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Plasma Nitrite/Nitrate Level Is Inversely Correlated with Plasma Low-Density Lipoprotein Cholesterol Level

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Summary

Background: Plasma nitrite/nitrate (NOx) is a stable end product of the vasodilator nitric oxide (NO). However, there are few reports about plasma NOx levels in humans.

Hypothesis: The purpose of this study was to assess the availability of plasma NOx for evaluating basal endogenously-synthesized or endothelium-derived NO, and to examine whether NOx levels are lowered in patients with coronary artery disease (CAD) or its risk factors.

Methods: Plasma NOx levels were measured using an automated system based on the Griess reaction. NOx levels for a 24-h period reproducibly became lowest at 6 A.M. in restricted healthy volunteers, and became stable in inpatient volunteers at 6 A.M. within 4 days after admission.

Results: Based on these findings, NOx levels at 6 A.M. in inpatients can be considered as the basal levels. In 40 inpatients suspected of CAD (28 men, 12 women; mean age 60 ± 11 years), the basal levels of NOx were not related to CAD and its risk factors, except for hypercholesterolemia. The NOx level of patients with hypercholesterolemia was significantly lower than that of patients with normal cholesterol ($n = 16$, 34 ± 16 μmol/l vs. $n = 24$, 49 ± 23 μmol/l, $p < 0.03$). Furthermore, the NOx levels correlated negatively with the total cholesterol and low-density lipoprotein cholesterol levels ($r = -0.40$, $p < 0.01$; $r = -0.47$, $p < 0.003$, respectively), but not with other lipid fraction levels.

Conclusion: The results suggest that the quantity of basal endothelium-derived NO synthesis may be decreased in the presence of hypercholesterolemia.

Key words: plasma nitrite/nitrate, endothelium-derived nitric oxide, Griess reaction, coronary artery disease, coronary risk factors, hypercholesterolemia, low-density lipoprotein

Introduction

Endothelium-derived relaxing factor (EDRF) is a potent arterial vasodilator regulating the vasomotor tone or blood flow, not only systemically but also locally,[1,2] and is now identified as either nitric oxide (NO) or a related nitrosylated compound. Endothelium-derived NO is synthesized from L-arginine by NO synthase and induces vasorelaxation through activation of cGMP in smooth muscle cells. On the other hand, its activity is quickly inactivated by superoxide anion, which is also synthesized in endothelial cells,[3] or by binding to hemoglobin in the blood stream.[4] Although the NO metabolism is very complicated and has not as yet been fully elucidated, it is thought to be metabolized eventually to a stable end product as nitrite/nitrate (NOx) in plasma through several intermediates,[5] after which it is eliminated from the kidney.[4]

In several recent studies using an NO synthase inhibitor, reduction of both agonist-induced and basal EDRF activities have been observed in patients with several disorders including coronary artery disease (CAD).[1,2] This endothelial dysfunction is explained by a decrease in NO generation due to depletion of substrate or impaired transduction system,[6,7] or by an enhanced inactivation of NO by superoxide anion after its release from endothelial cells.[8] However, the question of whether only enhanced NO destruction is related to the endothelial dysfunction or whether the quantity of NO generation is actually reduced is still unclear in vivo.

In the present study, we initially estimated the basal levels of plasma NOx in volunteers and then measured those in patients with CAD and its risk factors in order to examine if NO generation is decreased in such disorders.

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Methods

Evaluation of Basal Plasma NOx Level

To estimate the basal level of plasma NOx in subjects, we evaluated 24-h and day-to-day variations of plasma NOx level. For 24-h variations, plasma was drawn at 4-h intervals for 24 h from five healthy volunteers who had a free choice of diet at around 8 A.M., noon, 7 P.M., and who were not allowed to smoke, drink, or perform strenuous exercise for 48 h, including the 24 h prior to the samplings. To examine day-to-day variations, plasma was drawn from five healthy volunteers in the early morning at Days 1, 3, 4, 5, and 7, and from five inpatient-volunteers at 6 A.M. on Days 2, 4, 5, 6, and 8 of their hospital stays. The healthy volunteers were not restricted in their behavior, including eating habits, in any way. The inpatients took a well-balanced diet from the day of admission and continued with the hospital diet for 8 days. Patients with diseases potentially affecting plasma NOx levels, those being treated with vasoactive agents, or those expecting invasive examinations during the 8 days were excluded. The collected plasma samples were stored at −40°C until the day of NOx analysis.

