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Role of nitric oxide in maternal hemodynamics and hormonal changes in pregnant rats

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Normal pregnancy is characterized by a significant reduction in total peripheral vascular resistance and decreased pressor responsiveness to vasodilator agents. This review will consider whether nitric oxide (NO) contributes to these changes, and whether a deficiency of NO produces a pre-eclampsia like syndrome. The biosynthesis of NO increases in pregnant animals, as assessed by the raised plasma concentration, urinary excretion and metabolic production rate of guanosine 3',5'-cyclic monophosphate (cGMP), the second messenger of NO. In addition, urinary excretion of nitrate, the stable metabolites of NO, increases during pregnancy, paralleling the rise in cGMP. Several studies provide convincing evidence indicating that expression and activity of different NO synthases (NOS) are increased in gravid animals. Acute blockade of NOS causes a dose response increase in blood pressure and reverses the blunted vasopressor response to vasoconstrictor agents. Long-term NOS inhibition produces a pre-eclampsia like syndrome, characterized by maternal hypertension, proteinuria, thrombocytopenia, and renal damage, and lower litter size and fetal weight. Both acute and chronic responses are reduced when L-arginine, the substrate for NOS, is administered in high doses, indicating that these changes are specific to NO inhibition. In conclusion, present data suggest that a disturbance in NO release may contribute to the pathogenesis of pre-eclampsia.

***Key terms**: fetal growth, maternal hemodynamics, nitric oxide, nitric oxide synthase, pre-eclampsia like syndrome, rat pregnancy.*

INTRODUCTION

Normal pregnancy is characterized by a marked stimulation of the renin-angiotensin-aldosterone system (RAAS), which causes renal water and sodium retention, thus increasing plasma volume (Longo, 1983; Wilson *et al*, 1980). Plasma volume expansion is essential for normal fetal growth, as it allows a sustained elevation in cardiac output and, indirectly, in uterine blood flow. Despite the rise in blood volume and the activation of the vasoconstrictor RAAS, blood pressure does not increase during pregnancy (Wilson *et al*, 1980). At least two facts account for the normal blood pressure during pregnancy: there is a significant reduction in total peripheral vascular resistance and pressor responsiveness to vasoconstrictor agents is

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decreased (Gant *et al*, 1973; Phippard *et al*, 1986). The renal vascular bed also participates in this vasodilatory response to pregnancy. Despite the decline in mean arterial blood pressure (MAP), renal blood flow and glomerular filtration rate (GFR) increase about 50% above preconception levels. These changes underline the marked fall in renal vascular resistance that occurs during pregnancy (Conrad, 1984). The mechanisms responsible for these hemodynamic changes during pregnancy are still controversial.

In recent years, the role of nitric oxide (NO) as a potent vasodilator has become well accepted. The purpose of the present review is to provide evidence that NO participates in the cardiovascular adaptation to normal pregnancy. We will specifically discuss the changes in nitric oxide-nitric oxide synthase system in pregnant rats because this species offers many advantages. Cardiovascular and renal changes during rat pregnancy, such as increased uterine blood flow, decreased MAP, attenuation of pressor responsiveness to exogenous vasoconstrictor agents, and increased GFR are similar to those observed in pregnant women. In addition, rats are relatively inexpensive and easy to breed, making them suitable for their use in research.

BIOLOGY OF NITRIC OXIDE

Nitric oxide is an inorganic free radical gas that has, among other known functions, important cardiovascular effects related to its ability to inhibit platelet aggregation and relax vascular and uterine smooth muscle. It has a half-life of a few seconds and in biological systems it decomposes rapidly to nitrite and nitrate. NO is synthesized from L-arginine by different nitric oxide synthases (NOS), and L-citrulline is the byproduct (Vallance & Collier, 1994). So far, three isoforms of NOS have been identified: a neuronal type (nNOS or NOS I), a macrophage or inducible type (iNOS or NOS II), and an endothelial type (eNOS or NOS III). The neuronal isoform is found in central and peripheral neurons, macrophage type NOS is expressed after activation of cells with certain inflammatory mediators, and endothelial isoform is present in vascular endothelium, platelets and heart (Ignarro, 1991). NO produced in endothelial cells rapidly diffuses out into nearby target cells, such as vascular smooth muscle and blood platelets, where NO activates the soluble form of guanylate cyclase to raise the intracellular levels of guanosine 3', 5'-cyclic monophosphate (cGMP). Along with this paracrine role, NO may have an autocrine function by inhibiting the activity of NOS in endothelial cells (Fig 1).

