Effect of Prenatal Hypoxia in Transgenic Mouse Models of Preeclampsia and Fetal Growth Restriction

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Abstract

Mice lacking endothelial nitric oxide synthase (eNOS^{-/-}) or catechol-O-methyl transferase (COMT^{-/-}) exhibit a preeclampsia-like phenotype and fetal growth restriction. We hypothesized that a hypoxic insult would result in a more severe phenotype. Pregnant eNOS^{-/-}, COMT^{-/-} and control (C57BL/6J) mice were randomized to hypoxic (10.5% O₂) or normal conditions (20.9% O₂) from gestational day 10.5 to 18.5. Hypoxia increased the blood pressure in all genotypes and proteinuria in C57BL/6J and eNOS^{-/-} mice. Fetal survival was significantly reduced following hypoxia, particularly in eNOS^{-/-} mice. Birth weight was decreased in both C57BL/6J and COMT^{-/-} mice. Placentas from COMT^{-/-} mice demonstrated increased peroxynitrite. Despite similar hypoxia-induced effects on maternal blood pressure and proteinuria, eNOS^{-/-} embryos have a decreased tolerance to hypoxia. Compared to C57BL/6J, COMT^{-/-} mice exhibited less severe changes in proteinuria and fetal growth when exposed to prenatal hypoxia. This relative resistance to prenatal hypoxia was associated with a significant increase in placental levels of peroxynitrite.

Keywords

hypoxia, preeclampsia, fetal growth restriction

Introduction

Preeclampsia (PE) and fetal growth restriction (FGR) are pregnancy-specific disorders that complicate up to 10% of all pregnancies.¹ Together they are responsible for the majority of maternal and fetal morbidity and mortality associated with complicated pregnancies.^{2,3} The PE, characterized by the onset of hypertension and proteinuria during pregnancy, is a leading cause of maternal mortality, associated with an estimated 50 000 to 100 000 deaths globally each year.⁴ The FGR is defined as a fetus that fails to reach its genetic growth potential and is associated with a greatly increased risk of perinatal morbidity and mortality as well as long-term consequences such as an increased risk of developing cardiovascular disease later in life.^{5,6}

The etiologies of PE and FGR are complex and as yet poorly understood. There is evidence, however, to suggest that, there are a number of genetic and environmental factors common to both conditions, which contribute to increased uterine artery resistance and impaired placental development, resulting in increased peripheral vascular resistance and fetoplacental ischemia/hypoxia.⁷⁻¹⁰ Further, hypoperfusion of the placenta causes an increase in the production of a number of factors, including reactive oxygen species such as superoxide anions.¹¹ This increase in oxidative stress may reduce vasodilation further due to the scavenging of nitric oxide (NO), leading to increased

vascular contractility¹² and contributing to a self-perpetuating process which progressively exacerbates the acute and progressive endothelial dysfunction observed in women with PE.¹³

The lack of understanding of these pathologies can be attributed in part to the lack of appropriate animal models. Our group has experience in using genetically modified murine models that exhibit a PE/FGR-like phenotype, including mice lacking the endothelial NO synthase (eNOS^{-/-}) and mice deficient in the enzyme catechol-O-methyl transferase (COMT^{-/-}), both of which have been used to study these conditions.¹⁴⁻¹⁷ None of

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these models, however, develops what would be considered to be a severe PE-like phenotype.

From a clinical perspective, it has been suggested that both genetic and environmental factors play a fundamental role in the development of PE and FGR.^{18,19} Previous studies using murine models^{20,21} have observed that the interaction between altered genetic backgrounds, such as the deletion of the gene encoding for interleukin 10 (IL-10^{-/-}) and prenatal exposure to environmental insults, such as maternal hypoxia, could have a synergistic effect resulting in a more severe PE-like phenotype in mice. Together, these results suggest that animal models integrating genetic and environmental factors could be an interesting approach to develop more severe, consistent, and clinically relevant animal models of PE; such models would aid the investigation of potential therapeutic strategies.

