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SEX DIFFERENCES IN CONCENTRATIONS OF EXHALED NITRIC OXIDE AND PLASMA NITRATE

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Summary

Nitric oxide (NO) is generally considered as an endogenous vasoprotective agent. Various studies indicate that the female sex hormone estradiol, that contributes to the well known gender differences in cardiovascular disease, may enhance NO-production. Thus we studied sex differences in NO-generation by measuring single breath NO- exhalation and plasma levels of nitrate (NO3), the stable endmetabolite of NO. In this observational trial 22 male and 21 female volunteers, 19 to 38 years of age, were studied on 3 days at weekly intervals. Median concentrations of NO were 20 parts per billion (95% CI: 16 to 32 ppb) in women and 34 ppb (95% CI 31 to 58 ppb) in men. The median plasma concentrations of NO3 were 14 μM/L (95% CI: 11 to 23 μM/L) in women and 27 μM/L (95% CI: 24 to 47 μM/L) in men. Thus, men exhaled 59 % more NO ($p<0.001$) and had 99% higher NO3 levels than women ($p<0.0001$). Even when exhaled NO concentrations were corrected for body weight, men exhaled 50% more NO than women ($p=0.024$). No significant changes in measured endpoints were seen during the menstrual cycle ($p>0.05$) in women. In view of the diversity of NO-actions, the finding of marked sex differences in NO-production is basic to the elucidation of gender differences in a number of (patho)-physiologic conditions.

Key Words: nitric oxide, plasma nitrate concentration, gender, menstrual cycle

Cardiovascular disease is the leading cause of death in industrialized countries (1). The incidence of coronary artery disease is several fold higher in men as compared to premenopausal women (2). This difference is largely ascribed to differences in blood levels of the female sex hormone estradiol-17β (E2) which has well documented antiatherogenic-cardioprotective effects (3-5). Multiple regression analysis has shown that only 25 to 50% of this effect of estrogens can be attributed to the well known lipid-lowering activity of estrogen (3), suggesting that other mechanisms contribute to its cardioprotective effect.

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There is growing evidence that effects of estrogens on vascular function may be involved. Estradiol has vasodilatory properties: it relaxes blood vessels (7,8) and decreases vascular resistance and diastolic blood pressure (9). Further, estrogens seem to improve endothelium-dependent vasodilation in postmenopausal women (1) and augment endothelium-mediated dilation of atherosclerotic coronary arteries in monkeys (10) Some of these effects can be blocked by concomitant treatment with an inhibitor of nitric oxide synthase (NOS, 8) which catalyses formation of the endogenous vasodilator nitric oxide (NO; 11)

Basal release of NO is higher from aortic rings obtained from female than male rabbits and oophorectomy diminished both circulating E2-concentration and basal release of NO to levels seen in male rabbits (12). E2 -administration has been found to double NOS-activity in male and female rats (13). This effect of E2 probably accounts for the greater than 100 % increase in NO synthesis during pregnancy (14). It may further contribute to the observed vasodilation associated with pregnancy or the ovarian hyperstimulation syndrome, where E2-serum levels rise substantially (15). Preliminary results indicate that E2 at nanomolar concentrations may enhance expression of endothelial NOS-mRNA (16). Thus, basal NO synthesis is likely co-regulated by E2 but comparative data on NO-production under baseline conditions in healthy men and women are not available. However the above findings are in contrast to a single clinical trial, which showed higher NO3 -levels in men than women (17). This study is striking as it may indicate that whole-body endogenous NO-production may be even more pronounced in men than women.

Thus, we studied sex differences in NO-production as measured by NO-exhalation and by plasma levels of nitrate (NO3). NO3 is a stable end product of NO-synthesis and is considered an index of whole-body endogenous NO-production (18) under fasting conditions. We also tested whether physiologic fluctuations in E2-serum concentrations during the menstrual cycle may be accompanied by fluctuations in NO-production Additional aims of this trial were to quantify the day-to-day variability of NO-exhalation and NO3-plasma concentrations and to assess the degree of correlation of these measures of NO-production under baseline conditions.

Subjects and methods

The study was approved by the Ethics Committee on Human Subjects in Medical Research, University of Vienna Written informed consent was obtained from all volunteers prior to inclusion in the study.

