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Influence of sex and age on serum nitrite/nitrate concentration in healthy subjects

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Abstract

Measurement of serum nitrite/nitrate (NO*x*) concentrations has been considered useful to estimate nitric oxide production in humans. However little is known about the physiologic range and the factors affecting serum NO*x* levels. The aim of this study was, thus, to investigate the influence of sex and age on serum NO*x* levels in healthy subjects. We selected 263 healthy subjects (118 women and 145 men, 20–69 y) from 505 consecutive subjects who received annual medical checkups at our hospital. Serum NO*x* levels were determined using an analyzer employing the Griess method. The linear regression analysis showed that NO*x* increased significantly according to age in women ($r=0.22$, $P<0.05$), but did not in men ($P=$NS). Women of the younger age group (<40 y) showed significantly lower NO*x* levels than men of the same age group ($P<0.05$), whereas there was no significant difference between men and women of the older age (≥ 40 y). Then, to investigate whether menopause affects serum NO*x* levels, middle aged women (46–55 y) were selected. Multiple logistic regression analysis showed that menopause was an independent factor for increased serum NO*x* levels in middle aged women ($r=0.4$, $P<0.05$). These results suggest that the serum NO*x* concentration is affected by age in healthy women, possibly depending on menopausal state.

Abbreviations: NO*x*, nitrite/nitrate

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1. Introduction

Nitric oxide (NO) has been shown to play physiological and pathophysiological roles in widespread fields [1–3]. NO is not only a vasodilator but also a neurotransmitter and a killer molecule in the immune system, and is synthesized in various tissues and cells [1–3]. The major NO decay route in mammalian systems is the rapid reaction of NO with superoxide to form peroxynitrite [4]. NO cytotoxicity is associated with peroxynitrite decay products OH, NO_2 and NO_2^-. However, it is difficult to measure NO directly because NO is quite short lived (of the order of seconds to minutes). Recently, the direct measure of NO in the cardiovascular system has been available using the porphyrinic sensor [5]. Although this method is sensitive and informative, it is invasive and limited in the research field. Alternatively, NO*x* (nitrite and nitrate) is measured as an indirect marker of NO formation [6]. A number of investigators have reported that serum or plasma NO*x* concentrations are influenced by diseases, e.g., heart failure [7], sepsis [8] and liver cirrhosis [9]. These reports suggest that measurement of serum NO*x* concentrations is clinically useful to estimate NO formation. However, the physiologic range as well as the age- and sex-related change of serum NO*x* concentrations are unknown. In this study, we first examined whether serum NO*x* levels were different among vascular beds using patients who underwent the cardiac catheterization. Then, using healthy subjects, we investigated the distribution and the age- and sex-related change of serum NO*x* concentrations.

2. Materials and methods

2.1. Sampling from various vascular beds

To decide the vascular bed that represents the total body NO*x* measure, blood samples were obtained from six patients (58.2±4 y, mean±S.D.; 2 men and 4 women) who admitted to the Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo and underwent the cardiac catheterization due to suspected coronary artery disease. All the patients were stable: Three of the patients had significant coronary stenosis, and the other three did not. The patients were not taking any medication except that three were taking isosorbide diitrate, which was discontinued 3 days before catheterization. Blood sampling

was done during the cardiac catheterization when the patients were lying at rest for at least 1 h. Blood (2 ml) was collected from the antecubital vein by puncture, and then from other vascular beds through the catheter. Collected blood was centrifuged at 1000 *g* for 10 min, and the serum was stored at −20°C until the assay.

2.2. Sampling from healthy subjects

Five hundred and five consecutive subjects were enrolled who received annual medical checkups at the Division of Public Health, The Social Health Insurance Medical Center. The medical checkups consisted of physical examination, chest X-ray, electrocardiogram, urine analysis, complete blood cell counts, blood chemical analysis such as liver function, renal function, lipids and plasma glucose. Blood was collected after overnight fast, then serum samples for NO*x* assay were separated from blood chemistry and stored at −20°C. The subjects wrote the questionnaire on their medical history and the habits. The subjects who had any present illness and/or were taking regular medications were excluded. The subjects who showed any abnormality in the medical checkups were also excluded. Normal ranges of blood pressure, serum lipids and plasma glucose were determined as follows; systolic blood pressure <140 and diastolic blood pressure <90 mmHg, serum total cholesterol <220 mg/dl, serum triglyceride <150 mg/dl, fasting plasma glucose <110 mg/dl. Then 263 subjects (118 women and 145 men, 20–69 y), who were considered healthy, were selected.

