Long-Term Nitric Oxide Synthase Inhibition in Rat Pregnancy Reduces Renal Kallikrein

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Abstract—This study was performed to test the hypothesis that long-term nitric oxide synthase (NOS) inhibition during pregnancy may alter the predominance of the vasodilator kallikrein system. Sprague-Dawley rats were treated with the competitive inhibitor of NOS Nω-nitro-l-arginine (L-NNA, 50 mg · kg⁻¹ · d⁻¹, dissolved in water) from days 7 to 21 of pregnancy. Rats were studied before treatment (day 5), at days 11, 17, and 21 of pregnancy (during treatment), and at postpartum days 7 and 21 (after the drug was withdrawn at delivery). Each group (n=5 to 8) had its corresponding control group (C) that received only vehicle. Additional rats were treated with Nω-nitro-l-arginine methyl ester (L-NAME) alone or with an excess of L-arginine. At each study day, we measured blood pressure, collected urine overnight, obtained blood samples, and processed the kidneys for conventional histology and immunohistochemistry. In L-NNA rats, fetal and placental weights were reduced at days 17 and 21. Blood pressure was higher at days 17 and 21, returning to normal after L-NNA was removed. Urinary kallikrein activity was lower at days 11 and 17 (L-NNA=1147±213 and C=2317±146 nmol/16 h, P<0.001). Plasma renin activity was reduced at day 21 (L-NNA=9.6±2.1 and C=25.9±5 ng · mL⁻¹ · h⁻¹, P<0.05) and remained lower at postpartum day 7. L-NNA rats exhibited glomerular lesions and tubular atrophy, particularly of connecting tubules that displayed reduced kallikrein staining. Tubulointerstitial infiltrating macrophages (ED1+) were also observed. Renal lesions were present as early as day 11 and persisted at day 7 postpartum. L-NAME rats exhibited similar alterations that were attenuated with an excess of L-arginine. We postulate that the reduction in renal kallikrein may contribute to the hemodynamic alterations described in this model. (Hypertension. 1999;34[part 2]:865-871.)

Key Words: pregnancy ■ nitric oxide ■ plasma renin activity ■ renal kallikrein ■ renal lesions ■ macrophages

Normal pregnancy is characterized by a marked stimulation of the renin-angiotensin system (RAS), which causes renal water and sodium retention and increases plasma volume.1,2 In turn, plasma volume expansion allows a sustained elevation in cardiac output and, indirectly, in uterine blood flow, conditions necessary for normal fetal growth. Despite the rise in blood volume and the activation of the vasoconstrictor RAS, blood pressure decreases during pregnancy2 because of a marked reduction in peripheral vascular resistance associated with a predominance of vasodilator, such as kallikrein and nitric oxide (NO), over vasoconstrictor substances.3 Thus, systemic vasodilatation would initiate the series of hemodynamic changes that are important for fetal growth. Consistent with such a possibility, in normal pregnant rats we have demonstrated a rise in urinary kallikrein activity that precedes the increase in plasma renin activity (PRA).4 In addition, in conditions associated with a limited plasma volume expansion, such as idiopathic fetal growth restriction5 and preeclampsia,6 and in underweight mothers,7 we have demonstrated a significant reduction in urinary kallikrein activity. Kallikrein, through kinin formation, stimulates the NO–NO synthase (NOS) pathway, which is also activated in pregnancy. The plasma level, metabolic production, and urinary excretion of guanosine 3′,5′-cyclic monophosphate (cGMP), the urinary excretion and plasma levels of the stable NO metabolite nitrate, and the expression and activity of different NOSs are increased in gravid animals.8–10 Long-term administration of NOS inhibitors, such as Nω-nitro-l-arginine (L-NNA) and Nω-nitro-l-arginine methyl ester (L-NAME), to gravid rats causes sustained hypertension, reduced plasma volume, intrauterine growth retardation, proteinuria and thrombocytopenia, and renal morphological abnormalities that resemble those observed in women with preeclampsia.11–15

These observations led us to evaluate the hypothesis that long-term NOS inhibition during pregnancy may alter the predominance of the vasodilator kallikrein-kinin system (KKS), either by increasing renin or decreasing renal kallikrein, thus contributing to the hemodynamic alterations described in this model. In consequence, we explore the time

Received June 2, 1999; first decision July 1, 1999; revision accepted July 28, 1999.
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course of the changes on PRA, urinary kallikrein activity, and renal levels of renin and kallikrein as assessed by immunohistochemistry, associated with NOS inhibition in pregnant rats.

