectively, primarily in the endothelial layer when compared with that of nonpregnant (3.9±1.4 and 5.7±1.1 arbitrary units) and pregnant women (4.3±1.5 and 5.1±1.2 arbitrary units; Figure 1).
We also compared the expression of SSAO, an enzyme that generates MG. Interestingly, we observed an abundance of SSAO in arteries from women with preeclampsia when compared with arteries from nonpregnant and pregnant women (Figure 2).

Figure 1. Evidence for MG formation in preeclampsia. Representative images for CEL and CML staining (arrows) in the vasculature of nonpregnant (NON-PREG), pregnant (PREG), and preeclamptic (PE) women. Nuclei are shown in blue, whereas CEL and CML are shown in green. The images shown are magnified ×100.

Figure 2. SSAO expression in maternal vasculature. Representative images (×100) show SSAO expression in the vasculature of nonpregnant (NON-PREG), pregnant (PREG), and preeclamptic (PE) women. Nuclei are shown in blue and staining for SSAO is shown in green. The summary graph shows mean SSAO expression from 6 subjects per group. *P<0.05.

Arterial Expression of Glyoxalase I and II, GR, and Glutathione Peroxidase
We observed a significant decrease in the expression of glyoxalase I and II, the detoxifying enzymes in the vasculature of women with preeclampsia, when compared with arteries from normotensive pregnant women (Figure 3). Interestingly, GR that regenerates GSH, a cofactor necessary for the detoxification of MG, is reduced, whereas the expression of glutathione peroxidase, the enzyme that depletes GSH, is increased in the preeclamptic vasculature (Figure 4). These findings further strengthen the evidence for increased MG levels in the vasculature of women with preeclampsia.

Figure 3. Glyoxalase I and II expression in maternal vasculature. Representative figures (×100) for glyoxalase I (top) and II (bottom) expression in arteries from nonpregnant (NON-PREG), pregnant (PREG), and preeclamptic (PE) women. Nuclei are shown in blue and staining for glyoxalase I and II are shown in green. A summary graph from 6 subjects per group is shown. *P<0.05.

Endothelial Response to MG

Effect of MG on LOX-1 Expression
Endothelial cells treated with MG (0 to 1000 μmol/L) for 24 hours induced LOX-1 expression (≈100%, P<0.05) that was
maximal at a dose of 30 μmol/L (Figure 5A). In the next set of experiments, MG (30 μmol/L) induced ≈100% of the LOX-1 expression that was maximal at 24 hours (Figure 5B). Thus, MG increased LOX-1 expression in a dose- and time-dependent manner.

Effect of MG on Arginase Expression and Activity
Endothelial cells treated with MG (0 to 1000 μmol/L) for 6 hours showed significantly increased arginase II expression (≈70%; P<0.05) that was maximal at a dose of 30 μmol/L (Figure 6A). Interestingly, endothelial cells treated with 30 μmol/L of MG showed maximal expression as early as 6 hours (Figure 6B). Also, endothelial cells treated with MG (0 to 30 μmol/L) for 6 hours showed significantly increased arginase activity (0.053±0.020 to 0.133±0.020 U/mg of protein; P<0.05) in a dose-dependent manner that was maximal at 30 μmol/L.

Effect of Arginase and NOS Inhibition on MG-Induced Oxidative Stress
MG (30 μmol/L) treatment for 24 hours resulted in an increased generation of superoxide compared with untreated cells (62.3±5.7 versus 25.7±5.3; P<0.01) that was significantly reduced to similar levels after arginase (32.3±3.5) or NOS inhibition (29.4±2.9). In parallel to increased superoxide levels, nitrotyrosine staining was also increased by MG (30 μmol/L) treatment for 24 hours (78.28±4.90 versus 17.34±2.00; P<0.01) that was also reduced after arginase (38.13±3.70) and NOS inhibition (3.20±0.60).

Effect of Arginase and NOS Inhibition on MG-Induced LOX-1 Expression and 3,3′-Diocadecylindocarbocyanine–Labeled oxLDL Uptake
We wanted to determine whether arginase upregulation and, therefore, the subsequent generation of oxidative stress by...
NOS played a role in the upregulation of LOX-1 in response to MG. MG (30 μmol/L) treatment of endothelial cells for 24 hours upregulated LOX-1 expression by ≈100% (P<0.05), which was significantly reduced to control levels by arginase or NOS inhibition with BEC or L-NAME, respectively (P<0.05; Figure 7A). Because LOX-1 is involved in the binding and uptake of oxLDL, we also compared oxLDL uptake in response to MG using 3,3′-diododecylindocarbocyanine–labeled oxLDL (Figure S1 in the online Data Supplement). Treatment of endothelial cells with MG (30 μmol/L) for 24 hours significantly increased oxLDL binding and uptake that were prevented by arginase and NOS inhibition.

