eNOS knockout mouse as a model of fetal growth restriction with an impaired uterine artery function and placental transport phenotype

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Kusinski LC, Stanley JL, Dilworth MR, Hirt CJ, Andersson LJ, Renshall LJ, Baker BC, Baker PN, Sibley CP, Wareing M, Glazier JD. eNOS knockout mouse as a model of fetal growth restriction with an impaired uterine artery function and placental transport phenotype. Am J Physiol Regul Integr Comp Physiol 303: R86–R93, 2012. First published May 2, 2012; doi:10.1152/ajpregu.00600.2011.—Fetal growth restriction (FGR) is defined as the inability of a fetus to reach its genetically predetermined growth potential. In the absence of a genetic anomaly or maternal undernutrition, FGR is attributable to “placental insufficiency”: inappropriate maternal/fetal blood flow, reduced nutrient transport or morphological abnormalities of the placenta (e.g., altered barrier thickness). It is not known whether these diverse factors act singly, or in combination, having additive effects that may lead to greater FGR severity. We suggest that multiplicity of such dysfunction might underlie the diverse FGR phenotypes seen in humans. Pregnant endothelial nitric oxide synthase knockout (eNOS−/−) dams exhibit dysregulated vascular adaptations to pregnancy, and eNOS−/− fetuses of such dams display FGR. We investigated the hypothesis that both altered vascular function and placental nutrient transport contribute to the FGR phenotype. eNOS−/− dams were hypertensive prior to and during pregnancy and at embryonic day (E) 18.5 were proteinuric. Isolated uterine artery constriction was significantly increased, and endothelium-dependent relaxation significantly reduced, compared with wild-type (WT) mice. eNOS−/− fetal weight and abdominal circumference were significantly reduced compared with WT. Unidirectional maternofetal [14C]-methylaminoisobutyric acid (MeAIB) clearance and sodium-dependent [14C]-MeAIB uptake into mouse placental vesicles were both significantly lower in eNOS−/− fetuses, indicating diminished placental nutrient transport. eNOS−/− mice placentas demonstrated increased hypoxia at E17.5, with elevated superoxide compared with WT. We propose that aberrant uterine artery reactivity in eNOS−/− mice promotes placental hypoxia with free radical formation, reducing placental nutrient transport capacity and fetal growth. We further postulate that this mouse model demonstrates “uteroplacental hypoxia,” providing a new framework for understanding the etiology of FGR in human pregnancy.

nitric oxide; pregnancy; placental insufficiency

Fetal growth restriction (FGR) is defined as the inability of a fetus to achieve its genetically predetermined growth potential and is associated with high levels of perinatal mortality and morbidity (23). The main cause of FGR, in the absence of maternal undernutrition or fetal genetic anomaly, is placental insufficiency. The term placental insufficiency, denoting a reduced ability of the placenta to exchange nutrients and waste products between mother and fetus, was taken for many years as being synonymous with reduced blood flow through the uterine and/or fetoplacental circulations, arising from abnormal vascular development (15) or dysregulation of uterine and/or fetoplacental vessels (24). However, more recently, it has become clear that placental insufficiency can additionally involve physical abnormalities of the exchange barrier [e.g., decreased surface area and increased thickness (22)] and molecular abnormalities [e.g., reduced activity of key plasma membrane nutrient transporters in the epithelium of the placenta, the syncytiotrophoblast, such as the System A amino acid transporter (35)]. This information led us to propose that different placental phenotypes, or mixes of the abnormalities described above, might lead to different patterns of fetal growth (35). In this study, we have investigated the placental phenotype of the endothelial nitric oxide synthase (eNOS) knockout mouse (hereafter referred to eNOS−/−), as a mouse model of restricted fetal growth (9, 30, 39), with systemic vascular dysfunction and hypertension that persists throughout pregnancy (10) but with no evidence of altered placental histology (9). eNOS catalyzes the cellular conversion of arginine to nitric oxide (NO), which acts as a potent vasodilator by causing the relaxation of smooth muscle cells (25). During pregnancy, NO plays an important role in maternal cardiovascular adaptations and vasodilation of the systemic circulation, the increase in uterine and fetoplacental blood flow, the maintenance of low vascular resistance in the fetoplacental circulation, and the modulation of myogenic tone in mesenteric and uterine arteries; it is also proposed to be responsible for the reduced peripheral resistance in pregnant women (1, 36, 40). eNOS−/− dams have significantly reduced cardiac output in late gestation (16) and have elevated blood pressure, both prior to and throughout pregnancy, compared with wild-type (WT) controls (10), consistent with the concept that eNOS activity is involved in the regulation of blood pressure in the nonpregnant and pregnant states (1, 12, 29, 33). On day 17 of pregnancy, the placentas and fetuses of eNOS−/− mice are reported to be about 10% lighter (9). However, the placentas of eNOS−/− fetuses showed no histological abnormalities, leading Heffer et al. (9) to postulate that the underlying cause of the FGR in the absence of eNOS expression was reduced circulating NO leading to abnormal regulation of uteroplacental and/or fetoplacental blood flow. However, the function of uteroplacental or fetoplacental vessels was not investigated, and no changes in circulating maternal NO metabolites were found to support this
hypothesis (9). Such a notion would, however, be consistent with the evidence from human studies showing that NO does have an important role in regulating blood flow through both uterine and fetoplacental circulations (26, 27) and that such regulation might be abnormal in FGR (24).