Methods

Experimental Design
A total of 86 virgin Sprague-Dawley rats, weighing 230 to 260 g, were used and maintained at the Center for Medical Research animal care facilities, under constant temperature (24°C) in a 12-hour light/dark cycle, with free access to standard rat chow and water. All procedures were performed in accordance with the institutional and international guidelines for animal welfare.

Rats in estrous phase were mated overnight with fertile males, and day 0 of pregnancy was determined by the presence of spermatozoa in vaginal smears. Following a protocol previously described, on day 7 of gestation the rats were randomly assigned to either a control group or an experimental group that received 50 mg · kg⁻¹ · d⁻¹ of L-NNA dissolved in tap water.

Control and experimental groups were studied on days 11, 17, and 21 of pregnancy. Baseline values were obtained in another group of rats at day 5 of pregnancy, before randomization. Additional rats from both groups were allowed to deliver spontaneously and were studied on days 7 and 21 postpartum, after the drug was withdrawn at delivery. Five to 8 animals in each group per study day were used. Systolic blood pressure was measured by tail-cuff plethysmography, and rats were individually placed in metabolic cages with free access to food and water from 5 PM to 9 AM. On the following day, between 10 AM and 12 noon, the rats were weighed and anesthetized with pentobarbital sodium (40 mg/kg). Blood samples obtained from the abdominal aorta were placed in tubes containing either liquid EDTA or sodium heparin. An aliquot was used for microhematocrit determination, the remaining samples were centrifuged at room temperature, and the plasma was frozen for later determinations of sodium, protein, creatinine excretion, and kallikrein activity.

To determine the specificity of the lesions, additional rats (n=6 per group) received the NOS inhibitor L-NAME (50 mg · kg⁻¹ · d⁻¹), alone or associated with an excess of L-arginine (2.7 g · kg⁻¹ · d⁻¹), or received the inactive stereoisomer D-NAME (50 mg · kg⁻¹ · d⁻¹), from days 7 to 21 of pregnancy. Experimental and control groups were studied on day 21 of pregnancy according to the same protocol as described above.

Hormonal and Biochemical Measurements
Kallikrein activity was determined by the amidase method using the synthetic substrate DL-Val-Leu-arginine-p-nitroanilide. PRA was determined by radioimmunoassay of generated angiotensin I under control conditions. The concentration of urinary sodium was measured by flame photometry (IL 343). Colorimetric reactions were used to determine plasma and urine levels of creatinine (Beckman Autoanalyzer). Urinary protein concentration was determined by Bradford’s method (Bio-Rad protein assay).

Tissue Processing and Immunohistochemistry
Kidney slices 3 mm thick, including cortex, medulla, and papilla, were fixed by immersion in Bouin’s solution for 24 to 48 hours at room temperature, dehydrated, embedded in Paraplast, serially sectioned at 7-µm thickness, and mounted on glass slides until immunohistochemistry was performed. In addition, the tissue sections were studied with conventional staining, such as hematoxylin-eosin and periodic acid-Schiff (PAS). Immunohistochemical staining for renal kallikrein and renin was performed according to the peroxidase/antiperoxidase (PAP) method as previously described.

Briefly, the tissue sections were dewaxed, rehydrated, and rinsed in 0.05 mol/L Tris-phosphate-saline buffer, pH 7.6, and incubated overnight with rabbit antisera raised against rat tissue kallikrein (1:5000) or against renin (1:5000) at 22°C. The second antibody (goat anti-rabbit IgG, 1:20), and the corresponding PAP complex (1:150) were applied for 30 minutes each at 22°C. The immunoperoxidase reaction was visualized by incubating sections in 0.1% (wt/vol) 3,3'-diaminobenzidine and 0.03% (vol/vol) hydrogen peroxide. The antisera and PAP complex were diluted in Tris buffer containing 0.25% (vol/vol) Triton X-100 and 0.7% (wt/vol) l-carrageenan. To characterize the interstitial infiltrate, additional sections were incubated with ED1, a mouse monoclonal antisera raised against rat monocytes and macrophages (1:400). The second antibody (rabbit anti-mouse) was used at a dilution of 1:400, and the third (goat anti-rabbit) was diluted 1:20. The PAP complex was used as above. Controls for the immunostaining procedure were prepared by omission of the first antibody or by its replacement with preimmune serum. All sections were counterstained with hematoxylin and then dehydrated, cleared with xylene, and coverslipped.

