Endothelial Nitric Oxide Synthase Deficiency Reduces Uterine Blood Flow, Spiral Artery Elongation, and Placental Oxygenation in Pregnant Mice

Shathiyah Kulandavelu, Kathie J. Whiteley, Dawei Qu, Junwu Mu, Shannon A. Bainbridge, S. Lee Adamson

Abstract—Preeclampsia is associated with impaired uteroplacental adaptations during pregnancy and abnormalities in the endothelial NO synthase (eNOS)-NO pathway, but whether eNOS deficiency plays a causal role is unknown. Thus, the objective of the current study was to determine the role of eNOS in the mother and/or conceptus in uteroplacental changes during pregnancy using eNOS knockout mice. We quantified uterine artery blood flow using microultrasound, visualized the uteroplacental vasculature using vascular corrosion casts, and used pimonidazole and hypoxia-inducible factor 1α immunohistochemistry as markers of hypoxia in the placentas of eNOS knockout mice versus the background strain, C57Bl/6J (wild type). We found that increases in uteroplacental blood flow, uterine artery diameter, and spiral artery length were reduced, and markers of placental hypoxia in the junctional zone were elevated in late gestation in eNOS knockout mice. Both maternal and conceptus genotypes contributed to changes in uterine artery diameter and flow. Despite placental hypoxia, placental soluble fms-like tyrosine kinase 1 and tumor necrosis factor-α mRNA, and in maternal plasma, soluble fms-like tyrosine kinase 1 were not elevated in eNOS knockout mice. Thus, our results show that both eNOS in the mother and the conceptus contribute to uteroplacental vascular changes and increased uterine arterial blood flow in normal pregnancy. (Hypertension. 2012;60:231-238.)

Key Words: preeclampsia/pregnancy ▪ blood flow regulation ▪ NO ▪ hypoxia ▪ placenta ▪ endothelial NO synthase

The maternal cardiovascular system undergoes structural and functional changes to accommodate the increased circulatory requirements of the growing fetus. Nowhere is this more profound than in the uteroplacental vasculature, where a marked increase in uteroplacental blood flow is achieved by a large reduction in vascular resistance1,2 and pronounced enlargement and structural reorganization of the uterine and spiral arteries.3 Failure to make or to sustain these changes may result in preeclampsia, one of the most common and serious complications of human pregnancy. The mechanisms mediating uteroplacental changes during pregnancy are not fully understood. However, the L-arginine-NO pathway appears to play a central role in both normal pregnancy and in preeclampsia.4 In preeclampsia in humans, diverse elements of the L-arginine-NO signaling pathway may be abnormal, including reduced L-arginine substrate or cofactor availability, reduced endothelial NO synthase (eNOS) enzyme activity attributed to gene polymorphisms, or elevated levels of asymmetrical dimethylarginine.4 In pregnant rats5 and mice,6 NO synthase inhibition with N^G-nitro-L-arginine methyl ester induces preeclamptic signs. Despite considerable evidence implicating eNOS in preeclampsia, eNOS knockout (KO) mice do not develop the hallmarks of preeclampsia, gestational hypertension7–9 or proteinuria.9 Indeed, arterial pressure in eNOS KO mice decreases during pregnancy to become similar to that of pregnant wild-type controls.7–9 Nevertheless, there is a marked blunting of the normal increase in cardiac output7 and blunting of the enlargement of the heart and aorta10 and uterine artery10 in eNOS KO mice, suggesting an important role for eNOS in systemic cardiovascular changes in pregnancy. However, whether uteroplacental blood flow or placental oxygenation is abnormal in eNOS KO mice and whether the maternal and/or conceptus genotype is responsible are unknown. The goal of the current study was to use eNOS KO mice to determine the role of eNOS in mediating changes in the uteroplacental circulation during pregnancy, as well as the relative roles of eNOS expression in the mother versus the conceptus.

Methods

All of the procedures were approved by the animal care committee of Mount Sinai Hospital and were conducted in accordance with the guidelines of the Canadian Council of Animal Care.
C57Bl/6J (wild-type [WT]) controls (stock No. 000664) and eNOS KO mice (stock No. 002684) were obtained from Jackson Laboratories or raised in-house. Females were bred at 8 to 14 weeks of age and were studied in their first pregnancies. The presence of a sperm plug was defined as day 0.5 of gestation. WT and KO refer to the adult genotype, and ko, heterozygote (het), and wt refer to the conceptus genotype. In the first series, mice were bred with their own strain (N=5 WT; N=7 eNOS KO). They were studied at day 14.5 (end of organogenesis) or day 17.5 of gestation (2 days before normal term delivery). In the second series, WT males were bred with eNOS KO (N=7) females to obtain eNOS KO(het) pregnancies, and WT females (N=5) were bred with eNOS KO males to obtain WT(het) pregnancies. These pregnancies were studied at day 17.5 of gestation. Placentas were weighed with the yolk sac and amniotic membranes attached and after the myometrium (and any decidua that adhered to it) was removed. Fetal and maternal body weights and maternal organ weights were also recorded.