The study design was prospective, analyst-blinded and cross-sectional. Blood samples from female volunteers were also used for a different study (19)

A total of 22 healthy Caucasian men, aged between 19 and 35 years (26 ± 3 years, mean ± 1 SD) with a body mass index of 23.5 ± 2.2 kg/m^2 (mean ± 1 SD), and 21 Caucasian women, aged between 19 and 38 years (26 ± 5 years, mean ± 1 SD) with a body mass index of 21.9 ± 3.0 kg/m^2 (mean ± 1 SD), were included

All subjects passed a screening examination that included history, physical examination, 12 lead-electrocardiogram, blood pressure (BP), complete blood cell count with differential, clinical chemistry, liver and kidney function tests, serologic tests for hepatitis-viruses and HIV, urinalysis and urine drug-screening. Subjects were requested to maintain their usual exercise habits and diet and were instructed not to take any over-the-counter medication (OTC) throughout the study. Subjects were excluded if they were taking any prescription medication within the last 3 months, any OTC within the last 3 weeks, or if they had donated blood within the last 3 months prior to the first study day, were abusing alcoholic beverages, or smoking

Subjects were also excluded if they had had any clinically relevant illness in the 3 weeks before the first study day, or if an abnormality was found as part of the screening examination that the investigators considered to be clinically relevant. Drop out criteria were comedication with any drug, occurrence of any disease and non-compliance with the study protocol. All female subjects reported regular menstrual cycles and none of them had been taking oral contraceptives for at least 3 cycles.

Study design and experimental protocol: Male volunteers: Blood was drawn from a forearm vein and NO-exhalation was measured on 3 study days at weekly intervals.

Female volunteers: The female volunteers were requested to report at the research ward on day 7 of their menstrual cycle for blood sampling. The volunteers were instructed to use a commercially available test (Epignost Ovulationstest®, Sigma-Braumapharm, Vienna, Austria) for daily screening of their urine for the LH-surge at home. A positive test result was confirmed by a different test (Clearplan®, Much Pharma GmbH, Vienna, Austria). The volunteers were asked for additional blood sampling on the day after the tests turned positive and again 9 days thereafter, or to report for blood sampling if tests were not positive until the third morning after the expected ovulation, as estimated on the basis of their last cycles, the last blood sample was obtained 9 days later.

Blood samples were obtained by veinpuncture through a 21-gauge needle into Vacutainer® tubes containing lithium heparin, with the subjects in a seated position and after 30 minutes of rest, between 8:00 and 9:00 am after an overnight fast. All plasma samples were placed on ice for ten minutes, then centrifuged at 2000 g for 15 min at 4°C, aspirated and stored at -80°C until analysis in duplicates.

Exhaled NO was measured with a chemoluminescence detector (Monitor Labs Inc., Nitrogen oxides analyzer, Model 8840, USA) sensitive to NO from 1 to 10000 parts per billion (ppb) connected to a strip-chart recorder for on-line documentation. Calibration of the instrument was performed with a mixture of certified gases (NO, AGA-gas, Vienna, Austria) diluted in nitrogen by precision flow meters. A working pressure of 26 mmHg was obtained by connecting the analyzer to a vacuum pump and 1000 mL/min. of exhaled air was allowed to enter the inlet port. Three consecutive readings were made at 1 minute intervals. Results were compared to a baseline signal obtained with pure nitrogen. A mean of the three replicates was calculated and used for statistical analysis. Ambient air NO was recorded before each breath, although measured ambient air concentrations of 0-38 ppb did not affect readings in a previous study (20). Autoinhalation of NO from the nasopharynx (21) was avoided by application of a noseclip.

Subjects were instructed to fully inflate their lungs, to hold their breath for 10 seconds and to exhale for 10 seconds into a Teflon-tube which was connected to the inlet port. The end-expiratory concentrations were used for analysis. This assures that concentrations of exhaled NO reach a steady-state plateau and that inspired NO from the ambient air does not distort the results, because a previous study has shown that inhalation of 800 ppb NO did not change concentrations of exhaled NO after holding breath for 15 seconds (20). This indicates that exogenous NO is effectively removed from the respiratory tract within 15s.

Laboratory assays: Plasma concentrations of NO3 were determined colorimetrically as described in detail by Roth et al. (22). Concentrations were determined from a linear standard curve between 1 and 30 µmol/L sodium nitrite (r^2=0.9996); the intraassay coefficient of variation (CV%) was < 3%, and the day-to-day CV% was < 6%.

Serum levels of E2 and Progesterone were measured by radioimmunoassay (RIA) (Coat-A-Count® Diagnostic Products Corporation®, Los Angeles, CA, USA).

Statistical analysis: Nonparametric tests were chosen because data were non-normally distributed. Results are presented as medians and the 95% confidence intervals (CI) in parenthesis. The p-value was adjusted for multiple comparisons by the Bonferroni-method, where appropriate. The Spearman ranks correlation test was used for correlations between mean values of NO and NO3 within sexes.

Male volunteers: Intraindividual variability (CV%) was estimated from the day-to-day changes in exhaled NO and NO3-levels of the healthy men.