Quantitative Morphology
The tissue samples from all groups were coded and studied independently by 2 expert observers (S.P.S. and J.F.V.) in a blinded fashion. One section selected at random was used for each rat (5 to 8 rats per group), and at least 4 fields per section were studied. To estimate the intensity of the immunostaining at the cellular level, the observers made a single ordinal ranking (0 to 3), with 0 being absent, 1 being very faint yet distinguishable, 2 being moderate staining, and 3 the strongest staining. The comparison of staining intensity was done at a first antibody dilution of 1:5000. If any difference was observed, further dilutions of the first antibody were done until the staining disappeared. The tissue sections were observed and photographed on a Nikon Optiphot microscope, with a Nikon Microflex UFX IIA photographic system (Nikon Corp.).

Source of Antisera and Chemicals
L-NNA, L-NAME, D-NAMA, L-arginine, DL-Val-Leu-arginine-p-nitroanilide, l-carrageenan, Triton X-100, 3,3'-diaminobenzidine, and Trit were purchased from Sigma Chemical Co; Paraplast was purchased from Monolot Scientific; and the PAP complex was from Cappel. Rabbit antisera against kallikrein was obtained from our laboratory: the renin antibody was a gift from Dr Francois Alhenc-Gelas, INSERM U367, Paris, France; and a monoclonal antibody against macrophages (ED-1) was purchased from Serotec. The goat anti-rabbit IgG and the rabbit anti-mouse IgG were from Cappel.

Statistical Analysis
All data were expressed as arithmetic mean±SEM. Within each study day, we analyzed differences between the group means by an unpaired 2-tailed Student’s t test, with a significance level of 95%, using the computer program Stat View II (Abacus Concepts Inc).

Results
Net maternal weight at term (day 21 of pregnancy) was similar in both groups, whereas hematocrit values were higher in rats treated with L-NNA. No significant differences in creatinine clearance, urine volume, and urinary protein and sodium excretion were observed. Litter size was similar in both groups, and fetal and placental weights were significantly lower in L-NNA rats (Table). On day 17 of pregnancy, fetal weights (Control, 0.76±0.02 and L-NNA, 0.64±0.03 g, P<0.01) and placental weights (Control, 0.29±0.01 and L-NNA, 0.24±0.01 g, P<0.01) were also reduced. In contrast, no difference in pup weights was observed at postpartum days 7 (Control, 11.9±0.5 and L-NNA, 11.3±0.9 g) or 21 (Control, 35.7±1.8 and L-NNA, 38.2±2.2 g). However, litter size was significantly reduced in L-NNA rats compared...
with controls at postpartum days 7 and 21 (Control, 10.2±0.6 and L-NNA, 6.2±0.7, P<0.01). L-NNA dams allowed to deliver spontaneously did not show any difference in gestational length from control rats.

Systolic blood pressure was significantly higher in L-NNA rats at days 17 and 21 of gestation; this difference disappeared postpartum, after the drug had been withdrawn. PRA was reduced in L-NNA rats on days 21 of pregnancy and 7 postpartum. L-NNA treatment suppressed the fall in blood pressure as well as the marked increase in PRA observed normally at day 21. In L-NNA rats, urinary kallikrein activity was significantly reduced on days 11 and 17 of pregnancy, attaining normal values thereafter (Figure 1).