To investigate whether the menopausal state affects serum NO*x* levels, women aged 46–55 years were selected among healthy subjects mentioned above and divided into two groups based on the questionnaire. Premenopausal women were defined as having regular and monthly menstruation. Postmenopausal women were defined as not having menstruation for more than a year. Women whose menstrual cycles were irregular or unknown were excluded. Then, 31 women (18 premenopausal women and 13 postmenopausal women; 50.0±3.4 y) were enrolled.

2.3. Measurement of serum NOx concentration

Serum NO*x* levels were determined using an analyzer (TCI-NOX5000S, Tokyo Kasei Kogyo, Tokyo) as described previously [10]. Briefly, serum samples were centrifuged at 3000 *g* for 10 min. The supernatant (0.1 ml) was diluted with 0.4 ml of distilled water and 0.3 ml of 0.3 mol/l NaOH was added. After incubation for 5 min at room temperature, 0.3 ml of 5% (w/v) $ZnSO_4$ was added and incubated for another 5 min. The mixture was centrifuged at 2800 *g* for 10 min and the supernatant was applied to the analyzer. The analyzer

employs the technique of automated flow injection analysis. Nitrite reacts with a Griess reagent and forms a purple azo compound. The absorbance at 540 nm is measured. Nitrate is determined by reducing it to nitrite through a copperized cadmium reduction column.

We checked the accuracy of the assay by two ways. Six repeated measurements of 3 serum samples showed the values of 31.6±1.0, 68.6±2.0 and 90.2±3.1 μmol/l (mean±S.D.), then the coefficients of variation were calculated as 3.1, 2.9 and 3.4%, respectively. We next determined the recovery rates. Forty micro-molar nitrate was added to the above three samples. Then the recovery rates were calculated from the measured values and the above described values as 104.5±11.3, 99.8±3.2 and 108.7±6.4%, respectively (mean±S.D., $n=6$).

2.4. Statistical analysis

Serum NO*x* levels were analyzed after logarithmic transformation in order to normalize the distribution, and then back-transformed for presentation in the text, figure and table. Since the aging effects on NO*x* levels were different between men and women, they were analyzed separately in men and in women. Then linear regression analysis was performed between NO*x* and age as an explanatory variable. The difference in NO*x* levels between men and women was analyzed by unpaired two tailed Student's *t*-test. To investigate whether menopause affects serum NO*x* levels, multiple logistic regression analysis was performed with log-transformed serum NO*x* levels as a dependent variable, and menopause, age and body mass index (body weight/square body height; kg/m^2) as independent variables. All of the data were analyzed using the statistical software JMP ver3.2 (SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Serum NOx levels in various vascular beds

The sampling data from six patients were shown in Table 1. Serum NO*x* concentrations in the patients were 49.8±31.7 μmol/l (mean±S.D.) when blood was collected from the antecubital vein. NO*x* levels were similar among the samples from various vascular beds: pulmonary capillary, 104.8±21.0; main pulmonary artery, 100.9±16.5; right ventricle, 93.1±16.4; right atrium, 100.7±10.7; superior vena cava, 105.1±14.1; inferior vena cava, 96.3±12.9; femoral vein, 103.2±13.3; left ventricle, 94.7±8.1; ascending aorta, 95.9±4.9; femoral artery, 101.3±15.5 (% of antecubital vein, mean±S.D.). These results indicate that serum NO*x* levels are comparable between the sites of blood sampling at least in the chronic steady state.