The aim of this study, therefore, was to determine the interaction between genetic conditions known to cause a mild PE/FGR phenotype and prenatal exposure to a relevant perinatal hypoxic insult in the development of more severe PE/FGR-like phenotypes in already recognized murine models. We hypothesize that exposing pregnant COMT^{-/-} and eNOS^{-/-} mice will result in a more severe PE/FGR phenotype.

Methods

All protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care and conformed to the *Guide for the Care and Use of Laboratory Animals* (copyright 1996, National Academy of Science).

Animals and Treatments

Female COMT^{-/-} (obtained with agreement from Prof J Gogos, Columbia University), eNOS^{-/-}, and C57BL/6J mice (purchased from Jackson laboratories) aged 8 to 12 weeks were mated at night with males of a corresponding genotype. In the morning a plug was detected and was denoted as gestational day (GD) 0.5. Pregnant mice were randomized to prenatal hypoxia (10.5% $\pm$ 0.3% O₂) or normal conditions (20.9% O₂) from GD 10.5 to 18.5 (term is GD 19.5). A concentration of 10.5% O₂ was chosen, based on previous studies, as one at which control mice undergo a normal pregnancy, but which may have a synergistic effect when combined with the specific genetically modified mice. Previous studies observed no effect of hypoxia on either wild-type or knockout mice at a concentration of 11% O₂, but a significant effect on both the groups at 9.5% O₂ was observed.²¹ An intermediate concentration of 10.5% O₂ was therefore chosen. Mice assigned to the prenatal hypoxia group were placed inside a sealed acrylic chamber (Animal Chamber for Disease Modeling type A, Biospherix, Lacona, New York) that maintained an internal concentration of oxygen at 10.5% $\pm$ 0.3% by regulating nitrogen infusion. A plastic tray was placed in the lower level of the chamber with 250 g of soda lime (J. T. Baker, Phillipsburg, New Jersey) to scavenge excess CO₂ inside the chamber. After 3 days inside the chamber

(GD 14.5), mice were placed in clean cages and given fresh food and water during a rapid cage change.²²

Blood Pressure and Proteinuria

Blood pressure was measured using the tail-cuff method (IITC Life Science, Woodland Hills, California). Prior to mating, mice were placed in restraint tubes for 5 minutes on 3 consecutive days. Blood pressure was then recorded using tail-cuff plethysmography at GD 17.5.²³ Due to necessity, blood pressure was measured outside the hypoxic chamber; time taken to perform these measurements was minimized as much as possible. Urine was collected at GD 18.5 and stored at -80°C. Samples were then assayed to assess albumin concentration (AssayPro, St Charles, Missouri) as well as creatinine concentration (Cayman Chemical Company, Ann Arbor, Michigan).²⁴

Ultrasound Biomicroscopy

Both uterine and umbilical arteries and vein blood flow velocity were assessed at GD 17.5. Both left and right uterine arteries and the umbilical artery from at least 2 fetuses were assessed. Before the procedure, mice were anaesthetized with isoflurane (5%) in air. Maternal heart rate, respiratory rate, and rectal temperature were continuously monitored, and anesthetic concentration was adjusted (0.5%-1.5%) to maintain a constant maternal heart rate of 550 $\pm$ 50 bpm and a respiratory rate of 120 $\pm$ 20 cpm. Heating was adjusted to maintain rectal temperature of 37 $\pm$ 1°C. Hair was removed from the abdomen using chemical hair remover. Prewarmed gel was used as an ultrasound-coupling medium. Mice were then imaged transcutaneously using an ultrasound biomicroscope (model Vevo 770, VisualSonics, Toronto, Ontario, Canada) and a 30 MHz transducer operating at 100 frames/s, as described previously.²⁵ In Doppler mode, the high-pass filter was set at 6 Hz, and the pulse repetition frequency was set between 4 and 48 kHz. A 0.2 to 0.5 mm pulsed Doppler gate was used, and the angle between the Doppler beam and the vessel was <30°. Images were recorded for offline analysis. Doppler waveforms were obtained in both uterine arteries near the lateral-inferior margin of the uterocervical junction close to the iliac artery on each side. Peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured from at least 3 consecutive cardiac cycles that were not affected by motion caused by maternal breathing, and the results were averaged.

