Plasma and 24-h NO\textsubscript{x} and cGMP during normal pregnancy and preeclampsia in women on a reduced NO\textsubscript{x} diet

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\textsuperscript{1}Departments of Obstetrics, Gynecology and Reproductive Sciences, and of Cell Biology and Physiology, University of Pittsburgh, and Mage-Womens Research Institute, Pittsburgh, Pennsylvania 15213; and \textsuperscript{2}Departments of Physiology and Obstetrics and Gynecology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

Conrad, Kirk P., Laurie J. Kerchner, and Monique D. Mosher. Plasma and 24-h NO\textsubscript{x} and cGMP during normal pregnancy and preeclampsia in women on a reduced NO\textsubscript{x} diet. Am. J. Physiol. 277 (Renal Physiol. 46): F48–F57, 1999.—We tested the hypothesis that nitric oxide (NO) biosynthesis increases during normal human pregnancy and decreases in preeclampsia. The major metabolites of NO, nitrate and nitrite (NO\textsubscript{x}), were measured in both the plasma and 24-h urine of women subjected to a reduced NO\textsubscript{x} diet. In this way, the plasma and urinary levels mainly reflected endogenous production rather than dietary intake. Moreover, we assessed cGMP, a second messenger of NO, in the same samples. Both NO\textsubscript{x} and cGMP assays were validated in our laboratory. We first conducted a cross-sectional study of nonpregnant women (n = 15), normal pregnant women in the first (n = 9), second (n = 17) and third (n = 22) trimesters, as well as women with preeclampsia (n = 15) and transient hypertension of pregnancy (n = 7). We also performed a serial study in the same women (n = 9) before, during, and after pregnancy. Taken together, the results of the two investigations suggested marked increases in cGMP production especially during the first trimester when the maternal circulation is rapidly vasodilating. In contrast, whole body NO production as estimated by the plasma level and urinary excretion of NO\textsubscript{x} was not elevated during the first trimester. Finally, unequivocal demonstration of reduced NO biosynthesis in preeclampsia was not forthcoming.

nitric oxide; guanosine 3',5'-cyclic monophosphate; nitrate; human pregnancy; glomerular filtration

ONE OF THE EARLIEST maternal adaptations to pregnancy in women is profound vasodilation of nonreproductive organs such as the kidney. Similar vasodilation occurs in the circulation of gravid rats, and recent investigations demonstrated increased biosynthesis of nitric oxide (NO) and cGMP during pregnancy in this species, as well as a role for NO in the maternal vasodilatory response (reviewed in Ref. 31). Whether NO biosynthesis is increased in normal human gestation and contributes to maternal vasodilation are uncertain. Conversely, whether NO production is compromised in preeclampsia, thereby contributing to vasoconstriction, is also unresolved. Because NO is labile, measurement of the relatively stable metabolites, nitrate and nitrite (NO\textsubscript{x}), has been employed as an index of production (reviewed in Ref. 2). Using this approach, numerous investigators have examined NO production in both normal pregnancy and preeclampsia, but with mixed results. Three groups demonstrated an increase in either the plasma or urinary nitrate (or nitrite) level (23, 24, 26), and three reported no change (7, 13, 32), during normal pregnancy compared with the nonpregnant state. Similarly, three groups of investigators reported an increase in either the plasma or urinary nitrate (or nitrite) level (8, 26, 32), four reported a decrease (4, 14, 23, 29), and three showed no change (7, 13, 30), during preeclampsia compared with normal gestation. Thus the status of NO biosynthesis in women during normal pregnancy and preeclampsia remains unresolved.

