Endothelial NO Synthase Augments Fetoplacental Blood Flow, Placental Vascularization, and Fetal Growth in Mice

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

Abstract—It is not known whether eNOS deficiency in the mother or the conceptus (ie, placenta and fetus) causes fetal growth restriction in mice lacking the endothelial NO synthase gene (eNOS knockout [KO]). We hypothesized that eNOS sustains fetal growth by maintaining low fetoplacental vascular tone and promoting fetoplacental vascularity and that this is a conceptus effect and is independent of maternal genotype. We found that eNOS deficiency blunted fetal growth, and blunted the normal increase in umbilical blood flow and umbilical venous diameter and the decrease in umbilical arterial Resistance Index in late gestation (14.5–17.5 days) in eNOS KO relative to C57Bl/6J controls. On day 17.5, fetoplacental capillary lobule length and capillary density in vascular corrosion casts were reduced in eNOS KO placentas. Reduced vascularization may be a result of decreased vascular endothelial growth factor mRNA and protein expression in eNOS KO placentas at this stage. These factors, combined with significant anemia found in eNOS KO fetuses, would be anticipated to reduce fetal oxygen delivery and contribute to the fetal tissue hypoxia that was detected in the heart, lung, kidney, and liver by immunohistochemistry using pimonidazole. Although maternal eNOS deficiency impairs uteroplacental adaptations to pregnancy, maternal genotype was not a significant factor affecting growth in heterozygous conceptuses. This indicates that fetal growth restriction was primarily caused by conceptus eNOS deficiency. In mice, placental hemodynamic and vascular changes with gestation and growth restriction showed strong parallels with human pregnancy. Thus, the eNOS KO model could provide insights into the pathogenesis of human intrauterine growth restriction. (Hypertension. 2013;61:xx-xx.)

Key Words: angiogenesis  ■  pregnancy  ■  intrauterine growth restriction  ■  vascular growth factor  ■  hypoxia  ■ genetics-knockout models  ■  NO

Fetal intrauterine growth restriction (IUGR) adversely impacts ≈5% of all human pregnancies. IUGR increases perinatal mortality and morbidity and increases by ≈3-fold the risk of developing diverse adult-onset diseases, including coronary artery disease, diabetes mellitus, and hypertension.1 Despite its importance, effective treatments are lacking. Progress in the development of appropriate treatments for IUGR has been impeded by its multifactorial pathogenesis. Maternal factors such as preeclampsia increase the risk of IUGR.2 This disorder affects ≈5% of human pregnancies and is characterized by maternal hypertension, proteinuria, and reduced uteroplacental perfusion.2 However, IUGR is not well correlated with the severity of hypertension3 or the occurrence of abnormal uterine artery Doppler indices in preeclampsia.4 Thus, other factors are involved. IUGR can also be caused by a variety of fetal factors including infection and genetic abnormalities.5 Nevertheless, placental insufficiency is believed to underlie the majority of IUGR cases.5

The placenta in IUGR pregnancies often exhibits abnormalities, including fetoplacental hypovascularity based on placent al histomorphometry6 and increased fetoplacental vascular resistance as suggested by increased umbilical artery blood flow pulsatility.7 Impaired fetoplacental vascularization would be anticipated to increase fetoplacental vascular resistance, thereby decreasing fetoplacental perfusion, as well as decreasing the surface area for fetomaternal exchange. Both effects would tend to decrease the transfer of oxygen and nutrients thereby limiting fetal growth. The fetoplacental circulation lacks autonomic innervation8; therefore, fetoplacental vascular resistance is determined by angiogenesis and circulating and locally released vasoactive factors. NO is a potent vasodilator synthesized by the endothelial NO synthase (eNOS) isoform in endothelium of muscularized fetoplacental vessels.9 The importance of basal NO release in maintaining low vascular resistance in the fetoplacental circulation in humans has been shown in human placental arteries and perfused placental cotyledons in vitro,10,11 and in sheep, where NO5 inhibition increases fetoplacental vascular resistance in vivo.12 In addition, eNOS-derived NO may also maintain low fetoplacental vascular resistance by promoting angiogenesis.
and vasculogenesis as shown in other vascular beds studied in adult animals.\textsuperscript{13}