Table 1
Serum nitrite/nitrate (NO*x*) levels in various vascular beds[a]

Patient No.	Age (y)	Sex	NO*x* (μmol/l)										
			PCW	mPA	RV	RA	SVC	IVC	FV	LV	Ao	FA	CV
1	53	M	68.7	68.6	68.8	70.2	75.4	63.1	68.2	74.7	72.2	86.8	75.4
2	50	F	26.9	19.1	21.1	21.8	19.1	18.5	18.4	–	–	–	20.6
3	58	F	25.6	26.3	24.2	27.4	–	28.4	29.0	28.4	26.4	27.2	28.6
4	65	F	50.4	53.7	41.2	55.4	58.9	57.4	65.2	46.9	56.5	46.0	58.2
5	54	M	94.5	100.0	86.7	92.0	97.7	83.6	97.2	94.9	87.3	96.5	95.8
6	69	F	26.4	26.6	23.8	25.4	25.9	23.9	24.9	19.1	20.7	23.3	20.0

[a] PCW, pulmonary capillary wedge; mPA, main pulmonary artery; RV, right ventricle; RA, right atrium; SVC, superior vena cava; IVC, Inferior vena cava; FV, femoral vein; LV, left ventricle; Ao, ascending aorta; FA, femoral artery; CV, antecubital vein. Blank column indicates not examined.

3.2. Serum NOx levels in healthy subjects

The distribution of serum NO*x* concentrations in healthy subjects before and after log-transformation is shown in Fig. 1. Since the distribution of NO*x* was asymmetric and log-normal, NO*x* was analyzed statistically after log-transformation. Table 2 and Fig. 2 show serum NO*x* levels in 263 healthy subjects. The linear regression analysis showed that NO*x* increased significantly according to age in women ($r=0.22$, $P<0.05$), but did not in men ($P=$NS). When NO*x* levels were compared between the sexes, women of the younger age group (<40 y) showed significantly lower NO*x* levels than men of the same age group ($P<0.05$), whereas there was no significant difference between men and women of the older age (≥ 40 y).

To investigate the mechanism underlying the aging effect in women, the relationship between serum NO*x* levels and menopause was analyzed in middle aged women (46–55 y). Since postmenopausal women showed the significantly higher serum NO*x* levels, older age and higher body mass index than premenopausal women (data not shown), multiple logistic regression analysis was performed for serum NO*x* levels with menopause, age and body mass index as independent variables. This analysis showed that only menopause was an independent factor for increased serum NO*x* levels ($r=0.4$, $P<0.05$).

4. Discussion

In the present study, we investigated the sex difference and the aging change of serum NO*x* concentrations in healthy subjects. Serum NO*x* levels increased according to age in women but did not in men. Consequently, NO*x* levels were significantly lower in women of the younger age (<40 y) than men of the same

