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Hypertension. 1998;32:730-734
doi: 10.1161/01.HYP.32.4.730
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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

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Abstract—There is indirect evidence for a gender difference in nitric oxide (NO) synthesis from vascular endothelium. The aim of the present study was to determine NO production more directly in healthy women and men by the measurement of ^{15}N nitrate excreted in urine after the intravenous administration of L-$[^{15}N]_2$-guanidino arginine. Twenty-four healthy volunteers (13 men aged 22 to 40 years and 11 women aged 23 to 42 years) participated in this study. No subjects were receiving any medication. Women were studied between the 7th and 14th days of their menstrual cycles. Arterial blood pressure was measured oscillometrically, and 1.13 μmol L-$[^{15}N]_2$ arginine was administered intravenously after an overnight fast. Urine was collected for the next 36 hours in separate 12-hour periods. Urinary $^{15}N/^{14}N$ nitrate ratio was assessed by dry combustion in an isotope ratio mass spectrometer. Mean 36-hour urinary ^{15}N nitrate excretion was greater in women than in men (2111±139 versus 1682±87 ηmol; $P<0.05$). Furthermore, total urinary ^{15}N nitrate excretion was associated inversely with the mean arterial blood pressure in the whole group of subjects (coefficient of correlation, 0.47; $P=0.022$). The present data show that whole-body production of NO is greater in healthy premenopausal women than in men under ambulatory conditions. The cellular origin of NO measured in this study is unknown, but differences in endothelial production could underlie differences in vascular function between men and women. **(*Hypertension*. 1998;32:730-734.)**

Key Words: endothelium-derived relaxing factor ■ arginine ■ nitrates ■ gender ■ adults

Premenopausal women have less atheromatous arterial disease, including stroke or coronary artery disease, than men of similar age.[1] Synthesis of nitric oxide (NO) by the endothelium regulates vascular tone in the arterial bed and modulates interactions between the endothelium and circulating blood cells, including platelets and leukocytes.[2] Previous studies have suggested that a gender difference in the production of NO due to ovarian hormones (ie, estrogens) could contribute to this low risk of cardiovascular events in women of reproductive age. However, the role of NO is controversial because increased[3,4] or diminished[5,6] production in women compared with men has been reported. It is possible that the indirect nature and relative specificity of the methods used for the measurement of NO in those studies might account for these discrepancies. Measurement of urinary or serum nitrate is highly affected by diet.[7] Cyclic GMP is also the second messenger of atrial natriuretic peptide,[8] and exhaled NO reflects local biosynthesis in the lung and/or upper airways rather than in the whole body. We have developed a sensitive and specific method to measure more directly the conversion of L-arginine to NO.[9] The method is based on the measurement of ^{15}N nitrate (stable oxidation product of NO) excretion in urine after intravenous single administration of the stable isotope L-$[^{15}N]_2$-guanidino arginine. Using this methodology, we recently reported that the basal production of NO, was significantly higher in women than in men with essential hypertension. However, this difference was not statistically significant in the normotensive group, perhaps because of inadequate power.[9] Therefore, the aim of this study was to use this method to compare the activity of the L-arginine–NO system in a larger population of healthy women and men.

Methods

Study Protocol

The protocol was approved by the local ethics committee, and all subjects gave their written informed consent. The study sample consisted of 13 men (aged 22 to 40 years) and 11 premenopausal women (aged 23 to 42 years) recruited from our staff. All participants were healthy, normotensive, normocholesterolemic, nondiabetic, receiving no medication, and nonsmoking. The women reported regular menstrual cycles (26 to 32 days) for >6 months before the study, and none of them was taking oral contraceptives. Women were studied between the 7th and 14th days of their menstrual cycles. The subjects' characteristics are summarized in Table 1.

Received April 27, 1998; first decision May 12, 1998; revision accepted June 18, 1998.

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Presented as an oral presentation at the 5th International Meeting on Biology of Nitric Oxide, Kyoto, Japan, September 15–19, 1997; published in abstract form (*Jpn J Pharmacol.* 1997;75:16P).