In the current study, we hypothesized that eNOS sustains fetal growth by maintaining low fetoplacental vascular tone and promoting fetoplacental vascularity. We further hypothesized that this is a conceptus effect and is independent of maternal genotype. We used eNOS knockout (KO) pregnancies because eNOS KO fetuses exhibit fetal growth restriction in late gestation\textsuperscript{14–16} and pregnant eNOS KO mothers exhibit impaired uteroplacental remodeling and a blunted rise in uteroplacental blood flow and cardiac output.\textsuperscript{15,16} However, they neither overexpress sFlt1 mRNA in the placenta\textsuperscript{16} nor do they become more hypertensive during pregnancy.\textsuperscript{16–18} In this study, we quantified umbilical vein blood flow and umbilical artery Resistance Index using micro-ultrasound, visualized the fetoplacental vasculature using vascular corrosion casts, and evaluated hypoxia in the fetus in eNOS KO mice and in the background strain, C57Bl/6J (wild type [WT]). Whether IUGR is secondary to reduced uteroplacental perfusion or is a result of effects of eNOS deficiency in the conceptus in this model is not clear. Thus, crossbreeding experiments were performed to determine the impact of maternal genotype on the fetal phenotype.

**Methods**

Experiments were approved by the Animal Care Committee of Toronto Centre for Phenogenomics and Mount Sinai Hospital (Toronto, Ontario) and were conducted in accord with guidelines established by the Canadian Council on Animal Care.

C57Bl/6J (WT) controls (Stock 000664) and eNOS KO mice (KO) (Stock 002684) were obtained from Jackson Laboratories (Bar Harbor, ME) 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, heterozygous (het), and wt refer to the conceptus genotype. In the first series, mice were bred with their own

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Fetal growth and umbilical vein blood flow in mid- and late gestation. Fetal (A), placental (B), and umbilical vein (C) growth at 14.5 d and/or 17.5 d gestation in homozygous (WT[wt] and KO[ko]) mice and heterozygous (WT[het] and KO[het]) mice. Umbilical vein lumen diameter (C), mean blood velocity (D), blood flow (E), and blood flow normalized to fetal weight (F) were determined using micro-ultrasound in isoflurane-anesthetized mice on days 14.5 and 17.5 of gestation. Maternal genotype is in upper case and conceptus genotype is in lower case. Different letters (a and b) indicate significant changes over time within each strain (\(P<0.05\)). Significant differences between strains are indicated by \(P<0.05\) (for WT[wt] vs KO[ko] mice, or WT[het] vs KO[het] mice) or by \(P<0.05\) (for WT[wt] vs WT[het], or KO[ko] vs KO[het]). Mean±SEM. The conceptus number (n) is shown in bars. WT indicates wild type; KO, knockout; het, heterozygous.
Reduced Fetal Growth and Feto-placental Blood Flow at Mid- and Late Gestation in eNOS KO(ko) Fetuses

Between days 14.5 and 17.5 of gestation, WT(wt) fetuses exhibited significant increases in body weight of 4-fold, in placental weight of 1.2-fold, umbilical vein lumen diameter of 1.2-fold, and umbilical vein blood flow of 1.4-fold, whereas mean umbilical blood velocity did not change significantly (Figure 1A=IE). Fetal body weight and umbilical vein lumen diameter were not significantly different between the 2 groups on day 14.5 of gestation (Figure 1A and 1C), whereas on day 17.5 of gestation fetal weight was 14% lower and umbilical vein lumen diameter was 5% lower in eNOS KO(ko) fetuses compared with WT(wt) fetuses (Figure 1A and 1C). Fetal weight was lower even though litter size in KO(ko) pregnancies on day E17.5 (6.5±0.3; n=8) was smaller than WT(wt) (9.3±0.6; n=6; Figure S2 in the online-only Data Supplement), which might be anticipated to augment fetal growth. Nevertheless, umbilical venous blood flow was significantly reduced in eNOS KO(ko) fetuses on both days 14.5 and 17.5 (Figure 1E). When expressed per unit fetal weight, umbilical venous blood flow/g decreased markedly with advancing gestation. Like flow, flow/g was significantly reduced by ≈20% in eNOS KO(ko) fetuses relative to WT(wt) fetuses at both ages (Figure 1F). These findings indicate an essential role for eNOS in supporting feto-placental perfusion and feto-growth in late gestation in mice.