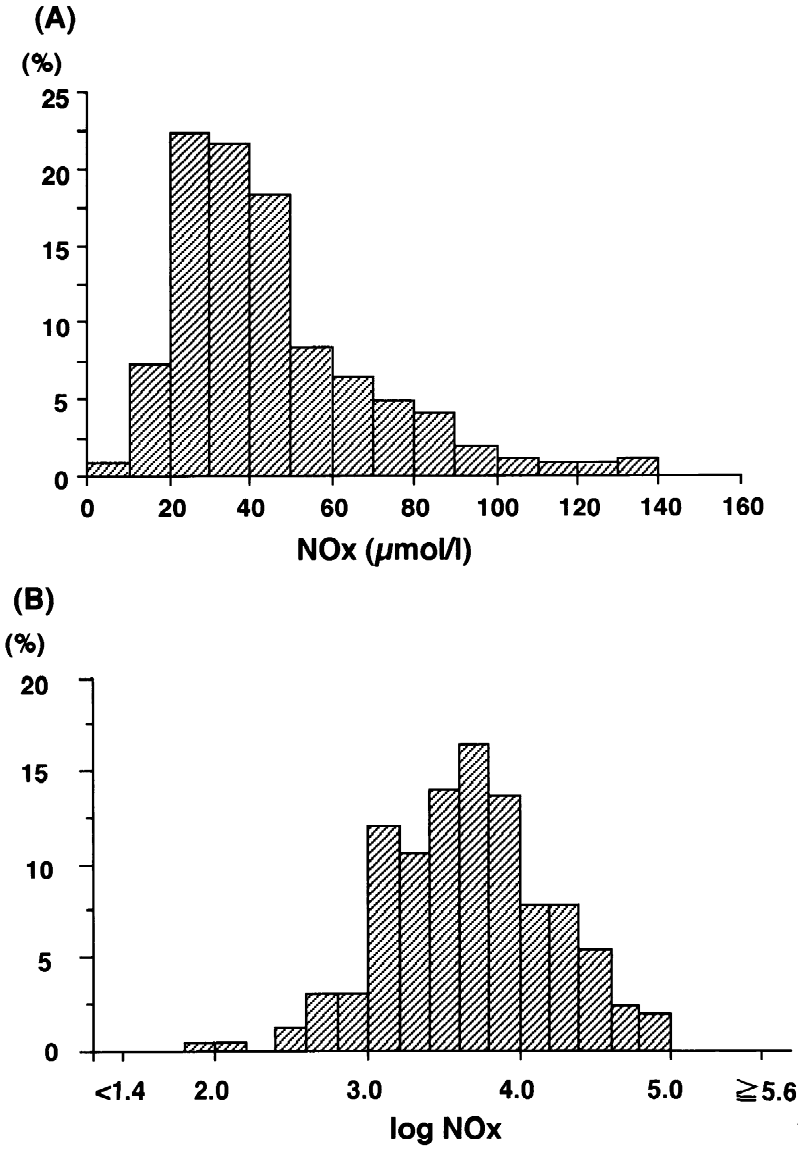

Fig. 1. Histograms showing the frequency distribution of serum NO*x* concentrations (A) and log-transformed NO*x* (B) in healthy subjects.

age but the sex difference was not observed in the older age groups. The aging effect of NO*x* in women may be attributed to menopause.

It has been reported that serum NO*x* concentrations differ in various pathological conditions [7–9]. Plasma NO*x* concentrations rise in septic shock, in which augmented NO production by endothelial cells and/or immune

Table 2
Serum nitrite/nitrate (NO*x*) levels in healthy subjects[a]

Age (y)	Women		Men	
	n	median (25 percentile, 75 percentile)	*n*	median (25 percentile, 75 percentile)
20~29	39	27.5 (23.3, 36.9)	39	38.1 (24.7, 49.8)
30~39	29	38.0 (29.3, 52.6)	40	47.5 (34.3, 65.0)
40~49	34	41.1 (29.0, 50.7)	34	38.1 (29.4, 56.0)
50~	16	46.8 (27.9, 68.7)	32	42.6 (34.4, 51.1)
Total	118	35.6 (24.8, 507)	145	41.0 (34.4, 51.1)

[a] The values of NO*x* are expressed as μmol/1. *n*; Number of subjects.

response cells including macrophages and neutrophils may play a role [8]. Serum NO*x* levels are increased in congestive heart failure [7] and liver cirrhosis [9] as well. In addition to endogenous NO production, serum NO*x* concentrations are affected by NO*x* excretion. Mackenzie et al. reported that serum NO*x* concentrations inversely correlated with renal function [11]. Renal function is frequently impaired in many disease states and this may affect NO*x* clearance. The effect of renal function on NO*x* concentrations should be accounted for in making any consideration on a given pathological condition and NO production as reflected in the NO*x* concentrations. Alternatively, serum NO*x* levels are expected to be markers for disease severity. However, little is known about the standard range and the physiological change of serum NO*x* concentrations.

There are two reports that studied the aging effect and the sex difference of

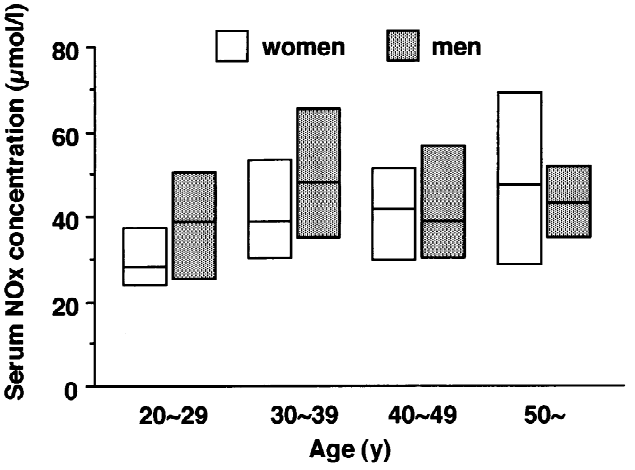

Fig. 2. Box-plot graph showing age- and sex-related changes in serum NO*x* concentrations in healthy subjects. Box represents the interquartile range (between the 25th and 75th percentiles), with the median value shown as a horizontal bar within each box.

serum NO*x* concentrations in humans [12,13]. Takahashi et al. investigated the correlation of plasma NO*x* concentrations with sex, age and atherosclerosis risk factors in office workers who were not taking any medication [12]. They demonstrated that plasma NO*x* levels were lower in women than in men and were increased according to age only in women. The other report by Jilma et al. showed that serum NO*x* concentrations and NO concentrations in exhaled gas were lower in healthy young women than in men (average 26 y, range 19–38 y, 22 men and 21 women) [13]. The results of our present study are consistent with these reports. However, the subjects with abnormal findings results such as hyperlipidemia, hyperglycemia and hypertension were not excluded in the study by Takahashi et al. [12]. Therefore, the present study is the first to show the sex difference and the aging effect of serum NO*x* concentrations in healthy subjects.