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TABLE 1. Subjects' Baseline Characteristics

Characteristic	Men	Women
n	13	11
Age, y	28.6±1.8	27.5±1.6
Body mass index, kg/m^2	23.9±1.1	23.1±0.82
Systolic blood pressure, mm Hg	125±1.53	117±4.45
Diastolic blood pressure, mm Hg	68.2±1.35	68.1±1.62
MAP, mm Hg	87.1±1.17	84.8±2.15
Total cholesterol, mmol/L	4.6±0.26	4.5±0.32
LDL cholesterol, mmol/L*	2.8±0.26	2.3±0.27
HDL cholesterol, mmol/L	1.3±0.09	1.6±0.11
Glucose, mmol/L	4.2±0.15	4.3±0.10
Triglyceride, mmol/L	1.2±0.12	1.2±0.27
Creatinine clearance, mL/min	104±8.2	101±6.7
Estradiol, pmol/L	. . .	272.1±37

Values are mean±SE.
*Calculated using Friedewald equation.

Materials

L-[^{15}N]$_2$-guanidino arginine (99 mol% ^{15}N) and sodium ^{15}N nitrate (99.3 mol% ^{15}N) were obtained from Tracer Technologies Inc and C/D/N Isotopes, respectively. IMAC HP555 was purchased from Merck Laboratories. Sulfanilamide, glycine, sodium chloride, phosphoric acid, Devarda's alloy, sodium hydroxide, and *N*-(1-naphthyl)ethylenediamine were of analytical reagent grade and were obtained from Sigma Chemical Co. Milli-Q+ water (mili pore; >18 mol/L S purity) was used for the preparation of aqueous solutions. Urine samples were stored at −80°C until analyzed. Nitrogen isotope ratio enrichments were analyzed using a continuous-flow gas isotope ratio mass spectrometry (20-20, Europa Scientific).

Tracer Infusion Study

Subjects received a limited nitrate diet (the diet excluded food items that contain a high concentration of nitrate, ie, cured meat, fruit, and particularly green leafy vegetables[7]) for 24 hours before and for 36 hours after the administration of L-[^{15}N]$_2$-arginine. The studies were conducted at 9 AM in a quiet, air-conditioned room maintained at a constant temperature (22°C to 24°C), with subjects in a recumbent position and after an overnight fast. Blood pressure was then measured 5 times using a Dinamap (Critikon) automatic recorder, with 3-minute intervals of rest between measurements. The values used in the study were the averages of the last 3 readings. Thereafter, an 18-gauge catheter was inserted into a left antecubital vein and 1.13 μmol sterile pyrogen-free L-[^{15}N]$_2$-arginine dissolved in 20 mL of 0.153 mol/L sodium chloride was administered over 10 minutes by means of a constant-rate infusion pump (Braun Perfusor ED 2). Baseline urine samples (before administration of the isotope) were collected to determine the natural enrichment of ^{15}N nitrate. Complete urine collections were made in prewashed (distilled water) 2-L polypropylene bottles containing 5 mL of 5 mol/L sodium hydroxide to prevent reduction of nitrate for the periods 0 to 12, 12 to 24, and 24 to 36 hours after dosing. The subjects did not exercise during the study period, but usual ambulatory activity was permitted. The urine volume was measured, and samples from each collection were frozen at −80°C until analysis.

Analytical Methods

Measurement of Total Nitrate

Total urinary nitrate was measured as previously described.[10] Briefly, nitrate was reduced to nitrite with a copper/cadmium reduction column and subsequent Griess reaction, modified by replacing carrier fluid with 0.2 mol/L glycine, pH 9.4. The detection limit of this method is 1 μmol/L, and the interday coefficient of variation over the measured concentration range (20 to 1000 μmol/L) was <3%.

Measurement of ^{15}N Nitrate

To determine ^{15}N enrichment of nitrate in urine, a modification of that procedure described by Brooks and colleagues[11] was followed.[9] Briefly, urinary nitrate was extracted using a selective ion exchange resin (IMAC HP555) and converted to ammonia using Devarda's alloy with subsequent conversion to nitrogen gas by combustion at 1000°C and analysis by continuous-flow gas isotope ratio mass spectrometry. The precision of the ratio ^{15}N/^{14}N measurement of this mass spectrometer is ±0.0004%. The linearity of the measurements was demonstrated across the range of the expected enrichments (0.368 to 1 atom %) with a correlation coefficient of 0.999 by linear regression analysis. The interday coefficient of variation ranged from 0.40% to 0.75%.