In human IUGR, lower placental blood flow per unit fetal weight is associated with higher blood flow pulsatility in the umbilical artery. Here, we, therefore, quantified pulsatility in the umbilical artery using Resistance Index on days 14.5 and 17.5 of gestation. In WT(wt) fetuses, peak systolic and end-diastolic velocities measured using Doppler ultrasound significantly increased with advancing gestation (Figure 2A and 2B). In eNOS KO(ko) fetuses, however, the increase in end-diastolic velocity was reduced over this interval with the result that the Resistance Index in the umbilical artery significantly decreased with age in the WT(wt) but not in the eNOS KO(ko) group (Figure 2C). On day 17.5 of gestation, Resistance Index tended to be higher in the eNOS KO(ko) group (P=0.06). Both end-diastolic (~27%) and peak systolic (~14%) blood velocities were significantly decreased in eNOS KO(ko) fetuses versus WT(wt) on day 17.5 of gestation (Figure 2A and 2B).

Given that the umbilical arterial Resistance Index failed to decrease significantly from mid- to late gestation in eNOS KO(ko) fetuses, we suspected that feto-placental vascularization might be reduced in late gestation in eNOS KO(ko) placentas. To investigate this possibility, we prepared vascular corrosion casts of the feto-placental circulation of eNOS KO(ko) fetuses on day 17.5 of gestation and found shorter capillary lobules at the chorionic surface (~25%; Figure 3A, 3B, and 3E) but similar mean capillary diameters (11.7±0.5 µm versus 12.6±0.9 µm in WT(wt)). Capillary density seemed to be decreased in the KO(ko) compared with WT(wt) placentas (Figure 3C and 3D). Quantification of the labyrinth component in histological sections revealed a significant 17% reduction in area fraction of fetal blood spaces in the eNOS KO(ko) placentas compared with WT(wt) placentas (Figure 3F). The area fractions of the labyrinth and of the other placental components (decidua, junctional zone, chorionic plate) were not significantly altered. These findings suggest that eNOS promotes feto-placental vascularization of the labyrinth.

Figure 2. Umbilical arterial Resistance Index in mid- and late gestation in endothelial NO synthase KO(ko) mice. Peak systolic (A) and end-diastolic (B) velocities in the umbilical artery were used to calculate Resistance Index (C) in lightly-anesthetized mice using micro-ultrasound on days 14.5 and 17.5 of gestation. Maternal genotype is in upper case and conceptus genotype is in lower case. Different letters (a and b) indicate significant changes over time within each strain (P<0.05). Significant differences between strains are indicated by *P<0.05 for WT(wt) vs KO(ko) mice. Mean±SEM. The conceptus number (n) is shown in bars. WT indicates wild type; KO, knockout.
To determine whether impaired angiogenesis is associated with a reduction in expression of the proangiogenic factor VEGF, we performed quantitative real-time polymerase chain reaction and immunohistochemistry to detect placental VEGF mRNA and protein expression, respectively. VEGF mRNA levels were similar between groups on day 14.5, but the significant increase on day 17.5 was blunted in eNOS KO (ko) placentas, resulting in VEGF mRNA levels that were significantly lower than WT (wt) on day 17.5 of gestation (Figure 4). VEGF protein immunoreactivity was also significantly lower in the junctional zone on day 17.5. Therefore, decreased VEGF levels may contribute to impaired fetoplacental vascularization in late gestation in eNOS KO (ko) placentas.