Please see the online-only Data Supplement for detailed methods regarding uterine arterial hemodynamic monitoring and vascular corrosion casting, indirect immunohistochemical detection of placental hypoxia and vascular smooth muscle cells, histochemistry of uterine natural killer (uNK) cells, quantitative RT-PCR of placental soluble fms-like tyrosine kinase (sFlt) 1 and tumor necrosis factor (TNF)-α mRNA, ELISA of maternal plasma sFlt1, and clinical biochemistry of maternal glucose levels.

Results are reported as mean±SEM, where N is the number of mothers. Significance (P<0.05) was tested using a Student t test or 2-way ANOVA followed by a multiple comparison test for >2 groups.

**Results**

### Fetal, Placental, and Maternal Growth in Late Gestation in eNOS KO(ko) Mice

Fetal body weight was similar at 14.5 days of gestation but was significantly lower (−14%) at 17.5 days of gestation in eNOS KO(ko) pregnancies compared with WT(wt) pregnancies. Placental weights were not different between the 2 strains and increased similarly during pregnancy (Table). Placental mRNA expressions of inducible NO synthase and neuronal NO synthase were not significantly different between the 2 groups, and eNOS expression was absent in the KO mice (data not shown). Maternal body weight increased similarly between 14.5 and 17.5 days of gestation in both groups (+21%) but was significantly lower (−10%) in eNOS KO(ko) than in WT(wt) mice at all times (Table). During pregnancy, there were significant increases in maternal kidney and liver weights in both strains, whereas maternal heart and spleen weights increased significantly only in WT(wt) mice. When expressed relative to maternal body weight, there were significant decreases in maternal kidney, spleen, heart, and brain weights in both strains during pregnancy (Table S1, available in the online-only Data Supplement).

### Reduced Uteroplacental Blood Flow and Elevated Uteroplacental Vascular Resistance at Midgestation and Late-Gestation in eNOS KO(ko) Mice

Uterine arterial blood flow was significantly reduced by 45% to 50% in eNOS KO(ko) mothers at 14.5 and 17.5 days of gestation (Figure 1C) even after expressing flow per unit of maternal body weight to account for their smaller maternal size (Figure S1A, available in the online-only Data Supplement). Both mean blood velocity (−15% to 20%) and uterine arterial diameter (−24% to 29%) were significantly reduced (Figure 1A and 1B). Reductions in uterine blood flow would be anticipated to reduce nutrient delivery to the conceptus unless there was a corresponding increase in nutrient content in blood. We, therefore, measured plasma glucose concentration in the maternal circulation at 17.5 days of gestation but found no significant differences between strains (8.3±0.6 mmol/L in WT(wt) and 8.9±0.2 mmol/L in KO(ko); P=0.05). Thus, results indicate that eNOS expression is required for normal increases in uterine artery diameter, uterine artery blood flow, and uterine glucose delivery during pregnancy in mice.

In human preeclampsia, reduced blood flow11 and increased blood flow pulsatility in the uterine artery12 are often present in the first half of pregnancy. Both changes are presumably a consequence of increased downstream vascular resistance. We, therefore, quantified pulsatility in the uterine artery. We observed significant increases in peak systolic velocity and end-diastolic velocity from 14.5 to 17.5 days of gestation in both strains (Figure S2A and S2B). However, end-diastolic velocity tended to be lower in eNOS KO(ko) pregnancies, which led to significant elevations in the resistance index and pulsatility index at both gestational ages (Figure S2C and S2D). This result suggested that uterine vascular resistance was elevated in eNOS KO(ko) pregnancies. This result is consistent with lower uterine artery blood flows (Figures 1 and S1) and similar arterial blood pressures during pregnancy7,8 in mice lacking the eNOS gene relative to WT controls.

### Reduced Growth of Spiral Arteries and Central Arterial Canals in eNOS KO(ko) Mice

eNOS deficiency also resulted in abnormalities in the uteroplacental circulation downstream of the uterine arteries,
Specifically, the spiral arteries and central arterial canals. In normal pregnancy, the spiral arteries that supply blood to the placental exchange region are coiled, large diameter vessels, with little or no vascular smooth muscle.\(^{13,14}\) However, spiral arteries are narrower, and they have more smooth muscle in preeclamptic pregnancies.\(^{13,14}\) Vascular corrosion casts showed reduced spiral artery coiling in the eNOS KO (ko) mothers relative to controls (Figure 2A and 2B). This may be attributed at least in part to a significant reduction in spiral artery length at 14.5 and 17.5 days of gestation (by \(-30\%\); Figure 2C), whereas the diameters of the spiral arteries were similar between the 2 strains at both gestational ages (140±6 \(\mu m\) in eNOS KO (ko) versus 169±2 \(\mu m\) in WT (wt) at 14.5 days; 176±6 \(\mu m\) in eNOS KO (ko) versus 170±8 \(\mu m\) in WT (wt) at 17.5 days). In addition, immunoreactivity to desmin, a marker of vascular smooth muscle, was positive around most of the spiral arteries in the decidua at 17.5 days in the eNOS KO (ko) placentas (Figure S3).

