Abstract—Alterations in nitric oxide (NO) production have been suggested to play a role in mediating changes in renal function during normal pregnancy and in pregnancy-induced hypertension. Although NO production is enhanced during normal pregnancy, the mechanisms for the increase are unknown. The purpose of this study was to determine whether the elevation in NO production during pregnancy is associated with increases in renal expression of endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) nitric oxide synthases. To achieve this goal we examined systemic and renal hemodynamics, urinary excretion of nitrate/nitrite, and renal protein expression of the three NOS isoforms in pre-pregnant rats, pregnant rats at days 6, 13, and 19 of gestation and at day 4 postpartum. Mean arterial pressure decreased by 14% in late pregnancy whereas the glomerular filtration rate and renal plasma flow increased by 21% and 24%, respectively, in mid pregnancy. Excretion of nitrate/nitrite increased throughout pregnancy with a 3.4-fold increase present at day 19 (12.2±0.7 to 41.1±1.3 μmol/24 h). Renal eNOS protein expression decreased by 39% during pregnancy with the lowest level resulting at day 19 and returning to virgin levels by day 4 post partum. In contrast, renal iNOS and nNOS protein expression increased 31% and 25%, respectively, with highest expression occurring for both at day 13 of pregnancy. These data suggest that the increased NO production and renal hemodynamics associated with pregnancy in rats may be caused by the upregulation of iNOS and nNOS in the kidney. (Hypertension. 1999;33[part II]:435-439.)

Key Words: kidney ■ glomerular filtration rate ■ renal blood flow ■ endothelial factors

Normal pregnancy in humans is associated with significant changes in cardiovascular and renal function. These physiological adaptations include decreases in mean arterial pressure that are accompanied by reductions in total peripheral resistance and increases in cardiac output. In addition to the changes in systemic hemodynamics, there are marked alterations in renal function. Increases in renal plasma flow (RPF) and glomerular filtration rate (GFR) of >40% are commonly observed in pregnant women. In addition, the sensitivity of the renal circulation to vasoconstrictors is significantly reduced during normal pregnancy. Although the exact mechanisms that are involved in mediating these physiological changes during pregnancy are unclear, recent studies suggest that activation of endothelial factors such as nitric oxide (NO) may play an important role.

Several lines of evidence support the concept that there is increased endogenous production of NO during pregnancy and that NO mediates the renal and cardiovascular adaptations during pregnancy. For example, increases in GFR and RPF and decreases in arterial pressure during pregnancy are attenuated by inhibition of systemic NO synthesis. During normal pregnancy increased mRNA expression for both neuronal (nNOS) and endothelial (eNOS) nitric oxide synthases has been measured in different tissues. In addition, increased plasma concentration and urinary excretion of cGMP, a secondary messenger of NO, and increased urinary excretion of nitrite and nitrate, metabolic products of NO, are both indicative of enhanced whole-body NO production.

Although there is ample evidence that whole-body NO production is elevated during normal pregnancy, it is unknown whether NO production in the kidney is increased. As a means of investigating the NO system in the kidney we examined protein levels of the enzyme, nitric oxide synthase, which catalyzes the formation of NO from l-arginine. Therefore, the purpose of this study was to determine whether the renal hemodynamic changes observed during normal pregnancy were associated with changes in the renal expressions of endothelial, neuronal, and/or inducible nitric oxide synthases. In addition, we examined changes in mean arterial pressure and renal hemodynamics during normal pregnancy. We also estimated whole-body NO production.

Methods

All studies were performed in 200- to 250-g female Sprague Dawley rats purchased from Harlan Sprague Dawley Inc (Indianapolis, IN).
Animals were housed 3 to a cage in a temperature-controlled room (23°C) with a 12:12 hour light/dark cycle. Rats intended to become pregnant were placed with a fertile male, and day 1 of pregnancy was determined by the presence of sperm in the vaginal smear. All experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for use and care of animals and the protocols were approved by the Animal Care and Use Committee at the University of Mississippi Medical Center.

Telemetric Measure of Arterial Pressure in Conscious Rats
Arterial pressure was chronically monitored in the same group of conscious rats (n=6) during prepregnancy, gestation, and postpartum by use of a telemetry system. While the rats were under anesthesia with oxyfluorane, a flexible catheter attached to a radio transmitter (Data Sciences) was inserted in the abdominal aorta just below the renal arteries. After surgery the rats were individually housed in cages with ad libitum access to food and water. The radio transmitter sent arterial pressure signals to a radio receiver positioned underneath the cage. The system cycled from animal to animal with data acquired at a rate of 500 Hz per 20 seconds every 10 minutes. Data were averaged in 60-minute blocks for analysis with arterial pressure averaged during 24-hour periods for daily values.