In part, the controversy may stem from methodological shortcomings. First, the dietary intake of nitrate can dramatically affect the plasma level and urinary excretion of NO\textsubscript{x}, and more reliable estimates of nitrate, and hence NO biosynthesis, may be obtained when dietary intake is reduced and controlled. Apparently, in the aforementioned studies of human pregnancy and preeclampsia such dietary measures were not implemented. Second, many of the studies relied on the urinary excretion of NO\textsubscript{x} in “spot” collections, which may reflect acute fluctuations in the renal tubular reabsorption of NO\textsubscript{x} as much as the production rate (34). The 24-h urine collection is preferable, because it better represents the steady state. Third, many of the studies relied on the measurement of NO\textsubscript{x} in the plasma. Clearly, the plasma level will be influenced by the clearance, as well as the production of these NO metabolites. (All of these caveats and pitfalls have been recently reviewed in Ref. 2.)

To circumvent these methodological shortcomings, we measured the plasma level and 24-h urinary excretion of NO\textsubscript{x} during normal pregnancy and preeclampsia in women on a diet containing reduced NO\textsubscript{x}. We also measured the second messenger of NO, cGMP, in the same biological specimens. In addition, we undertook the assessment of NO biosynthesis taking an entirely different approach; namely, the analysis of the NO-hemoglobin adduct by electron paramagnetic resonance (EPR) spectroscopy (12).

METHODS

Human subjects. In a cross-sectional study design, blood samples and 24-h urinary collections were obtained from nonpregnant women (n = 15), from women at various stages of normal pregnancy (1st (n = 9), 2nd (n = 17), and 3rd (n = 22) trimesters), and from women with preeclampsia (n = 15) and transient hypertension of pregnancy (n = 7). During late...
pregnancy, all samples were obtained before the onset of labor. All subjects gave informed consent for blood and urine collection, as well as the dietary measures (described below), which were approved by the University of New Mexico Investigational Review Board.

The diagnosis of preeclampsia was made by strict criteria: onset of hypertension during late gestation with systolic and diastolic blood pressure greater than 140/90 mmHg on at least two occasions and urinary protein excretion greater than 0.3 g/24 h (25). Furthermore, these subjects were normotensive during the first trimester and had no history of chronic hypertension. Normal pregnant women in the third trimester were recruited as control subjects. For an additional control group, blood and urine samples were also obtained from women in late pregnancy with transient hypertension (hypertension without proteinuria), which is believed to be a disease different from preeclampsia (25).

A longitudinal study design was also conducted in normal pregnancy, so that each woman would serve as her own control. Moreover, the dietary measures were more precisely controlled in this protocol (see below). Blood samples and 24-h urinary collections were obtained from women before conception in the follicular phase of the menstrual cycle, then once during each trimester, as well as postpartum. (We attempted to obtain postpartum samples several weeks after cessation of breast-feeding, but with only partial success, because some women breast-fed for as long as 18 mo. Therefore, for some of these subjects, we actually obtained postpartum samples during but at least toward the end of lactation, when breast-feeding was more infrequent.) We recruited 13 women, and 9 completed the study. All subjects gave informed consent which was approved by the Investigational Review Board of the Magee-Womens Hospital.

Women who smoked were excluded from both the cross-sectional and longitudinal studies, as were women with any underlying chronic illnesses such as hypertension, renal disease, or diabetes mellitus. If signs and symptoms of acute infection were evident such as an upper respiratory tract or gastrointestinal infection, then whenever possible the study was delayed until after resolution of the illness, because infection can markedly stimulate NO production (21). Indeed, one woman in the longitudinal design protocol during the