The pulsatility index (PI) and the resistance index (RI) were used as pulsed-wave Doppler measurements of downstream uterine and umbilical artery resistance and were calculated as PI = (PSV - MDV)/MV and RI = (PSV - MDV)/PSV, where PSV = peak systolic velocity, MDV = minimum diastolic velocity and MV = mean velocity (time averaged velocity). When MDV = 0, a velocity of 0.1 mm/s was used for calculation of PI and RI.

Fetal and Placental Measurements

Mice were culled on GD 18.5 and pups and placentas dissected out. Pups from the right uterine horn were blotted and weighed

Table 1. Maternal Body Weight, Systolic Blood Pressure, and Proteinuria at Gestational Day 18.5.

	C57BL/6J		COMT ^{-/-}		eNOS ^{-/-}		ANOVA		
	Normoxia (n = 9) Mean (SEM)	Hypoxia (n = 8) Mean (SEM)	Normoxia (n = 10) Mean (SEM)	Hypoxia (n = 10) Mean (SEM)	Normoxia (n = 7) Mean (SEM)	Hypoxia (n = 9) Mean (SEM)	Int	Geno	Hypx
Maternal body weight GD 18.5, g	30.9 (0.8)	24.7 (0.9) ^a	32.0 (0.9)	28.5 (0.8) ^a	27.3 (0.4)	21.8 (1.0) ^a	b	b	b
Maternal systolic blood pressure, mm Hg	122.9 (5.3)	134.7 (3.3)	118.8 (7.0)	132.8 (4.3)	138.3 (5.9)	150.9 (1.9)		b	b
Albumin/creatinine ratio	0.034 (0.02)	0.108 (0.03) ^a	0.102 (0.01)	0.063 (0.02)	0.080 (0.01)	0.207 (0.02) ^a	b	b	b

Abbreviations: ANOVA, analysis of variance; COMT^{-/-}, catechol-O-methyl transferase; eNOS^{-/-}, endothelial nitric oxide synthase; GD, gestational day; Geno, genotype; Hypx, hypoxia; Int, interaction.

^a $P < .05$ versus animals with the same Geno exposed to normoxia following Bonferroni post-hoc test.

^b $P < .05$ when the effect of Geno, exposure to Hypx, or their Int was evaluated by 2-way ANOVA.

Figure 1. Exposure to hypoxia during gestation significantly reduced pup survival. A, There was a significant effect of genotype and exposure to hypoxia on number of surviving pups at GD 18.5. The number of viable pups was significantly reduced in all groups of mice compared with their normoxic controls. B, Compared with their normoxic controls, eNOS^{-/-} mice showed the largest increase in fetal demise. * $P < .05$, 2-way ANOVA; [†] $P < .05$, Bonferroni post-hoc test. The P value on panel B was determined using a Kruskal-Wallis test. ANOVA indicates analysis of variance; eNOS^{-/-}, endothelial nitric oxide synthase; GD, gestational day.

and fetal crown to rump length and abdominal circumference determined. The gross anatomy of the pups was also examined. Placentas were blotted dry and weighed.

Detection of Superoxide and Nitrotyrosine

Cryosections of placentas and uterine arteries were taken (20 μ m), and dihydroethidium (DHE) was used to assess the presence of superoxide as described previously.²⁶ Immunohistochemistry was used to detect nitrotyrosine, the footprint of peroxynitrite, in 8 μ m cryosections of placenta and uterine arteries. Sections were incubated at room temperature for 1 hour with an antinitrotyrosine antibody (Millipore, Billerica, Massachusetts; 1:125 dilution). This was followed by an 1-hour incubation with a goat antirabbit secondary antibody (AlexaFluor, Life Technologies, Burlington, Canada; 1:250 dilution). Sections were then counterstained with diamidino-2-phenylindole. Images were then obtained using a fluorescence microscope (Olympus IX 81,

Olympus Canada, Richmond Hill, Canada) using the CY3 filter. Images were analyzed using Adobe Photoshop to determine mean fluorescence intensity/pixel. Four sections per animal were used, and the mean value was determined.