Calculations and Statistical Analysis

Urinary nitrate excretion was calculated from the volume of urine excreted and duplicate measurement of nitrate concentration. The ^{15}N isotope enrichment of nitrate was calculated according to Hauck and colleagues[12]: atom % ^{15}N=100/(2R+1), where R is the ratio of ions with m/z 28 and 29. Urinary excretion of ^{15}N nitrate was determined by measuring the urinary nitrate excretion multiplied by the measured atom percent excess of urinary ^{15}N nitrate. A 1-compartment pharmacokinetic model was used to analyze the urine data obtained in this study. The ^{15}N nitrate elimination rate was determined by a single-pool kinetic equation.[13] Extracellular body water was estimated using the following formulas: 0.135×W+7.35 (males) and 0.135×W+5.27 (females), where W is body weight (kilograms).[14] All values are summarized as mean±SE. Stepwise regression analysis was performed to determine the relationship between the 36-hour urinary ^{15}N nitrate excretion and gender, body mass index, mean arterial blood pressure (MAP), total cholesterol, HDL and LDL cholesterol, serum glucose, triglyceride, and 17β-estradiol levels. Differences were sought using repeated-measures ANOVA of urinary ^{15}N nitrate excretion for each 12-hour period. A value of $P<0.05$ was considered significant.

Results

Thirty-six-hour total urinary nitrate excretion was similar in the female and male groups (2481±285 versus 2537±288 μmol, respectively; P=NS). However, the mean 36-hour urinary ^{15}N nitrate excretion was greater in women than in men (2111±139 versus 1682±87 ηmol; $P<0.05$). These values represent 0.172±0.012% and 0.140±0.008% of ^{15}N nitrogen administered ($P<0.05$), respectively, assuming that 1 labeled guanidino nitrogen per arginine molecule is converted to nitrate. The difference in urinary ^{15}N nitrate excretion was statistically significant during the first 12 hours but not for the second and third 12-hour periods. The urinary excretion of ^{15}N nitrate in each 12-hour period after the administration of L-[^{15}N]$_2$-arginine is shown in Table 2. The cumulative recovery of ^{15}N nitrate in urine is shown graphically in the Figure. LDL cholesterol concentration was lower in women compared with men, albeit not statistically significant. In addition, stepwise regression analysis showed an inverse correlation between MAP and 36-hour urinary ^{15}N nitrate excretion (coefficient of correlation, 0.47; P=0.022) in all subjects studied. Multiple regression analysis showed that the regression coefficient associated with gender was still significant even when blood pressure and LDL cholesterol concentration analyzed separately were taken into account (r=0.61, $P<0.001$ and r=0.81, $P<0.001$, respectively). In addition, stepwise multiple regression analysis showed no

TABLE 2. Urinary Excretion of Total Nitrate and ^{15}N Nitrate at Each 12-Hour Period After Intravenous Dose of L-[$^{15}N]_2$-Arginine, Tabulated by Gender

Time, h	NO_3, μmol	Atom %	Atom % Excess	^{15}N NO_3, ηmol	^{15}N Recovered, %
Women					
0–12	862±114	0.545±0.022	0.182±0.024	1407±102*	0.116±0.008*
12–24	824±103	0.431±0.008	0.065±0.008	479±36	0.039±0.003
24–36	795±127	0.401±0.003	0.032±0.003	225±21	0.019±0.002
Total (0–36)	2481±285			2111±139†	0.172±0.012†
Men					
0–12	986±150	0.478±0.027	0.105±0.027	1059±53	0.087±0.004
12–24	732±94	0.436±0.010	0.062±0.010	434±46	0.036±0.004
24–36	815±125	0.399±0.005	0.028±0.005	201±34	0.016±0.003
Total (0–36)	2537±288			1682±87	0.140±0.008

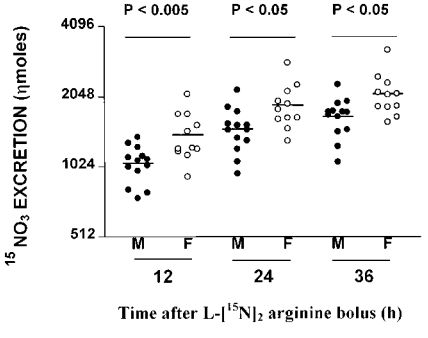
Values are mean±SE for 13 men and 11 women.
*$P<0.005$, †$P<0.05$.

association between extracellular body water and 36-hour urinary ^{15}N nitrate excretion ($r=0.08$, $P=$NS). There was a positive correlation between serum 17β-estradiol levels and 36-hour total urinary ^{15}N nitrate excretion (coefficient of correlation, 0.66; $P=0.038$) in the female group. The mean elimination rates of ^{15}N nitrate in men and women were similar (-0.084 hour^{-1} and -0.083 hour^{-1}, respectively).

