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Hideaki Higashino
Department of Pharmacology,
Kinki University School of
Medicine,
372-2, Ohno-Higashi,
Osaka-Sayama,
Osaka 589-8511, Japan

Serum Nitric Oxide Metabolite Levels in Groups of Patients with Various Diseases in Comparison of Healthy Control Subjects

^{1,2}H. Higashino, ¹M. Tabuchi, ³S. Yamagata, ¹T. Kurita,
²H. Miya, ²H. Mukai and ²Y. Miya

Since Nitric Oxide (NO) is produced by three types of Nitric Oxide Synthases (NOSs), rapid changes in stable oxidized metabolites (nitrite and nitrate, NOx) in the tissues and blood should be represented by the amount of stable forms in serum and may reflect vascular activities and circulatory or inflammatory changes in the body. Therefore, serum NOx levels in patients with various diseases were measured and compared to healthy controls. Four hundred and sixty five in- and outpatients aged 14 to 96 years were included in this study and 49 healthy hospital workers were included in the control group. The NOx levels of both groups were measured at rest in the morning using an ozone chemiluminescence method. When compared with the control group, serum NOx levels were higher in patients consist of around 40 or more in numbers diagnosed with cardiovascular diseases including myocardial infarction, hyperlipaemia, gastrointestinal diseases including acute enteritis, chronic liver diseases including viral B and C type hepatitis and liver cirrhosis, diabetes in males, hypertension with comorbid diseases. In addition, patient groups with renal disorders, hyperuricemia, osteoporosis, untreated cancers, autoimmune diseases such as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) were also higher, although the each number might be not enough to compare between the groups. Patient groups with acute and chronic inflammation had significantly higher serum NOx levels. Therefore, measurement of serum NOx in patients may be useful for understanding the status and pathophysiology of inflammatory diseases and those in which inflammation is a component.

Key words: Nitric oxide metabolites (NOx), serum, patients, inflammatory diseases, pathophysiology

¹Department of Pharmacology, Kinki University School of Medicine, Osaka, Japan

²Department of Internal Medicine, Higashi-Yodogawa Hospital, Osaka, Japan

³Department of Respiratory and Allergic Medicine, Kinki University School of Medicine, Osaka, Japan

INTRODUCTION

Since Nitric Oxide (NO) is produced by three types of Nitric Oxide Synthases (NOSs), rapid changes in stable oxidized metabolites (nitrite and nitrate, NOx) in the tissues and blood should be represented by the amount of stable forms in serum and may reflect vascular activities and circulatory or inflammatory changes in the body (Van den Hove *et al.*, 2002). NO is produced in all tissues and organs by constitutive NOS (eNOS), which includes endothelial NOS (eNOS; isoform III) and neuronal NOS (nNOS; isoform I) and inducible NOS (iNOS; isoform II) (Förstermann *et al.*, 1994). Therefore, pathophysiological changes such as atherosclerosis with coronary artery diseases (Matsubara *et al.*, 2003; Ekmekçi *et al.*, 2006), endothelial dysfunction (Akarasreenont *et al.*, 2001), pro-inflammation and inflammation seen in various diseases (Kawashima *et al.*, 2004; Oekonomaki *et al.*, 2004; Kamiya *et al.*, 2005; Loukovaara *et al.*, 2005) may be to some extent studied by measuring NO metabolites in the peripheral blood (Chou *et al.*, 1998; ter Steege *et al.*, 1998a, b; Choi, 2003). Our previous findings from hypertensive patients (Higashino *et al.*, 2006) showed that high blood pressure does not directly affect the serum NOx levels and that serum NOx levels may be linked to the developmental stages of hypertensive patients with co-morbidities. We have now done a more extensive study that includes patients with many different types of diseases to understand more about the pathophysiological changes such as atherosclerosis, endothelial dysfunction and diseases involving inflammation that might be reflected in NOx levels, compared with those of the healthy control.

MATERIALS AND METHODS

Study design: In brief, serum NOx levels were obtained from 465 (207 males aged 14-88 years and 258 females aged 18-96 years) in and outpatients from whom blood samples were collected. To avoid adverse effects on patients, those receiving various drug treatments were not eliminated from the study to survey the involvement of NO in the diseases as the first step of study, even though some drugs may affect NO producing or eliminating processes and could change the pathophysiological status of some patients. The hospital was a common private one with 200 beds in size which mainly treated internal diseases such as cardiovascular, digestive, metabolic, inflammatory and immune ones at urban Osaka in Japan. Serum NOx levels were also obtained from 49 healthy hospital workers during their regular legal health check. No pregnant female was included in this study. This experiment to measure NOx levels in the serum of

patients was started at March 12, 1998 and ended at July 17, 2005. Measurements were made at rest for both groups with an ozone chemiluminescence method using a Nitric Oxide Analyser (FES-450; Scholartec, Osaka, Japan). Permission to use blood samples for our experimental use was obtained from all subjects. Consent was obtained from the subjects after explanation of the non-therapeutic use and their signatures were placed on the agreement form by their own will. Furthermore, permission to conduct this study was granted by the ethics committee review board in the hospital as a third-party organization.