Tissue Homogenization and Immunoblotting

Placentas were collected immediately after euthanasia and frozen in liquid nitrogen. Subsequently, frozen tissue homogenates were prepared in ice-cold sucrose homogenization buffer. Protein content of each homogenate was determined using a Bradford protein assay. Bands for eNOS and inducible NO synthase (iNOS; mouse monoclonal antibodies from BD Biosciences, Mississauga, Canada; 1/250) were normalized to actin (rabbit polyclonal antibody from Abcam Inc, Toronto, Canada; 1/2000).

Statistical Analysis

All normally distributed data are expressed as mean $\pm$ standard error of the mean (SEM) and were analyzed using Graphpad Prism 5.0 software. Statistical analyses included Student t test, 1-way analysis of variance (ANOVA), or 2-way ANOVA that included genotype and exposure to hypoxia as sources of variation followed by Bonferroni post hoc test. A P value $< .05$ was considered statistically significant.

Results

Maternal Body Weight, Systolic Blood Pressure, and Proteinuria

The effect of exposure to hypoxia on maternal body weight, systolic blood pressure, and proteinuria, as assessed at GD 18.5, is summarized in Table 1. There was a significant effect of both genotype and exposure to hypoxia as well as a significant interaction of these 2 factors, on maternal body weight ($P < .05$, 2-way ANOVA). Maternal body weight of mice exposed to hypoxia was significantly reduced in all groups of mice compared to their normoxic genotype control ($P < .05$; Table 1). Maternal systolic blood pressure was significantly affected by both genotype and exposure to hypoxia ($P < .05$, 2-way ANOVA; Table 1). There

Figure 2. Uterine artery Doppler indices were not affected following exposure to hypoxia. A, Example images of uterine artery Doppler waveforms from C57BL/6J and COMT^{-/-} mice with and without exposure to hypoxia. There was no effect of genotype or exposure to hypoxia on either mean uterine artery blood flow velocity (B) or uterine artery resistance index (C). COMT^{-/-} indicates catechol-O-methyl transferase.

was also a significant effect of genotype and exposure to hypoxia, as well as a significant interaction of these factors, on maternal proteinuria ($P < .05$, 2-way ANOVA; Table 1). As indicated, proteinuria was increased in normoxic COMT^{-/-} mice, and a statistically significant effect of genotype was observed in line with previous studies.^{16,17} Interestingly, however, this was not exacerbated by exposure to hypoxia. Proteinuria was significantly increased, following exposure to hypoxia, in both C57BL/6J and eNOS^{-/-} mice ($P < .05$).

Pup Survival

No cases of maternal mortality were observed in dams exposed to hypoxia. Both fetal ultrasound assessment and fetal surgical extraction at term demonstrated a significant effect of maternal hypoxia on pup survival ($P < .05$, 2-way ANOVA; Figure 1A). In C57BL/6J mice, the number of viable pups was significantly reduced in mice exposed to hypoxia compared with their normoxic controls (Figure 1A). The relative increase in fetal mortality following exposure to hypoxia is shown in Figure 1B; eNOS^{-/-} mice were most affected with a survival rate below

10%. Given the low survival rate of pups in these mice, no further determinations were performed in the eNOS^{-/-} group.

Uterine and Umbilical Artery Blood Flow Velocity

Example images of uterine artery Doppler waveforms from C57BL/6J and COMT^{-/-} mice are shown in Figure 2A. There was no effect of genotype or exposure to hypoxia on uterine artery mean velocity in either C57BL/6J (497 ± 50 vs 474 ± 44 mm/s) or COMT^{-/-} mice (497 ± 30 vs 450 ± 46 mm/s) exposed to normoxia or hypoxia, respectively (Figure 2B). Additionally, there was no effect of genotype or exposure to hypoxia on the uterine artery RI in C57BL/6J mice (0.56 ± 0.05 vs 0.48 ± 0.03) or COMT^{-/-} mice (0.51 ± 0.026 vs 0.54 ± 0.03) exposed to normoxia or hypoxia, respectively (Figure 2C).

