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

L.C. Kusinski¹, J.L. Stanley², M.R. Dilworth¹, C.J. Hirt², I.J. Andersson², L.J. Renshall¹, B.C. Baker¹, P.N. Baker², C.P. Sibley¹, M. Wareing¹, J.D. Glazier¹.

¹Maternal and Fetal Health Research Centre, School of Biomedicine, Manchester Academic Health Science Centre, The University of Manchester, St Mary’s Hospital, Manchester M13 9WL, UK. ²Faculty of Medicine and Dentistry, University of Alberta, Edmonton T6G 2S2, Canada.

Corresponding author:
Dr J. D. Glazier
Maternal and Fetal Health Research Centre
School of Biomedicine, University of Manchester
5th Floor (Research)
St Mary’s Hospital
Oxford Road
Manchester
M13 9WL
Tel: 0161 276 6485
Fax: 0161 701 6971
e-mail: j.glazier@manchester.ac.uk

Running title: Placental function in the eNOS knockout mouse
Abstract

Fetal growth restriction (FGR) is the inability of a fetus to reach its genetically predetermined growth potential. In the absence of 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, as compared to wild-type (WT) mice. eNOS−/− fetal weight and abdominal circumference were significantly reduced compared to 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−/− mouse placentas demonstrated increased hypoxia at E17.5, with elevated superoxide compared to 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 aetiology of FGR in human pregnancy.

**Key words:** nitric oxide, pregnancy, placental insufficiency
**Introduction**

Fetal growth restriction (FGR) is defined as the inability of a fetus to achieve its genetically pre-determined 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 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 catalyses 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, the modulation of myogenic tone in mesenteric and uterine arteries, and is 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 to wild-type (WT) controls (10); consistent with the concept that eNOS activity is involved in the regulation of blood pressure in the non-pregnant 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 Hefler 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 allied 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 multi-faceted investigation in pregnant eNOS−/− mice and performed the following investigations: (i) measurement of maternal blood pressure and urine protein excretion; (ii) determination of the degree of FGR in eNOS−/− mice by constructing fetal weight distribution curves similar to those used in human pregnancy; (iii) measurement of the reactivity of uterine and umbilical vessels using wire myography; (iv) assessment of whether placental transport capacity was altered by measuring the expression and activity of the System A amino acid transporter; (v) evidence was sought for 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-Nos3\(^{tm1Unc}\)/J). Homozygous eNOS\(^{−/−}\) mice were mated and the presence of a copulation plug denoted as day 0.5 of pregnancy. C57/Bl6J 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 tissue harvested.

Genotyping

DNA was extracted from maternal and fetal tail tips using DNeasy kit (Qiagen, Sussex, UK) and genotype 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'; 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 non-pregnant 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; St Charles, MO, USA) and creatinine concentration was measured by a creatinine assay kit (Cayman Chemical Company, Ann Arbor, USA).

Fetal and placental measurements

Fetal and placental wet weights were taken after blotting following laparotomy. Fetal anthropometric 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 performed as described previously (17). Constriction was measured using phenylephrine (PE, \(10^{-10}\) to \(10^{-5}\)M) and in vessels pre-constricted with PE, endothelial-dependent relaxation was assessed with acetylcholine (ACh, \(10^{-10}\) to \(10^{-5}\)M). Myography was also performed on umbilical arteries as previously described (17), with the modification that vessels were pre-constricted to an EC\(_{80}\) of U46619.

System A amino acid transporter activity and expression

System A activity in isolated mouse placental vesicles

Mouse placental vesicles at E17.5 were prepared from the maternal-facing plasma membrane of syncytiotrophoblast layer II and purity 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 maternofetal transfer of ¹⁴C-MeAIB

Pregnant mice were anaesthetised at E17.5 using an intra-peritoneal injection of 0.3ml Hypnorm/water/midazolam (1:2:1), and surgical procedures performed as described previously (5). A 100μl bolus of PBS containing ¹⁴C-MeAIB (3.5μCi) was injected into the tail vein of the dam which was sacrificed between 1 to 6 min post-injection. Unidirectional maternofetal clearance (K mf) 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 extracted and cDNA 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