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eNOS KO (ko) Pups Are Hypoxic and Anemic

Decreased fetoplacental vascularity and decreased umbilical venous blood flow would be anticipated to decrease fetal oxygen delivery unless there is a compensatory increase in the oxygen carrying capacity of the fetal blood. To test this, fetal cord blood was collected and subjected to hematological analysis. Surprisingly, the erythrocyte count, hematocrit, and hemoglobin were significantly lower in eNOS KO (ko) fetuses (Table), suggesting an increase in the proportion of immature erythrocytes. An increased proportion of immature erythrocytes is also observed in human IUGR fetuses.19

Fetal Growth Is Determined by Conceptus Genotype

Uterine arterial blood flow in eNOS KO (ko) mothers was half that of WT controls in late pregnancy,16 but whether this contributed to IUGR in eNOS KO (ko) fetuses was not clear. To determine the extent to which maternal genotype determines fetal phenotype, we performed a crossbreeding study and examined heterozygous placentas and fetuses on day 17.5 of gestation. In this study, heterozygous fetuses had either homozygous eNOS KO mothers (KO (het)) or control mothers (WT (het)). In het fetuses, fetal body weight and umbilical venous blood flow/g fetal weight (parameters from which it was derived [ie, mean venous velocity, venous lumen diameter, and fetal weight]) were not significantly affected by maternal genotype on day 17.5 of gestation (Figure 1). Fetal body weight and umbilical venous blood flow/g in het fetuses were intermediate and significantly different than both homozygous KO (ko) and WT (wt) fetuses. Thus, deficits in fetoplacental perfusion and fetal growth were primarily determined by the genotype of the conceptus (fetus and placenta).

Discussion

In the current study, we observed strong parallels in mice with previous work in humans in normal pregnancy and in pregnancies complicated by IUGR. During normal late gestational
development in mice (current study), as in humans, umbilical venous blood flow increased in association with an increase in umbilical vein diameter, whereas mean blood velocity remained constant. Also observed was a marked decrease in umbilical blood flow expressed per unit fetal weight with advancing gestation in mice (current study) as in humans. This was likely permitted by enhanced placental exchange efficiency as a result of the maturation and thinning of the placental barrier and by the increase in fetoplacental vascularity that is observed in late gestation in both species.

Reduced fetal growth in eNOS KO fetuses was associated with reduced umbilical blood flow per unit fetal weight, thus modeling human IUGR. eNOS KO fetuses also exhibited fetal hypoxia, fetal erythrocyte immaturity, and reduced fetoplacental capillaries, thereby further mimicking IUGR in humans. Interestingly, reduced NOS activity and expression occurs in subset of human IUGR placentas. VEGF expression may also be reduced in some cases of human IUGR, and thus similar to the reduction observed in eNOS KO placentas. Mechanistically, therefore, this mutant may model a specific subset of human IUGR in which, for whatever cause, there is a functional deficit in the placental eNOS/VEGF pathway.

As well as being a vasodilator, VEGF is also a key mediator of angiogenesis and is thought to play an important role in promoting angiogenesis during normal placental development in humans. VEGF-mediated angiogenesis is predominantly

Table. Hematology Parameters in Fetal WT (wt) and KO (ko) Mice at 17.5 d of Gestation

<table>
<thead>
<tr>
<th>Hematology Parameter</th>
<th>WT (wt) (n=18 Fetuses)</th>
<th>KO (ko) (n=23 Fetuses)</th>
<th>Significance P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (×10^{12}/L)</td>
<td>3.52±0.07</td>
<td>2.97±0.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hgb, g/L</td>
<td>125±2</td>
<td>110±5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hct, L/L</td>
<td>0.40±0.01</td>
<td>0.35±0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MCHC, g/L</td>
<td>315±1.32</td>
<td>309±1.94</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>113±1</td>
<td>121±1</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MCH, pg/cell</td>
<td>35.5±0.35</td>
<td>37.2±0.37</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>WBC (×10^{9}/L)</td>
<td>137±5</td>
<td>137±6</td>
<td>NS</td>
</tr>
<tr>
<td>Plt (×10^{9}/L)</td>
<td>348±19</td>
<td>317±17</td>
<td>NS</td>
</tr>
</tbody>
</table>

RBC indicates red blood cell count; Hgb, hemoglobin concentration; Hct, hematocrit; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; WBC, white blood cell count; Plt, platelet count; NS, not significant.