Example images of umbilical artery Doppler waveforms from C57BL/6J and COMT^{-/-} mice are shown in Figure 3A. There was a significant effect of both genotype and exposure to hypoxia on umbilical artery minimum velocity as well as a significant interaction of these 2 factors ($P < .05$, 2-way ANOVA; Figure 3B). It was significantly reduced in C57BL/

Figure 3. Umbilical artery Doppler indices were altered following exposure to hypoxia. A, Example images of umbilical artery Doppler waveforms from C57BL/6J and COMT^{-/-} mice with and without exposure to hypoxia. B, Umbilical artery minimum blood flow velocity was significantly reduced in COMT^{-/-} compared with C57BL/6J mice. There was also a significant effect of exposure to hypoxia; minimum blood flow velocity was significantly reduced in C57BL/6J mice exposed to hypoxia compared with normoxic controls. C, Umbilical artery resistance index was also significantly affected by both genotype and exposure to hypoxia; this was significantly increased in C57BL/6J mice compared with their normoxic controls. * $P < .05$, 2-way ANOVA; † $P < .05$, Bonferroni post-hoc test. ANOVA indicates analysis of variance; COMT^{-/-}, catechol-O-methyl transferase.

6J mice exposed to hypoxia in comparison with their normoxic genotype controls (8.9 ± 2.1 vs 21.7 ± 2.9 mm/s, $P < .05$; Figure 3B). In COMT^{-/-} mice exposed to hypoxia, however, there was no significant difference in minimum velocity when compared with their normoxic controls (2.5 ± 0.8 vs 6.8 ± 2.0 mm/s; Figure 3B).

A significant effect of both genotype and hypoxia on umbilical artery RI was also observed ($P < .05$, 2-way ANOVA; Figure 3C). Interestingly, the deleterious effect of hypoxia on this parameter reached significance in C57BL/6J mice (0.84 ± 0.02 vs 0.91 ± 0.02) but not in COMT^{-/-} mice (0.94 ± 0.04 vs 0.98 ± 0.01 ; Figure 3C).

A complete assessment of uterine and umbilical artery hemodynamic parameters, as assessed by ultrasound biomicroscopy, is further detailed in Supplemental Table 1.

Pup and Placental Growth Characteristics

There was a significant effect of exposure to hypoxia on the average litter weight, and a significant interaction between

genotype and exposure to hypoxia ($P < .05$, 2-way ANOVA; Figure 4A). Litter weight was significantly reduced in mice exposed to hypoxia in comparison with their normoxic genotype controls in both C57BL/6J (0.68 ± 0.02 vs 1.06 ± 0.03 g) and COMT^{-/-} mice (0.75 ± 0.03 vs 0.94 ± 0.05 g, $P < .05$; Figure 4A). The reduction in fetal weight was also calculated as a percentage of normoxic controls; this was significantly lower in COMT^{-/-} mice compared with C57BL/6J mice ($19.8\% \pm 5.0\%$ vs $36.0\% \pm 2.8\%$, $P < 0.05$; Figure 4B).

A similar significant effect of exposure to hypoxia was seen when crown to rump length ($P < .05$; Figure 4C) and abdominal circumference ($p < 0.05$; Figure 4E) were assessed. Crown to rump length was significantly reduced in C57BL/6J mice exposed to hypoxia compared with normoxic controls (25.2 ± 0.5 vs 30.0 ± 0.4 mm, $P < .05$; Figure 4C), although there were no changes observed in COMT^{-/-} mice (27.0 ± 0.6 vs 28.0 ± 0.6 mm). When calculated as a percentage of normoxic controls, the reduction in crown to rump length was significantly higher in C57BL/6J compared with COMT^{-/-} mice ($16.0\% \pm 1.4\%$ vs $3.6\% \pm 2.0\%$; $P < .01$, Figure 4D).

Figure 4. Exposure to hypoxia significantly reduced pup growth. A, Pup weight was significantly reduced in both C57BL/6J and COMT^{-/-} mice following exposure to hypoxia. B, Reduction in body weight attributable to hypoxia was significantly greater in C57BL/6J compared with COMT^{-/-} mice. C, Exposure to hypoxia significantly reduced crown to rump length in C57BL/6J mice; again the reduction attributable to hypoxia was significantly greater than that seen in COMT^{-/-} mice. (D) Abdominal circumference was significantly reduced in both groups of mice (E); the reduction in abdominal circumference attributable to hypoxia was significantly greater in C57BL/6J mice than COMT^{-/-} mice (F). **P* < .05, ***P* < .01, 2-way ANOVA/Student's *t* test; †*P* < .05, Bonferroni post-hoc test. ANOVA indicates analysis of variance; COMT^{-/-}, catechol-O-methyl transferase.