**Hypoxyprobe staining**

Mice were treated at E17.5 with an intra-peritoneal injection of pimonidazole (60mg/kg Hypoxyprobe-1; Hypoxyprobe, Int. Massachusetts, USA) 2h prior to sacrifice. Placental tissue was harvested, fixed and processed as described previously (4). 5µm sections were incubated with Hypoxyprobe mouse IgG monoclonal antibody 1:100 (0.7µg/ml) overnight at 4°C. Non-immunized mouse IgG serum replaced primary antibody in negative controls. A biotinylated F(ab’)_2 fragment of rabbit anti-mouse IgG (Dako, Cambridgeshire) was applied for 1h at RT (1:200). Following signal amplification for 30min with 100µl ABC elite kit (Vector Laboratories, Peterborough), immunoreactivity was detected with diaminobenzidine followed by counterstaining with Mayer’s Haematoxylin. For quantitative immunohistochemical analysis, 4 sections from 4 placentas per litter were assessed for staining intensity. Staining intensity in the junctional and labyrinth zones of the placenta was scored by 3 individuals blinded to sample identity on a scale ranging from 0 (no stain) to 3 (intense stain).

**Superoxide and nitrotyrosine staining**

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

Statistical Analysis
Data were analysed using a Mann Whitney 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/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<sup>−/−</sup> mice both prior to, and during pregnancy (Table 2). Heart rate was significantly lower in non-pregnant eNOS<sup>−/−</sup> mice compared to WT controls, but this trend was not maintained during pregnancy (Table 2). Heart rate increased during pregnancy in eNOS<sup>−/−</sup> 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<sup>−/−</sup> mice (4.28 (3.54, 6.30)) compared to WT (0.93 (0.82, 1.82); P<0.01, Mann Whitney test, n=8 and 7 for eNOS<sup>−/−</sup> and WT respectively) as was urinary albumin:creatinine ratio (0.68 (0.57, 1.3) and 0.20 (0.11, 0.29) respectively; P<0.05, Mann Whitney test).

Fetal and placental weight

Mean weight of eNOS<sup>−/−</sup> fetuses at E17.5 was ~10% lower than that of WT fetuses, whilst placental weight was not altered, resulting in a significantly reduced fetal:placental weight ratio (Table 3). In order to characterise the observed FGR in more detail, fetal weight distribution curves were constructed for both eNOS<sup>−/−</sup> and WT groups (Figure 1). Fetal weight distribution of eNOS<sup>−/−</sup> fetuses was shifted to the left (indicative of a lower weight) and 32% of the eNOS<sup>−/−</sup> fetuses had a weight below the 5<sup>th</sup> centile of the normal WT distribution. This reduction in eNOS<sup>−/−</sup> fetal weight was associated with a significantly reduced abdominal circumference whilst 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<sup>−/−</sup> and WT dams (Figure 2A); constriction was significantly increased in arteries from eNOS<sup>−/−</sup> mice at concentrations of 10<sup>−6</sup> and 10<sup>−5</sup>M (P<0.05; two-way ANOVA). In pre-constricted uterine arteries, ACh elicited endothelium-dependent relaxation in uterine artery loops from both eNOS<sup>−/−</sup> and WT dams, although this was significantly attenuated in the vessels from eNOS<sup>−/−</sup> dams (Figure 2B; P<0.001; two-way ANOVA). Fetal umbilical artery constriction in response to U46619 was comparable in eNOS<sup>−/−</sup> and WT mice (Figure 3A). Umbilical arteries pre-constricted with U46619 at EC<sub>80</sub>, and then treated with incremental doses of the endothelial-dependent vasodilators histamine and substance P (SP) (Figures 3B and C), failed to elicit relaxation in either eNOS<sup>−/−</sup> or WT vessels. In contrast, when NO donation was provided by sodium nitroprusside (SNP; Figure 3D), umbilical arteries of both groups showed a similar dose-dependent response in endothelial-independent relaxation.

System A amino acid transporter activity

Uptake of 14C-MeAIB into placental plasma membrane vesicles was significantly higher in the presence of Na<sup>+</sup> compared to the absence of Na<sup>+</sup> (P<0.001, two-way ANOVA with Bonferroni’s post-test). Na<sup>+</sup>-dependent uptake of 14C-MeAIB was linear over 15-60s in both eNOS<sup>−/−</sup> and WT placental vesicles (r<sup>2</sup>=0.94 and r<sup>2</sup>=0.95 respectively; Figure 4A). However, uptake into placental vesicles from eNOS<sup>−/−</sup> fetuses was significantly lower than that into vesicles from WT fetuses (P<0.0001; F-test); at 60s, System A activity in eNOS<sup>−/−</sup> placentas was ~27% of that in WT. To test whether this marked reduction in placental System A activity in eNOS<sup>−/−</sup> mice in vitro was
mirrored by a reduced maternofetal transport of $^{14}$C-MeAIB in vivo, we measured unidirectional maternofetal transfer of $^{14}$C-MeAIB ($^{14}$C-MeAIB $K_{mf}$; Figure 4B). At E17.5, $K_{mf}$ for $^{14}$C-MeAIB in eNOS$^{-/-}$ mice was significant lower as compared to WT (P<0.05; Mann Whitney 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 respectively was (0.92 (0.64, 1.71), 0.85 (0.73, 1.19)) for $Slc38a1$, (0.69 (0.50, 0.93), 0.36 (0.21, 0.69)) for $Slc38a2$, (1.08 (0.82, 1.61), 0.87 (0.81, 1.05)) for $Slc38a4$ and (1.15 (0.78, 1.64), 1.06 (1.00, 1.25)) for $YWHAZ$.