The unusual morphology of the spiral artery is promoted by granulated uNK lymphocytes, which are abundant in the mouse placenta in midpregnancy and then decline in number from day 15 to 18.\(^{15}\) We examined the distribution of uNK cells at 14.5 days of gestation using Dolichos Biflorus Agglutinin (DBA) lectin staining and found a 30% reduction in their number in the decidua in eNOS KO (ko) placentas (Figure 3). Thus, abnormal spiral arteries may be secondary to impaired uNK cell activity, recruitment, and/or retention in eNOS KO (ko) pregnancies.

The maternal spiral arteries supplied between 1 and 4 central arterial canals in the placentas in both strains (2.7±0.5 in eNOS KO (ko); 2.7±0.5 in WT (wt)). Despite having similar numbers of canals, the mean diameter of the arterial canals was significantly lower in eNOS KO (ko) pregnancies (250±1 \(\mu m\)) than in controls (330±3 \(\mu m\)) at 17.5 days of gestation. These findings suggest that eNOS plays an important role in elongation of the maternal spiral arteries and in promoting enlargement of the conceptus-derived arterial canals during pregnancy in mice.

**Role of Maternal Versus Fetal Genotype in Determining Uteroplacental Phenotype**

To understand the extent to which the maternal and fetal genotypes determined the uteroplacental phenotype, we performed a crossbreeding study so that the mothers were either eNOS KO or WT and their fetuses were all heterozygotes. Importantly, the genotypes of all of the fetuses in both crosses were identical, allowing us to evaluate the influence of the maternal genotype on her litter and, by comparing with homozygous breeding described above, the influence of the litter’s genotype on the mother. Heterozygotes would be anticipated to have an intermediate phenotype as a consequence of intermediate levels of eNOS expression.\(^{16}\)

We found that maternal genotype was a significant factor in determining uterine arterial lumen diameter and blood flow (Figure 1) and spiral artery length (Figure 2) but not fetal body weight (Table). Fetal genotype was a significant factor in determining fetal body weight but did not significantly influence spiral artery length (Figure 2C). Interestingly, although mean fetal body weight was significantly greater in heterozygotes than in homozygotes in KO pregnancies (Table), this was not associated with significantly augmented maternal uterine arterial lumen diameter or blood flow (Figure 1), suggesting that fetal growth may have been achieved by greater placental and/or fetal efficiency. This contrasts with WT pregnancies, where significantly blunted fetal growth in heterozygotes (Table) was associated with a corresponding reduction in uterine arterial lumen diameter and flow (Figure 1).
Increased Placental Hypoxia in eNOS KO(ko) Mice

Decreased uterine arterial blood flow would be anticipated to decrease oxygen delivery to the placenta. We, therefore, predicted that the placentas in eNOS KO(ko) pregnancies would be hypoxic in vivo. To test this, we used the hypoxia markers pimonidazole and hypoxia-inducible factor 1 (HIF-1). As shown in Figure 4, strong immunoreactivity was detected in spongiotrophoblast and trophoblast giant cell layers of the junctional zone of the eNOS KO(ko) placentas, whereas faint staining primarily in the spongiotrophoblast cell layer was detected in controls. Quantification of the hypoxic cells in the junctional zone revealed increased accumulation of both pimonidazole and hypoxia-inducible factor 1α in eNOS KO(ko) placentas (Figure 4). These findings suggest that eNOS KO(ko) placentas were hypoxic in late gestation.

Maternal Plasma sFlt1 Levels and Placental Expression of sFlt1 and TNF-α mRNA Were Unaltered in eNOS KO(ko) Mice

Hypoxia increases placental production of the antiangiogenic factor, sFlt1, and the inflammatory cytokine, TNF-α, in human placental explants. Placental hypoxia is thought to cause the elevation in sFlt1 in maternal plasma in preeclamptic pregnancies. Whether placental hypoxia causes the high TNF-α in maternal plasma is less clear. However, despite apparent placental hypoxia in eNOS KO(ko) pregnancies (Figure 5), there were no significant increases in placental sFlt1 or TNF-α mRNA levels (Figure 5B and 5C). Maternal plasma sFlt1 (Figure 5A) was not elevated in pregnant eNOS KO(ko) mice, whereas it is often elevated in women with preeclampsia.