### Table 1. Clinical characteristics of subjects in cross-sectional study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nonpregnant</th>
<th>Normal Pregnancy/Trimester</th>
<th>Preeclampsia</th>
<th>Transient Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>9</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>Maternal age, yr</td>
<td>31 ± 2</td>
<td>29 ± 1</td>
<td>28 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>Maternal wt, kg</td>
<td>65 ± 3</td>
<td>69 ± 3</td>
<td>71 ± 3</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>10 ± 1c</td>
<td>18 ± 1c</td>
<td>30 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>Birth wt, g</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>113 ± 4</td>
<td>112 ± 4</td>
<td>104 ± 3</td>
<td>109 ± 2</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>75 ± 2</td>
<td>65 ± 3</td>
<td>59 ± 3</td>
<td>60 ± 3</td>
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<tr>
<td>Uprot/V, mg/24 h</td>
<td>105 ± 11</td>
<td>105 ± 12</td>
<td>128 ± 10</td>
<td>166 ± 12</td>
</tr>
<tr>
<td>Plasma uric acid, mg/dl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of subjects. SBP, systolic blood pressure; DBP, diastolic blood pressure; Uprot/V, urinary protein excretion; NA, not available. *P < 0.05 vs. nonpregnant. **P < 0.05 vs. 1st trimester. *P < 0.05 vs. all other groups. *P < 0.05 vs. all other groups except transient hypertension of pregnancy. *P < 0.05 vs. all other groups excluding preeclampsia. *P < 0.05 vs. 3rd trimester. *P < 0.05 vs. all groups; *P < 0.05 vs. nonpregnant, 1st trimester, and preeclampsia. *P < 0.05 vs. 1st trimester. *P < 0.05 vs. all groups; *P < 0.05 vs. nonpregnant, 3rd trimester and preeclampsia. *P < 0.05 vs. nonpregnant. *P < 0.05 vs. 100%.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nonpregnant</th>
<th>Normal Pregnancy/Trimester</th>
<th>Preeclampsia</th>
<th>Transient Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>9</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>V, ml/24 h</td>
<td>1,391 ± 193</td>
<td>1,642 ± 288</td>
<td>1,731 ± 161</td>
<td>1,364 ± 116</td>
</tr>
<tr>
<td>Uprot/V, mg/24 h</td>
<td>1,234 ± 70</td>
<td>1,035 ± 79</td>
<td>1,211 ± 86</td>
<td>1,104 ± 49</td>
</tr>
<tr>
<td>Pcr, μM</td>
<td>0.89 ± 0.03</td>
<td>0.69 ± 0.04c</td>
<td>0.69 ± 0.03f</td>
<td>0.71 ± 0.03f</td>
</tr>
<tr>
<td>GFR, ml/24 h</td>
<td>141 ± 8</td>
<td>155 ± 15</td>
<td>179 ± 12</td>
<td>164 ± 9</td>
</tr>
<tr>
<td>UNOX/V, μmol/24 h</td>
<td>541 ± 45d</td>
<td>401 ± 42e</td>
<td>589 ± 52</td>
<td>640 ± 29</td>
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<tr>
<td>UNOX/UCr, μmol/mg</td>
<td>0.45 ± 0.04</td>
<td>0.40 ± 0.05</td>
<td>0.51 ± 0.05</td>
<td>0.60 ± 0.03f</td>
</tr>
<tr>
<td>PNOx, μM</td>
<td>36 ± 3</td>
<td>32 ± 3</td>
<td>34 ± 2</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>CNOx, I24 h</td>
<td>113 ± 17</td>
<td>132 ± 13</td>
<td>181 ± 19</td>
<td>199 ± 12</td>
</tr>
<tr>
<td>GFR × PNOx, μmol/24 h</td>
<td>5,019 ± 395</td>
<td>4,990 ± 795</td>
<td>6,190 ± 630</td>
<td>5,530 ± 401</td>
</tr>
<tr>
<td>FENOX, %</td>
<td>11.7 ± 1.3</td>
<td>8.6 ± 0.8</td>
<td>10.0 ± 0.8</td>
<td>12.6 ± 0.9</td>
</tr>
<tr>
<td>UGH,V, mmol/24 h</td>
<td>843 ± 55b</td>
<td>1,215 ± 143</td>
<td>1,397 ± 121</td>
<td>1,311 ± 94</td>
</tr>
<tr>
<td>UGH/V, μmol/mg</td>
<td>0.73 ± 0.05b</td>
<td>1.21 ± 0.16</td>
<td>1.21 ± 0.07</td>
<td>1.18 ± 0.06</td>
</tr>
<tr>
<td>PNOx, mmol/d</td>
<td>6.3 ± 0.5</td>
<td>7.1 ± 1.1</td>
<td>8.1 ± 0.9</td>
<td>6.9 ± 0.7</td>
</tr>
<tr>
<td>CNOx, I24 h</td>
<td>144 ± 13</td>
<td>200 ± 29</td>
<td>178 ± 15</td>
<td>234 ± 30</td>
</tr>
<tr>
<td>GFR × FENOX, mmol/24 h</td>
<td>891 ± 67</td>
<td>1,068 ± 164</td>
<td>1,613 ± 191c</td>
<td>1,110 ± 127</td>
</tr>
<tr>
<td>FENOX, %</td>
<td>104 ± 9</td>
<td>132 ± 20</td>
<td>106 ± 11</td>
<td>142 ± 16e</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of subjects. V, urinary flow rate; Uprot/V, urinary excretion of substance; Pcr, plasma concentration of substance; GFR, glomerular filtration rate; Ccr, renal clearance of substance; FENOX, fractional excretion of substance; NOx, nitrate + nitrite; PNOx, plasma concentration of substance; GNOx, nitrate + nitrite.
postpartum period demonstrated a plasma level and urinary excretion rate of NOx that exceeded the average values by twofold. From the General Clinical Research Center (GCRC) nursing notes, we discovered that she experienced sudden onset of a gastrointestinal distress on the day of the 24-h urine collection and blood draw; these data were therefore excluded from the analysis. Fortunately, she agreed to another postpartum study after resolution of her gastrointestinal illness.