Bearing in mind the maternal cardiovascular abnormalities observed in the pregnant eNOS−/− mouse, as well as the evidence that NO appears to have a role in controlling vascular tone in human placental vessels, the overarching aim of this study was to test the hypothesis that FGR in the eNOS−/− mouse was associated with impaired vascular function in uteroplacental and/or umbilical circulations and that this leads to allayed changes in placental nutrient transport function. Placental system A amino acid transporter activity was selected as a model nutrient transporter for study, as a reduction in its activity has previously been shown to be associated with FGR and related to its severity (8, 14). To address this hypothesis, we carried out a multifaceted investigation in pregnant eNOS−/− mice and performed the following investigations: 1) measurement of maternal blood pressure and urine protein excretion; 2) determination of the degree of FGR in eNOS−/− mice by constructing fetal weight distribution curves similar to those used in human pregnancy; 3) measurement of the reactivity of uterine and umbilical vessels using wire myography; 4) assessment of whether placental transport capacity was altered by measuring the expression and activity of the System A amino acid transporter; and 5) investigation for evidence of a potential mechanism linking aberrant uterine artery contractility with reduced System A activity, by measuring tissue hypoxia and the generation of superoxide free radicals in eNOS−/− placentas.

METHODS

Mice and Ethical Approval

Animal care and experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. eNOS−/− mice were obtained from Jackson Laboratories (strain B6.129P2-Nos3tm1Unc/J). Homozygous eNOS−/− mice were mated, and the presence of a copulation plug was denoted as day 0.5 of pregnancy. C57/B6J mice, the background strain, were used as WT control mice for comparison. Animals had free access to food (Beekay Rat and Mouse Diet; Bantin & Kingman) and water and were maintained on a 12:12-h light-dark cycle at 21–23°C. All animals were killed at either embryonic day 17.5 (E17.5) or E18.5 by Schedule 1 procedure in accordance with the UK Animals (Scientific Procedures) Act 1986, and their tissues were harvested.

Genotyping

DNA was extracted from maternal and fetal tail tips using DNeasy kit (Qiagen), and genotype was determined by a triplex PCR reaction using the following primers F: 5′-ATT TCC TGT CCC CTG CCT TC-3′, Mut: 5′-TGG CTA CCC GTG ATA TTG CT-3′, and WT: 5′-GGC CAG TCT CAG AGC CAT AC-3′. Genotyping of pregnant eNOS−/− dams and randomly selected fetuses from each litter confirmed all animals were homozygous for deletion of the eNOS gene.