Abdominal circumference was significantly reduced in both C57BL/6J (22.4 ± 0.4 vs 27.0 ± 0.4 mm) and COMT^{-/-} (23.4 ± 0.4 vs 25.2 ± 0.4 mm) mice exposed to hypoxia compared with their normoxic controls (*P* < .05; Figure 4E). As was observed with other measurements of pup growth, the relative reduction in abdominal circumference following exposure to hypoxia was significantly higher in C57BL/6J compared with COMT^{-/-} mice (16.9 ± 1.4 vs $7.3 \pm 1.5\%$, *P* < .01; Figure 4F).

Although there was an effect of genotype (*P* < .05, 2-way ANOVA) on placental weight, there was no effect of exposure to hypoxia (Figure 5A). Similarly, there was a significant effect of genotype on body weight–placental weight ratio (*P* < .05, 2-way ANOVA; Figure 5B); there was, however, no effect of exposure to hypoxia.

Assessment of Oxidative Stress

Example images of DHE staining are shown in Figure 6A. There was no effect of genotype or exposure to hypoxia on superoxide production in the placentas of C57BL/6J or COMT^{-/-} mice (Figure 6B). Interestingly there was, however,

a significant effect of both genotype and exposure to hypoxia on peroxynitrite production (*P* < .05, 2-way ANOVA); this was significantly increased in COMT^{-/-} mice exposed to hypoxia (*P* < .05; Figure 7B). Example images of nitrotyrosine staining are shown in Figure 7A.

Placental eNOS and iNOS Expression

Representative Western blots illustrating placental eNOS (Figure 8A) and iNOS (Figure 8C) expression are shown. There was no effect of genotype or exposure to hypoxia (*P* > .05; 2-way ANOVA) on placental eNOS (Figure 8B) or iNOS (Figure 8D) expression.

Discussion

PE and FGR are pregnancy conditions that are responsible for the majority of maternal and perinatal morbidity and mortality; the lack of curative treatments for either of these conditions presents an important unmet clinical need. The complex and likely multifactorial etiology of these conditions has undoubtedly contributed to the dearth of treatments. One further hurdle, however,

Figure 5. Exposure to hypoxia did not affect placental weight. A, Placental weight was significantly greater in COMT^{-/-} compared with C57BL/6J mice. There was no effect of exposure to hypoxia on placental weight in either group of mice. B, Pup weight/placenta weight ratio was significantly lower in COMT^{-/-} compared with C57BL/6J mice; there was, however, no effect of exposure to hypoxia in either group. * $P < .05$, 2-way ANOVA. ANOVA indicates analysis of variance; COMT^{-/-}, catechol-O-methyl transferase.

has been a lack of appropriate animal models with which to test potential new therapies. Recently, the development of genetically modified mice, which recapitulate some or all of the signs of PE and/or FGR, has provided potential new models of these conditions. Using 2 particular models, the eNOS^{-/-} and COMT^{-/-} mice, we observed some of the signs of PE and/or FGR, including increased proteinuria, FGR, and abnormal uterine and umbilical Doppler waveforms. We used these models to investigate novel treatment strategies;^{15,16} however, the phenotype observed in both models is subtle, and this subtlety constrains our ability to test potential new therapies.

A number of environmental factors have been suggested to play a role in the development of PE and FGR. Of these, fetoplacental hypoxia has been implicated in the pathogenesis of

both FGR and PE.²⁷⁻²⁹ Recently, it has been observed that exposure to hypoxia during gestation in a mouse model of PE has a synergistic effect, resulting in a significantly stronger phenotype.²¹ We hypothesized that exposure of both eNOS^{-/-} and COMT^{-/-} mice to hypoxia during gestation would strengthen the PE/FGR phenotype as observed previously. This would result in a model that was not only clinically relevant but also provides a greater distinction between control and “PE/FGR” animals.