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 (Figures 5A and B). Following quantitative assessment of staining intensity, the spongiotrophoblast of eNOS$^{-/-}$ placentas demonstrated significantly greater staining intensity compared to WT placentas (P<0.05, Mann Whitney test; Figure 5D). There was no difference in the intensity of staining in the labyrinth between the groups (Figure 5E). Immunoreactive specificity was confirmed by the absence of staining in negative controls where primary antibody was replaced by non-immune mouse serum (Figure 5C). At E18.5, DHE immunofluorescent staining intensity (detecting
superoxide) was significantly increased in eNOS<sup>−/−</sup> mice (126 ± 15% intensity of WT control, mean ± SEM, 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 ± 14 and 70 ± 14 for eNOS<sup>−/−</sup> and WT, mean ± SEM, n=6-7).
Discussion

Several phenotypic features observed here in eNOS<sup>−/−</sup> mice such as lower fetal weights (9, 30, 39), pre-pregnancy 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<sup>−/−</sup> mice at late gestation arising from improper cardiovascular remodelling and adaptations (16). We have also demonstrated that eNOS<sup>−/−</sup> 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<sup>−/−</sup> mice towards late pregnancy that was not observed in the C57Bl6/J background strain (10). The hemodynamic and metabolic characteristics of pregnant eNOS<sup>−/−</sup> mice, with the existence of pre-pregnancy hypertension, deviate from the classification of pre-eclampsia 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 pre-eclampsia.

This study, for the first time, has examined the vascular reactivity of both uterine and umbilical arteries in eNOS<sup>−/−</sup> mice, allowing differential effects of eNOS gene ablation on the utero-placental and fetoplacental circulations to be explored. Our evidence of increased vasoconstriction to PE and reduced relaxation to the endothelial-dependent vasodilator ACh of eNOS<sup>−/−</sup> uterine arteries (Figure 2) is consistent with previous observations in different vascular beds of non-pregnant 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<sup>−/−</sup> mice; myometrial vessels isolated from human FGR pregnancies showed increased constriction (U46619 and arginine vasopressin) and reduced endothelial-dependent relaxation (bradykinin) when compared to 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<sup>−/−</sup> mice (Figure 3) showing 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 endothelial-dependent agonists that usually elicit vascular relaxation having no effect on pre-constricted 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<sup>−/−</sup> mice.

At E17.5, eNOS<sup>−/−</sup> fetuses displayed a significantly smaller abdominal circumference (Table 3) in agreement with others (32), with a significantly reduced trunk diameter of eNOS<sup>−/−</sup> 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 g fetus produced per g placenta as compared to 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 14C-MeAIB, a specific substrate of system A. We demonstrated that in the placentas of eNOS−/− fetuses, maternofetal clearance of 14C-MeAIB was significantly reduced. This paralleled observations in vitro where System A-mediated uptake of 14C-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 14C-MeAIB as a substrate to the fetus (Figure 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, 2 and 4 that mediate system A activity, suggesting that the reduced system A activity was attributable to post-transcriptional regulation.
As system A is downregulated by reduced oxygen concentration in human placental cytотrophoblast cells (28), we explored the possibility that dysfunctional regulation of uterine artery contractility in eNOS<sup>−/−</sup> 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 spongiotrophoblast of placentas from eNOS<sup>−/−</sup> fetuses was relatively higher as compared to WT. However, there was no evidence of increased labyrinthine hypoxia in eNOS<sup>−/−</sup> placentas (Figure 5). We consistently observed that the spongiotrophoblast was relatively hypoxic compared to labyrinth in both groups. This maintenance of relative hypoxia and oxygen gradients between neighbouring cellular layers is intriguing and accords well with previous reports describing relatively low levels of oxygen in trophoblast giant cells, spongiotrophoblast and glycogen cells of the junctional zone as compared to 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 ≤10mm Hg; 1.5% oxygen). A relative lack of labyrinthine hypoxia in the eNOS<sup>−/−</sup> 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<sup>−/−</sup> 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 signalling (13). The reduced placental system A activity observed here with both *in vitro* (vesicle uptake) and *in vivo* (maternofetal clearance) approaches using MeAIB as 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 SNAT 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 characterisation of functional phenotype in eNOS−/− mice and also advance knowledge regarding the mechanisms that underlie the aetiology of FGR in this model. Uterine arteries of eNOS−/− mice exhibited greater vasoconstriction and impaired vasodilatory capacity as compared to 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\textsuperscript{-/-} placentas. The highly novel finding that placentas of eNOS\textsuperscript{-/-} 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 aetiological 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 \textit{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 aetiological pathway of FGR as proposed here for the eNOS\textsuperscript{-/-} mouse. This suggests that the eNOS\textsuperscript{-/-} mouse model might be usefully applied to characterising 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.
Author contributions