Due to its short half-life, it has been difficult to measure NO directly. Therefore, researchers have used different strategies to determine NO-NOS activation, such as: measurement of NOS enzyme activity by using the [^{3}H]-citrulline conversion assay; immunohistochemical localization of different NOS isoforms; detection of

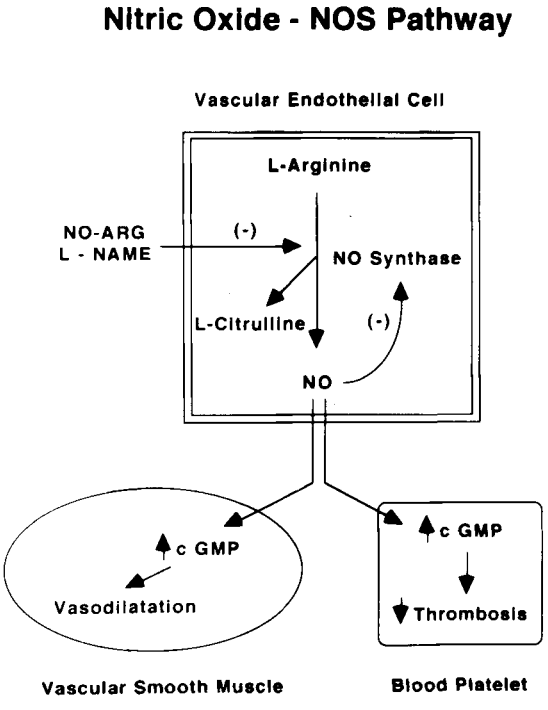

Fig 1. Schematic representation of the transcellular mechanisms by which NO communicates with nearby target cells. A vascular endothelial cell that generates NO from L-arginine is represented. NO diffuses out of the endothelial cell into vascular smooth muscle cells and blood platelets, where NO activates cytosolic guanylate cyclase to raise intracellular levels of cGMP. In addition, NO may have an autocrine function by inhibiting NOS in its endothelial cell of origin. (Adapted from Ignarro, 1991).

messenger RNA for NOS; measurement of nitrite/nitrate, the stable metabolites of NO, and measurement of cGMP, the second messenger for NO. In addition, either acute or chronic administration of structural analogs of L-arginine, such as Nω-nitro-L-arginine or nitro-L-arginine methyl ester (L-NAME), competitively inhibit the formation of nitric oxide, which can be reversed by excess amounts of L-arginine, thus providing a valuable tool to study the role of NO in different biological systems.

EVIDENCE THAT NO-NOS ARE INCREASED IN RAT PREGNANCY

Changes in cGMP and in nitrite-nitrate excretion

Results available in the literature strongly suggest that production of endogenous NO is increased in gravid rats. Plasma levels and urinary excretion rates of cGMP are increased during rat gestation. These findings most probably reflect increased tissular production of cGMP. A metabolic study demonstrated increased entry of cGMP into the plasma compartment, rather than decreased clearance (Conrad & Vernier, 1989). Interestingly, pseudopregnant rats also exhibit enhanced urinary cGMP excretion, suggesting that the proliferative activity that accompanies feto-placental maturation, as well as placental hormones, are not necessary for the rise in urinary excretion of cGMP. Although these findings are considered as indirect evidence of increased NO-NOS activity during rat pregnancy and pseudopregnancy, it is worth noting that other mediators, such as atrial natriuretic peptide, may also produce elevations of cGMP and vasodilation during pregnancy.

The urinary excretion and plasma levels of the stable NO metabolite nitrate are increased in pregnant and pseudo-pregnant rats, paralleling the rise in urinary cGMP excretion. Chronic treatment with L-NAME inhibits the increase in urinary nitrate excretion, indicating that it is a consequence of NO activity (Conrad *et al*, 1993).

Nitric oxide synthases during pregnancy

Several studies provide convincing evidence indicating that NOS expression and activity increase during pregnancy. The gene expression of endothelial constitutive nitric oxide synthase is elevated in rat aorta during pregnancy. Estradiol supplementation to gonadectomized rats reproduces this change, whereas progesterone and testosterone administration has no effect (Goetz *et al*, 1994). Another study showed increased levels of calcium-dependent NOS activity in uterine artery, heart, kidney and skeletal muscle obtained from near-term pregnant Guinea pigs. These changes were also mimicked when ovariectomized animals were treated with estradiol, thus supporting the idea that the increment in NOS activity is under estrogen influence (Weiner *et al*, 1994).