Maternal Blood Pressure and Heart Rate

Blood pressure and heart rate measurements in nonpregnant mice and pregnant mice at E10.5 and E17.5 were made using a previously validated tail-cuff system (IITC Life Science) (42).

Urine Albumin and Creatinine Concentration

Urine albumin concentration was measured at E18.5 using a mouse albumin ELISA kit (AssayPro), and creatinine concentration was measured by a creatinine assay kit (Cayman Chemical).

Fetal and Placental Measurements

Fetal and placental wet weights were taken after blotting following laparotomy. Fetal anthropology measurements—crown-rump length (from top of head to start of the tail following the curve of the spine), abdominal circumference (taken from where the umbilical cord inserts), and head circumference (above the eyes and ears)—were performed by a single observer using cotton thread.

Maternal Uterine and Umbilical Artery Function

Main loop uterine arteries were dissected from eNOS−/− and WT dams at E17.5 and wire myography were performed as described previously (17). Constriction was measured using phenylephrine (PE; 10−10 to 10−3M) and in vessels preconstricted with PE, endothelium-dependent relaxation was assessed with ACh (10−10 to 10−5M). Myography was also performed on umbilical arteries, as previously described (17), with the modification that vessels were preconstricted to an EC50 of U46619.

System A Amino Acid Transporter Activity and Expression

System A activity in isolated mouse placental vessels. Mouse placental vessels at E17.5 were prepared from the maternal facing plasma membrane of syncytiotrophoblast layer II, and purity was measured by enrichment of alkaline phosphatase, as described previously (18). Enrichment of alkaline phosphatase was not significantly different between groups, confirming comparable membrane purity (11.7 ± 1.9 and 10.5 ± 0.8 in eNOS−/− and WT, respectively; n = 6). System A activity was measured as the Na+-dependent uptake of 14C-MeAIB, as described previously (18).

Unidirectional maternofoetal transfer of 14C-MeAIB. Pregnant mice were anesthetized at E17.5 using an intraperitoneal injection of 0.3 ml Hypnorm-water-midazolam (1:2:1), and surgical procedures were performed as described previously (5). A 100-μl bolus of PBS containing 14C-MeAIB (3.5 μCi) was injected into the tail vein of the dam, which was killed between 1 and 6 min postinjection. Unidirectional maternofoetal clearance (Km0) of 14C-MeAIB was calculated as described previously (5).

mRNA expression of system A transporter isoforms. Primers were designed for the three genes encoding system A isoforms: Slc38a1, Slc38a2, and Slc38a4 (Table 1). Placentas were harvested at E17.5, RNA was extracted, and cDNA was generated, as described previously (4), mRNA expression was measured using real-time quantitative PCR (qPCR) and quantified as described previously (4). All amplicons were of predicted size, and gene identity was confirmed by sequencing.

Placental Hypoxia and Free Radical Generation

Hypoxpyrobe staining. Mice were treated at E17.5 with an intraperitoneal injection of pimonidazole (60 mg/kg Hypoxyprobe-1; Hypoxyprobe) 2 h prior to death. Placental tissue was harvested, fixed, and processed as described previously (4). Five-micrometer sections were incubated with Hypoxyprobe mouse IgG monoclonal antibody 1:100 (0.7 μg/ml) overnight at 4°C. Nonimmunized mouse IgG serum replaced primary antibody in negative controls. A biotinylated F(ab')2 fragment of rabbit anti-mouse IgG (Dako) was applied for 1 h at room temperature (RT: 1:200). Following signal amplification for 30 min with 100 μl ABC elite kit (Vector Laboratories), immunoreactivity was detected with diaminobenzidine followed by counterstaining with Mayer’s hematoxylin. For quantitative immunohistochemical analysis, four sections from four placentas per litter were assessed for
staining intensity. Staining intensity in the junctional and labyrinth zones of the placenta was scored by three individuals blinded to sample identity on a scale ranging from 0 (no stain) to 3 (intense stain).