**Role of Peroxynitrite in MG-Induced LOX-1 Expression**

Because arginase upregulation can ultimately lead to peroxynitrite formation by NOS in response to MG, we wanted to determine whether peroxynitrite was involved in the upregulation of LOX-1 in response to MG. MG (30 μmol/L) treatment for 24 hours increased LOX-1 expression in endothelial cells that was significantly reduced to control levels by FeTPPS (peroxynitrite scavenger), confirming a role for peroxynitrite in MG-induced LOX-1 expression (Figure 7B).

**Discussion**

In this study, we demonstrate for the first time evidence for MG formation in the vasculature of women with preeclampsia. This is supported by increased levels of CML and CEL, which are markers of MG formation in the vasculature.
Furthermore, SSAO, the enzyme that generates MG, is increased in preeclampsia, whereas the expressions of the glyoxalase enzymes that degrade MG are reduced. Moreover, the expression of GR that generates GSH is also reduced. In fact, this is the first report of AGE formation in the vasculature of women with preeclampsia. We further show that MG by upregulates LOX-1 expression via arginase.

Overexpression of glyoxalase I has been shown to inhibit AGE formation in bovine endothelial cells. Our study demonstrates that decreased glyoxalase I and II expression in the vasculature of women with preeclampsia could have led to increased MG in the vasculature and, therefore, increased formation of CML and CEL. We also show an increased expression of SSAO in the vasculature of women with preeclampsia. Normally SSAO is involved in the deamination of aminoacetone but in the process generates MG. Thus, we have evidence for greater production and reduced degradation of MG in the vasculature of women with preeclampsia.

MG plays a role in the development of hypertension and oxidative stress. It is suggested that MG may induce hypertension via different mechanisms, such as increasing vascular contractility, generating reactive oxygen species, and proliferating vascular smooth muscle in resistance arteries leading to vascular remodeling, ultimately resulting in enhanced vasoconstriction and, therefore, hypertension. However, the role of MG in inducing hypertension and oxidative stress in preeclampsia is unknown. We have previously shown increased LOX-1 and arginase expressions in the maternal vasculature of women with preeclampsia, when compared with normotensive pregnant women. We, therefore, explored the possibility that MG can affect these pathways.

Here we show that MG could be one factor involved in the upregulation of arginase and LOX-1 in preeclamptic vasculature. In response to MG, arginase inhibition significantly reduced MG-induced LOX-1 expression, suggesting a role for arginase in the upregulation of LOX-1. Importantly, in response to MG, arginase upregulation is maximal as early as 6 hours, whereas LOX-1 upregulation is maximal at 24 hours. This suggests that products generated because of arginase upregulation early in the response to MG may play a role in the upregulation of LOX-1. It is well known that NOS can generate superoxide. Our data show that increased superoxide production in response to MG is inhibitable by BEC or L-NAME to a similar extent, suggesting a role for arginase and, therefore, for NOS in possibly mediating the generation of reactive oxygen species. Inhibition of superoxide production by L-NAME suggests a role for uncoupling of NOS as a source of superoxide in response to MG. In our study, we have used L-NAME as an inhibitor of all isoforms of NOS and, therefore, cannot identify the isoform of NOS that generates superoxide. Uncoupling of NOS can occur because of a deficiency of either L-arginine, the substrate, or tetrahydrobiopterin, the cofactor, depending on the isoform of NOS. Although all isoforms of NOS undergo uncoupling because of deficiency of tetrahydrobiopterin, it is primarily inducible NOS and neuronal NOS that undergo uncoupling because of L-arginine deficiency. In our study, we show that MG can also induce peroxynitrite formation in endothelial cells. It is possible that peroxynitrite can oxidize tetrahydrobiopterin and, thus, create deficiency leading to uncoupling of NOS. Thus, in addition to the direct effect of arginase upregulation on depleting L-arginine, peroxynitrite generated because of arginase upregulation can also cause uncoupling of NOS. However, further studies are needed to characterize the mechanisms of uncoupling of NOS attributed to upregulation of arginase in endothelial cells in response to MG. Because arginase inhibition with BEC reduced superoxide generation by endothelial cells in response to MG, it is reasonable to speculate that arginase could be a source of superoxide. Interestingly, MG has also been shown to inactivate human Cu, Zn superoxide dismutase that can also result in increased superoxide levels.

Our data show that MG can upregulate LOX-1 in endothelial cells; therefore, MG might directly or indirectly play a role in the development of atherosclerosis in cardiovascular diseases and also in inducing such abnormalities in the vasculature of women with preeclampsia. This could also partly explain the accumulation of oxLDL in the vasculature of women with preeclampsia, a feature that is analogous to atherosclerosis. Other than oxLDL, AGEs can also bind to LOX-1 and can provide a feedforward progression to induce oxidative stress via the upregulation of LOX-1.