Maternal genotype is in upper case and conceptus genotype is in lower case. Values are mean±SEM.
mediated by eNOS. NO is required for the effects of VEGF on endothelial cell differentiation, migration, and formation of capillary networks in vitro. In vivo, NO is also required for the angiogenic effects of VEGF because in eNOS KO mice, recombinant VEGF protein or adenovirus-mediated VEGF gene transfer failed to improve the impaired angiogenesis found in the hindlimb of these mutants. In the current study in mice, reduced capillary lobule length and capillary density were observed in eNOS KO mice in late gestation. This is consistent with previous work showing reduced capillary density in eNOS KO mice in the perinatal period in the lungs and the myocardium, and in nonpregnant adults, in hindlimb skeletal muscle, and the myocardium. eNOS-derived NO is not only an important downstream mediator of VEGF, it is also an upstream promoter of VEGF expression. Administration of an NO donor or transfection with a DNA plasmid encoding eNOS increased VEGF protein levels in vascular smooth muscle cells in humans and rats, and in skeletal muscle in rats. Therefore, the decreases in perfusion leading to impaired placental vascularity, and umbilical blood flow in late gestation.

Perspectives

In the present study we demonstrated that eNOS plays an essential role in augmenting fetal growth, fetoplacental vascularity, and umbilical blood flow in late gestation in mice. This is likely a result of the dual roles of NOS in maintaining low vascular resistance and in promoting vascularization in the fetoplacental and fetal body circulations. These factors, along with decreased erythropoiesis in eNOS KO fetuses, most likely contributed to reduced fetal tissue oxygenation and reduced fetal growth at term. Intriguingly, we further demonstrated that, although maternal eNOS deficiency is known to have uteroplacental blood flow, maternal genotype was not a significant factor affecting fetal growth. Similarly, poor correspondence between fetal growth and abnormal uterine artery hemodynamics, and between fetal growth and severity of preeclampsia is observed in human pregnancy. We also found that changes in umbilical blood flow, blood velocity, and vein diameter in late gestation in mice were similar to normal human pregnancy, and that abnormalities in umbilical flow, placental vascularity, and fetal growth in eNOS KO pregnancies showed strong parallels with human IUGR pregnancies. These findings suggest the use of the eNOS KO mouse model for future studies exploring the pathogenesis and potential treatment of IUGR.

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Disclosures

References


What Is New

- Endothelial NO synthase (eNOS) deficiency blunted the normal increases in umbilical blood flow, and eNOS fetuses exhibited fetal hypoxia, fetal erythrocyte immaturity, and reduced fetoplacental capillaries. These factors likely contributed to the observed reduction in fetal tissue oxygenation and reduced fetal growth at term.

- Although maternal eNOS deficiency is known to halve uteroplacental blood flow, maternal genotype was not a significant factor affecting growth of heterozygous fetuses, suggesting that fetal intrauterine growth restriction (IUGR) was primarily a result of conceptus eNOS deficiency.

What Is Relevant?

- Fetal IUGR is associated with increased risk of developing diverse adult-onset diseases including hypertension. Mechanisms involved are unknown, but maternal, fetal, and placental factors may be involved. In this current study, we showed that fetal IUGR in the eNOS knockout mouse model was primarily caused by conceptus eNOS deficiency.

Summary

- eNOS plays an essential role in augmenting fetal growth, fetoplacental vascularity, umbilical blood flow, and fetal oxygen delivery in late gestation in mice.