Superoxide and nitrotyrosine staining. For superoxide staining, 20-μm cryosections were taken of the midplacenta at E18.5, washed, and incubated for 30 min at 37°C with Hanks balanced salt solution (HBSS). Sections were then incubated with 20 μM dihydroethidium (DHE; Sigma) for 30 min at 37°C. Samples were then washed with HBSS. Superoxide was indicated by oxidative fluorescence of DHE. For nitrotyrosine staining, 8-μm cryosections were incubated with rabbit anti-nitrotyrosine antibody (1:125, 5.7 μg/ml; Millipore) for 1 h at RT. Following application of an Alexa Fluor goat anti-rabbit secondary antibody (1:250) for 1 h at RT, immunofluorescent staining intensity was measured.

Statistical Analysis

Data were analyzed using a Mann Whitney U-test, Student's t-test, and one- or two-way ANOVA with post hoc test as appropriate. Data are presented with n representing individual fetuses and placentas or the mean value calculated for an individual litter, as indicated in the legends.

RESULTS

Maternal Blood Pressure and Urinary Protein Concentration

Systolic and diastolic blood pressures were significantly higher in eNOS−/− mice both prior to, and during pregnancy (Table 2). Heart rate was significantly lower in nonpregnant eNOS−/− mice compared with WT controls, but this trend was not maintained during pregnancy (Table 2). Heart rate increased during pregnancy in eNOS−/− mice but decreased in WT (P < 0.05, Kruskal-Wallis test). At E18.5, urinary albumin concentration [mg/dl; median (quartiles)] was significantly higher in eNOS−/− mice [4.28 (3.54, 6.30)] compared with WT [0.93 (0.82, 1.82); P < 0.01, Mann Whitney U-test, n = 8 and 7 for eNOS−/− and WT, respectively] as was urinary the albumin:creatinine ratio [0.68 (0.57, 1.3) and 0.20 (0.11, 0.29), respectively; P < 0.05, Mann Whitney U-test].

Fetal and Placental Weight

Mean weight of eNOS−/− fetuses at E17.5 was ~10% lower than that of WT fetuses, while placental weight was not altered, resulting in a significantly reduced fetal:placental weight ratio (Table 3). To characterize the observed FGR in more detail, fetal weight distribution curves were constructed for both eNOS−/− and WT groups (Fig. 1). Fetal weight distribution of eNOS−/− fetuses was shifted to the left (indicative of a lower weight) and 32% of the eNOS−/− fetuses had a weight below the 5th centile of the normal WT distribution. This reduction in eNOS−/− fetal weight was associated with a significantly reduced abdominal circumference, while head circumference and crown-rump length were unaltered (Table 3).

Maternal Uterine and Umbilical Artery Vascular Function

At E17.5, PE induced a dose-dependent constriction in uterine artery loops from both eNOS−/− and WT dams (Fig. 2A); constriction was significantly increased in arteries from eNOS−/− mice at concentrations of 10⁻⁶ and 10⁻⁵ M (P < 0.05; two-way ANOVA). In preconstricted uterine arteries, ACh elicited endothelium-dependent relaxation in uterine artery loops from both eNOS−/− and WT dams, although this was significantly attenuated in the vessels from eNOS−/− dams (Fig. 2B; P < 0.001; two-way ANOVA). Fetal umbilical artery constriction in response to U46619 was comparable in eNOS−/− and WT mice (Fig. 3A). Umbilical arteries preconstricted with U46619 at EC₅₀ and then treated with incremental doses of the endothelium-dependent vasodilators histamine and substance P (SP)

### Table 1. Sequences (5’→3’) and annealing temperatures for primers targeting system A transporters and the housekeeping gene YWHAZ