Nerve fibers containing NOS have been localized in mid-term pregnant rat uterus; the greatest number of NOS positive nerve fibers was localized near the cervix, whereas endothelial cell NOS activity appeared to distribute uniformly. Interestingly, the presence of NOS activity in the pregnant rat uterus declined near term, as shown by histochemical and biochemical assays (Natuzzi *et al*, 1993). Another study demonstrated the presence of iNOS staining in cells at the fetal-maternal interface of the rat placenta; this staining was greatly reduced during labor (Purcell *et al*, 1997). In addition, using immunohistochemical techniques, Riemer and coworkers (1997) revealed the expression of two NOS isoforms in the pregnant rat uterus: eNOS was localized in the vascular endothelium, and iNOS in the myometrial and vascular smooth muscles, as well as in the decidual epithelium; the expression of both isoforms declined significantly in laboring rats. Similarly, NOS activity, evaluated by measuring the difference in radiolabeled arginine to citrulline conversion, decreased between days 15 and 21 of gestation (Sladek & Roberts, 1996). The changes in placental and uterine NOS expression and activity suggest a paracrine role for NO in regulating uterine contractility, blood flow and immunosuppression required for pregnancy maintenance. NO withdrawal at term may also be involved in the initiation of

labor. A brief summary of the evidence indicating increased NO biosynthesis during pregnancy in rats is provided in Table I.

MATERNAL AND FETAL EFFECTS OF ACUTE AND LONG-TERM NOS INHIBITION

Maternal effects of acute NOS inhibition

Acute administration of L-NAME or Nω-nitro-L-arginine to conscious rats produces a dose-response increase in MAP of significantly greater magnitude in pregnant rats in late gestation than in either non-pregnant rats, or in pregnant rats in mid gestation (Molnár & Hertelendy, 1992; Nathan *et al*, 1995). This response is abolished by L-arginine administration, providing strong evidence that the action of this inhibitor is due specifically to the inhibition of NO synthesis from L-arginine. In addition, continuous infusion of NOS inhibitors to pregnant rats reverses the blunted vasopressor response to angiotensin II (ANG II), vasopressin, and norepinephrine obtained during gestation, suggesting that NO is involved in the vascular refractoriness observed during pregnancy (Molnár & Hertelendy, 1992).

The renal circulation also participates in the vasodilatory response to pregnancy. Baseline GFR and effective renal plasma flow are significantly increased, and effective renal vascular resistance is decreased in chronically instrumented gravid rats, as compared with virgin controls. During infusion of NOS inhibitors, these three parameters equalized in the pregnant and virgin rats, suggesting that pregnant animals are more responsive to NOS inhibition than virgin rats (Danielson & Conrad, 1995).

In summary, the data from acute studies suggest that NO contributes to the attenuated pressor responses of vasoconstrictor agents, as well as to the renal vasodilatation and hyperfiltration observed during pregnancy.

Maternal effects of long-term NOS inhibition

Different researchers have investigated the effects of prolonged NO inhibition on maternal hemodynamics and fetal growth, by treating pregnant rats with NOS inhibitors, either diluted in their drinking solution (Baylis & Engels, 1992; Diket *et al*, 1994; Pierce *et al*, 1995; Salas *et al*, 1995), given by continuous endovenous infusion (Molnár *et al*, 1994) or by osmotic minipumps placed subcutaneously (Buhimschi *et al*, 1995; Helmbrecht *et al*, 1996; Yallampalli & Garfield, 1993). Results obtained by these different routes are quite comparable, and will be discussed together.