Measurement of Renal Hemodynamics in Conscious Rats With Use of Chronic Protocols
Renal hemodynamics were determined in conscious virgin (n=5) and pregnant rats at days 6 (n=8), 13 (n=7), and 19 (n=6) of gestation, and rats at day 4 post partum (n=3). During isoflurane anesthesia, rats were surgically instrumented with catheters (PE 50 tubing) in the femoral vein and carotid artery for blood sampling and blood pressure monitoring. A midline lower abdominal incision was made, and the bladder was cannulated with flare-tipped PE 90 tubing for urine collection. All catheters were tunneled to the back of the neck and exteriorized. Animals were allowed to recover for 3 days before renal function measurements. On the day of renal function measurements, the rats were removed from their metabolic cages and placed in modified restraining cages. The femoral vein catheter was connected to an infusion pump that delivered isotonic saline containing [125I]iothalamate (Isotex Diagnostics; 0.05 mCi/kg-1·min-1) and [131I]iodohippurate (Syncor International Corporation; 0.1 mCi/kg-1·min-1) at a fixed rate of 3 mL/h. Arterial pressure was monitored with a pressure transducer connected to a Grass model 7B chart recorder (Grass Instrument Co.) for continuous recording. After a 1-hour equilibration period, 2 consecutive 20-minute urine collections were obtained in each rat. Urine volume was determined gravimetrically. GFR and RPF were calculated from concentrations of [125I] and [131I] in plasma and urine.

Measure of Urinary Nitrite/Nitrate Excretion
Urinary nitrite/nitrate excretion rates were determined in one group of rats (n=10) with measurements made during the prepregnant state, at days 6, 13, and 19 of pregnancy, and at day 4 post partum. Animals were fed a low nitrite/nitrate diet (AIN76, ICN Biomedicals, Inc.) throughout the experimental protocol. Escherichia coli was the source of nitrate reductase for conversion of nitrate to nitrite; sodium nitrate was the standard to verify that all nitrate was converted to nitrite. The concentration of nitrite was measured colorimetrically with the Griess reagent. Sodium nitrite was used as the standard, and the data were expressed as millimoles of nitrate/nitrite excreted in 24 hours in the rat.

Determination of Serum L-Arginine Levels
Serum was collected and stored at −20°C for the measurement of serum L-arginine levels from virgin rats (n=10), pregnant rats at days 6 (n=10), 13 (n=10), and 19 (n=10) of pregnancy, and rats at day 4 post partum (n=10). After precipitation with 25% salicylic acid, serum proteins were centrifuged, and the supernatant was chromatographed by high-performance liquid chromatography (Beckman 6300 Autoanalyzer, Beckman Instruments). Data were recorded and calculated by the Maxima software program (Waters) and expressed as nanomoles of L-arginine per milliliter of serum.

Isolation of Total Cellular Proteins and Western Blot Analyses
Kidneys were removed from virgin rats (n=10), pregnant rats at days 6 (n=10), 13 (n=10), and 19 (n=10) of pregnancy, and rats at day 4 postpartum (n=10) and quick frozen in liquid nitrogen and stored at −80°C. Each kidney was ground with use of a mortar and pestle chilled in liquid nitrogen and stored in a sterile tube at −70°C. Kidneys were homogenized 20% (wt/vol) in buffer containing 20 mM HEPES, pH 7.5, 100 mM NaCl, 100 mg/mL pepstatin, 10 mM EDTA, 100 mg/mL leupeptin, 1 mM EDTA, 100 mg/mL phenanthrolone, and 1 mM EDTA E-64 (Sigma Chemical Co.). Total protein concentration was determined with use of the Sigma Protein Determination kit (P5656, Sigma Chemical Co.). Equivalent amounts of total protein from each rat kidney sample were separated by electrophoresis with a 7.5% polyacrylamide resolving gel. Recommended positive controls were used for proper analysis (human endothelial, mouse macrophage, and rat pituitary; Transduction Laboratories). After transfer to nitrocellulose, membranes were probed with either the mouse monoclonal antibody ECNOS (Transduction Laboratories) for quantification of eno or with the mouse monoclonal antibody nNOS (Transduction Laboratories) for quantification of both nNOS and inducible nitric oxide synthase (iNOS). Actin (actin antibody, Amersham) was used as an internal control, and NOS expression was normalized relative to actin. Horseradish peroxidase conjugated goat anti-mouse IgG (Amersham) was used as a secondary antibody. Bound antibody was detected by chemiluminescence (ECL Plus kit, Amersham) with quantification by densitometry (BioRad).