Figure 2. Spiral artery remodeling is reduced in pregnant endothelial NO synthase (eNOS) knockout (KO(ko)) mice. Spiral artery morphology was evaluated from vascular corrosion casts. Scanning electron micrograph of maternal vascular cast filled from the arterial side at 17.5 days of gestation in wild-type (WT(wt)) (A) and KO(ko) (B) mice. C, Spiral arteries were significantly shorter in eNOS KO mothers as compared with WT mothers. Maternal genotype is in upper case and conceptus genotype is in lower case. *P<0.05 WT(wt) versus KO(ko) or WT(het) versus KO(het). Mean±SEM for N shown in bars. Scale bars, 1 mm.

Figure 3. Reduced uNK expression in endothelial NO synthase (eNOS) knockout (KO(ko)) placentas at 14.5 days of gestation. A and B, Dolichos Biflorus Agglutinin (DBA) lectin histochemistry was used to detect uNK cells in wild-type (WT(wt)) and eNOS KO(ko) placentas at 14.5 days of gestation. Number of uNK cells was similar in the metrial triangle (C) but significantly reduced in the decidual region (D) in eNOS KO(ko) placentas as compared with WT(wt) controls. Maternal genotype is in upper case and conceptus genotype is in lower case. MT indicates metrial triangle; D, decidua; uNK, uterine natural killer. Mean±SEM for N shown in bars. *P<0.05 WT(wt) vs KO(ko). Scale bars, 250 μm.
Discussion

As in humans and other species, we observed a near doubling of uterine arterial blood flow between 14.5 and 17.5 days of pregnancy, suggesting that a large decrease in vascular resistance also occurs in this vasculature in late gestation in mice. Mechanisms reducing vascular resistance at this site include vascular remodeling to enlarge the uterine artery and downstream vascular tree, enhanced vasodilation of uterine and uteroplacental vessels, angiogenesis, and growth of the low-resistance, maternally perfused blood

Figure 4. Increased markers of placental hypoxia in endothelial NO synthase (eNOS) knockout (KO(ko)) mice. Pimonidazole (A through D) and hypoxia-inducible factor (HIF) 1α (E) immunohistochemistry were used to identify hypoxic regions in the placenta at 17.5 days of gestation. Representative images are shown. B, D, and E, Strong immunoreactivity was detected in the spongiotrophoblast and trophoblast giant cell layer in the eNOS KO(ko) placentas. The percentage area of positive cells (brown staining) per junctional zone was determined using Visiomorph (F and G). C, Negative control. D indicates decidua, JZ, junctional zone, L, labyrinth, Sp, spongiotrophoblast, arrow indicates trophoblast giant cell. Scale bars, 200 μm. *P<0.05 wild-type (WT(wt)) vs KO(ko).

Figure 5. No significant elevation in placental soluble fms-like tyrosine kinase (sFlt) 1 or tumor necrosis factor (TNF)-α mRNA levels and plasma sFlt1 levels in endothelial NO synthase (eNOS) knockout (KO(ko)) mice. A, Plasma sFlt1 by ELISA for mice when nonpregnant and at 17.5 days of gestation; □, WT(wt); ■, KO(ko). B and C, Placental mRNA levels expressed relative to wild-type (WT(wt)) are shown for sFlt1 and TNF-α in WT(wt) and KO(ko) mice. Maternal genotype is in upper case and conceptus genotype is in lower case. Mean±SEM for N shown in bars; Different letters (a and b) indicate significant changes over time within each strain (P<0.05). *P<0.05 WT(wt) vs KO(ko).
spaces of the placenta. Elevated uterine arterial vascular resistance in eNOS KO pregnancies may, therefore, be mediated by the roles of eNOS in dilution and remodeling of blood vessels in response to mechanical and/or hormonal signals\textsuperscript{30} and in angiogenesis.\textsuperscript{21} As in human preeclampsia,\textsuperscript{12-14,22,23} eNOS KO mice fail to exhibit the normal decrease in uterine arterial vascular resistance in pregnancy, fail to exhibit normal spiral artery morphology, and exhibit placental hypoxia. Nevertheless, placental sFlt1 and TNF-\(\alpha\) mRNA levels and maternal plasma sFlt1 levels were not elevated in pregnant eNOS KO(ko) mice. This result may explain why pregnant eNOS KO mice do not show maternal clinical signs of preeclampsia. If so, we infer that eNOS may play a role in the progression from placental hypoxia (stage 1) to maternal clinical signs (stage 2) in this disorder.\textsuperscript{22} Importantly, maternal clinical signs of preeclampsia are elicited in numerous other mouse models,\textsuperscript{24} making their absence in eNOS(ko) mice all the more intriguing.