Reduced nitrate diet. In the cross-sectional study, the subjects were requested to forgo foodstuffs rich in nitrate for 24 h before and during the 24-h urine collection. Specifically, cured meats, fish, cheese, vegetables, melons, herb or black teas, beer, wine, and malted beverages were excluded from the diet (18). To monitor compliance, the women completed a Diet Record Book. We found that the 24-h washout period was sufficient to reach baseline levels of NOx excretion (see RESULTS).

In the longitudinal study, two days of food and beverages were prepared for the subjects by the GCRC Kitchen of the University of Pittsburgh in consultation with a Registered Dietitian. The diet contained 20–25 mg of nitrate per day. By comparison, the average daily nitrate intake in the United States is 100 mg (36). The subjects had some choice in the selection of the meals, but whatever they chose in the initial study before pregnancy was provided in all subsequent studies, so that the dietary intake of nitrate was constant at each time point. These subjects were also asked to forgo all medications including vitamin supplements during the 48-h study, particularly since vitamin C may interfere with the measurement of urinary NOx (2).

Sample collection. Peripheral venous blood was drawn into 10-ml tubes containing either EDTA or heparin. The plasma fraction was separated from blood cells by centrifugation, 2,000 g for 20 min at 4°C. After washing the red blood cells three times in Ringer solution, both the plasma and cells were frozen at −80°C. Ten milliliters of blood was also drawn into a tube containing no additives. This blood was allowed to clot at room temperature for 30 min. It was then centrifuged as above, and the serum stored at −80°C. The blood samples were obtained at the end of the 24-h urine collection.

Each subject was instructed on the proper collection of a 24-h urine. Urine collection containers and “plastic hats” (to facilitate urine collection) were provided to each subject. Because many bacteria can reduce nitrate to nitrite and nitrogen (1) and nitrite is the endpoint of our determination of nitrate (see below), we added 0.1 g of tetracycline, as well as 0.2 g each of chloramphenicol and ampicillin to each urine container, to prevent bacterial growth (Sigma Chemical, St. Louis, MO). As well, the subjects were instructed to keep the urine in the refrigerator whenever possible. These antibiotics did not interfere with the measurement of NOx (see below). None of the urine collections demonstrated detectable nitrite as determined by Chemstrip 9 (Boehringer Mannheim Diagnostics, Indianapolis, IN) indicating that we were successful in circumventing bacterial formation of nitrite.