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Ta, °C</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc38a1</td>
<td>NM_134086</td>
<td>55</td>
<td>GAGCAAGTCTTGGGACCCAC</td>
<td>CACCACATCACCAGAAGTCG</td>
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<tr>
<td>Slc38a2</td>
<td>NM_175121</td>
<td>55</td>
<td>GCTGTTGCCATTGAATAGC</td>
<td>CAGTTGATGACCGTCGCC</td>
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<tr>
<td>Slc38a4</td>
<td>NM_029705</td>
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<td>CAAATAGGAAGGAGAACGAGG</td>
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<td>YWHAZ</td>
<td>NM_011740</td>
<td>60</td>
<td>AGCAGGACAGGATATAGC</td>
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<td></td>
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<tr>
<td>Ta, annealing temperature.</td>
<td></td>
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</table>

### Table 2. Maternal systolic and diastolic blood pressure and heart rate in nonpregnant and pregnant WT and eNOS−/− mice at E10.5 and E17.5

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>eNOS−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>119.3 ± 2.6 (25)</td>
<td>132.7 ± 3.1** (20)</td>
</tr>
<tr>
<td>E10.5</td>
<td>118.1 ± 2.2 (26)</td>
<td>132.6 ± 3.3** (24)</td>
</tr>
<tr>
<td>E17.5</td>
<td>123.0 ± 6.0 (8)</td>
<td>138.9 ± 3.7* (11)</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>76.9 ± 3.9 (25)</td>
<td>92.3 ± 4.7* (20)</td>
</tr>
<tr>
<td>E10.5</td>
<td>67.3 ± 2.2 (26)</td>
<td>84.2 ± 3.8** (24)</td>
</tr>
<tr>
<td>E17.5</td>
<td>79.9 ± 7.6 (8)</td>
<td>97.9 ± 4.8 (11)</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>523 ± 14 (25)</td>
<td>442 ± 12** (20)</td>
</tr>
<tr>
<td>E10.5</td>
<td>498 ± 14 (26)</td>
<td>472 ± 12 (24)</td>
</tr>
<tr>
<td>E17.5</td>
<td>447 ± 30 (8)</td>
<td>497 ± 17 (11)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = number of dams in parentheses). NP, nonpregnant; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; WT, wild type; eNOS, endothelial nitric oxide synthase. *P < 0.05, **P < 0.01 eNOS−/− vs. WT (two-way ANOVA with Bonferroni post hoc test).

### Table 3. Fetal and placental weights, fetal:placental weight ratio and fetal anthropometric measurements in WT and eNOS−/− mice at E17.5

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>eNOS−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal weight, g</td>
<td>0.88 ± 0.01 (136)</td>
<td>0.79 ± 0.01*** (242)</td>
</tr>
<tr>
<td>Placental weight, g</td>
<td>0.09 ± 0.001 (136)</td>
<td>0.09 ± 0.001 (242)</td>
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<tr>
<td>F:P ratio</td>
<td>10.23 ± 0.19 (136)</td>
<td>9.08 ± 0.14*** (242)</td>
</tr>
<tr>
<td>Abdominal circumference, mm</td>
<td>25.23 ± 0.24 (43)</td>
<td>25.09 ± 0.18 (56)</td>
</tr>
<tr>
<td>Cervix length, mm</td>
<td>24.40 ± 0.30 (43)</td>
<td>20.73 ± 0.26** (56)</td>
</tr>
<tr>
<td>Head circumference, mm</td>
<td>23.37 ± 0.22 (43)</td>
<td>23.00 ± 0.26 (56)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = number of fetuses in parentheses). F:P, fetal-to-placental weight. ***P < 0.01, ***P < 0.001 eNOS−/− vs. WT (unpaired t-test).
mirroring the in vitro observations. Placental eNOS and Slc38a4 revealing 32% of eNOS
The vertical dashed line represents the 5th centile on the WT curve (725 mg),
with Bonferroni’s post hoc test). Na\(^+\)\(\text{P}\) (Fig. 4B). At E17.5, \(K_{\text{mf}}\) for \(^{14}\text{C-MeAIB}\) in eNOS\(^{-/-}\) mice was significantly lower compared with WT \(P < 0.05\) (Mann Whitney \(U\)-test), mirroring the in vitro observations. Placental Slc38a1, Slc38a2, and Slc38a4 mRNA expression showed no differences between eNOS\(^{-/-}\) and WT groups, nor was mRNA expression of the reference gene YWHAZ altered. Relative to a placental calibrator sample, mRNA expression [median (quartiles)] in
eNOS\(^{-/-}\) \((n = 9)\) and WT \((n = 6)\) groups was 0.92 (0.64, 1.71) and 0.85 (0.73, 1.19) for Slc38a1, 0.69 (0.50, 0.93) and 0.36 (0.21, 0.69) for Slc38a2, 1.08 (0.82, 1.61) and 0.87 (0.81, 1.05) for Slc38a4, and 1.15 (0.78, 1.64) and 1.06 (1.00, 1.25) for YWHAZ, respectively.