We found an important and previously unrecognized role of eNOS in spiral artery morphology. The spiral arteries, which normally have a thin or absent smooth muscle cell coat, retained their smooth muscle cell layer and were also less elongated and less tortuous than in control mice. Cross-breeding studies suggested that maternal eNOS deficiency was responsible, because elongation was abnormal in eNOS KO(ko) and eNOS KO(het) pregnancies but normal in WT(het) pregnancies. Although abnormalities in spiral artery morphology could be secondary to systemic effects in eNOS KO mice (eg, altered hormone levels), evidence supports a local role for NO synthase within the decidua. In human preeclamptic pregnancies, there is reduced NO synthase activity in the uterine placental bed.\textsuperscript{25} In mice, maternal deficiency of uNK cells within the decidua results in spiral arteries that are more muscular and less coiled\textsuperscript{15,26} and, hence, are similar to those found in eNOS KO(ko) mice. Thus fewer uNK cells recruited and/or retained at 14.5 days of gestation in the decidua of eNOS KO(ko) mice may have caused spiral artery abnormalities. uNK cells in mice express inducible NO synthase,\textsuperscript{27} and spiral artery morphology is abnormal in inducible NO synthase KO mice.\textsuperscript{28} However, uNK cells may also express eNOS, as has been reported in rats,\textsuperscript{29} and if so, the loss of this enzyme may prevent the uNK cells that are there from functioning normally. Furthermore, uNK cells release proangiogenic factors, including vascular endothelial growth factor and factors that destabilize blood vessels, including interferon-\(\gamma\), which acts by antagonizing transforming growth factor-\(\beta\).\textsuperscript{26,30} Both vascular endothelial growth factor and transforming growth factor-\(\beta\) stimulate expression of eNOS from endothelial cells\textsuperscript{31,32} and, therefore, act in part via the NO pathway. Thus, the absence of eNOS may blunt the response of cells to uNK-released mediators, and this may also contribute to abnormal spiral artery morphology. Interestingly, as in eNOS KO pregnancies, mice with abnormal spiral artery morphology caused by uNK cell deficiency\textsuperscript{15,33} or inducible NO synthase deficiency\textsuperscript{8,28} also fail to show maternal hypertension during gestation. These results suggest that spiral artery abnormalities alone are insufficient to cause maternal signs. This is consistent with the finding that similar spiral artery abnormalities are observed, in the absence of preeclamptic signs, in human pregnancies with preterm labor, miscarriage, placental abruption, or fetal intrauterine growth restriction.\textsuperscript{14}

In the normal mouse placenta, we found that pimonidazole staining was highest in the spongios tropheoblast and trophoblast giant cell layers of the junctional zone, confirming previous results.\textsuperscript{34,35} This region is primarily perfused by maternal blood in venous channels draining the labyrinthine sinusoids of the placenta.\textsuperscript{36} This blood is depleted of nutrients and oxygen and enriched in wastes from the fetus, which may explain why this region is relatively hypoxic. In eNOS KO(ko) placentas, the junctional zone and the adjacent labyrinth region showed signs of increased hypoxia, as indicated by increased pimonidazole immunoreactivity in comparison with WT(wt) placentas (current study). Increased hypoxia could be attributed to reduced uteroplacental blood flow observed in eNOS KO(ko) mice, which would tend to reduce oxygen delivery to these regions. This effect may be exacerbated in eNOS KO(ko) tissues, because low vascular NO blunts the adaptive reduction in oxygen consumption that normally occurs at lower oxygen levels.\textsuperscript{37} Thus, in eNOS KO(ko) placentas, increased hypoxia markers in the junctional zone may be attributed to decreased uteroplacental oxygen delivery and augmented cellular oxygen consumption caused by a failure to reduce tissue oxygen consumption in accord with availability.

In conclusion, we found that increases in uteroplacental blood flow, uterine artery diameter, and spiral artery length were reduced, and placental hypoxia in the junctional zone was elevated in late gestation in eNOS KO mice. Both maternal and conceptus genotypes contributed to changes in uterine artery diameter and flow. It is interesting that a similar 50% reduction in uterine blood flow caused by \(N^\text{6}-\text{nitro-L-arginine methyl ester}\) administration or by a reduction in uterine arterial perfusion pressure in rat pregnancy leads to maternal preeclampsic signs, including hypertension and increased levels of sFlt1 in the maternal circulation.\textsuperscript{5,38,39} In contrast, in eNOS KO(ko) pregnancies, despite placental hypoxia, sFlt1 levels in the maternal circulation and sFlt1 and TNF-\(\alpha\) mRNA expressions in the placenta were not elevated. Furthermore, superoxide detected by dihydroethidium in eNOS KO(ko) placentas was elevated by only +26\%.\textsuperscript{30} Thus, a minimal placental response to hypoxia may explain why pregnant eNOS(ko) mice do not develop gestational hypertension\textsuperscript{7–9} or proteinuria.\textsuperscript{9}