Discussion

The results of this study provide evidence that the whole-body conversion of L-[$^{15}N]_2$-arginine to ^{15}N nitrate is greater in healthy women than in men, although the total urinary nitrate excretion does not differ between groups. Furthermore, 36-hour urinary ^{15}N nitrate excretion is associated inversely with the MAP over the whole group of subjects studied and positively with serum 17β-estradiol levels in the female group. ^{15}N nitrate is derived from oxidation of ^{15}N NO produced from L-[$^{15}N]_2$-arginine by NO synthase. However, the enzyme isoform(s) and tissue(s) in which this is occurring are unknown and cannot be addressed by measurement of urinary nitrate excretion.

Direct measurement of NO production is extremely difficult because of NO'ss short half-life in vivo.[2] NO is rapidly oxidized to nitrate by oxygenated hemoglobin, molecular oxygen, and superoxide anions and is excreted as such into the urine.[15] In mammalian cells, NO is synthesized from the guanidino nitrogen atoms of L-arginine, and this is the only known route by which these nitrogen atoms may be incorporated into nitrate.[16] Therefore, determination of the urinary excretion of ^{15}N nitrate after intravenous administration of L-[$^{15}N]_2$-guanidino-arginine is a more specific measure of whole-body NO synthesis than measurement of total nitrate, cyclic GMP, and L-citrulline.



Cumulative urinary excretion of ^{15}N nitrate after the intravenous administration of L-[$^{15}N]_2$-guanidino arginine in men (solid symbols) and women (open symbols). Values are mean±SE for 13 men and 11 women.

In this study, we found that the total urinary nitrate excretion did not differ between men and women; however, the mean 36-hour urinary ^{15}N nitrate excretion was significantly higher in women compared with men after systemic administration of L-[$^{15}N]_2$-arginine. The main problem with the use of total urinary nitrate excretion as a measure of NO synthesis is, however, that nitrate may arise from sources other than that generated from the metabolism of NO, and dietary intake of nitrate may exceed endogenous production.[7] The subjects received a limited rather than a free nitrate diet before and after the administration of the stable isotope; the purpose of using a limited nitrate diet in this study was to reduce the body nitrate pool background to achieve urinary nitrate enrichments in the range of 0.5 to 0.8 atom %, during the first 12 hours after the tracer infusion. The measurement of urinary ^{15}N nitrate generated from L-[$^{15}N]_2$-arginine is independent of nitrate excretion from dietary sources and other unknown sources. The possibility that the renal clearance of nitrate may be lower in men than in women could explain this finding. However, the analysis of the rate of urinary ^{15}N nitrate excretion was assessed over 36 hours (>90% of generated nitrate was excreted), and the mean elimination constants of nitrate were very similar in both groups, as was the creatinine clearance. Therefore, differences in renal epithelial handling of nitrate are unlikely to explain the difference in ^{15}N nitrate excretion. It would also be of interest to measure the concentration of ^{15}N nitrate in plasma to corroborate that the increased urinary ^{15}N nitrate excretion in the female group was due to higher production rather than altered renal excretion. Although the isotope ratio mass spectrometer used in this study for the determination of ^{15}N nitrate enrichment has a very high precision (eg,

±0.0004), it suffers from the disadvantage that relatively large amounts of sample (55 μg nitrogen) are required. Because the plasma nitrate concentration is ≈30 μmol/L,[17] the required plasma volume containing this amount of nitrogen-nitrate would be approximately 130 mL, which represents a limitation for ethical reasons and because of the difficulty of handling such a large volume of sample. In such instances, other spectrometric techniques such as gas chromatography–mass spectrometry on selected ion monitoring may be suitable. However, the precision of measurements with this procedure is rarely better than 1%,[18] and this limitation precludes reliable measurements on plasma nitrate enrichment in the range of 0.5 to 0.8 atom %.