Female volunteers: Changes in exhaled NO and NO3-levels during the menstrual cycle were analyzed with Friedman ANOVA.

Sex differences: The Mann-Whitney rank sum test was used for comparing the average values obtained from the three study days of men and women.

Results

Three of 21 women were not included in the final analysis because of urinary tract infection, evidence of Epstein Barr virus infection, or protocol violation, respectively.

E2-serum concentrations increased from a median of 145 pmol/l (CI: 113 to 184 pmol/L) during the follicular phase, to 232 pmol/L (CI: 223 to 464 pmol/L) during midcycle, and 392 pmol/L (CI: 324 to 513 pmol/L) in the luteal phase. Progesterone serum concentrations increased from a median of 1.6 nmol/L (CI: 1.3 to 1.8 nmol/L) during the follicular phase, to 4.3 nmol/L (CI: 4.1 to 11.7 nmol/L) during midcycle, and 27.1 nmol/L (CI: 19.1 to 34.7 nmol/L) during the luteal phase.

However, there were no significant changes in NO concentrations in exhaled air nor any changes in NO3-plasma levels throughout the menstrual cycle in women ($p>0.05$). Median concentrations of NO were 20 ppb (CI: 16 to 32 ppb) in women and 34 ppb (CI: 31 to 58 ppb) in men. The median plasma concentrations of NO3 were 14 µM/L (CI: 11 to 23 µM/L) in women and 27 µM/L (CI: 24 to 47 µM/L) in men. This resulted in a significant 59 % ($p<0.001$) sex difference in exhaled NO and a 99% between-gender difference in NO3 -levels ($p<0.0001$, figure 1). When concentrations of NO were normalized for body weight or body surface, endexpiratory NO concentrations of women (median: 0.32 ppb/kg, CI: 0.25 - 0.51 or 12 ppb/m^2, CI: 9 - 18) were still substantially lower than those in men (median: 0.48 ppb/kg, CI: 0.40 - 0.79; $p=0.024$ or 18 ppb/m^2, CI: 16 - 30, $p=0.019$, respectively). Further, there was no significant correlation between body weight or body surface and exhaled NO or NO3-levels in either sex ($p>0.05$).

The mean CV% of the day-to-day variability for NO and NO3 measurements were 24% and 28%, respectively. NO and NO3 measurements did not correlate in men ($r^2=0.061$; $p=0.532$) or women ($r^2=0.253$; $p=0.068$).

Female subjects had significantly higher diastolic BP (70 mmHg; CI: 69 to 74 mmHg) than men (66 mmHg; CI: 62 to 70 mmHg, $p=0.018$) but significantly lower systolic BP (115 mmHg, CI: 113 to 119 mmHg) than men (120 mmHg, CI: 118 to 126 mmHg; $p=0.010$). Thus, a lower pulse pressure was observed in women (45 mmHg; CI: 42 to 47 mmHg) as compared to men (59 mmHg; CI: 53 to 59 mmHg, $p<0.001$).

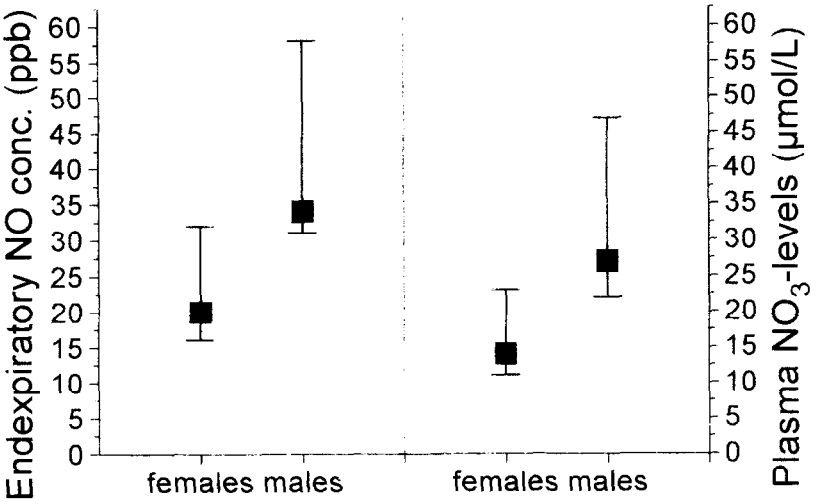

FIG. 1

Sex differences in concentrations of endexpiratory nitric oxide (NO; $p<0.001$) of single-breath NO measurements (left) and in concentrations of plasma nitrate (NO3; $p<0.0001$, right). Data are presented as medians and the 95% confidence intervals as error bars.

