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Pathophysiology of Chronic Nitric Oxide Synthase Inhibition-Induced Fetal Growth Restriction in the Rat

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Abstract

Objective—To evaluate the pathophysiology of chronic nitric oxide synthase (NOS) inhibition-induced fetal growth restriction (FGR) in the rat.

Methods—Timed-pregnant rats received L-NAME (2.5 mg/kg/h) with or without endothelin (ET-1) receptor A (ET_A) antagonist from day 14 to 21 of gestation. In separate groups, ET_A antagonist and/or L-NAME were discontinued on day 18. On day 21 fetal and placental weights, and maternal and fetal plasma nitrate/nitrite (NO_x) were determined.

Results—L-NAME led to FGR, and decreased maternal and fetal NO_x. Maternal NO_x was further decreased when ET_A antagonist was co-administered with L-NAME. ET_A antagonism along with L-NAME did not impact fetal growth. Discontinuation of L-NAME on day 18 resulted in normal fetal and placental growth at day 21 and an increase of maternal NO_x. Simultaneous cessation of both NOS inhibition and ET_A antagonism on day 18 produced FGR at day 21, whereas continuation of ET_A antagonism after discontinuation of L-NAME resulted in normal fetal growth.

Conclusions—NOS inhibition in the pregnant rat leads to decreased maternal and fetal nitric oxide (NO) production and FGR. The effects of NOS inhibition on fetal growth are reversible, and are mediated at least in part by ET-1. With chronic NOS inhibition, ET_A antagonism improves but does not normalize fetal growth, and may allow increased access of L-NAME to the fetal compartment. Continued access of L-NAME to the fetal compartment may limit the effect on fetal growth of any therapeutic intervention in this model of FGR.

Keywords

Endothelin antagonism; Fetal growth restriction; Nitric oxide synthase inhibition; Rat

INTRODUCTION

Nitric oxide synthase (NOS) inhibition in the pregnant rat has been used as a model for the study of fetal growth restriction (FGR) (1–3). NOS inhibition causes a decrease in the production of nitric oxide (NO), a vasodilator. During pregnancy, administration of the NOS inhibitor L-NAME results in decreased uterine and placental perfusion and ultimately leads to fetal and placental growth restriction (1,2,4,5). Fetal growth is compromised as early as day four, and placental growth as early as day one, of a seven day infusion of L-NAME

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(days 15 through 21 of gestation) (5). We and others have demonstrated that L-NAME administration also results in increased maternal circulating endothelin-1 (ET-1), a potent vasoconstrictor (6,7). We have established that this increased ET-1 is of primary importance in the pathophysiology of the decreased uterine and placental perfusion observed in this model. ET receptor antagonism in the presence of continuing NOS inhibition normalizes placental perfusion (and fetal growth) early in the treatment period, but this improvement in perfusion and growth is not sustained (5). This finding led us to investigate other mechanisms by which chronic NOS inhibition may impact perfusion and growth. These could include irreversible effects of L-NAME on vascular integrity or fetal growth and augmented transport of L-NAME to the fetus. If early L-NAME exposure led to irreversible effects on fetal growth in spite of L-NAME being discontinued, it would suggest an underlying vascular insult that may not be responsive to therapeutic interventions intended to improve perfusion. Similarly, if therapeutic interventions led to increased placental perfusion and consequently to increased transport of L-NAME to the fetal compartment, fetal growth may paradoxically be adversely affected. These factors would need to be taken into account when evaluating the effects of therapeutic interventions.

The purpose of this study was to determine whether the effects of chronic L-NAME administration are reversible and whether the maternal administration of L-NAME has an impact on fetal NO production. By answering these questions, we can correctly evaluate the impact of therapeutic interventions in this model of FGR.

MATERIALS AND METHODS

Nitric oxide synthase inhibitor and endothelin antagonist

The NOS inhibitor, nitro-L-arginine methyl ester (L-NAME), was obtained from Sigma (St. Louis, MO). The ET_A antagonist ABT-546, a nonpeptide ET_A receptor antagonist with 28,000-fold selectivity for ET_A over ET_B (8), was provided by Abbott Laboratories, Abbott Park, IL.