**Perspectives**

The current findings suggest that the absence of eNOS may inhibit placental production of sFlt1, TNF-\(\alpha\), and/or the generation of reactive oxygen species in response to hypoxia. We speculate that without eNOS protein in the hypoxic placenta, the placental production and release of sFlt1 and other factors into the maternal circulation may be prevented, thereby preventing stage 1 of preeclampsia (ie, placental hypoxia) from progressing to stage 2 (ie, maternal clinical signs). It is possible that the absence of eNOS reduces the generation of reactive oxygen species in response to placental hypoxia\textsuperscript{41} by preventing eNOS uncoupling.\textsuperscript{42} This would imply that eNOS in the hypoxic placenta may contribute to
dysregulated oxidative stress. In that case, increased placental eNOS expression observed in some preeclamptic human pregnancies (eg, References 43 and 44) instead of playing a compensatory role as previously thought may actually contribute to the maternal disease. It is interesting that elimination of eNOS protein appears to block the progression from stage 1 to stage 2 of preeclampsia (current study), whereas blocking the enzymatic activity of eNOS by N\textsuperscript{G}-nitro-L-arginine methyl ester does not. Possibly it is the loss of the eNOS protein and its ability to contribute to protein-protein complexes that is the important factor that is involved in blocking the progression to stage 2 of preeclampsia in eNOS-deficient mice. Thus, future studies to examine the role of eNOS in elevated sFlt1 expression in placental hypoxia are warranted and may lead to novel insights and, ultimately, therapies for preeclampsia in human pregnancy.

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Disclosures

References


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**Novelty and Significance**

**What Is New?**

- In eNOS knockout mice, eNOS deficiency blunted normal changes in the uteroplacental arterial vasculature, reduced decidual uNK number, and reduced uterine arterial blood flow, resulting in placental hypoxia.
- Despite hypoxia, placental sFlt1 and TNF-α mRNA and maternal plasma sFlt1 were not elevated in eNOS knockout mice.

**What Is Relevant?**

- Placental hypoxia is thought to underlie preeclampsia, a hypertensive disorder of human pregnancy, by increasing placental expression of factors such as sFlt1 and/or TNF-α.

- eNOS knockouout mice display placental hypoxia but not gestational hypertension, suggesting that this interaction requires eNOS protein.

**Summary**

eNOS is required for normal uteroplacental blood flow and placental oxygenation during pregnancy in mice.
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Shathiyah Kulandavelu, PhD1,2, Kathie J Whiteley, MSc1, Dawei Qu, MD, PhD1, Junwu Mu, MD, PhD1,2, Shannon A Bainbridge, PhD1,2 and S. Lee Adamson, PhD1,2

1Samuel Lunenfeld Research Institute of Mount Sinai Hospital, 2Departments of Physiology, and Obstetrics and Gynaecology, University of Toronto, Toronto, Ontario, Canada.

Address for correspondence:

Dr. S. Lee Adamson
Samuel Lunenfeld Research Institute of Mount Sinai Hospital
60 Murray St, Box 42, 6th floor, Rm 6-1016-3
Toronto, Ontario, Canada, M5T 3L9
E-mail: adamson@lunenfeld.ca
Tel: 1-416-586-8377
Fax: 1-416-586-5993
Supplemental Materials and Methods:

Uterine arterial hemodynamics

In the first and second series of animals, the uteroplacental circulation was examined using transcutaneous micro-ultrasound (Model 770 with a 30-MHz transducer; VisualSonics, Toronto, Canada) while pregnant mice were anesthetized with 1-2% isoflurane in oxygen. Rectal temperature was maintained between 37 and 38°C. Doppler waveforms were obtained in the uterine artery near the internal iliac artery. Peak systolic velocity (PSV), and end-diastolic velocity (EDV), area under the peak velocity-time curve, and R-R interval were measured from three consecutive cardiac cycles and the results were averaged. Uterine artery luminal (i.e. inner) diameter was measured from vascular casts or from micro-ultrasound B-mode images and results were combined. Preliminary analysis showed no significant difference in diameters measured by the two methods. For example, at E17.5 in eNOS KO(ko) mice, mean diameters were not significantly different whether measured by echo (0.16 ± 0.01 mm (N=3) or by casting (0.19 ± 0.02 mm (N=4), P=0.13). Preliminary results were in accord with our prior work showing no differences between umbilical artery and vein diameters measured by micro-ultrasound in vivo or by micro computed tomography of casts ex vivo. Mean velocity (MV) over the cardiac cycle was calculated by dividing the area under the peak velocity-time curve by the R-R interval. A parabolic blood flow velocity distribution was assumed so that flow was determined by the formula: \( F = \frac{1}{2} \pi \frac{MV}{D/2} \) (where MV = mean peak velocity over the cardiac cycle (cm/s); D = diameter (cm); F = blood flow (ml/min)). Uterine artery Resistance Index (RI = (PSV-EDV)/PSV) and Pulsatility Index (PI = (PSV-EDV)/MV) were calculated to quantify the pulsatility of blood velocity waveforms.

Uteroplacental vascular casts

In the first and second series of animals at 14.5 and 17.5 d of gestation, vascular corrosion casts of the uteroplacental vasculature were prepared using published methods. Casts were imaged using light or scanning electron microscopy. Spiral artery lengths were traced on 2D images and measured using Image J (NIH, Maryland), and diameters were measured at 30-50 arbitrarily selected points in each image (magnification 50x) using SIS XL DOCU image software (Olympus, Munster, Germany). Proximal, middle, and distal diameters of the central arterial canals were measured (Figure S4) and results averaged.