Chronic administration of NOS inhibitors to gravid rats results in a dose-dependent

Table I

Evidence of increased nitric oxide biosynthesis during pregnancy

Observations	References
Increased plasma cGMP levels	Conrad & Vernier, 1989
Increased urinary cGMP excretion	Conrad & Vernier, 1989
	Conrad *et al*, 1993
Increased metabolic production rate of cGMP	Conrad & Vernier, 1989
Increased urinary excretion and plasma levels of nitrite/nitrate	Conrad *et al*, 1993
Increased expression of eNOS mRNA in aorta	Goetz *et al*, 1994
Increased NOS activity in different tissues	Weiner *et al*, 1994
NOS expression in uterus and placenta changes towards parturition	Natuzzi *et al*, 1993
	Purcell *et al*, 1997
	Riemer *et al*, 1997

increase in systemic blood pressure in all but one study (Diket *et al*, 1994), thus confirming the major role of NO in the maintenance of normal vascular tone during pregnancy (Baylis & Engels, 1992; Molnár *et al*, 1994; Salas *et al*, 1995). An excess of L-arginine, but not of the inactive stereoisomer D-arginine, reduced L-NAME effects on blood pressure (Buhimschi *et al*, 1995).

Marked proteinuria and thrombocytopenia, and reduced plasma volume expansion were also observed after NOS inhibition (Baylis & Engels, 1992; Molnár *et al*, 1994; Salas *et al*, 1995; Yallampalli & Garfield, 1993). Long-term Nω-nitro-L-arginine administration to pregnant rats produced a dose-dependent decrease in plasma renin activity (PRA) without changes in either serum or urinary aldosterone levels (Salas *et al*, 1995, 1997) (Fig 2). Because NO synthesis inhibition increases blood pressure and consequently withdraws sympathetic activity (both renin inhibitory signals), the reduced PRA levels observed in this condition might be an indirect consequence of the hemodynamic changes induced by NO synthesis blockade. To address this issue, control rats were instrumented with an intra-aortic balloon catheter (to control renal perfusion pressure) and pretreated with propranolol (to eliminate beta-adrenergic effect). These rats exhibited an increased PRA in response to L-NAME treatment (Sigmon *et al*, 1992). Direct influence of nitric oxide on renin release was also explored in an isolated perfused juxtaglomerular apparatus preparation, in which influences from both the baroreceptor and the sympathetic nervous system are eliminated. In this preparation, addition of Nω-nitro-L-arginine to the external bath fluid increased renin release. When the inhibitor was administered to the luminal fluid at the macula densa, renin secretion was decreased by making it less sensitive to the stimulatory effect of a low luminal NaCl concentration (He *et al*, 1995).

Three different studies report no change in either urinary or serum aldosterone levels after NO synthesis blockade, despite marked reductions in PRA (Arnal *et al*, 1992; Salas *et al*, 1995; Salazar *et al*, 1992). The possible mechanisms of this apparent dissociation between PRA and aldosterone levels need to be further explored.

Although NO stimulates soluble guanylate cyclase activity, NOS blockade did not cause significant reductions in cGMP. cGMP levels, measured in amniotic fluid, did not display a dose-dependent reduction with L-NAME (Diket *et al*, 1994). Similarly, urinary cGMP excretion was not reduced either in non-pregnant or pregnant rats, or in rats treated with NOS inhibitors (Arnal *et al*, 1992; Salas *et al*, 1997) (Fig 3), suggesting that cGMP levels