Ovarian hormones could account for the sex difference and the sex-related aging change of serum NO*x*. Estrogen, an ovarian hormone, has anti-atherogenic actions [14–17]. One of the mechanisms is considered to stimulate NO production by vascular endothelial cells [18,19]. If serum NO*x* reflects the NO production by vascular endothelial cells, increased estrogen secretion might result in the increase in serum NO*x* concentrations. The data that serum NO*x* levels were decreased in ovariectomized female rats [20] support this idea. In humans, however, there are conflicting reports on the menstrual cycle fluctuation of serum NO*x* concentrations [13,21], and thus it is unknown whether estrogen affects serum NO*x* levels.

Our findings that serum NO*x* levels were lower in young women than in men of the same age, and that postmenopausal women showed higher NO*x* levels than premenopausal women are inconsistent with the stimulatory effect of estrogen on NO production [18,19]. Also, aging is associated with the decrease in NO production by vascular endothelial cells [22,23], which might result in the decreased serum NO*x* concentration. However, in the present study, serum NO*x* levels did not decrease with age in men but rather increased with age in women. Why are our results on serum NO*x* concentrations apparently contradictory to the previous reports on endothelial NO production? One possible explanation for this is the findings in rats that aortic endothelial cell but not pulmonary artery endothelial cell NO production is reduced with age [25]. If serum NO*x* is derived more from pulmonary circulation than from systemic circulation, serum NO*x* might not be reduced with age as was the case in men in this study. To explore the site or the organ that contributes to determining serum NO*x* concentrations, we compared the serum samples obtained at various vascular beds. Although it has been reported that NO production measured directly by the porphyrinic sensor in the left ventricular myocardium markedly fluctuated in response to left ventricular loading on a beat-to-beat basis [26], NO*x* concentrations were equivalent among the sites of sampling. This may be due to the long half-life of serum nitrate (8 h) [24]. This result suggests that measurement of serum NO*x* is not adequate to estimate the dynamic change of NO

production. Rather, because of its simplicity and inexpensiveness, serum NO*x* measurement can be applied in the widespread clinical field to grossly evaluate the disease state.

Food intake influences serum NO*x* levels as an exogenous source of nitrate [6]. Although blood sampling was performed after overnight fast, the sex- and age-related difference in daily food intake might have affected serum NO*x* concentrations. Non-enzymatic NO synthesis, nitric oxide synthase-independent pathway, is one source of 'extra-endothelial' NO production. For example, NO is converted from nitrite under highly acidic condition or disease states, such as ischemia [27]. Renal function also influences serum NO*x* concentrations by modulating NO*x* excretion [11]. In our study, subjects with serum creatinine ≥ 1 mg/dl or with any abnormality in urinary analysis were excluded. Finally, there was no difference in serum creatinine concentrations between men and women, although we had no data on the creatinine clearance. Previous reports have demonstrated creatinine clearance is decreased with age [28], and is lower in females than in males in all age groups [29]. Therefore, if serum NO*x* concentrations are simply reflections of renal function, serum NO*x* levels should be higher in women. Also, it is reported that there is no difference in urinary NO*x* concentrations regardless of age and sex in healthy subjects [30]. Thus, although we did not measure creatinine clearance and NO*x* clearance in this study, these findings could allow us to regard that the difference of renal function had a minimal effect on serum NO*x* concentrations in healthy subjects. Taken together, serum NO*x* levels may, in some cases, reflect endothelial NO production but be regulated by many factors including extra-endothelial production. In conclusion, we demonstrated the distribution, sex difference and aging change of serum NO*x* concentrations in healthy subjects. This study provides the basic data necessary to evaluate the serum NO*x* concentrations in various disease states.

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