Measurement of NOx. For the cross-sectional study, we measured NOx in heparinized plasma and urine as previously reported and extensively validated by us (12). Briefly, nitrate was reduced to nitrite using nitrate reductase (Aspergillus species; Boehringer, Mannheim, Germany), and the cofactors, FAD and NADPH. Then, after addition of the Greiss reagent, nitrite was measured by spectrophotometry at 546 nm.
Plasma proteins were first precipitated with zinc sulfate. For urine, each sample was assayed at dilutions of 40×, 60×, and 80× made with distilled water, and for plasma, each sample was assayed at dilutions of 4×, 8×, and 10×. After multiplication by the appropriate dilution factor, the three determinations were averaged. Nitrite was also measured without prior reduction of nitrate, and the plasma levels and 24-h urinary excretion were generally <1 µM and <2 µmol/24 h, respectively. When the antibiotics were added to water in concentrations comparable to those used to inhibit bacterial growth in the 24-h urine, they did not produce absorbency at 546 nm. Nor did the addition of antibiotics to nitrate standards affect either the efficiency of conversion to nitrite by nitrate reductase or the final absorbency. The interassay coefficient of variation was determined in urine from subjects assayed four to six times over a 4-mo period. The NOx values of the four samples ranged from 500 to 1,000 µM, and the coefficient of variation was between 7.2 and 15.6%. “Spike and recovery” tests were also performed on a pool of plasma from four subjects. When the 1.0-ml plasma pool was spiked with 15, 30, and 60 nmol of nitrate and each measured on four occasions over a 3-mo period, the mean recoveries were 85 ± 5%, 74 ± 2%, and 78 ± 7%, respectively.

For the longitudinal study, we measured NOx in EDTA plasma and urine using a nitrate/nitrite colorimetric kit according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI). The principle of measurement was the same as described above, insofar as nitrate was first converted to nitrite by nitrate reductase, and the nitrite was then determined by the Greiss reaction, which produced a colorimetric product at 550 nm. Plasma proteins were initially removed by using Amicon filters and centrifugation. Measurements were performed on plasma and urine each in duplicate that were diluted 2× and 20×, respectively, prior to assay. For validation of the Cayman nitrate/nitrite assay, plasma and urine pools were each analyzed five times within the same assay yielding intra-assay coefficients of variation of 3.5 and 4.4%, respectively. Plasma and urine pools were also analyzed on four separate occasions over several months, and the inter-assay coefficients of variation were 8.5 and 8.3%, respectively. These intra- and interassay coefficients of variation were determined for levels of NOx that fell in the low to middle (working) portion of the standard curve. Dilutional parallelism was assessed in pooled plasma and urine. Nitrate was added to the plasma pool, which was then diluted 2×, 4×, 8×, 16×, and 32× for assay. Nitrate was also added to the urine pool, which was diluted 10×, 20×, 40×, and 80× for assay. After multiplying by the dilution factors, the coefficients of variation were 11.2 and 6.5%, respectively, for these plasma and urine pools. Finally, when plasma and urine pools were spiked with nitrate each at two different levels, the recoveries ranged from 89 to 98%.

Measurement of cGMP. cGMP in EDTA plasma and urine was measured by specific radioimmunoassay as previously described by us (11, 12).

NO-hemoglobin. The washed red blood cells from the heparinized blood specimens were frozen at −80°C. Any NO-hemoglobin in the cellular fraction is stable indefinitely.

Fig. 2. Plasma levels (A) and urinary excretion (B and C) of nitrate and nitrite (NOx) in nonpregnant women, in women during normal pregnancy, as well as in patients with preeclampsia and transient hypertension of pregnancy. Boxes, horizontal lines (medians), solid squares (means), and percentile lines and flags are described in legend to Fig. 1. For the sake of clarity, not all statistically significant comparisons are indicated (see Table 2 for additional statistical comparisons).
at such temperatures, and the samples can also be shipped long distances on dry ice without deterioration. NO-hemoglobin was measured by EPR spectroscopy as previously reported (12).