**Hypoxia and the Generation of Superoxide Free Radicals in the eNOS\(^{-/-}\) Mouse Placenta**

Figure 5 shows representative examples of immunostaining detecting hypoxic adducts in the placentas of eNOS\(^{-/-}\) and WT mice following injection of Hypoxyprobe into the pregnant dams. All placentas exhibited immunoreactive product in both the spongiotrophoblast of the junctional zone, as well as the labyrinth. In all placentas, staining intensity in the spongiotrophoblast was consistently more intense than that in the labyrinth (Fig. 5, A and B). Following quantitative assessment of staining intensity, the spongiotrophoblast of eNOS\(^{-/-}\) placentas demonstrated significantly greater staining intensity compared with WT placentas \((P < 0.05\) Mann Whitney \(U\)-test; Fig. 5D). There was no difference in the intensity of staining in the labyrinth between the groups (Fig. 5E). Immunoreactive specificity was confirmed by the absence of staining in negative controls, where primary antibody was replaced by nonimmune mouse serum (Fig. 5C). At E18.5, DHE immunofluorescent staining intensity (detecting superoxide) was significantly increased in eNOS\(^{-/-}\) mice \((126 \pm 15\%\) intensity of WT control, means \(\pm SE, n = 10\) litters; \(P < 0.05\) one-sample \(t\)-test), indicative of oxidative stress. However, no differences in nitrotyrosine staining (arbitrary values), as a permanent footprint of peroxynitrite, were observed between groups \((92 \pm 14\) and 70 \(\pm 14\) for eNOS\(^{-/-}\) and WT, means \(\pm SE, n = 6\) or 7; \(P > 0.05\)).

**DISCUSSION**

Several phenotypic features observed in eNOS\(^{-/-}\) mice, such as lower fetal weights \((9, 30, 39),\) a prepregnancy hypertension \((2, 12, 29, 33)\) that was maintained throughout pregnancy \((10),\) and an increase in heart rate over gestation \((16),\) agree well with previous observations. The increase in heart rate may be invoked to try and compensate for the lower stroke volume and cardiac output in eNOS\(^{-/-}\) mice at late gestation, arising from improper cardiovascular remodeling and adaptations \((16)\). We have also demonstrated that eNOS\(^{-/-}\) mice at E18.5 had proteinuria, contrasting with the observations of others who did not observe any difference in urine protein concentration but did report an increase in urine protein excretion in eNOS\(^{-/-}\) mice toward late pregnancy that was not
observed in the C57BL6/J background strain (10). The hemodynamic and metabolic characteristics of pregnant eNOS\(^{-/-}\) mice, with the existence of prepregnancy hypertension, deviate from the classification of preeclampsia in human pregnancy of “gestational hypertension that was not present prior to pregnancy coupled with proteinuria” (37), suggesting this model might be better regarded as a model of human chronic hypertension rather than preeclampsia.