Acknowledgements

This work was supported by the BBSRC (studentship to L.C.K.) and a MRC Programme Grant (92495; C.P.S., P.N.B. and M.W.). We are very grateful to staff in the Manchester Biological Services Facility for their cooperation. We would like to thank Dr S. Greenwood for critical review of the manuscript.
References


Figure Legends

Figure 1. Fetal weight distribution curves at E17.5. Mean fetal weight of eNOS<sup>−/−</sup> mice (dashed line, r²=0.79; n=242 fetuses, 41 litters) was significantly lower than in WT control mice (solid line, r²=0.97; n=136 fetuses, 20 litters). The vertical dashed line represents the 5<sup>th</sup> centile on the WT curve (725mg), revealing 32% of eNOS<sup>−/−</sup> fetuses fall below this.

Figure 2. Maternal uterine arterial reactivity at E17.5. Constriction of loop uterine arteries in response to PE (A) was significantly greater, and relaxation by ACh (B) significantly lower, in eNOS<sup>−/−</sup> mice (dashed line; n=10 from 5 dams) compared to WT controls (solid line; n=8 from 4 dams). *P<0.05, **P<0.001; two-way ANOVA with Bonferroni post-hoc test.

Figure 3. Umbilical arterial reactivity at E17.5. Maximum constriction of umbilical arteries in response to U46619 in eNOS<sup>−/−</sup> mice (open circles; n=22 arteries from 7 litters) was similar to WT controls (solid circles; n=13 arteries from 7 litters) (A). Each data point represents the mean constriction from two vessels from the same animal. The horizontal line represents the median. Relaxation of umbilical arteries of eNOS<sup>−/−</sup> mice (dashed line; n=22 arteries from 7 litters) compared to WT controls (solid line; n=13 arteries from 7 litters) (B-D). The endothelium-dependent vasodilators histamine (B) and SP (C) did not elicit relaxation in arteries pre-constricted to U46619 (EC<sub>80</sub>) in either eNOS<sup>−/−</sup> or WT mice. Arteries from both groups of mice showed a dose-dependent relaxation to the NO donor SNP; there was no significant difference between eNOS<sup>−/−</sup> and WT mice (D).

Figure 4. System A amino acid transporter activity at E17.5. System A activity (Na<sup>+</sup>-dependent <sup>14</sup>C-MeAIB uptake) in placental vesicles of eNOS<sup>−/−</sup> (dashed line; n=6 vesicle isolates from 17 dams with 98 fetuses) mice compared to WT controls (solid line; n=6 vesicle isolates from 19 dams with 144 fetuses) (A). Lines were fitted with least squares linear regression and uptake was significantly lower in eNOS<sup>−/−</sup> mice at all time points (*P<0.001; F test). Unidirectional maternofetal clearance of <sup>14</sup>C-MeAIB
\(^{14}\text{C-\text{MeAIB} K_{mf}}\) across placentas of eNOS\(^{\sim/-}\) (open circles; \(n=10\) litters with 59 placentas) and WT (solid circles; \(n=9\) litters with 67 placentas) (B) mice. Each data point corresponds to the mean value of \(^{14}\text{C-\text{MeAIB} K_{mf}}\) calculated for one litter. Horizontal line denotes the median. \(*P<0.05;\) Mann Whitney test.