Animals

Female and male Sprague-Dawley rats (Harlan Sprague Dawley, Madison, WI) received a standard laboratory rodent diet (PMI Feeds, St. Louis, MO), water *ad libitum,* and were kept on a 12-hour light/12-hour dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee. Rats were bred at an age of 11–20 weeks and a weight of 225–250 g. Date of sperm positivity was designated day 0 of a 22-day gestation.

Nitric oxide synthase inhibition and ET receptor antagonism

On day 14 of gestation, two Alzet osmotic pumps (model 2ML1, output=10 μl/h, Durect Corp., Palo Alto, CA) were placed subcutaneously on the back of the rat between the scapulae. One pump was used to infuse either L-NAME (2.5 mg/kg/h) or normal saline vehicle. L-NAME infusion continued through gestation day 21 (7 days) in rats used for perfusion and fetal growth studies (n=6 per group). For studies of fetal growth and NO_x recovery after NOS inhibition, the L-NAME infusion continued through day 18 (4 days), at which time the pump was removed under anesthesia and the rats continued their gestation until day 21 (n=6 per group).

The second pump was used to infuse ABT-546 (20 mg/kg/day), or vehicle (20% ethyl alcohol, 40% propylene glycol, and 0.04 M NaOH in H_2O). This dose of the ET_A antagonist has proven effective in *in vivo* pseudoefficacy studies to block ET-1-induced increases in mean arterial pressure in rats (9). ABT-546 administration was continued for 7 days (to

determine the impact of ET_A antagonism on L-NAME-induced FGR), or for 4 days (to determine the impact of simultaneous cessation of NOS inhibition and ET_A antagonism).

Nitrate/nitrite assay in maternal and fetal plasma

Blood for nitrate/nitrite assay was collected on gestation day 21. Maternal blood was drawn from the abdominal vena cava into a heparin-treated syringe at laparotomy and fetal blood was collected by heparin-treated capillary tube after incision across the carotid and jugular vessels in anesthetized pups. Fetal blood from each litter was pooled.

For the study of NO_x recovery after NOS inhibition, maternal rats were infused with L-NAME or vehicle for 4 days. Maternal blood was collected from a tail vein on gestation day 14 (before L-NAME pump insertion), day 18 (before pump removal), and from the vena cava on day 21 at laparotomy.

Plasma was prepared from the blood samples and was frozen at −80°C until assayed. Residual L-NAME, which interferes with NO analysis on the NO analytic instrument, was removed from the plasma on a Dowex 50WX8-400 ion exchange column (Sigma, St Louis, MO). Plasma nitrate/nitrite (NO_x) was evaluated using a Sievers 280i NO analyzer (GE Analytical Instruments, Boulder, CO). After conversion of nitrate to nitrite, total NO_x was quantified using a dilution series of nitrate standards. Results were expressed as nmol NO_x/ml plasma.

Statistical analyses

Results are presented as mean ± standard error of the mean (SE). Statistical comparisons were made using an analysis of variance (ANOVA) with *post hoc* Newman-Keuls test (Figures 1 and 2), a repeated measures ANOVA (Figure 3, intragroup), or a Mann-Whitney U test (Figure 3, between groups on specific days). All statistical tests were two-tailed and results were considered statistically significant at $P<0.05$.

RESULTS

Fetal and placental weights

Administration of L-NAME for 7 days resulted in significant reduction of fetal and placental weights. Co-administration of ABT-546 had no impact on fetal growth, although placental growth was normalized (Figure 1). ET_A antagonist administration without L-NAME resulted in significantly increased fetal weights.

In rats treated with L-NAME alone, when this NOS inhibitor was discontinued after 4 days (gestation day 18), fetal weights from L-NAME-treated rats did not differ significantly from control fetal weights at day 21 (Figure 1). In contrast, placental weights remained significantly reduced after the cessation of NOS inhibition. In rats treated with both L-NAME and ABT-546, neither fetal nor placental weights improved when ET_A antagonism was discontinued at the same time as the discontinuation of NOS inhibition (gestation day 18). However, when ET_A antagonism was continued through day 21, after discontinuation of NOS inhibition at day 18, fetal weights, but not placental weights, were the same as normal control fetal weights.