Statistical analysis. In Tables 1–4, data are presented as means ± SE. In Figs. 1–4, the median, 10, 25, 75, and 90 percentiles, as well as the mean are depicted. All of the clinical characteristics were analyzed by one-factor randomized block design ANOVA, and if overall significance was observed, then individual group means were contrasted by the Fisher protected least significant difference test. For the laboratory data in the cross-sectional study, the Kruskal-Wallis test was applied, and if overall significance was reached, then individual groups were compared by the Mann-Whitney test. In the longitudinal study, data not normally distributed were first log transformed. Then, an unbalanced repeated measures ANOVA was performed to accommodate missing data (only 2 of 45 total cells). If a significant effect of time was obtained, then group means were contrasted by post hoc tests. \( P < 0.05 \) was considered to be significant (37).

RESULTS

Cross-sectional study. Table 1 summarizes the clinical characteristics of the subjects in the cross-sectional study. The women with preeclampsia and transient hypertension of pregnancy were significantly younger than the other subject groups. At the time of study, there was no significant difference in maternal weight or gestational age among the three groups of women in the third trimester. As expected, women with preeclampsia demonstrated significant hypertension, proteinuria, and hyperuricemia, and 73% were nulliparous. Many of the women with preeclampsia were treated with a variety of antihypertensive agents, \( \beta \)-methylamines, and anticonvulsants, and four of these women were receiving magnesium sulfate at the time of study, and two of the women with transient hypertension were also receiving the drug.

Table 2 depicts the laboratory data for the subjects in the cross-sectional study. On the basis of the urinary excretion of creatinine, several of the 24-h collections were inadequate, and these subjects were excluded from the analysis. Plasma creatinine was significantly reduced during normal pregnancy, reflecting the gestational increase in glomerular filtration rate (GFR), the calculated value of which tended to be elevated in this cross-sectional study, but did not reach statistical significance. Moreover, the urinary flow rate was significantly higher in the women with preeclampsia most likely due to bedrest, treatment, and the mobilization of peripheral edema.

The urinary excretion of cGMP was markedly increased during normal and pathological pregnancies, whereas plasma values were not significantly altered (Table 2; Fig. 1). The fractional excretion of cGMP exceeded 100% in the normal third trimester women and women with preeclampsia (\( P < 0.05 \) by Mann-Whitney test). When expressed as micromoles per 24 h, the urinary excretion of NOx was significantly reduced during the first trimester compared with the nonpregnant level. When expressed either as micromoles per 24 h or micromoles per milligram creatinine, the urinary excretion of NOx was significantly elevated during the third trimester compared with the nonpregnant level. It was also significantly reduced in preeclampsia compared with the normal third trimester, when expressed as micromoles per milligram creatinine (Table 2; Fig. 2). Linear regression analyses failed to demonstrate a significant relationship between the urinary excretion of cGMP and NOx within any of the subject groups. When all subject groups were combined, a weak, albeit significant, relationship was observed (\( r = 0.24, P < 0.05 \)).

Longitudinal study. The women were first studied before pregnancy during the follicular phase of the menstrual cycle, then on 8.7 ± 0.8 wk of gestation, and finally in the postpartum period. In five of the women, the postpartum study was conducted after breast-feeding; in three subjects during, but near the end of breast-feeding; and in one subject, both during and after breast-feeding. Before the index pregnancy, three of nine women were nulliparous.
The laboratory data for the longitudinal study are portrayed in Table 3. The GFR was significantly elevated and the plasma creatinine reciprocally reduced during pregnancy. The urinary excretion of cGMP markedly increased during pregnancy beginning in the first trimester, and plasma levels were significantly elevated by the second trimester (Fig. 3). These changes were accompanied by increased filtered load, fractional excretion, and renal clearance of cGMP. In contrast to cGMP, the urinary excretion of NOx tended to decline during the first trimester, whereas plasma NOx was significantly reduced and the renal clearance increased at this stage of pregnancy (Table 3; Fig. 4). Interestingly, there was a significant correlation between the urinary excretion of NOx and cGMP, but only during the three trimesters of pregnancy ($r = 0.70$, $P < 0.05$). When all time points were combined, a significant relationship was also observed ($r = 0.46$, $P < 0.01$).