We also contemplated the possibility that a different body handling of L-[$^{15}N]_2$-arginine between genders could explain the sex difference in the urinary excretion of ^{15}N nitrate. For instance, a difference in the size of L-arginine pools within the peripheral circulation and tissues could alter the enrichment of L-[$^{15}N]_2$-arginine where NO is synthesized and therefore account for the differences observed. However, Beaumier et al[19] and Castillo and colleagues[20] explored the effect of a high L-arginine intake on the conversion of L-[$^{15}N]_2$-arginine to NO in humans by measuring ^{15}N nitrate and total nitrate excretion in urine. Although the plasma L-arginine flux increased approximately 3-fold with arginine supplementation, the arginine-supplemented diet did not alter the total daily rate of conversion of plasma L-[$^{15}N]_2$-arginine to urinary ^{15}N nitrate in the normal and supplemented diets. Furthermore, at the dose of tracer used in our study (1.13 μmol), it is unlikely that different pharmacokinetics of the infused L-[$^{15}N]_2$-arginine in each group could be responsible for the observed findings. Indeed, Van Haeften and colleagues[21] have reported that constant intravenous infusions of L-arginine at a rate of 3, 9, and 15 mg/kg per minute during 30 minutes did not modify the half-life and volume of distribution of L-arginine in humans. Moreover, as the volume of distribution of L-arginine is very similar to the extracellular volume (290 mL/kg),[21] we explored the association between extracellular body water and urinary ^{15}N nitrate excretion. Stepwise multiple regression analysis showed no association between these variables. However, whether the increased urinary ^{15}N nitrate excretion observed in healthy women, which these data suggest, is due primarily to a change in the level and regulation of NO synthase and/or to changes in the metabolism and tissue availability of L-arginine cannot be determined from this study.

We also found in the present study an inverse relationship between MAP and urinary ^{15}N nitrate excretion throughout the blood pressure range, a result consistent with ours[9] and other previous findings[22,23] that NO synthesis is reduced in patients with essential hypertension. Because gender and blood pressure were significantly correlated with urinary ^{15}N nitrate excretion, we examined the possibility that blood pressure acted as a confounding variable. Furthermore, as the LDL cholesterol concentration was slightly lower in women compared with men, this may also have affected the results obtained. However, multiple regression analysis showed that the regression coefficient associated with gender was still significant even when blood pressure and LDL concentration were taken into account.

We detected a positive correlation between serum 17β-estradiol and the levels of urinary ^{15}N nitrate excretion during the follicular development in women, which agrees with previous findings of an association between serum total nitrate and 17β-estradiol levels.[24] However, the contribution of other sex hormones such as estrone, follicle-stimulating hormone, luteinizing hormone, or other endogenous substances involved in follicular development may also modulate the conversion of L-[$^{15}N]_2$-arginine to ^{15}N nitrate. Further studies are needed before a direct cause-and-effect relationship between serum 17β-estradiol concentration and urinary ^{15}N nitrate excretion can be established.

Taken together, since subjects were of similar age, body mass index, and levels of blood pressure, serum cholesterol, and glucose, the most likely explanation of our findings is that the production of ^{15}N nitrate after the intravenous administration L-[$^{15}N]_2$-arginine is higher in healthy women than in men. In line with this conclusion, these results are in agreement with our previous findings in which the total urinary ^{15}N nitrate excretion was also higher in hypertensive women than in men.[9] In addition, these findings are compatible with those of Chowienczyk et al[3] and Kharatinov and colleagues.[4] However, our results differ from those of Jilma et al[5] and Takahashi and colleagues,[6] who reported that the plasma levels of nitrate were greater in men than in women. A confounding factor in the interpretation of their findings could be the contribution of nitrite and nitrate to the plasma pool from the diet. In another report, Giovannoni and colleagues[25] did not find any significant difference in the mean serum nitrate and nitrite between healthy men and women. However, it is worth highlighting that plasma nitrate levels do not aid in elucidating the finer differences in NO production, since the rate of nitrate synthesis and elimination and its volume of distribution are all factors that modify the plasma concentration. Moreover, because of its large volume of distribution (extracellular fluid volume)[17] and a background plasma level of 30 μmol/L, it is possible that the sample studied was of inadequate power to detect differences in the activity of the constitutive NO synthase, which produces NO in the nanomolar range.

In summary, the present data suggest that the most likely explanation of this gender difference in urinary ^{15}N nitrate excretion is that under ambulatory conditions, whole-body NO biosynthesis is higher in healthy premenopausal women than in men. However, in view of the limitations in the interpretation of whole-body metabolic tracer studies, further investigations aimed to assess the in vivo significance and relationship between NO production and vascular function are essential. It is possible that a difference in endothelial NO production contributes to differences in vascular function and predisposition to arterial disease in men compared with women.

Acknowledgments

This study was supported by the Joint Research Board at St Bartholomew's Hospital (Project XMLH) and the British Heart Foundation.

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