Exposure to hypoxia had a significant effect on the characteristic signs of PE, namely, systolic blood pressure at the end of pregnancy in all groups and proteinuria in control and eNOS^{-/-} mice. This suggests that exposure to hypoxia during gestation is able to increase the PE-like phenotype previously observed. Interestingly, it was observed that proteinuria was increased in normoxic COMT^{-/-} mice, which is in line with results previously published by ourselves and other investigators.^{16,17} Indeed, Kanasaki et al demonstrated that this was associated with glomerular endotheliosis.¹⁷ In this study, exposure to hypoxia did not exacerbate the proteinuria observed in normoxic COMT^{-/-} mice; from the results obtained, it is not clear why this is the case, but this provides preliminary evidence that pregnant COMT^{-/-} mice are relatively protected against hypoxic insult.

We also observed that exposure to hypoxia exacerbated FGR in COMT^{-/-} mice and induced FGR in control mice. Interestingly, although COMT^{-/-} pups from normoxic pregnancies were slightly smaller than their C57BL/6J controls, the relative reduction in pup weight was greater in control mice. Further, it was demonstrated that eNOS^{-/-} pups display a greatly reduced tolerance to maternal exposure to hypoxia, greatly limiting our study of this model as described here. It is possible to speculate on the possible causes of increased fetal demise; previous studies have observed that uterine artery remodeling is significantly impaired in pregnant eNOS^{-/-} mice, which likely contributes to reduced pup survival and the growth restriction observed by limiting the supply of oxygen and nutrients to the fetus.³⁰ It may be that by placing the mother in an hypoxic environment, as in this study, delivery of oxygen to the fetus is diminished to a point at which survival is severely limited.

Further investigation of the phenotype allowed the observation that control mice were more severely affected by exposure to hypoxia than COMT^{-/-} mice. Investigation of both the uterine and umbilical artery Doppler waveforms demonstrated that, although exposure to hypoxia significantly affected the indices measured, absolute reduction in blood flow velocity or increase in the RI was significantly greater in control compared with COMT^{-/-} mice. This result is somewhat surprising, given hypoxia can induce increased release of vasoconstrictor catecholamines. Previous studies, however, highlight that in some conditions, hypoxia can increase the activity of monoamine oxidases,^{31,32} which may be able to compensate for the lack of COMT in the knockout model.

In order to determine the possible mechanisms that may mediate the relative protection of COMT^{-/-} mice to a hypoxic

Figure 6. Exposure to hypoxia did not affect placental superoxide production. A, Example placental sections, taken from C57BL/6J and COMT^{-/-} mice with or without exposure to hypoxia, following staining with DHE. B, There was no effect of genotype or exposure to hypoxia on placental superoxide production. COMT^{-/-} indicates catechol-O-methyl transferase; DHE, dihydroethidium.

Figure 7. Peroxynitrite formation was significantly increased in placentas of COMT^{-/-} mice exposed to hypoxia. A, Example of placental sections, taken from C57BL/6J and COMT^{-/-} mice with or without exposure to hypoxia, stained with nitrotyrosine (the permanent footprint of peroxynitrite). B, There was a significant effect of both genotype and exposure to hypoxia on placental peroxynitrite production; this was significantly increased in COMT^{-/-} mice following exposure to hypoxia. **P* < .05, 2-way ANOVA; †*P* < .05, Bonferroni post-hoc test. ANOVA indicates analysis of variance; COMT^{-/-}, catechol-O-methyl transferase.