- Pregnancy in eNOS knockout mice exhibits strong parallels with human IUGR and, therefore, provides a model for future explorations of the pathogenesis and potential treatment of IUGR.

Novelty and Significance
Endothelial NO Synthase Augments Fetoplacental Blood Flow, Placental Vascularization, and Fetal Growth in Mice

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eNOS expression augments fetoplacental blood flow, placental vascularization, and fetal growth in mice.

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Expanded method:

**Umbilico-placental hemodynamics**

In the first series, mice were bred with their own strain. They were studied at days 14.5 (end of organogenesis) (N=5 WT mothers; N=7 eNOS KO mothers) or 17.5 d of pregnancy (2 days before normal term delivery) (N=6 WT mothers; N=9 eNOS KO mothers). In the second series, WT females (N=5) were bred with eNOS KO males to obtain WT(het) mice and eNOS KO females (N=9) were bred with WT males to obtain KO(het) mice. Crossbred pregnancies were studied at 17.5 d of gestation. In both series of animals, the fetoplacental circulation was examined using transcutaneous micro-ultrasound (Model 770 with 30-MHz transducer; VisualSonics, Toronto, ON) while pregnant mice were lightly anesthetized with ~ 1.5% isoflurane in oxygen by face mask and rectal temperature was maintained between 37-38 °C. Doppler waveforms in the umbilical vein and artery were obtained near the placental end of the umbilical cord (Figure S1B and S1C). Peak systolic velocity (PSV), 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. Umbilical venous diameter was measured from power Doppler images (Figure S1A). Mean velocity (MV) over the cardiac cycle was calculated by dividing the area under the peak velocity-time curve by the R-R interval. Umbilical artery Resistance Index (RI = (PSV - EDV)/PSV) was calculated to quantify arterial blood flow pulsatility. A parabolic blood velocity distribution was assumed so that umbilical venous blood flow was determined by the formula: F = ½ π MV (D/2)^2 (where MV = mean peak velocity (cm/s); D = diameter (cm); F = blood flow (ml/min)).

**Placental histomorphometry and vascular corrosion casts**

In the first series of animals, placental morphometry (newCAST™; Visiopharm, Hoersholm, Denmark) and placental vascular corrosion casts were obtained at 17.5 d of gestation. Histomorphometry was performed on CD34 stained histological cross-sections at the placental midline (1:100, Serotec; Raleigh, NC) to identify fetal endothelium (N=5-8 placentas per strain). Vascular corrosion casts of the fetoplacental vasculature were obtained using published methods.1 Casts were examined by scanning electron microscopy. The lengths of the capillary lobules (mag. 250x) were measured at four orthogonal locations on the chorionic surface of each cast, and diameters of fetal capillaries (mag. 1200x) were measured at 20-40 arbitrary locations on each cast. The results were averaged for 6 placental casts per group for WT(wt) mice (1-2 each from 4 pregnancies) and eNOS KO(ko) mice (2 each from 3 pregnancies).

**Immunohistochemical detection of fetal hypoxia and vascular endothelial growth factor (VEGF) protein**

In a third series of pregnant WT(wt) and eNOS KO(ko) mice, the hypoxia marker, pimonidazole hydrochloride (Hypoxyprobe–1™, 60 mg/kg mice, Chemicon, Temecula, CA) was injected intraperitoneally at day 17.5. Two hours later, the mother was euthanized and fetuses were collected and processed for pimonidazole immunohistochemistry following the manufacturer's protocol. The percentage area of pimonidazole staining has been shown to be
well correlated with oxygen electrode measurements. One sagittal section per fetus per pregnancy was analyzed using a Leica DM 4500 microscope (N=5-7 fetus in total from 5 pregnancies from each strain). The percentage area of positive cells (brown staining) in each organ (kidney, liver, heart, lung) was determined using Visiomorph (Visiopharm, Hoersholm, Denmark) analysis software.