**Figure 5.** Immunohistochemical staining for placental hypoxia. Hypoxyprobe was used to localise hypoxic adducts by pimonidazole binding at E17.5 in WT control (A) and eNOS\(^{\sim/-}\) (B) mice (X40 magnification). Negative control showed absence of immunoreactive staining (C). Assessment of Hypoxyprobe staining intensity in the spongiotrophoblast (D) and labyrinth (E) in mouse placentas from WT (closed circles; \(n=16\) placentas from 4 litters) compared to eNOS\(^{\sim/-}\) (open circles; \(n=20\) placentas from 5 litters). Staining intensity in the spongiotrophoblast layer was significantly higher in eNOS\(^{\sim/-}\) mice compared to WT, but this trend was not observed in the labyrinth. Horizontal line represents the median. \(*P<0.05;\) Mann Whitney test. **Key:** BS, Maternal blood space; D, Decidua; GC, Giant cell; L, Labyrinth; Sp, Spongiotrophoblast.
**Tables**

Table 1. Sequences (5’→3’) and annealing temperatures (Ta) 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><em>Slc38a1</em></td>
<td>NM_134086</td>
<td>55</td>
<td>GAGCAAGTCTTCGGCACCAC</td>
<td>CACCATCACCACCAACACTCG</td>
</tr>
<tr>
<td><em>Slc38a2</em></td>
<td>NM_175121</td>
<td>55</td>
<td>GCCTGGCATTCAATAGC</td>
<td>CGTTCATCATCCGTCTCC</td>
</tr>
<tr>
<td><em>Slc38a4</em></td>
<td>NM_027052</td>
<td>55</td>
<td>CAATAGAAGACGGAAGG</td>
<td>GCTGTCCATGAATCTGTC</td>
</tr>
<tr>
<td><em>YWHAZ</em></td>
<td>NM_011740</td>
<td>60</td>
<td>AGCAGGCAGAGCGATATG</td>
<td>TCAGCACCTCCGTCTTC</td>
</tr>
</tbody>
</table>
Table 2. Maternal systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) in non-pregnant (NP) and pregnant WT and eNOS<sup>−/−</sup> mice at E10.5 and E17.5.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>eNOS&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</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</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</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</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>523±14 (25)</td>
<td>444±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 mean ± S.E.M. (n, no. dams)

*P<0.05, **P<0.01 eNOS<sup>−/−</sup> vs. WT (Two-way ANOVA with Bonferroni post-hoc test).
Table 3. Fetal and placental weights, fetal: placental weight (F:P) ratio and fetal anthropometric measurements in WT and eNOS<sup>−/−</sup> at E17.5

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>eNOS&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal weight (g)</strong></td>
<td>0.88 ± 0.01 (136)</td>
<td>0.79 ± 0.01*** (242)</td>
</tr>
<tr>
<td><strong>Placental weight (g)</strong></td>
<td>0.09 ± 0.001 (136)</td>
<td>0.09 ± 0.001 (242)</td>
</tr>
<tr>
<td><strong>F:P ratio</strong></td>
<td>10.23 ± 0.19 (136)</td>
<td>9.08 ± 0.14*** (242)</td>
</tr>
<tr>
<td><strong>Crown rump length (mm)</strong></td>
<td>25.23 ± 0.24 (43)</td>
<td>25.09 ± 0.18 (56)</td>
</tr>
<tr>
<td><strong>Abdominal circumference (mm)</strong></td>
<td>24.70 ± 0.30 (43)</td>
<td>23.23 ± 0.30** (56)</td>
</tr>
<tr>
<td><strong>Head circumference (mm)</strong></td>
<td>23.37 ± 0.22 (43)</td>
<td>23.00 ± 0.26 (56)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M (n, no. fetuses).

**P<0.01, ***P<0.0001 eNOS<sup>−/−</sup> vs. WT (Unpaired t-test).
Figures

Figure 1

![Graph showing distribution of fetal weight](image)

*Fetal weight (mg)*
Figure 2

A. 

B. 

Constriction
(% of control)

Relaxation
(% max PE constriction)

Log [PE] (M)

Log [ACh] (M)
Figure 4

A. Na⁺ dependent \(^{14}\)C-MeAIB uptake (nmol/mg protein) vs. Time (s) for WT and eNOS⁻/⁻ cells.

B. \(^{14}\)C-MeAIB K\text{mf} (µl/min/g placenta) for WT and eNOS⁻/⁻ cells.
Figure 5

A. GC, BS, Sp, D

B. GC, D, Sp, L

C. GC, D, Sp, L

D. Score

E. Score

WT vs. eNOS−/−