Study Patients

Plasma level of NOx was measured in consecutive patients suspected of suffering from ischemic heart disease. These patients were examined for CAD, hypertension, diabetes mellitus, and hypercholesterolemia. CAD was defined as the presence of >75% stenosis in at least one vessel on coronary angiogram. Hypertension was defined as a diastolic blood pressure >90 mmHg. Diabetes mellitus was diagnosed by the results of 75 g oral glucose tolerance testing. Hypercholesterolemia was defined as a plasma cholesterol level >220 mg/dl. None of the patients had renal dysfunction, infectious diseases, or immune disorders. All of the women were in the postmenopausal stage and were not receiving estrogen therapy. Patients with heart failure, myocardial infarction, vasospastic angina, variant angina, and arrhythmia, and those from whom vasoactive agents could not be withheld, were excluded from study. All patients were taking a well-balanced 1,600 to 1,800 Kcal/day diet during hospitalization. Cholesterol-lowering or vasoactive agents were withdrawn 48 h before blood sampling. Blood was collected from the cubital vein early in the morning before cardiac catheterization after overnight fasting at least 4 days after admission and stored at −40°C until the day of NOx analysis. Informed consent was obtained from all patients and the study protocol was approved by our institutional committee on human research.

Measurement of Plasma NOx

Venous blood samples were collected in tubes containing ethylenediamine tetra-acetic acid. To deproteinize plasma, 0.5 ml of 0.6 N perchloric acid was added to 0.5 ml of plasma. The mixture was incubated at 0°C for 10 min and then centrifuged at 10,000 rpm at 2°C for 10 min. A 800 µl sample of the supernatant was neutralized with 5M KOH / 1M Tris-HCl, and the sample was recentrifuged to remove any additional precipitation. The pH of the supernatant was adjusted to between 8.0 and 9.0 by the addition of 5 mol/l KOH. Plasma levels of NOx were measured using an autoanalyzer system (TCl-NOX 1000, Tokyo Kasei Kogyo Co., Tokyo) based on the method of Kanno *et al.*[9] Deproteinized samples 100 µl was injected into the autoanalyzer using a Rheodyne syringe. Samples were passed through a copperized cadmium reduction column to reduce nitrate to nitrite at a flow rate of 1 ml/min in the carrier solution (0.07% ethylenediamine tetra-acetic acid and 0.3% NH4Cl) and then reacted with a Griess reagent (1% sulfonamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, 5% HCl). Absorbance of a purple azo dye was measured at 540 nm with a flow-through visible spectrophotometer (Model S/3250; Tokyo Kasei Kogyo Co., Tokyo) connected to a chart recorder. Authentic nitrite and nitrate were used to assess the reduction efficiency of the cadmium column. Quantitative analysis was performed using $NaNO_2$ and $NaNO_3$ as external and internal standards in duplicate. Coefficients of variation for intra-assay and interassay variations were 4.2% and 5.5%, respectively. The limit of detection of was 0.5 µmol/l (99% confidence limit).

Measurement of Plasma Lipid Level

Plasma levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured enzymatically by previously described methods.[10] Lipoprotein(a) was measured with a commercially available ELISA kit (Biopool AB, Umeå, Sweden). Concentrations of apolipoprotein AI, apolipoprotein B were determined using the single radial immunodiffusion method based on the method of Mancini *et al.*[11] The diffusion plate, antiserum, and standard human serum were purchased from Daiichi Pure Chemical Co., Ltd., Tokyo.

Statistical Analysis

Data are expressed as mean ± SD. The influence of CAD, diabetes mellitus, hypertension, hypercholesterolemia, smoking status, and a family history of CAD on plasma NOx levels was determined by analysis of variance, respectively. Correlations between NOx levels and age and lipid levels were determined by linear regression analysis. A stepwise multivariate linear regression analysis was employed to examine the influence of the following coronary risk factors on NOx levels: age, gender, weight, serum creatinine, diabetes mellitus, hypertension, hypercholesterolemia, smoking status, and a family history of CAD. A p value <0.05 was considered to indicate statistical significance.