Statistical Analysis
All data are expressed as mean±SEM. Comparisons of pregnant rats and post partum rats with virgin rats were analyzed by use of repeated measures ANOVA followed by Dunnett’s test for serial measurements which included arterial pressure and excretion of nitrate/nitrate. For renal hemodynamics, serum L-arginine, and renal protein NOS expression studies, comparisons of pregnant rats and post partum rats with virgin rats were analyzed by use of factorial ANOVA followed by Scheffe’s test. A value of P<0.05 was considered statistically significant.

Results
Changes in Mean Arterial Pressure During Pregnancy
The changes in arterial pressure that occurred during pregnancy are illustrated in Figure 1. MAP averaged approximately 109±0.5 mm Hg during prepregnancy and was con-
sistent until late pregnancy, when it decreased to a low of 94±6.0 mm Hg at day 21 of gestation (P<0.05). After delivery, MAP returned to prepregnant values. Mean systolic and diastolic pressures also decreased in late pregnancy from an average of 126±0.7 mm Hg for systolic and 92±0.4 mm Hg diastolic pressures during prepregnancy to lows of 114±3.0 mm Hg and 77±7.0 mm Hg for systolic and diastolic pressures, respectively, by day 21 of pregnancy (P<0.05 for both).

Changes in Renal Hemodynamics During Pregnancy
Figure 2 demonstrates the renal hemodynamic gestational increases observed during pregnancy for both GFR and RPF. Both GFR and RPF peaked at day 13 or mid pregnancy (2.71±0.14 and 7.62±0.97 mL/min, respectively) compared with virgin values (2.15±0.27 and 5.77±0.71 mL/min, respectively) representing a 21% increase in GFR and a 24% increase in RPF during pregnancy. By day 19 or late pregnancy, both GFR and RPF had returned to virgin values.

Changes in Urinary Nitrite/Nitrate Levels During Pregnancy
Urinary nitrate and nitrate excretion was measured to estimate whole-body production of NO. As shown in Figure 3, excretion of nitrite/nitrate doubled by mid gestation (day 13, 25.8±0.73 mmol/24 h, P<0.05) compared with prepregnant values (12.2±0.69 mmol/24 h). Nitrite/nitrate excretion peaked at late pregnancy (day 19, 41.1±1.34 mmol/24 h, P<0.05) and returned to prepregnant values by day 4 post partum (11.7±1.86 mmol/24 h).

Changes in Serum l-Arginine Levels During Pregnancy
Because l-arginine is the endogenous source of NO, serum l-arginine levels were also measured. During pregnancy the observed increase in nitrite/nitrate excretion (Figure 3) coincided with a decrease in serum l-arginine levels (Figure 4) with the lowest levels observed by mid pregnancy (day 13, 114.5±13.99 nmol/mL, P<0.05) compared with prepregnant levels (162.1±6.60 nmol/mL). By day 4 post partum, serum l-arginine levels had returned to prepregnant levels.

Protein Expression of the NOS Isoforms During Pregnancy
Renal levels of eNOS gradually decreased during pregnancy with the lowest level observed by late pregnancy (day 19, P<0.05) compared with virgin levels (Figures 5 and 6). Subsequently, renal eNOS protein levels returned to virgin levels after delivery of the pups (day 4 postpartum). Therefore, renal eNOS protein levels decreased by approximately 40% during pregnancy. In contrast, both iNOS and nNOS showed a gradual increase in renal protein expression during pregnancy (Figures 5 and 6). iNOS renal protein expression reached a peak by mid pregnancy (day 13, P<0.05) compared with virgin values. This same trend was observed for

![Figure 4. Serum l-arginine levels during pregnancy. All data are expressed as mean±SEM.](http://hyper.ahajournals.org/Downloaded from

![Figure 5. Representative Western blot of renal protein expression of NOS isoforms during normal pregnancy. Whole kidney protein (100 µg) was probed in separate blots with either the mouse monoclonal antibody specific for endothelial NOS (for detection of eNOS) or neuronal NOS (for detection of nNOS and iNOS) as described in Methods.](http://hyper.ahajournals.org/Downloaded from

![Figure 2. Renal hemodynamic changes during pregnancy. All data are expressed as mean±SEM.](http://hyper.ahajournals.org/Downloaded from

![Figure 3. Nitrite/nitrate excretion rates during pregnancy. All data in the experiment are expressed as mean±SEM.](http://hyper.ahajournals.org/Downloaded from

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plays an important role in controlling renal hemodynamics, isoforms in rats during pregnancy. Because endothelial NOS in the kidney we examined the protein expression of the NOS renal NO system. As a means of investigating the NO system pregnancy in the rat are associated with alterations in the whether the renal hemodynamic changes during normal production in the kidney is increased. However, it is unknown if NO changes during pregnancy occur concurrently with increases strongly suggest that the systemic and renal hemodynamic with previous studies.4,11,16 Taken together, these data mediate the renal adaptations during pregnancy. For exam- in the present study we found that MAP decreased during late pregnancy and that GFR and RPF increased to a maximum by mid pregnancy with a decrease toward virgin levels postpartum. Whereas nNOS mid to late pregnancy and returned to virgin values after delivery of the pups (day 4 postpartum). Whereas nNOS protein expression did not decrease significantly during late pregnancy and remained elevated at day 4 postpartum.