Maternal and fetal NO_x

Maternal and fetal NO_x on day 21 were both reduced in response to NOS inhibition (Figure 2). Maternal NO_x was further decreased when the highly ET_A-selective antagonist, ABT-546, was present with L-NAME, but fetal NO_x was not. The antagonist administered alone caused a slight reduction in maternal but not fetal NO_x.

Maternal NO_x did not change significantly over time throughout pregnancy in control rats (Figure 3). In rats treated with L-NAME for 4 days only (days 14–18), maternal NO_x was significantly reduced on day 18, but recovered and was not significantly different from control values by the end of pregnancy

DISCUSSION

NOS inhibition in the pregnant rat is a model that has been used to study both FGR and preeclampsia (1,2,4,5,10,11). Nitric oxide is a vasodilator that plays an important role in the regulation of vascular tone in normal and pathologic circumstances. A deficiency of NO in the uteroplacental circulation may contribute to the pathophysiology of FGR and preeclampsia in humans. Therefore, understanding the physiologic response to NOS inhibition becomes central to understanding the model, particularly when evaluating potential therapeutic interventions in this model.

As expected, NOS inhibition leads to decreased maternal NO production. We confirmed decreased NO production by demonstrating decreased maternal NO_x in this model. It had not previously been determined whether the effects of NOS inhibition on fetal growth during pregnancy were reversible. If the effects were irreversible, this would indicate that the vasculature was no longer capable of responding to vasoactive mediators and would explain the inefficacy of ET_A antagonism. If irreversibility had been demonstrated, this would need to be taken into account when evaluating the effect of a therapeutic intervention. To investigate this possibility, we administered L-NAME only for days 14–18 and then evaluated NO production and fetal growth on day 21. We found that fetal growth returned to normal by the end of gestation (day 21). Therefore, the effects of L-NAME administration on fetal growth do in fact appear to be reversible. Of note, placental weights remained decreased despite discontinuation of L-NAME. However placental function was apparently adequate in these pups, as evidenced by normal fetal weights. Maternal NO production after discontinuation of L-NAME was increased. This may be a compensatory response contributing to the improvement in fetal growth after discontinuation of L-NAME.

NOS inhibition in the pregnant rat leads to decreased uterine and placental perfusion (5) as well as increased circulating ET-1 levels (6,7). ET-1, acting via the ET_A receptor may further decrease uteroplacental perfusion beyond that caused by decreased NO production. L-NAME has the capacity to cross the placenta from the maternal to the fetal vasculature and may have an adverse impact on fetal growth independent of the known adverse impact of L-NAME on maternal uteroplacental perfusion. Therefore, any intervention that increases uteroplacental perfusion (e.g. ET_A antagonism) and/or the ability of L-NAME to access the fetal compartment could paradoxically have an adverse impact on fetal growth. To evaluate this possibility, we measured NO metabolites in the maternal and fetal vasculature. Administration of the ET_A antagonist alone resulted in slightly decreased maternal NO_x. This is expected, given that NO balances the physiologic effects of ET-1 and inhibition of ET-1 activity by ET_A receptor blockade would reduce the necessity for NO production. We did not observe this decrease in our previous study with A-127722, another ET_A antagonist (5). This may be a result of lower selectivity of this agent. Administration of L-NAME alone resulted in decreased maternal and fetal NO_x. In our prior study, we reported that fetal NO_x was not significantly decreased with L-NAME administration. This is likely because the variation in our measurements was higher in that study than in this study. Administration of an ET_A antagonist in the setting of L-NAME administration did not have a significant impact on fetal NO_x production compared to administration of L-NAME alone. We have shown previously that ET_A antagonism in the setting of NOS inhibition normalizes uterine and placental perfusion through day 4 of antagonist infusion (5). Increased tissue perfusion may allow persistent access of L-NAME into the fetus, allowing for continued suppression

of fetal NO_x. NOS inhibition in the fetal compartment likely leads both to systemic fetal effects and to reduced fetal-placental perfusion that result in growth restriction. L-NAME also leads to increased uterine and placental apoptosis (12) which may contribute to the observed FGR. The decreased fetal NO_x does not appear to be an effect of the ET_A antagonist per se, given that fetal NO_x was not impacted by administration of the ET_A antagonist alone. In this model, the potentially beneficial vasodilatory effects of ET_A antagonism on placental perfusion paradoxically allow continued, and possibly increased access of L-NAME to the fetal compartment. This limitation should be taken into consideration when evaluating the effects of any therapeutic intervention that is intended to increase placental perfusion in this model of FGR.