Results

Variations of Plasma NOx

The plasma NOx levels of the early morning varied greatly from day to day in the healthy nonrestricted volunteers, and coefficient variations ranged from 0.23 to 0.63 (raw data are

not shown). In restricted healthy volunteers, although the NOx levels showed a variation from 22 to 40 μmol/l at the beginning (6 A.M.), the patterns of individual variations were similar over 24 h: they increased in the daytime and then returned to the initial levels by 6 A.M. on Day 2 (Fig. 1A). The lowest level of NOx was observed at around 6 A.M. in all subjects. Therefore, for day-to-day variations of inpatient volunteers, therefore, plasma was collected at 6 A.M. As shown in Figure 1B, the NOx of inpatient volunteers tended to become lower after admission and stabilized within 4 days. Coefficient variation from Day 3 to Day 7 were small (from 0.04 to 0.10) in five inpatients. Based on these observations, for evaluating basal NOx level, plasma was collected from the study population at 6 A.M. at least 4 days after admission.

Basal Level of Plasma NOx

In all, 40 patients were recruited for this study. Their profiles, including plasma NOx levels, are summarized in Table I.

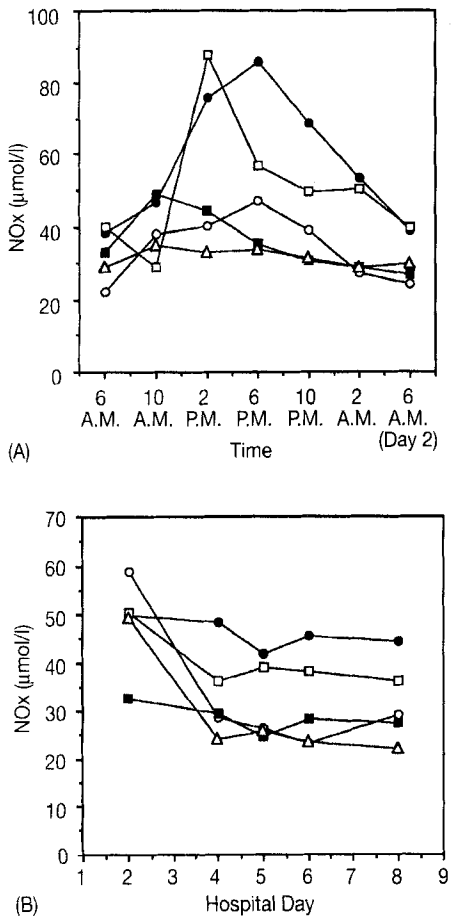

FIG. 1 (A) Twenty four-hour variations of plasma NOx (nitrite/nitrate) level in five healthy volunteers whose lifestyles were subject to restrictions. Blood sampling was performed every 4 h from 6 A.M. on Day 1 to 6 A.M. on Day 2. (B) Day-to-day variations of plasma NOx (nitrite/nitrate) level in five inpatient volunteers. Blood sampling was performed at 6 A.M. on Days 2, 4, 5, 6, and 8 of their hospital stays.

TABLE I Patient profile and plasma NOx levels

			NOx (μmol/l)
No. of patients		40	42 ± 21
Male		28	44 ± 21
Female		12	39 ± 22
Age (years)	60 ± 11		
Body weight (kg)	61 ± 10		
Serum creatinine (mg/dl)	0.8 ± 0.2		
Coronary artery disease	(+)	19	37 ± 21
Single-vessel		11	41 ± 24
Double-vessel		8	35 ± 19
	(−)	21	47 ± 20
Diabetes mellitus	(+)	13	42 ± 29
	(−)	27	43 ± 17
Hypertension	(+)	7	40 ± 27
	(−)	33	43 ± 20
Hypercholesterolemia	(+)	16	34 ± 16[a]
	(−)	24	49 ± 23
Smoking status	(+)	23	43 ± 25
	(−)	17	41 ± 16
Family history of CAD	(+)	5	48 ± 27
	(−)	35	42 ± 20

[a] $p<0.03$ vs. hypercholesterolemia (−).
Abbreviations: NOx=nitrite/nitrate, CAD=coronary artery disease.