Discussion

The main purpose of this study was to quantify sex differences in production of endogenous NO and to study the influence of cyclic changes in E2 -levels on NO-production. The most evident finding was the markedly higher NO-exhalation and NO3-levels in men as compared to premenopausal women (figure 1).

As NO production cannot be directly quantified in vivo, we have employed two individual measures of NO-generation: NO-exhalation and NO3-plasma levels. Conceivably, endexpiratory NO-concentrations reflect a number of processes including synthesis of NO, partitioning into the airspace, diffusion in the gas phase and degradation.
Despite this limitation, effects of NO-releasing substances or inhibitors of NOS have previously been quantified by measuring NO-exhalation (20, 23). The same method can be used to measure physiologic changes in NO-exhalation during exercise (24). Thus it is possible to directly measure by this approach pharmacological and physiologic changes in NOS-activity.

Although the origin of expired NO remains to be determined, the site of origin is expected in close proximity to, and in rapid equilibrium with, the alveolar air space (24). Exhaled NO could derive from endothelium (25) or bronchial epithelial cells (26) or macrophages (24) and finally neurons in the lung.

While respiratory frequency can change the apparent peak NO concentration because of excessive dead volume ventilation, this effect has never been observed under moderate changes of respiratory frequency for endexpiratory NO-exhalation. Further, we have used an artificial breathing maneuver as outlined in the methods section, to guarantee that even small between-subject variations in respiratory frequency do not have an impact on measured NO-concentrations.

We have used plasma nitrate levels as a second indicator of NO-production, as nitrate is a stable end product, and has frequently been used to demonstrate changes in NO-generation in various disease states or after pharmacological intervention (27-29).

The lack of correlation between methods may indicate that the measurements of exhaled NO and NO3-levels reflect NO-synthesis from different endogenous sources or that a certain amount of NO3 derived from dietary sources. However, our results reflect a physiologic setting rather than an artificial state of zero-NO3 intake. In view of the rapid clearance (22 mL/min) of NO3 (28) and the 12 hours fasting period prior to blood sampling, it is rather unlikely that dietary NO3 has contributed to the gender differences in NO3 -levels. Nevertheless, sex differences in measured expired NO-levels and plasma NO3-levels were of similar direction and magnitude (Fig. 1). Thus we believe that results from our surrogate endpoints reflect true sex differences in NO-production. We found no previous reports on sex differences in exhaled NO, but our finding of higher NO3-levels in men is in principle agreement with the results of another larger trial (17). These authors found 65% higher NO3- levels in men (n=126) than in women (n=79), in an older population (17). Thus, the consistency in results makes it unlikely that our results are false positive. Further it has been shown by Green et al. (18) that changes in fiber or protein intake do not substantially alter 24 hour urinary nitrate excretion. For this reason potential sex differences in dietary composition or nitrate intake were probably not responsible for sex differences in NO3- levels.

Are our results compatible with the higher incidence of cardiovascular disease in men? Formation of endothelial NO inhibits platelet aggregation and induces vasodilation which may be important mechanisms in preventing thrombus formation and vasospasm (30). Nonetheless, we have found indices of higher NO-production in men than in women. At first glance these experimental findings seem in conflict with epidemiologic data that men are more prone to developing atherosclerosis early in their life than premenopausal women. Conceivably, the higher NO-production in men may be explained as a physiologic counterregulatory action in response to decreased vascular function in men; as yet undefined mechanisms may reactively enhance NO-production in men. Candidates for such an NO production-enhancing effect could be the higher concentrations of cholesterol (31) or higher levels of the atherogenic hormone endothelin (32-34) in men as compared to women (35). The paradox that risk factors of atherosclerosis such as hypercholesteremia, endothelin or male sex increase NO-production may thus be explained by NO acting in a negative feedback loop.

A further explanation could be, that in vivo most of the measurable NO-production occurs in inflammatory cells and the higher NO-production could reflect more extensive oxidative burst in men as compared to women. This may lead to oxidation of sulfhydryl groups that are either involved in NO-storage (31) or NO-mediated vasodilation (36).

Finally a limitation of this cross-sectional trial is that we cannot exclude that sex differences of unknown potential confounders such as cholesterol levels, hemoglobin levels, respiratory frequency, basic metabolic rate, etc., have resulted in our findings.

In conclusion our results do not support the concept that NO contributes directly to the lower cardiovascular morbidity in women as compared to men. However, gender is another physiologic determinant of NO-production beside age (37).

The finding of sex differences in NO-production appears basic to the elucidation of gender differences in a number of (patho)-physiologic conditions, because NO acts as a vasoactive, platelet-regulatory, cytotoxic, neurotransmitting, immuno-and broncho-modulatory substance (11, 20). Further investigation is warranted to enhance our understanding of the underlying mechanisms of the observed gender differences in NO-generation.

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