This study provides further evidence that ET-1 has an important role in NOS inhibition-induced FGR. When NOS inhibition and ET_A antagonism were both stopped at 4 days, fetal growth was restricted. However, when ET_A antagonism was continued after NOS inhibition was discontinued, fetal weights were normal. Reduced fetal weights when NOS inhibition and ET_A antagonism are discontinued simultaneously are evidence of the importance of ET-1 in the pathophysiology of NOS inhibition-induced FGR. ET receptor antagonism results in increased ET-1 production both in humans (13–15) and in rats (16). NOS inhibition also leads to increased ET-1 production (6,7). When both ET_A antagonism and L-NAME are discontinued, the upregulated ET-1 is unopposed, and fetal growth is restricted. The observed full recovery of normal fetal weights when ET_A antagonism is continued beyond the cessation of NOS inhibition is further evidence for the involvement of ET-1. In spite of the increased production of ET-1, continued presence of the antagonist blocks the action of ET-1 and prevents it from affecting fetal growth.

NOS inhibition in the pregnant rat is an established model for the study of FGR. In this model, decreased maternal NO production leads to decreased uteroplacental perfusion and ultimately to FGR. The effects of L-NAME on fetal growth are reversible. L-NAME can cross the placenta and may independently contribute to FGR. We have shown previously that ET-1, through its vasoconstrictive activity, is of primary importance in the pathophysiology of this model (5). Any intervention to circumvent the vasoconstriction and improve uteroplacental perfusion in this model has the potential to increase access of L-NAME to the fetal compartment, thus confounding the evaluation of the impact of that intervention on fetal growth.

Acknowledgments

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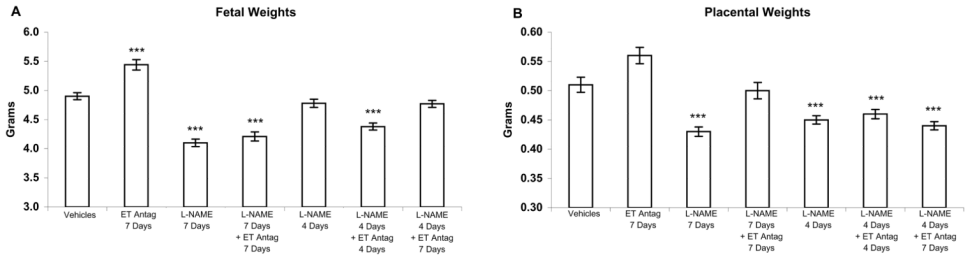

Figure 1.

Fetal and placental weights from pregnant rats in response to L-NAME and an ET_A antagonist. Infusion of the NOS inhibitor L-NAME (2.5 mg/kg/h), and the ET_A antagonist ABT-546 (20 mg/kg/day) was for 4 or 7 days (as indicated on the chart), gestation days 14–18 or 14–21, and weights were recorded on day 21. **(A)** Fetal weights were decreased by L-NAME and were not improved by ET_A antagonist in combination with L-NAME when infusion of both agents was for 7 days. Discontinuation of L-NAME at day 18 resulted in normal fetal growth. When L-NAME and ABT-546 were infused together, simultaneous discontinuation of both L- NAME and ET_A antagonist at day 18 produced growth restriction at day 21 whereas continuation of ET_A antagonism after discontinuation of NOS inhibition resulted in normal growth. **(B)** Placental weights were also decreased by 7 days of L-NAME but were significantly improved by the ET_A antagonist. Neither discontinuation of L-NAME nor continuation of ET_A antagonism after cessation of NOS inhibition improved placental weights. Results are presented as mean weight in grams ± SE (n=6 maternal rats per group; litter sizes (range = 10.7–14.3 fetuses/litter) did not differ significantly among groups). *** $P<0.001$ by ANOVA compared to vehicle-treated rats.