This study, for the first time, has examined the vascular reactivity of both uterine and umbilical arteries in eNOS\(^{-/-}\) mice, allowing differential effects of eNOS gene ablation on the uteroplacental and fetoplacental circulations to be explored. Our evidence of increased vasoconstriction to PE and reduced relaxation to the endothelium-dependent vasodilator ACh of eNOS\(^{-/-}\) uterine arteries (Fig. 2) is consistent with previous observations in different vascular beds of nonpregnant mice, showing that deletion of eNOS resulted in enhanced PE-induced constriction and reduced vasodilatory capacity to ACh (3, 7, 12, 21). It is of interest that myometrial arterial reactivity in human FGR mimics the responses observed here in uterine arteries of eNOS\(^{-/-}\) mice; myometrial vessels isolated from human FGR pregnancies showed increased constriction (U46619 and arginine vasopressin) and reduced endothelium-dependent relaxation (bradykinin) compared with controls (41).

In keeping with our previous findings (17), both histamine and substance P, as endothelial-dependent agonists, were ineffective in eliciting vasodilation of umbilical arteries in both WT and eNOS\(^{-/-}\) mice (Fig. 3), showing that this lack of response was unrelated to eNOS activity and NO production. However, umbilical vessels from both groups demonstrated vasodilation in response to donated NO following SNP treatment, confirming NO signal transduction mechanisms were intact. This phenomenon has been observed in human umbilical arteries, with endothelium-dependent agonists that usually elicit vascular relaxation having no effect on preconstricted arteries (38). These results suggest that agonist-induced NO release may not be an important regulator of fetoplacental arterial tone in mice, and it is possible that, in common with the
human fetoplacental vasculature, flow-induced NO release might be more important (19). Collectively, our observations point to dysfunctional regulation of the uteroplacental, rather than the fetoplacental, circulation in eNOS^−/− mice.

At E17.5, eNOS^−/− fetuses displayed a significantly smaller abdominal circumference (Table 3) in agreement with others (32), with a significantly reduced trunk diameter of eNOS^−/− embryos detectable as early as day 8.5 of pregnancy (31). However, in contrast with others, we did not observe a reduced crown-rump length (9, 32) or head circumference (32). Overall, our data suggest the growth restriction of eNOS^−/− fetuses is asymmetric, with evidence of reduced placental efficiency, as exemplified by the lower fetal:placental weight ratio (i.e., lower weight fetus produced per gram of placenta compared with WT). These characteristics are similar to those seen in human FGR (6).

One of the novel aspects of this study in eNOS^−/− mice was that placental amino acid transport was examined, allowing mechanistic insights regarding whether nutrient transport was altered in this model of FGR. This aspect was investigated in vivo by measuring placental System A transporter activity as the maternofetal clearance of $^{14}$C-MeAIB into isolated plasma membrane vesicles as well as in vitro where System A-mediated uptake of $^{14}$C-MeAIB into isolated plasma membrane vesicles was also significantly reduced in the placentas of eNOS^−/− fetuses. As the vesicles were derived from the maternal facing plasma membrane of syncytiotrophoblast layer II of mouse placenta, this stands as a plausible plasma membrane locus underpinning the aberrant system A activity (18). Both approaches clearly demonstrated that the placenta of eNOS^−/− fetuses has reduced System A transporter activity and a diminished ability to transport $^{14}$C-MeAIB as a substrate to the fetus (Fig. 4). This phenomenon was not associated with a change in expression of Slc38a1, Slc38a2, or Slc38a4 genes, which encode for the three sodium-coupled neutral amino acid transporter (SNAT) isoforms, SNAT1, SNAT2, and SNAT4 that mediate system A activity, suggesting that the reduced system A activity was attributable to posttranscriptional regulation.