The patients with CAD showed a trend toward lower NOx levels compared with the patients without CAD, but the difference was not statistically significant. There was no difference in the level of plasma NOx between gender, diabetes mellitus, hypertension, smoking status, and a family history of CAD. However, the NOx level was significantly lower in patients with hypercholesterolemia than in patients with normal cholesterol. It was also confirmed by a stepwise multivariate regression analysis that the factors of gender, age, weight, serum creatinine, diabetes mellitus, hypertension, smoking status, and a family history of coronary artery disease were not related to the NOx levels ($F = 0.52, 0.45, 0.02, 0.01, 0.04, 0.09, 0.12, 0.34$, respectively).

Relation between NOx and Lipid Levels

In order to ascertain which lipoprotein fractions are related to plasma NOx levels, we further analyzed the lipid profile of the study population. Mean triglyceride, total cholesterol, LDL cholesterol, HDL cholesterol, and lipoprotein(a) were 159 ± 97 mg/dl (12 to 563 mg/dl), 213 ± 37 mg/dl (147 to 286 mg/dl), 136 ± 39 mg/dl (49 to 215 mg/dl), 27 ± 10 mg/dl (8 to 51 mg/dl), 32 ± 20 mg/dl (2 to 87 mg/dl), respectively. No patients with familial hypercholesterolemia were included in this study. When the population was divided into two groups using 150 mg/dl as the cutoff point for triglyceride, 150 mg/dl for LDL cholesterol, 40 mg/dl for HDL cholesterol, and 30 mg/dl for lipoprotein(a), the NOx level was significantly lower in the high LDL group than in the low LDL group (high LDL group:

n = 12, 29 ± 16 μmol/l; low LDL group: n = 28, 49 ± 20 μmol/l, $p < 0.005$), but no such difference was observed in other lipid fractions. Moreover, both the total cholesterol and LDL cholesterol levels negatively correlated with plasma NOx levels (Fig. 2), but other lipids had no significant correlation with plasma NOx levels (Table II).

Discussion

NOx in plasma is the primary metabolite of NO. This has been reported previously by several laboratories.[4, 12, 13] Exogenous nitrogen obtained by food ingestion or inhalation of ambient air is also the source of plasma nitrate. NOx is primarily excreted from the kidney with a half life of 3–5 h,[14] and the level of plasma NOx is determined by the balance between rates of production and excretion. As a result, nonelective blood sampling showed variable plasma NOx levels; it was found, however, that a sampling from restricted healthy volunteers in the early morning yielded a stable NOx level. The variation observed between individuals under these conditions may indicate the difference in basal level of accumulated endogenously and exogenously derived NOx. Specifically in inpatients, the NOx level should reflect only the differences in the basal level of endogenously derived NOx, because exogenously derived NOx may show little individual variation due to similar exogenous nitrogen intake or physiologic activities.

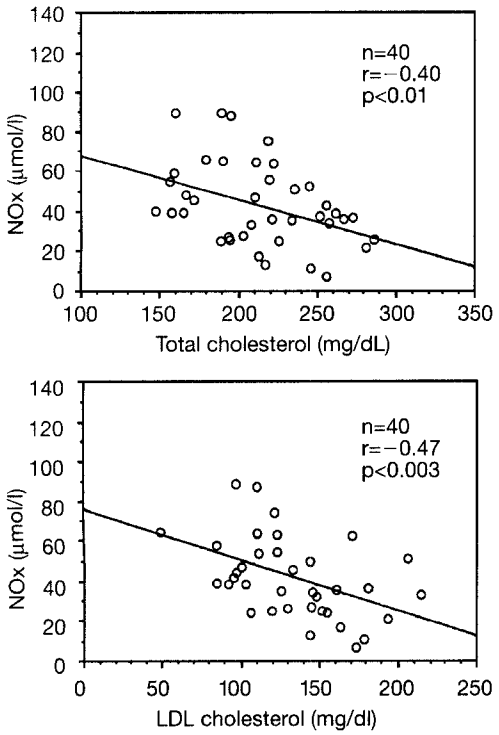

FIG. 2 Correlation between plasma NOx (nitrite/nitrate) levels and total cholesterol levels or LDL (low-density lipoprotein) cholesterol levels.