At day 21 of pregnancy, rats treated with L-NAME had a rise in blood pressure similar to that of L-NNA rats (128±7.4 mm Hg) and also exhibited reduced fetal weight (5.0±0.2 g). Pregnant rats treated with D-NAME or l-arginine did not exhibit an increase in blood pressure as well as the marked increase in PRA observed normally at day 21. In L-NNA rats, urinary kallikrein activity was significantly reduced on days 11 and 17 of pregnancy, attaining normal values thereafter (Figure 1).

Figure 1. Line graphs show systolic blood pressure, PRA, and urinary kallikrein activity in control rats (C) and in rats that received L-NNA 50 mg · kg⁻¹ · d⁻¹ from days 7 to 21 of pregnancy (L-NNA). Values are mean±SEM. *P<0.05; **P<0.01; ***P<0.001 vs control rats.

### Morphological Study

Representative morphological features of renal sections from control and L-NNA–treated rats are shown in Figure 2. A through H. Tissues from sections 2A through 2F and 2H were obtained at day 21 of pregnancy, whereas section 2G was obtained at postpartum day 7. Figure 2A shows normal glomeruli and tubules from a control pregnant rat. Figure 2B shows a section from an L-NNA rat, depicting dilatation of some tubules and flattening of the tubular epithelium. Kallikrein immunostaining was observed exclusively in the connecting tubule cells of the distal nephron (Figure 2C and 2D) and was reduced in L-NNA rats at both days 17 and 21 of pregnancy (Figure 2D). Comparison of staining intensity was done at a first antibody dilution of 1:5000. Five of 8 control rats exhibited the maximum staining, scoring ++++, and 3 had moderate staining (++). Three of 6 L-NNA rats exhibited faint staining, scoring +; 2 had moderate staining, scoring ++; and only 1 had maximum staining (+++). Further dilution of the first antibody showed that the kallikrein staining disappeared in L-NNA kidneys at a dilution of 1:80 000, whereas in control kidneys, it persisted even at a dilution of 1:160 000. Compared with control rats (Figure 2C), the kallikrein-containing cells from L-NNA rats were smaller (Figure 2D), although the cross-sectional area of the cells was not measured. No differences in renin staining were observed; at a dilution of 1:5000, all sections from both groups displayed the maximum intensity and were ranked as ++++ (Figure 2E and 2F). Kidneys from L-NNA rats exhibited interstitial cellular inflammatory infiltration, particularly at postpartum day 7. The infiltration was predominantly monocytes-macrophages (ED1+) (Figure 2G). Tubular and glomerular alterations were observed as early as day 11 of pregnancy (4 days with NOS inhibition). At this time, some glomeruli were hypercellular and Bowman’s capsule was thickened. These lesions progressed until day 21 of pregnancy, when segmental areas of hyalinization, segmental fibrinoid necrosis of the glomerular tuft, and cellular vacuolization were observed. In addition, intraglomerular mitosis was also observed (Figure 2H). Some tubules exhibited the presence of eosinophilic intraluminal material, as well as thickening of basal membrane, containing PAS-positive material. These changes were decreased at postpartum day 7 and...
Figure 2. Color plate showing representative morphological features of renal sections from control and L-NNA–treated rats, obtained at day 21 of pregnancy (A through F and H) or at postpartum day 7 (G). A and B, Sections from control (A) and L-NNA–treated (B) rats stained with hematoxylin and eosin. Note the atrophy of tubules (**). C and D, Sections from control (C) and L-NNA–treated (D) rats immunostained for kallikrein. Kallikrein is present in connecting tubules (*), which are dilated, atrophic, and with reduced kallikrein staining in L-NNA rats. E and F, Sections from control (E) and L-NNA–treated (F) rats immunostained for renin. Renin is restricted to the afferent arterioles in the vicinity of glomeruli, without evident changes between groups. G, Section from L-NNA rat, showing the presence of abundant monocytes/macrophages, as identified with the specific ED1 antigen (arrows). H, Intraglomerular mitosis in an L-NNA rat, stained with PAS. Bars in A, B, E, and F=50 μm; in C, D, G, and H=20 μm.
were undetectable at postpartum day 21 (data not shown). Similar alterations were observed with the administration of L-NAME, another inhibitor, and were attenuated with the administration of an excess of L-arginine. Rats treated with D-NAME did not exhibit renal alterations (data not shown).