Indirect immunohistochemical detection of placental hypoxia and vascular smooth muscle cells

In a third series of pregnant WT(wt) and eNOS KO(ko) mice, the hypoxia marker, pimonidazole hydrochloride (Hypoxyprobe®1™, 60 mg/kg maternal body weight, Chemicon, Temecula, CA) was injected intraperitoneally at day 17.5 (N=5-6 mothers in each group). Two hours later, the mother was sacrificed and placentas with the myometrium still attached were collected and processed for Hypoxyprobe-1 immunohistochemistry following the manufacturer’s protocol. Binding of Hypoxyprobe-1 has been shown to be well correlated with oxygen electrode measurements. One midline section per placenta per pregnancy was examined by light microscope. Placentas were also stained for HIF1alpha (1:100, Rabbit anti-HIF1alpha, Novus Biologicals, Littleton, CO). The junctional zone and labyrinth regions of each placenta
were identified and analyzed using a Leica DM 4500 microscope. The percentage area of positive cells in each region was determined using Visiomorph (Visiopharm, Hoersholm, Denmark) analysis software (Figure S5).

Placentas were also stained for desmin (1:200, Rabbit anti-desmin, ABCAM, Cambridge, MA) to identify vascular smooth muscle cells. Biotinylated goat anti-rabbit IgG (Vector Lab, Vurlingame, CA) diluted 1:200 was used as the secondary antibody. One midline section per placenta per pregnancy was examined by light microscopy (N=4 pregnancies per group).

**Histochemistry of uNK cells**

In a fourth series of pregnant WT(wt) and eNOS KO(ko) mice, implantation sites (i.e. placentas with the myometrium still attached) were collected at 14.5 d of gestation and stained for lectin (50 µg/ml biotinylated *Dolichos biflorus agglutinin* (DBA); Sigma, St-Louis, MO) to identify uterine natural killer (uNK) cells. One midline section per implantation site per pregnancy was examined using a Leica DM 4500 microscope at 100x magnification (N= 4-5 placentas per group). The decidua and the metrial triangle regions of each implantation site were identified and the number of lectin-positive cells per region per section was counted.

**RT-qPCR for sFlt1 and TNF-α mRNA**

In a fifth series of pregnant WT(wt) and eNOS KO(ko) mice, placentas were collected for RNA isolation at 14.5 d and 17.5 d of gestation (N=3-6 pregnant mice per age per group). The myometrium (and any adherent decidua) and the fetal membranes were removed from the placenta before flash freezing in liquid nitrogen. There were no significant changes with gestational age so results were combined to test for effect of genotype. sFlt1, TNF-α, nNOS, iNOS, eNOS and β-actin were measured by RT-qPCR and results expressed as fold-change relative to WT(wt) controls.

**ELISA of maternal plasma sFlt1**

In a sixth series of WT(wt) and eNOS KO(ko) mice, blood was collected from isoflurane-anesthetized mice by cardiac puncture into heparinized-coated capillary tubes at two stages; non-pregnant (N=8 mice per group) and day 17.5 (N=5 mice per group). Plasma from non-pregnant mice was diluted 2-fold and plasma from 17.5 d pregnant mice was diluted 10-fold so that values were within the range of the standard curve. Plasma sFlt1 was measured in duplicate using an ELISA kit (R&D Systems, Minneapolis, MN).

**Maternal glucose levels**

In a seventh series of mice, blood (~120 µL) was collected from the saphenous vein of awake fed mice prior to pregnancy and on 17.5 d of gestation (N=7-12 mice per group) and analyzed using Nova stat profile M7 (Nova Biomedical, Waltham, MA) for glucose levels.
Statistical analysis

Results are reported as mean ± SEM, where N is number of mothers. Significance was tested using 2-way ANOVA with strain and gestational age, or maternal and paternal strains as factors. This was followed by a Holm-Sidak test for multiple comparisons. Number of uNK cells, central arterial canal diameters and mRNA levels were analyzed for statistical significance between strains using a Student’s t-test. P<0.05 was considered statistically significant.
References:


Table S1. Maternal organ weights in non-pregnant and 17.5 d of gestation WT(wt) and KO(ko) mice.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Non-pregnant</th>
<th></th>
<th></th>
<th>17.5 d of gestation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actual (g)</td>
<td>WT(wt)</td>
<td>KO(ko)</td>
<td>WT(wt)</td>
<td>KO(ko)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Actual (g)</td>
<td>0.117 ± 0.003(^a)</td>
<td>0.115 ± 0.004(^a)</td>
<td>0.142 ± 0.003(^b)</td>
<td>0.133 ± 0.005(^b)</td>
</tr>
<tr>
<td></td>
<td>Per maternal body wt. (x 10(^{-3}))</td>
<td>5.9 ± 0.2(^a)</td>
<td>5.7 ± 0.3(^a)</td>
<td>4.3 ± 0.2(^b)</td>
<td>4.2 ± 0.2(^b)</td>
</tr>
<tr>
<td>Liver</td>
<td>Actual (g)</td>
<td>0.90 ± 0.02(^a)</td>
<td>0.91 ± 0.04(^a)</td>
<td>1.60 ± 0.07(^b)</td>
<td>1.61 ± 0.14(^b)</td>
</tr>
<tr>
<td></td>
<td>Per maternal body wt. (x 10(^{-3}))</td>
<td>45.8 ± 0.8</td>
<td>45.2 ± 2.6</td>
<td>47.6 ± 1.2</td>
<td>49.6 ± 3.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>Actual (g)</td>
<td>0.075 ± 0.006(^a)</td>
<td>0.072 ± 0.006</td>
<td>0.091 ± 0.005(^b)</td>
<td>0.080 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>Per maternal body wt. (x 10(^{-3}))</td>
<td>3.8 ± 0.2(^a)</td>
<td>3.6 ± 0.4(^a)</td>
<td>2.8 ± 0.2(^b)</td>
<td>2.5 ± 0.2(^b)</td>
</tr>
<tr>
<td>Lung</td>
<td>Actual (g)</td>
<td>0.16 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.19 ± 0.04</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Per maternal body wt. (x 10(^{-3}))</td>
<td>8.0 ± 0.9</td>
<td>9.1 ± 1.3</td>
<td>5.9 ± 1.1</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td>Heart</td>
<td>Actual (g)</td>
<td>0.104 ± 0.002(^a)</td>
<td>0.115 ± 0.003</td>
<td>0.129 ± 0.003(^b)</td>
<td>0.129 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>Per maternal body wt. (x 10(^{-3}))</td>
<td>5.3 ± 0.2(^a)</td>
<td>5.7 ± 0.2(^a)</td>
<td>3.9 ± 0.2(^b)</td>
<td>4.1 ± 0.3(^b)</td>
</tr>
<tr>
<td>Brain</td>
<td>Actual (g)</td>
<td>0.45 ± 0.01</td>
<td>0.43 ± 0.02</td>
<td>0.46 ± 0.01</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Per maternal body wt. (x 10(^{-3}))</td>
<td>23.0 ± 0.6(^a)</td>
<td>21.3 ± 1.2(^a)</td>
<td>14.4 ± 0.8(^b)</td>
<td>13.7 ± 0.2(^b)</td>
</tr>
</tbody>
</table>

Maternal genotype in upper case and conceptus genotype in lower case. Values are mean ± SEM, N=5-8 mothers per strain; Different letters (a,b) indicate changes over time within each strain (P<0.05). There were no significant differences between strains.
**Figure S1.** Uterine arterial blood flow normalized to maternal body weight during pregnancy. Uterine arterial blood flow expressed per kg of maternal body weight in WT and KO mice with homozygous or heterozygous litters at 14.5 and 17.5 d of gestation. Maternal genotype is in upper case and conceptus genotype is in lower case. Different letters (a,b) indicate significant changes over time within each strain (P<0.05). * P<0.05, WT(wt) vs. KO(ko) mice. † P<0.05, WT(wt) vs WT(het). Mean ± SEM for N shown in bars.
Figure S2. Uterine arterial Resistance Index and Pulsatility Index are elevated in eNOS KO(ko) mice at mid- and late gestation. Peak systolic (A) and end-diastolic (B) velocities were used to calculate Resistance Index (C) and Pulsatility Index (D) at 14.5 and 17.5 d of gestation in WT(wt) and KO(ko) mice. Maternal genotype is in upper case and conceptus genotype is in lower case. Different letters (a,b) indicate significant changes over time within each strain (P<0.05). * P<0.05, WT(wt) vs KO(ko). Mean ± SEM for N shown in bars.
Figure S3. Immunohistochemistry detection of vascular smooth muscle cell protein in spiral arteries. Desmin staining was used to detected vascular smooth muscle cells around the spiral arteries in WT(wt) and KO(ko) mice at 17.5 d of gestation. +, indicate spiral artery lumens.
Figure S4. Light micrograph image of a fetoplacental vascular cast of central arterial canal at 17.5 d of gestation. Proximal, middle and distal diameters of the central arterial canals were measured. Bar, 1 mm.
**Figure S5.** Quantification of HIF-1α staining using Visiomorph analysis software. Visiomorph software was used to superimpose a blue color in the location of HIF-1α positive brown staining as shown in these examples. The percentage area of positive cells (blue color) for HIF-1α staining was determined in the junctional zone for WT(wt) and KO(ko) placentas at 17.5 d of gestation. D, decidua, JZ, junctional zone, L, labyrinth.