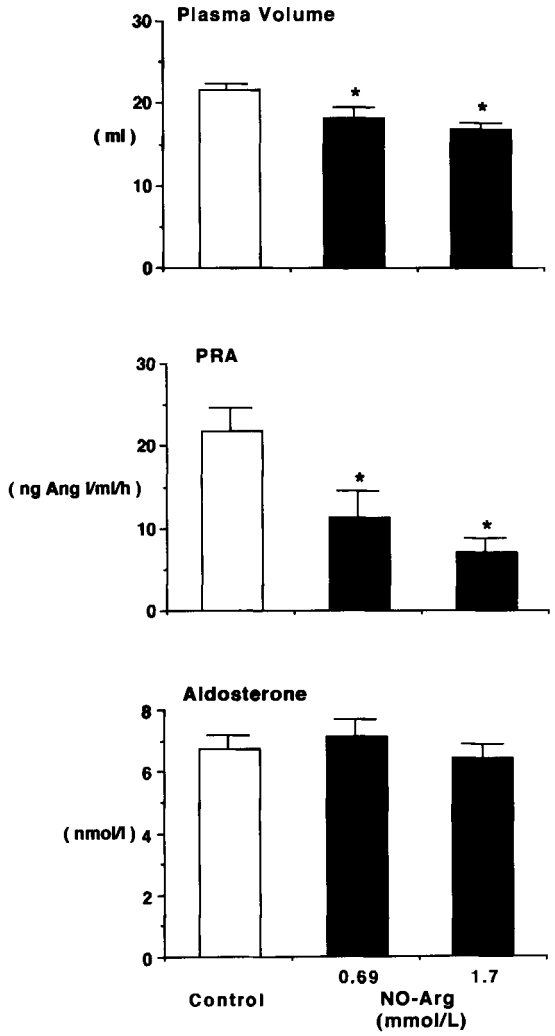

Fig 2. Effects on plasma volume, PRA and serum aldosterone levels of long-term administration of two doses of Nω-nitro-L-arginine to pregnant rats from days 7 to 21 of gestation. Data are presented as means ± SEMs. * $P < 0.05$ *vs* control group by ANOVA. (Adapted from Salas *et al*, 1995).

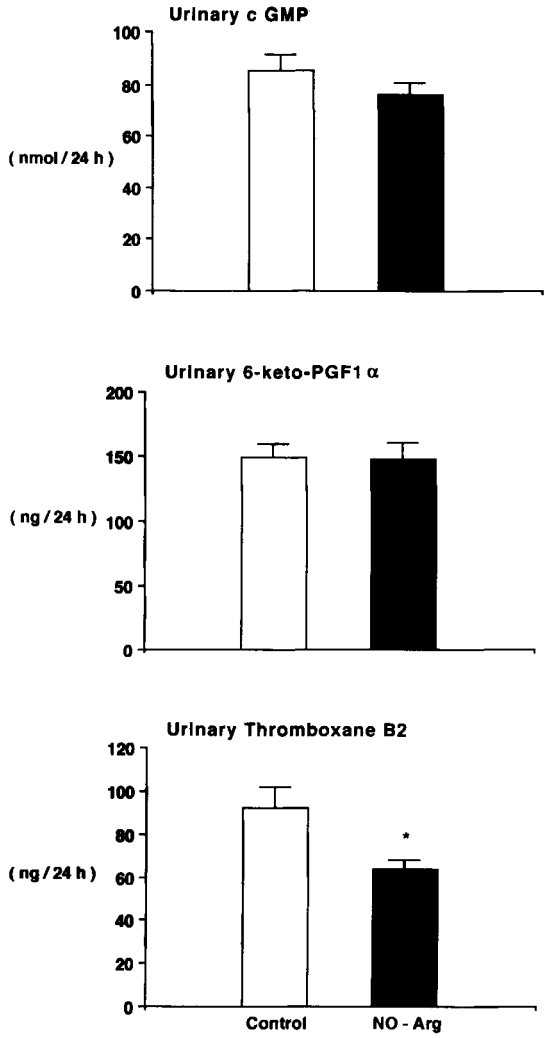

Fig 3. Effects on urinary excretion of cGMP, 6-keto-PGF1α and thromboxane B2 of long-term administration of 1.7 mmol/L of Nω-nitro-L-arginine to pregnant rats from days 7 to 21 of gestation. Data are presented as means ± SEMs. * $P < 0.05$ *vs* control group by unpaired Student's t-test. (Adapted from Salas *et al*, 1997).

may be influenced by activators of particulate guanylate cyclase, such as atrial natriuretic peptide (ANP). In this respect, it is worth noting that the administration of L-NAME to non-pregnant rats increased basal ANP levels and enhanced stretch-induced ANP release (Leskinen *et al*, 1995).

Chronic NOS blockade also influences maternal levels of other vasoactive agents. Pregnant rats treated with Nω-nitro-L-arginine exhibited a reduced urinary excretion of thromboxane B_2 (TxB_2), without significant changes in 6-keto-prostaglandin F1α (6-keto-PGF1α) excretion (Fig 3); in consequence, the 6-keto-PGF1α/TxB_2 ratio increased (Salas *et al*, 1997). *In vitro* studies have demonstrated that NO produced by endothelial cells increased the production of 6-keto-PGF1α and TxB_2, through activation of prosta-glandin H synthase, and that L-NAME significantly diminished prosta-glandins production (Davidge *et al*, 1995). Whether this is the mechanism involved in our observations remains to be elucidated.