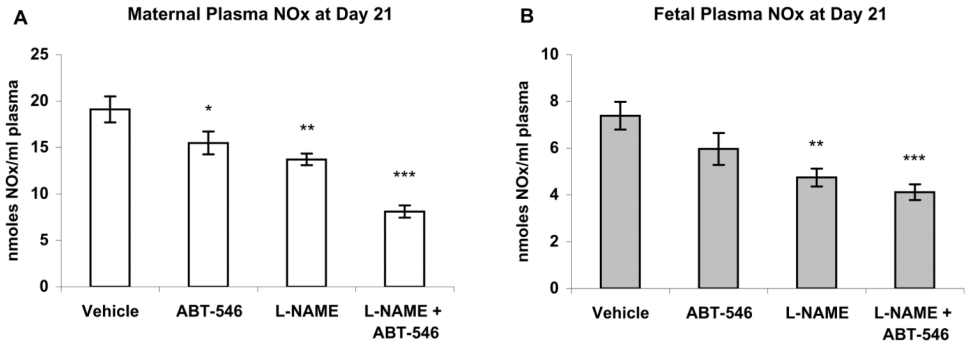

Figure 2.

Maternal (**A**) and fetal (**B**) plasma nitrate/nitrite (NO_x) at gestation day 21 in rats treated for 7 days (days 14–21) with the NOS inhibitor L-NAME (2.5 mg/kg/h), and the ET_A antagonist ABT-546 (20 mg/kg/day). L-NAME significantly lowered both maternal and fetal NO_x. ABT-546 in combination with L-NAME produced a further decrease in both maternal and fetal NO_x. A slight lowering of maternal NO_x was produced by the antagonist alone. Results are presented as mean nmoles NO_x/ml plasma ± SE (n=6 maternal rats per group). **P*<0.05, ** *P*<0.01, ****P*<0.001, by ANOVA compared to vehicle-treated rats.

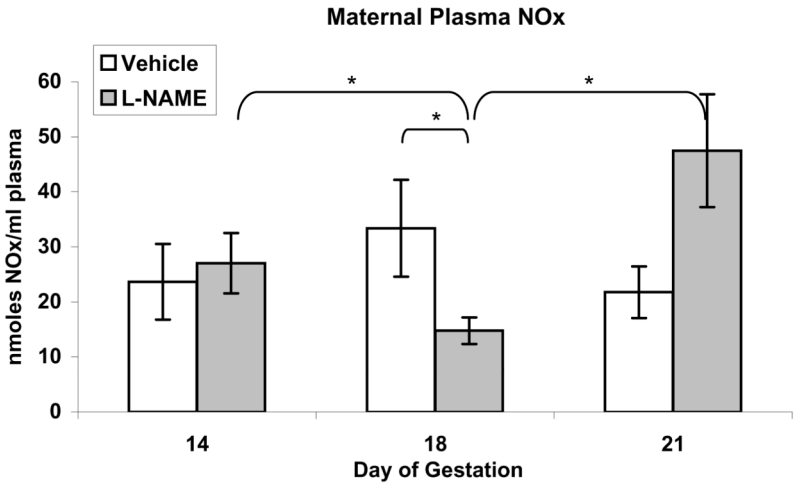

Figure 3.

Maternal plasma nitrate/nitrite (NO_x) in pregnant rats treated with the NOS inhibitor L-NAME (2.5 mg/kg/h) for 4 days, (gestation days 14–18), followed by 3 days without L-NAME. Plasma was collected on gestation day 14 just before L-NAME treatment was begun (baseline), on day 18 just before L-NAME pump removal, and on day 21 at laparotomy. Maternal plasma NO_x did not change over time in vehicle-treated rats. L-NAME significantly reduced maternal plasma NO_x. Cessation of NOS inhibition at day 18 resulted in a significant increase in maternal plasma NO_x at day 21 that was higher than the baseline value at day 14. Results are presented as mean nmoles NO_x/ml plasma ± SE (n=6 maternal rats per group). *P<0.05, by repeated measures ANOVA for comparisons between times within each group and by Mann-Whitney U for comparison between groups at each time.