insult, the effect of maternal exposure to hypoxia on the placenta was determined. It has been well documented that O₂ levels play a key role in regulating normal placental development and function (for review, see reference 33). Further, placental hypoxic injury has been observed in both PE²⁸ and FGR³⁴ and is likely to play a key role in mediating the pathophysiology of both the conditions. As was previously observed,¹⁶ placentas from normoxic COMT^{-/-} mice were heavier than those from C57BL/6J mice, with a concomitant reduction in pup body weight–placenta weight ratio. Interestingly, there was no effect of exposure to hypoxia on these measures in either genotype. Previous studies of mice exposed to hypoxia during gestation have observed a small but significant decrease in placental weight.³⁵ Given the severity of the hypoxic insult used in this study, it is unclear why placental weight was not affected. This result indicates, however, that changes in placental growth did not mediate

the increased PE/FGR-like phenotype observed. Although placental weight was not affected by exposure to hypoxia, levels of placental oxidative stress were assessed, given that an increase in this measure is a pathological finding in both PE and FGR.^{15,36,37} Measurement of superoxide production, which is elevated following ischemia/reperfusion type injury, indicated that oxidative stress did not differ in placentas from control and COMT^{-/-} mice at GD 18.5. It was observed, however, that peroxynitrite formation was increased in placentas from COMT^{-/-} mice. Peroxynitrite is a product of nitric oxide scavenging by superoxide, and increased formation may be the result of an increase in superoxide and/or nitric oxide production. Given that levels of superoxide did not differ between the 2 groups, this result suggests that nitric oxide bioavailability was increased in placentas from COMT^{-/-} mice. Peroxynitrite formation was assessed in these studies using antibodies against nitrotyrosine

Figure 8.. Exposure to hypoxia did not alter placental expression of eNOS or iNOS. Example Western blots of placental eNOS (A) and iNOS (C) expression in COMT^{-/-} and C57BL/6J mice. There was no effect of genotype or exposure to hypoxia on placental eNOS (B) or iNOS (D) expression. COMT^{-/-} indicates catechol-O-methyl transferase; eNO, endothelial nitric oxide synthase; iNOS, inducible NO synthase.

residues, the permanent footprint of peroxynitrite. It is therefore possible that an increase in superoxide production occurred prior to GD 18.5 but this had returned to normal prior to assessment, leaving only evidence of increased nitrative stress. However, following our observation of the severely decreased tolerance of eNOS^{-/-} mice to hypoxia during gestation, we further hypothesized that the relative protection of COMT^{-/-} mice to a hypoxic insult was mediated by increased nitric oxide bioavailability. Investigation of placental expression of both eNOS and iNOS demonstrated that expression of these enzymes did not differ between the 2 groups. This does not, however, indicate activity levels of either enzyme. Umbilical artery Doppler waveforms indicate increased placental resistance in COMT^{-/-} mice, which was further exacerbated following exposure to hypoxia. Any increases in the bioavailability of NO in these mice, as hypothesized previously, might be expected to mediate vasodilation in the placenta, therefore producing a decrease in placental resistance. That the opposite was observed, however, may be attributable to the placental pathology previously observed in these mice; namely widespread vascular thrombosis¹⁷ and reduced vascular density.³⁸ It is unlikely that any increase in NO bioavailability would be able to overcome these pathological alterations. Additionally, clinical studies of pregnancies complicated by FGR, which display abnormal umbilical artery Doppler waveforms, have observed an increase in fetoplacental NO production.³⁹ The hypothesis that placental nitric oxide bioavailability is increased and has a protective effect in COMT^{-/-} mice exposed to a hypoxic insult may therefore still hold true and should be investigated further.

In conclusion, exposure to hypoxia during gestation did increase the severity of the PE-like and FGR phenotype observed in eNOS^{-/-} and COMT^{-/-} mice. Exposure to hypoxia, however, also significantly altered the phenotype of control C57BL/6J mice, suggesting that the hypoxic insult did not interact with the specific genetic modifications to mediate the increased pathophysiology observed. We did not attain our objective of developing a useful experimental model of severe PE/FGR. The eNOS^{-/-} mice demonstrated a severely impaired tolerance to the hypoxic insult (reflected in the markedly increased fetal mortality), such that further investigation of this model (eg, FGR) was impossible, and COMT^{-/-} mice demonstrated a relative protection to hypoxic insult when compared with control mice. Thus, this latter finding is intriguing. The suggestion that increased placental nitric oxide bioavailability may mediate this effect is exciting; further studies using this model may enhance our understanding of placental responses to hypoxic insult.

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Supplemental Material

Supplemental Table 1 is available at <http://rs.sagepub.com/supplemental>.

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