As reported in acute experiments, chronic NO blockade increased renal vascular resistance and reduced GFR in near term pregnant rats (Baylis & Engels, 1992; Molnár *et al*, 1994). In addition to an abnormal renal function, chronic NO blockade produced renal histological abnormalities, such as focal glomerular sclerosis, occlusion of glomerular capillary lumens by eosinophilic material, and mesangial cell proliferation with preservation of the epithelial foot processes. The proportion of abnormal-appearing glomeruli was reduced with the addition of L-arginine (Helmbrecht *et al*, 1996). A mild diffuse interstitial edema and infiltrate of lymphocytes were also observed. Although Molnár *et al* (1994) reported that this alteration is unique to pregnancy, other authors, including us (unpublished data), have also observed renal damage in virgin rats (Baylis *et al*, 1992). This discrepancy may have been related to the early and more prolonged exposure of virgin rats to NOS inhibitors in the latter studies.

Despite the evidence suggesting that NO might be involved in maintaining uterine quiescence during pregnancy, chronic NO inhibition did not alter the day of spontaneous delivery, thus suggesting that other mechanisms are responsible for controlling the length of pregnancy (Molnár *et al*, 1994; Yallampalli & Garfield, 1993).

Fetal effects of long-term NOS inhibition

Chronic NO blockade, in a dose-dependent manner, caused a significant reduction in the weight and size of the pups, as well as reduced litter size and increased fetal mortality (Baylis & Engels, 1992; Salas *et*

al, 1995; Yallampalli & Garfield, 1993). We have proposed that the mechanisms causing fetal growth retardation are related to altered maternal vasodilation, which limits plasma volume expansion and, secondarily, reduces cardiac output and utero-placental blood flow. Nevertheless, a direct role of NO deficiency in placental perfusion cannot be excluded, since it is well known that the placental villus vascular tree has the ability to both generate and respond to NO (Myatt *et al*, 1991). A progressive and dose dependent hind limb disruption in the pups was observed after NO blockade (Diket *et al*, 1994; Salas *et al*, 1995). This alteration corresponded to hemorrhagic necrosis, with marked cellular infiltration and loss of structure. It was reversed with the administration of the nitric oxide donor sodium nitroprusside or with an excess of L-arginine. When dams were treated with the inactive enantiomer, D-NAME, none of the pups presented with hind limb defects. These results strongly suggest that hind limb disruption is specifically related to NO synthesis inhibition (Diket *et al*, 1994; Pierce *et al*, 1995). Most probably, this alteration is caused in the post-organogenic period since it is not produced when the drug is given during the first week of pregnancy (Salas *et al*, 1995). In addition, no defect was observed when the dams received aminoguanidine, a selective inhibitor of iNOS (Pierce *et al*, 1995), providing evidence supportive of specific inhibition of endothelial (cNOS) NO release in the pathogenesis of this defect.

The finding that lesions induced by chronic inhibition of NO synthesis, such as maternal hypertension, proteinuria, and renal glomerular injury and fetal growth restriction are reversed by treatment with L-arginine lends support to the potential use of NO donors in the treatment and prevention of pre-eclampsia. Nevertheless, the administration of the endogenous NO synthase-independent NO donor, molsido-mine, to pregnant rats treated with L-NAME, unexpectedly worsened pregnancy outcome by increasing fetal reabsorption and reducing litter size and fetal weight (Richer *et al*, 1996). Thus, data available provide a caveat regarding the use of this NO donor in pregnant women with pre-eclampsia.

OVERVIEW

There is strong evidence that suggests that increased NO synthesis occurs during pregnancy and that nitric oxide-nitric oxide synthases pathway is involved in maintenance of vascular tone and altered vascular responses to pressor agents during pregnancy.

Taking altogether, present data may have important clinical implications. Gant *et al* (1973) have shown that the blunting of the ANG II response in pregnancy is reversed in pregnant women destined to develop preeclampsia. In addition, NO is also increased in normal human pregnancy and seems to be decreased in women with pre-eclampsia. Moreover, umbilical arteries and veins from patients with pregnancy-induced hypertension had a significantly lower release of endothelium-derived relaxing factor than tissues from normotensive pregnant women (Pinto *et al*, 1991). In addition, inhibition of nitric oxide synthesis in rats during pregnancy produces signs similar to those of preeclampsia (Yallampalli & Garfield, 1993). It is, therefore, possible that a disturbance in NO release, either primary or secondary to endothelial dysfunction, may contribute to the pathogenesis of pre-eclampsia.

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