Although the numbers are small, the $P_{NOx}$, $U_{NOx}$, and $U_{NOx}/UCr$ during ($n = 4$) and after ($n = 6$) lactation were $17.2 \pm 1.9$ vs. $12.1 \pm 2.1 \mu M$, $824 \pm 73$ vs. $547 \pm 76 \mu mol/24$ h, and $0.92 \pm 0.35$ vs. $0.52 \pm 0.07 \mu mol/mg$, respectively. For $P_{cGMP}$, $U_{cGMP}$, and $U_{cGMP}/UCr$ during and after lactation, the values were $4.68 \pm 0.18$ vs. $3.91 \pm 0.10 \text{nM}$, $927 \pm 205$ vs. $693 \pm 71 \text{nmol/24h}$, and $0.67 \pm 0.14$ vs. $0.71 \pm 0.07 \text{nmol/mg}$, respectively.

NO-hemoglobin adduct. The frozen red blood cells of five or six subjects from each group in the cross-sectional study were analyzed by EPR spectroscopy. Despite scanning each sample 36 times to boost sensitivity, the 3-line hyperfine absorption spectrum indicative of NO-hemoglobin was not detected in any of the samples (data not shown).

Dietary intake and urinary excretion of NOx. Three nonpregnant women initially consumed a diet rich in nitrate for 3 days, immediately followed by a medium- and then low-nitrate diet each for 3 days (Table 4). The 24-h urinary excretion of NOx was measured on the third day of the high-nitrate diet and on both days 2 and 3 of the medium- and low-nitrate diets. The urinary excretion of NOx varied over a fourfold range depending on the diet. A 24-h washout period proved to be sufficient, as the NOx excretion rates were comparable on days 2 and 3 of either the medium- or low-nitrate diet protocols.

**DISCUSSION**

A major and surprising finding of this work was the divergence of both plasma concentration and urinary excretion of cGMP and NOx during early pregnancy in women at a time when the maternal circulation is undergoing rapid vasodilation. In contrast to rat pregnancy, where cGMP and NOx in both plasma and urine rose in parallel (12), we found that cGMP but not NOx increased during early gestation in women. In fact, the urinary excretion and plasma level of NOx fell significantly during the first trimester in the cross-sectional and longitudinal studies, respectively. Urinary excretion of NOx subsequently rose throughout the remain-
order of pregnancy in the cross-sectional study to significantly exceed nonpregnant values in the third trimester, albeit only by a small degree.

Previous reports on plasma level or urinary excretion of NOx during normal human pregnancy are controversial, with some showing an increase (23, 24, 26) and others no change (7, 13, 32). The present results may be particularly reliable, because both plasma concentration and 24-h urinary excretion of NOx were determined concurrently in women subjected to a reduced nitrate diet. Indeed, implementation of dietary measures is critical; in the present work, we demonstrated that the urinary excretion of NOx could vary over a fourfold range depending on the dietary intake of NOx (Table 4). Moreover, both cross-sectional and longitudinal studies were conducted involving two different populations of women (see METHODS for details). As noted above, a small, but significant increase in urinary excretion of NOx may have occurred during the third trimester, but there was no increase in either plasma or urinary NOx during early pregnancy when both the increase in cGMP and active maternal vasodilation are marked.

Our hypothesis was that, analogous to the gravid rat (12), cGMP and NOx in both plasma and urine would rise in parallel, suggesting increased NO biosynthesis during human pregnancy. On balance, however, the plasma and urinary NOx levels suggest unchanged or reduced NO biosynthesis at least during early pregnancy. Consistent with this conclusion was the absence of detectable NO-hemoglobin in the red blood cells of pregnant women, which contrasts with our observations in gravid rats where we detected the adduct in at least one-half of the late pregnant animals (12).