Discussion

The present study shows that NOS inhibition in pregnant rats produces a significant reduction in urinary kallikrein activity and in renal kallikrein immunostaining, associated with renal morphological abnormalities, high blood pressure, reduced PRA levels, and reduced fetal weight. These functional and morphological alterations seem to be a consequence of NOS inhibition rather than the effect of L-NAME or L-NNA per se, for several reasons. First, they were observed with the use of 2 different inhibitors (L-NNA and L-NAME); second, they were attenuated with the administration of an excess of substrate (L-arginine); and third, they were not observed when the inactive stereoisomer D-NAME was administered.

An interesting finding of our study is that NOS inhibition in pregnant rats reduced urinary kallikrein activity and renal kallikrein immunostaining. The KKS is known to be activated in normal pregnancy, and reduced urinary kallikrein activity has been demonstrated in pregnant women with fetal growth restriction or preeclampsia.\(^5,6\) conditions characterized by a reduced plasma volume expansion and increased total peripheral vascular resistance. The renal KKS seems to participate in complex events such as regulation of blood pressure and control of the extracellular volume, sodium and water excretion, renal vascular resistance, and renin release.\(^3\) Several findings provide anatomic and functional frameworks for a role of the KKS in the hemodynamic events in the kidney. Among them, (1) immunohistochemical studies demonstrate an anatomic relationship between kallikrein-containing tubules and the juxtaglomerular apparatus\(^20\,21\); (2) kallikrein is observed in the basolateral membrane of connecting tubule cells,\(^22\) and Madin-Darby canine kidney distal tubule cells transfected with rat kallikrein cDNA secrete kallikrein into both the apical and basolateral poles,\(^23\) suggesting that renal kallikrein can reach the circulation; and (3) there is evidence for renal kinins as mediators of the amino acid–induced renal hyperperfusion and hyperfiltration in the rat.\(^24\) Furthermore, kinins contribute to the control of renin secretion.\(^25\)

Previous studies have shown that L-NAME partially or completely blocks glomerular filtration rate and renal plasma flow responses to amino acid infusion in rats, suggesting that NO is a downstream modulator of the actions of kinin on the glomerulus.\(^26\) The reduced urinary kallikrein activity observed in the present study may be caused directly by NOS inhibition or may be a consequence of the morphological alterations observed in the connecting tubule cells. In non-pregnant rats, a 3-week treatment with L-NAME increased hepatic kininogen and renal kallikrein mRNA levels.\(^27\) In the same study, the use of HOE-140, a Bradykinin B\(_2\)-receptor antagonist, produced a greater rise in blood pressure in L-NAME–treated rats, suggesting that an increased activity of the KKS plays a protective role in this model of hypertension. Several factors may account for this apparently contrasting result. First, in our study we gave the NOS inhibitor for only 2 weeks, a period of time in which no significant changes in the components of the KKS were observed in the study by Chao et al.\(^27\) Second, the interaction between the NO-NOS pathway and the KKS may be different in pregnant animals. And third, it cannot be ruled out that the stimulation of the KKS reported in that study\(^27\) was caused by the increment in blood pressure, which was >200 mm Hg after 3 weeks of L-NAME treatment and not a direct effect of NOS inhibition. In our study, the highest blood pressure observed was within the normal range for virgin rats (134 mm Hg) and presumably did not activate compensatory mechanisms. We were surprised to find that urinary kallikrein activity was within the normal range on day 21 of pregnancy, despite continuous administration of L-NNA and decreased renal kallikrein immunostaining. Because kallikrein excretion in control pregnant rats had returned toward nonpregnant values, we could speculate that NOS inhibition attenuates the pregnancy-induced rise in kallikrein but has no effect on basal kallikrein. Alternatively, the ratio of active to total kallikrein may have increased to overcome the reduction in renal kallikrein synthesis observed in this model. This possibility was not investigated in the present study. Independently of the mechanism that caused reduced renal kallikrein synthesis, this change may contribute to the rise in blood pressure and the reduction in plasma volume expansion described previously in this model.\(^14\)