Placentas from WT(wt), eNOS KO(ko), WT(het) and eNOS(het) mice with the myometrium still attached were processed for VEGF immunohistochemistry using standard methods (1:200, Rabbit anti-VEGF, Thermo Scientific, Fremont, CA). Biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 was used as the secondary antibody. One midline section per placenta per pregnancy was examined. The junctional zone and labyrinth regions of each placenta were identified and percentage area of positive cells per each region was determined using Visiomorph analysis software.

Quantitative RT-PCR for VEGF mRNA

In a fourth series of pregnant WT(wt) and eNOS KO(ko) mice, placentas were collected for RNA isolation at 14.5 and 17.5 d of gestation (N=3 per age per group). The myometrium (with any adherent decidua) and the fetal membranes were removed from the placenta before flash freezing in liquid nitrogen. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. RNA samples were column purified using RNeasy MinElute Cleanup Kit (Qiagen, Mississauga, ON) and treated with 2.5 uL DNase I (Qiagen). 1 μg of total RNA was primed with random hexamer and single-strand cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). 10ng of cDNA was subjected to quantitative PCR in an optical 96-well plate with the Eppendorf Mastercycler Ep Realplex (Brinkmann, Mississauga, ON) using SYBR Green detection chemistry. Quantitative PCR primers were designed specifically to include all splice variants of VEGF-A (Forward: GAG CAG AAG TCC CAT GAA CTG, Reverse: TGT CCA CCA GGG TCT CAA TC). β-actin and HPRT1 were used as reference genes. β-actin (Forward: TCG TGC GTG ACA TCA AAG AGA, Reverse: GAA CCG CTC GTT GCC AAT A) and HPRT1 (Forward: TCT TTG CTG ACC TGC TGG ATT, Reverse: TAT GTC CCC CGT TGA CTG ATC). Samples were run in duplicates. The data was analyzed using the ΔΔ-Ct method with VEGF expression in the KO(ko) placentas normalized to the geometric mean of two housekeeping genes (B-actin and HPRT1) and expressed relative to the expression in the WT(wt) control at 14.5 d of gestation.

Hematology of fetal blood

In a fifth series of pregnant WT(wt) and eNOS KO(ko) mice (N=3-7 mothers), the uterus was removed, chilled, and individual conceptuses were exposed and re-warmed to resume cardiac function and placental blood flow. An EDTA-coated capillary tube was inserted into an umbilical vessel to obtain fetal blood at 17.5 d of gestation. The blood was assessed for hematological parameters (red blood cell, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, white blood cell, platelet count) using a hematology analyzer (AcT Diff, Beckman Coulter, Toronto, ON)
References:


Figure S1. Ultrasound evaluation of umbilico-placental vascular structure and hemodynamics. Umbilical vessels were identified using micro-ultrasound in isoflurane-anesthetized mice. Power Doppler images of the fetoplacental circulation were obtained (A) for caliper measurements of umbilical vein luminal diameter (average of 2-5 measurements). Doppler blood velocity waveforms were obtained from the umbilical artery (B) and umbilical vein (C) and peak systolic and minimum diastolic velocities measured. Em, embryo; P, placenta; PSV, peak systolic velocity; EDV, end-diastolic velocity.
Figure S2: Litter size was significantly lower in eNOS KO(ko) than WT(wt) mice at 17.5 d of gestation only. Maternal genotype is in upper case and conceptus genotype is in lower case. Mean ± SEM. Number of pregnancies (N) shown in bars. Significant differences between strain is indicated by *P<0.05 for WT(wt) vs. KO(ko) mice.
Figure S3: Pimonodazole detection of tissue hypoxia in eNOS KO(ko) fetuses at 17.5 d of gestation. Representative images from N=5-8 fetuses from 5-6 different litters are shown on left. Quantification of % area of positive pimonidazole staining in the fetal heart, lung, kidney and liver showed elevated immunoreactivity (*P<0.05) in KO(ko) versus WT(wt) mice (in graphs on right, N in bars shows number of fetuses).