TABLE II Correlation between plasma levels of NOx and lipids (n=40)

Lipid component	r-Value	p-Value
HDL cholesterol	−0.18	NS
Triglyceride	0.01	NS
Apolipoprotein B	−0.29	NS
Apolipoprotein AI	−0.01	NS
Lipoprotein (a)	−0.14	NS

Abbreviations: NOx = nitrite/nitrate, HDL = high-density lipoprotein, NS = not statistically significant.

Since NOx is expected to be distributed in the whole extracellular space, it should be considered that measured plasma levels would be underestimated.[14]

Nitric oxide is biosynthesized in several cell types.[15] Although we cannot identify the sites responsible for NO synthesis by measuring plasma NOx, as suggested by Wennmalm,[16] it may be assumed that the vascular endothelium, weighing around 1.5 kg and thus being the largest endocrine organ in the body, is the source of a considerable part of the total NO synthesis. Thus, plasma NOx levels of inpatients in the early morning should be useful for the rough evaluation of basal NO generation by endothelial cells.

The present study was initially conducted based on the speculation that basal levels of plasma NOx may be decreased in patients with CAD. However, the level observed in those patients was not significantly lower than that in patients without CAD. When the NOx level of patients with CAD is compared with our previously assessed basal NOx level of healthy controls (45 ± 13 μmol/l, n = 51; age: 45 ± 7; unpublished data), a significant difference is again not obtained. Since the coronary blood flow is only 5% of cardiac output and the resultant NOx would be diffused to the whole extracellular space, it may be supposed that the basal plasma NOx, which may reflect the basal whole endothelium-derived NO, only superficially reflects the local NO generation from the coronary vascular bed. With respect to hypertension, diabetes mellitus, and smoking, which are also related to the impaired EDRF activity, it remains unclear whether the NO generation is decreased in the presence of these disorders, particularly in hypertension; diminished, unchanged, and even increased NO generation has been reported.[17–19] The extracellular distribution might obscure the small steady changes of NOx associated with these disorders.

It is interesting that, in contrast, the basal plasma NOx level was decreased in hypercholesterolemic patients; moreover, in all patients NOx levels correlated negatively with LDL levels. There are many lines of evidence to show that EDRF activity is reduced in hypercholesterolemic patients. Recently, attention has been focused on superoxide anion in explaining this reduced activity.[8] Superoxide anion scavenges NO to an active intermediate of peroxinitrite, and eventually NO is thought to be metabolized to NOx. Such an accelerated NO destruction might have led to the plasma NOx elevation observed in this

study. However, the amount of NOx metabolized may primarily depend on the production rate of NO by endothelial cells. Moreover, so far there have been few in vivo reports showing an elevated NO generation in hypercholesterolemia.

On the other hand, Drexler *et al.* have demonstrated that hypercholesterolemia reduces the availability of an NO substrate, L-arginine.[6] Another group showed that oxidized LDL inhibits NO synthesis by degenerating endothelial G protein or G protein-dependent pathways.[7] More recently, Liao *et al.* and others have shown that oxidized LDL decreases the expression of endothelial NO synthase.[20,21] All these reports indicate that NO generation is decreased in hypercholesterolemia. These in vitro findings, taken together with our results, indicate that the decreased NOx levels observed in the present study may reflect the reduced endothelium-derived NO generation in hypercholesterolemic patients. Similarly, Böger *et al.* demonstrated that urinary NOx excretion is decreased in cholesterol-fed rabbit showing impaired endothelium-dependent relaxation.[22] Our previous observation that cholesterol-fed rabbit showed lower plasma NOx levels than controls (unpublished data) also supports the results presented here. To exclude a possibility of failure in NOx analysis, we confirmed that LDL does not modify the results of NOx analysis based on the Griess reaction (data not shown).

Conclusion

In the present study, we regarded NOx levels in the early morning as a basal level in inpatients and suggested the possibility that NO generation is decreased in hypercholesterolemia. However, several issues, for example, the source of plasma NOx, the entire metabolic pathway of NO, and NOx distribution in the whole body, have yet not been fully understood. Therefore, to estimate NO generation by plasma NOx more precisely, further studies to examine the blood flow of a specific organ or the whole body and arterial-venous NOx difference will be needed.

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