Inhibition of NO formation substantially attenuated the stimulatory effect of pregnancy on PRA. The reduced PRA levels are in keeping with previous studies in pregnancy and in nonpregnant conditions.\(^28\,30\) Because L-NNA administration increases blood pressure, thus withdrawing sympathetic activity (both renin inhibitory signals), the lower PRA levels may be an indirect consequence of the hemodynamic changes induced by NO inhibition. Decreased PRA levels have also been observed in pregnancies associated with reduced plasma volume expansion, such as in idiopathic fetal growth restriction and in preeclampsia.\(^5\,6\) It is interesting to note that this difference in PRA between the control and L-NNA groups was not accompanied by substantial changes in renal immunostaining at the juxtaglomerular apparatus. This paradox of low PRA levels without apparent changes in renal renin has been described in a model of experimental diabetes\(^15\) and can be explained by the fact that the immunohistochemical technique is less sensitive to detecting small changes in renin content than is PRA.

Rats treated with NOS inhibitors during pregnancy exhibited glomerular, tubular, and interstitial abnormalities. Several studies have shown that NOS inhibition causes a marked increase in the number of abnormal glomeruli in pregnant and in virgin rats. Here, we report regression of these lesions after drug removal. Renal changes were present as early as day 11 of pregnancy (4 days of L-NNA treatment), before the onset of increased systolic blood pressure. The mechanisms causing renal injury during chronic NO blockade are under discussion. Previous studies have shown that the NO-NOS system controls both the tone of the glomerular afferent arteriole by modulating its myogenic response and the glomerular hemodynamics indirectly through tubuloglomerular feedback and renin release.\(^33\) Therefore, renal alter-
ations may be secondary to intrarenal ischemia or glomerular hypertension and seem not to be caused by the increment in maternal systolic blood pressure. Another possible mechanism contributing to renal damage is the presence of infiltrating macrophages, because in some models of glomerulonephritis, local macrophage proliferation has been associated with the progression of glomerular and tubulointerstitial injury.

However, macrophage infiltration in the present study was a rather late event, observed mainly at postpartum day 7.

In keeping with previous studies, the present results confirm that NOS inhibition during pregnancy reduces fetal and placental weights (for review, see Reference 35). We have proposed that the mechanisms that cause fetal growth restriction are related to an altered maternal vasodilation, which limits plasma volume expansion and, secondarily, reduces cardiac output and uteroplacental blood flow. A direct role of NO deficiency in placental perfusion may also be involved, because it is well known that the placental villous vascular tree has the ability to both generate and respond to NO.

Thus, chronically inhibiting the release of NO from placental vessels would result in vasoconstriction and reduced blood flow that indirectly contribute to reduced fetal weight. Surviving pups were capable of catch-up growth, probably favored by the reduction in litter size.

Despite the existence of renal morphological abnormalities and reduced levels of PRA and kallikrein excretion, the present results show no significant changes in diuresis, creatinine clearance, and proteinuria after 2-week L-NNA treatment. Although sodium excretion doubled during NOS inhibition, this difference was not statistically significant. The present results are in disagreement with previous ones that demonstrated increased protein excretion and reduced glomerular filtration rate during NOS inhibition in pregnant rats. A possible explanation for these differences relates to the fact that in our study, pregnant rats exhibited only a moderate increase in systolic blood pressure that was within the normotensive range for virgin rats, whereas in the previous studies, blood pressure reached much higher values (mean arterial blood pressure of 150 mm Hg). Presumably these contrasting results are caused by differences in the dose of the inhibitor used and/or in the route of administration (oral versus intravenous infusion).

In conclusion, the present results demonstrate that long-term NOS inhibition during pregnancy causes reduced renal kallikrein synthesis, associated with renal morphological alterations, increased blood pressure, decreased PRA, and reduced fetal weight. We postulate that the reduction in the vasodilator KKS could contribute to the hemodynamic alterations described in this model by interfering with the normal vasodilator response of pregnancy.

Acknowledgments

This work was partially supported by grant 1.96.0779 from the Fondo Nacional de Desarrollo Cientifico y Tecnológico (Chile), FONDECYT. The excellent technical assistance of María Alcohado and Carlos Céspedes is gratefully acknowledged.

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Hypertension. 1999;34:865-871
doi: 10.1161/01.HYP.34.4.865

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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