Several explanations for the apparent divergence of cGMP and NOx during early human gestation are possible. First, unlike the rat in which NO mediates vasodilation of the renal and perhaps other vascular beds during pregnancy (reviewed in Ref. 31), another primary vasodilator such as carbon monoxide (15) or C-type natriuretic factor (16) may be primarily responsible both for the increase in cGMP as well as the maternal vasodilation in women. Second, NO biosynthesis may increase in the vasculature during human pregnancy, albeit only by a small amount. Hemodynamically speaking, however, this small increase may be potent. In support of this possibility, the typical dosage of sublingual nitroglycerin used clinically, which has profound hemodynamic consequences, would not be easily detected as nitrate in the urine or plasma against the backdrop of ~500 and 25 µM, respectively, even if one assumes that the NO released from this agent was all metabolized to nitrate. Furthermore, the NO-hemoglobin adduct was detected by EPR spectroscopy in only 10 of 24 patients administered intravenous nitroglycerin for several hours (9). Thus it is possible that plasma level and 24-h urinary NOx are largely unrelated to the NO produced that is hemodynamically relevant. Recent preliminary work by Baylis and colleagues (33) supports this concept for the gravid rat.
A third potential explanation is that the half-life of NO is prolonged in normal pregnancy, which could amplify its hemodynamic action and the production of cGMP without necessarily affecting steady-state formation of nitrate. Similar to this line of reasoning, a fourth possibility is that the sensitivity of the vasculature to NO is exaggerated in pregnant women. However, the scant amount of data available addressing this issue indicate that the sensitivity of the vasculature to relaxation by NO donors is similar in the nonpregnant and pregnant condition (reviewed in Ref. 31).

Whether plasma and urinary NO\textsubscript{x} levels are altered in preeclampsia relative to normal pregnancy is also controversial; some investigators reported increases (8, 26, 32) or decreases (4, 14, 23, 29), whereas others observed no change (7, 13, 30). Our hypothesis was that the renal handling of NO\textsubscript{x} in humans (35). In the present study, the renal clearance in the nonpregnant condition was on average 16 and 39 l/24 h in the cross-sectional and longitudinal studies, respectively. The fractional excretion was 12 and 26%, respectively. Thus nitrate undergoes considerable tubular reabsorption. The variation between the two studies was not due to significant differences in the urine excretion, but rather to differences in the plasma levels (cf. Tables 2 and 3, and Figs. 2 and 4).

To our knowledge, there is little information on the renal handling of NO\textsubscript{x} in humans (35). In the present work, the renal clearance in the nonpregnant condition was ~16 and 39 l/24 h in the cross-sectional and longitudinal studies, respectively. The fractional excretion was 12 and 26%, respectively. Thus nitrate undergoes considerable tubular reabsorption. The variation between the two studies was not due to significant differences in the urine excretion, but rather to differences in the plasma levels (cf. Tables 2 and 3, and Figs. 2 and 4).

### Table 4. Dietary intake and urinary excretion of NO\textsubscript{x}

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Relative Dietary Intake of Nitrate</th>
<th>Urinary Excretion of NO\textsubscript{x}, (\mu\text{mol}/24\text{ h})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High/day 3</td>
<td>Medium/day 2</td>
</tr>
<tr>
<td>1</td>
<td>3,235</td>
<td>755</td>
</tr>
<tr>
<td>2</td>
<td>785</td>
<td>346</td>
</tr>
<tr>
<td>3</td>
<td>1,913</td>
<td>1,328</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1,976 ± 709</td>
<td>810 ± 285</td>
</tr>
</tbody>
</table>

Subjects consumed foodstuffs rich in nitrate for the “high” nitrate diet (e.g., leafy vegetables, cured meats and cheese). These foodstuffs were reduced or completely eliminated for the “medium” and “low” nitrate diets, respectively. See Methods for details.
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