The role of MG has been extensively studied in diabetes mellitus and its complications, such as diabetic nephropathy. This is important because renal lesion, called glomerular endotheliosis, is known to occur in preeclampsia. Because MG is involved in renal complications of diabetes mellitus, it is tempting to speculate that MG could be involved in the renal complications of preeclampsia as well. In addition, AGEs have primarily been associated with complications of diabetes mellitus, and we have now evidence to suggest that vascular complications of preeclampsia may also be attributable to AGEs. In fact, we have previously shown increased expression of receptor for AGEs in the vasculature of women with preeclampsia. All of these further support a role for MG and possibly AGEs in the vascular complications and oxidative stress that are features of preeclampsia.

**Perspectives**

The role of MG in causing vascular complications of diabetes mellitus is well established. The role of MG in the development of hypertension is emerging. Diabete mellitus, atherosclerosis, and hypertension are known risk factors for the development of preeclampsia. Therefore, factors that are involved in the above conditions may also play a role in the initiation and propagation of the disease process in preeclampsia. Our study provides evidence for increased levels of MG in the vasculature of women with preeclampsia. Moreover, novel mechanisms for MG-induced oxidative stress through arginase and LOX-1 in endothelial cells have been described. Recent studies suggest that preeclamptic women are prone to cardiovascular disease later in life. Increased MG levels in the vasculature of these women might partly explain their susceptibility to develop cardiovascular complications. Our study has potential therapeutic
implications not only to prevent vascular complications of preeclampsia but also other hypertensive vascular disorders.

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Disclosures
None.

References


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EVIDENCE FOR INCREASED METHYLGLYOXAL IN THE VASCULATURE OF WOMEN WITH PREECLAMPSIA: ROLE IN UPREGULATION OF LOX-1 AND ARGINASE

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Methods

Subjects

Pregnant subjects were recruited at the time of delivery and non-pregnant 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, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All subjects provided informed consent prior to inclusion in the study. Six 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 two occasions 6h apart and proteinuria of > 500 mg in a 24 hour collection or > +2 on a dip stick.1 Normal pregnant subjects (n=6) were normotensive throughout pregnancy. Non-pregnant subjects (n=6) were also normotensive. None of the subjects had a history of chronic hypertension, renal, liver or other metabolic diseases and were not on any medications. Fat biopsies were collected from these subjects as detailed below. Omental fat biopsies were obtained at the time of caesarean section for normotensive pregnant and preeclamptic women, or during abdominal surgeries for non-pregnant women who were admitted for indications such as dysfunctional uterine bleeding, ovarian cyst, menorrhagia and dysmenorrhea, then snap frozen in liquid nitrogen and stored in – 80°C.2 The patient characteristics are shown on Table S1. In the non-pregnant group, all subjects were, non-smokers, were not previously pregnant and were not on any medications.

Immunohistochemistry

Omental fat biopsies that were stored at -80°C were cut into about 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 arteries in the section were counted. The slides were immunostained using rabbit monoclonal antibodies for CML, CEL, GPx and GR (1:100; Abcam Inc, Cambridge, MA), SSAO, glyoxalase I and II (1:100; Santa Cruz Biotechnology Inc, Santa Cruz, CA ) and were counter stained with anti-rabbit secondary antibody (1: 200 Alexa fluor 488, Invitrogen Canada Inc, Burlington, ON ) to detect the primary antibody and visualized using a FITC filter.

The arterial expression of these enzymes was quantified based on the intensity of the fluorescent staining. Mean fluorescence intensity was obtained by dividing fluorescence intensity by the area of the artery and reported as arbitrary units.

Cell Culture

HUVEC Isolation

For our cell culture studies we chose human umbilical vein endothelial cells (HUVECs) because they are of human origin, easily available, and importantly express both LOX-1 and arginase.3, 4 HUVECs were isolated from umbilical cords obtained from normotensive pregnant women according to a previously described procedure.5 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, cells were plated in six-well plates and incubated in M199 medium (without phenol red) containing 1% FBS and no endothelial cell growth supplement.
Superoxide Imaging

DHE is a cell permeable dye, highly selective for superoxide and very minimally oxidized by other oxygen derived free radicals and reactive oxygen species. We have used this method in our previous study. Briefly, DHE (20 μmol/L) was added during the last 45min of a 24h incubation with MG. The cells were washed thrice with Hank’s balanced salt solution and imaged using CY3 filter in a Olympus IX81 microscope. Cells in three different fields per well were imaged and the mean fluorescence intensity per cell was calculated from three independent experiments.
References


Table S1.

Characteristics of subjects from whom fat biopsies were obtained

<table>
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<tr>
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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.
Figure S1. Effect of MG on Dil-labeled oxLDL uptake

Representative images showing Dil-labeled oxLDL uptake in response to MG (30 µmol/L) treatment for 24h in the presence or absence of BEC and L-NAME.