As System A is down-regulated by reduced oxygen concentration in human placent al cytotrophoblast cells (28), we explored the possibility that dysfunctional regulation of uterine artery contractility in eNOS^−/− mice leads to diminished oxygen delivery and tissue hypoxia, contributing to the reduced System A activity. Using Hypoxyprobe, we found the degree of hypoxia in the syncytiotrophoblast of placentas from
eNOS−/− fetuses was relatively higher compared with WT. However, there was no evidence of increased labyrinthine hypoxia in eNOS−/− placentas (Fig. 5). We consistently observed that the spongiosotrophoblast was relatively hypoxic compared with labyrinth in both groups. This maintenance of relative hypoxia and oxygen gradients between neighboring cellular layers is intriguing and accords well with previous reports describing relatively low levels of oxygen in trophoblast giant cells, spongiosotrophoblast, and glycogen cells of the junctional zone compared with closely apposed cells or the labyrinth (20, 43). The physiological significance of this is incompletely understood but may relate to the sustainment of endocrine and paracrine functions performed by cells comprising the junctional zone (11). We cannot exclude the possibility that regions of the labyrinth experienced a mild hypoxia, which was not detectable within the threshold of sensitivity by this approach (pimonidazole binds at oxygen tensions of ≤10 mmHg; 1.5% oxygen). A relative lack of labyrinthine hypoxia in the eNOS−/− placenta might also reflect reduced fetal extraction of oxygen by the growth-restricted fetus, as suggested in human FGR (34). Nevertheless, our observation of increased superoxide generation in the placenta of eNOS−/− mice is certainly compatible with the notion of a hypoxic period followed by reperfusion injury and subsequent increased cellular oxygenation (13) related to abnormal uterine vascular reactivity. Various cytotoxic events arise from the cellular accumulation of superoxide, including lipid peroxidation, protein denaturation, DNA oxidation, and perturbed intracellular signaling (13). The reduced placental system A activity observed here with both in vitro (vesicle uptake) and in vivo (maternofetal clearance) approaches using MeAIB as a substrate, suggests that the defect giving rise to this effect in eNOS−/− mice was retained following plasma membrane isolation. This implicates either an intrinsic change in the plasma membrane of syncytiotrophoblast layer II that influences System A catalytic activity and/or that SNAT4 proteins have been modified in some way that alters transporter activity. We cannot distinguish between these possibilities, and this clearly merits further investigation. However, it is noteworthy that the magnitude of the reduction observed in System A activity in vesicles from eNOS−/− mice in vitro (~73%) was markedly greater than that observed in vivo, as measured by maternofetal clearance (~21%). Direct comparison of these observations is difficult given the disparate methodologies, but it is tempting to speculate that endogenous intracellular and/or extracellular factors present in vivo modulate system A activity further.

**Perspectives and Significance**

Two major observations are reported in this study: FGR in eNOS−/− mice is associated with impaired uterine artery function and diminished placental System A amino acid transporter activity. These observations extend the characterization of functional phenotype in eNOS−/− mice and also advance knowledge regarding the mechanisms that underlie the etiology of FGR in this model. Uterine arteries of eNOS−/− mice exhibited greater vasoconstriction and impaired vasodilatory capacity compared with WT, implicating dysregulation of the uteroplacental circulation. This observation generated the postulate that altered uterine arterial reactivity leads to ischemia/reperfusion events, evidenced by the increased placental hypoxia and superoxide generated in eNOS−/− placentas. The highly novel finding that placentas of eNOS−/− fetuses exhibited impaired nutrient transporter capacity, as measured by System A amino acid transporter activity, mirrors the trend found in human FGR, where the reduction in placental System A amino acid transporter activity is related to the severity of FGR. Collectively, our data suggest a new framework for understanding the etiological pathway linking maternal cardiovascular dysfunction to FGR: abnormal uterine arterial function reduces fetal growth predominantly by causing hypoxia and free radical production in the placenta with effects on placental nutrient transport capacity, rather than through reduced oxygen delivery to the fetus per se. Three different models of human FGR have previously been proposed by Kingdom and Kaufmann (15) based on phenotypes describing placental villous development and placental and fetal oxygenation. One of these models, uteroplacental hypoxia, is compatible with the etiological pathway of FGR as proposed here for the eNOS−/− mouse. This suggests that the eNOS−/− mouse model might be usefully applied to characterizing a subpopulation of women with heterogeneous disease, which is currently described by the single term of FGR. Using this and other gene knockout mice could provide a novel framework to explore different FGR aetiologies, thus providing an experimental classification of the disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**