Discussion
In the present study we found that MAP decreased during late pregnancy and that GFR and RPF increased to a maximum by mid pregnancy with a decrease toward virgin levels by late gestation. Nitrite/nitrate excretion gradually increased to a maximum in late pregnancy, and serum L-arginine levels decreased to a low at mid pregnancy, with both returning to virgin levels postpartum. The data for systemic and renal hemodynamics and excretion of nitrite/nitrate are consistent with previous studies.4,11,16 Taken together, these data strongly suggest that the systemic and renal hemodynamic changes during pregnancy occur concurrently with increases in whole-body NO synthesis. However, it is unknown if NO production in the kidney is increased.

The main purpose of this investigation was to determine whether the renal hemodynamic changes during normal pregnancy in the rat are associated with alterations in the renal NO system. As a means of investigating the NO system in the kidney we examined the protein expression of the NOS isoforms in rats during pregnancy. Because endothelial NOS plays an important role in controlling renal hemodynamics, we first measured renal eNOS protein expression levels. We observed a gradual decrease of 40% in renal protein expression of eNOS during pregnancy with a subsequent return to virgin levels after delivery of the pups. This observed decrease in renal eNOS protein expression was unexpected because eNOS is localized to regions of the renal vasculature and glomerulus that are important for dilation of renal blood vessels.21

The observed decrease in renal protein expression of eNOS during normal pregnancy could be caused by negative feedback regulation of eNOS by NO. NO has been shown to decrease eNOS activity.27,28 eNOS protein and mRNA levels have been reported to be decreased by LPS in cultured endothelial cells suggesting induction of iNOS and indirect negative feedback of eNOS.29 Therefore, NO produced by the other NOS isoforms, iNOS and nNOS, could have an inhibitory effect on eNOS caused by negative feedback regulation. Additionally, decreases in renal eNOS protein expression could also reflect decreases in serum L-arginine in favor of utilization by the other NOS isoforms. However, a decrease in eNOS protein expression does not necessarily represent a decrease in NO production by that NOS isoform. An increase in eNOS enzyme activity could result in no net change in NO production by the eNOS isoform in the kidney. Therefore, the eNOS isoform could still be an important source of NO in the kidney during pregnancy.

We next examined the renal protein expression of the iNOS and nNOS isoforms. Renal protein expression of both iNOS and nNOS increased by 30% and 25%, respectively, by day 13 of pregnancy. These increases corresponded to the peak increases noted for GFR and RPF measurements during pregnancy.

Because these measurements represent whole-kidney NOS protein expression, they are not indicative for specific NOS isoform expression in distinct regions of the kidney. These isoforms have been localized in different regions of the kidney20 –25 and may differ in their pattern of induction and inhibition.15,16,26,27 Therefore, NOS isoforms differ in their localization in the kidney, differential local expression of the 3 different isoforms may occur during pregnancy, and NO synthesis in different regions of the kidney may vary for maintenance of renal function. Further studies will be necessary to determine whether the isoforms differ in their expression in different regions of the kidney during pregnancy.

Several lines of evidence support the concept that NO mediates the renal adaptations during pregnancy. For example, increases in GFR and RPF during pregnancy are attenuated by the nonselective NOS inhibitor, L-NAME.9,10 Because iNOS and nNOS are upregulated during normal pregnancy, they may be important in mediating the renal hemodynamic changes during normal pregnancy. However, the quantitative importance of these specific isoforms in mediating the changes in renal function during pregnancy has yet to be fully elucidated. Further studies with specific NOS inhibitors will be necessary to determine the relative importance of each isoform in the regulation of renal hemodynamics during normal pregnancy.

In summary, we found that pregnancy was associated with a decline in MAP in late pregnancy, increases in GFR and
RPF in mid pregnancy, and an increase in nitrite/nitrate excretion in late pregnancy. Pregnancy was associated with a decrease in serum l-arginine. Changes in renal hemodynamics and whole-body NO production were associated with significant changes in expression of the different NOS isoforms. Renal eNOS decreased but iNOS and nNOS expression increased. Although differences in NOS enzyme activity have not been measured, these data suggest that the increased renal NO production and changes in renal hemodynamics associated with normal pregnancy in rats may be caused by the upregulation of iNOS and nNOS in the kidney.

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