The healthy control group: Forty-nine volunteer hospital workers (8 males and 41 females, average age: 37.1 ± 1.8 years, range: 21-61 years) without any clinical abnormalities were included in the control group after their authorized health checks. Clinically normal values are: blood pressure ($<140/90$ mmHg), serum total cholesterol (T-cholesterol; <220 mg dL⁻¹), serum glucose (<126 mg dL⁻¹), serum triglyceride (<150 mg dL⁻¹), serum creatinine (<1.0 mg dL⁻¹), serum uric acid (<6.5 mg dL⁻¹) and serum alanine aminotransferase (ALT; <50 I.U. L⁻¹).

Disease group criteria: Each patient with given diseases was assigned to one or two patient groups (Table 1).

Analysis of NOx concentration in serum: Blood was drawn from the median vein of the upper limb into a vacuum collecting tube at early morning before medication and smoking after overnight fasting of at least 10-12 h to avoid the influence of drugs, smoking and nitro-compounds from food. The concentrations of NOx (nitrite and nitrate) in the serum were measured as described in our previous report (Higashino *et al.*, 2006). Briefly, the serum was ultrafiltered using Ultrafree-MC: Biomax-10 filters (Millipore, Bedford, MA, USA) and then, NO₃⁻ in the filtrate was reduced to NO₂⁻ with nitrate reductase (Dojindo, Kumamoto, Japan). NO₂⁻ in the 50-100 µL solution was converted into NO gas with a saturated-ascorbic acid solution and finally NO gas was placed into a Nitric Oxide Analyzer FES-450 (Scholartec, Osaka, Japan) and measured using the ozone chemiluminescence method. All glass and plastic tubes and containers used for sample preparation were carefully washed out with enough volume of deionized water to avoid contamination.

Analytical methods: Data expressed as the mean (SEM) were compared with an Analysis of Variance (ANOVA) test (Girden, 1992) of the various diseases, followed by Fisher's Protected Least Significant Difference (PLSD) method (Agresti, 1992) as a post-hoc test. Correlation and

Table 1: Criteria of disease groups

Disease groups	Criteria ages (No. of patients; Male: Female)
1: Cardiovascular diseases	Experienced or recovered from myocardial infarction, angina pectoris, heart failure, or aortic aneurysm 75.6±1.7 years (n=39; 21:18)
2: Hyperlipaemia	Serum T-cholesterol level >240 mg dL ⁻¹ , serum triglyceride level >150 mg dL ⁻¹ , or LDL >140 mg dL ⁻¹ , 68.2±1.7 years (113; 46:67)
3: Gastrointestinal diseases	1: Gastritis with symptoms such as gastric pain, nausea, or uncomfortable epigastric sensation 2: Acute enteritis with symptoms such as abdominal pain, diarrhea, with or without positive endoscopic and/or abnormal X-ray findings, and/or leukocyte count >10,000 mm ⁻³ , CRP >1 mg dL ⁻¹ in the peripheral blood, 54.3±2.7 years (41; 15:26)
4: Hepatitis and cirrhosis	Serum alanine aminotransferase (ALT) >100 IU L ⁻¹ , HBs-Ag(+), HCV-Ab(+), or findings of liver cirrhosis 68.0±1.4 years (91; 62:29)
5: Diabetes	Serum glucose level >126 mg dL ⁻¹ at fasting or >200 mg dL ⁻¹ at any time, and/or hemoglobin A1c level > 6.5%, 66.1±1.3 years (62; 35:27)
6: Hypertension	Blood pressure >140/90 mmHg at rest/sitting position, 70.7±0.8 years (175; 79:96)
7: Immune diseases	Autoimmune diseases and allergies, 64.8±2.6 years (44; 8:36)
8: Respiratory diseases	Any respiratory system dysfunction including asthma, bronchitis and COPD, 62.5±3.6 years (48; 25:23)
9: Disease groups seemed to consist of insufficient numbers to compare accurately between the groups	
Renal disorders	Serum creatinine level >2.0 mg dL ⁻¹ with symptoms such as hyperazotemia, edema, proteinuria, anuria, hypertension, anaemia and pericarditis, 74.5±2.7 years (n = 15 ; 9:6)
Hyperuricemia	Serum uric acid level >7.5 mg dL ⁻¹ , 64.2±3.2 years (10; 7:3)
Osteoporosis	Calcium levels <0.5 g cm ⁻² in the radius by a DXA exam, or bone fractures in the vertebrae without any trauma, 76.0±1.9 years (26; 2:24)
Cancers	Having any cancer or experienced cancer, 74.3±1.6 years (15; 11:4)
Thyroiditis	Acute or chronic thyroiditis including Basedow's and Hashimoto's diseases, 67.5±3.4 years (22; 4:18)
Stroke	History of stroke such as hemorrhages, thrombosis, or embolism, 80.0±1.6 years (20; 4:16)
Miscellaneous diseases	Other cases such as common cold, headache and fatigue, 47.7±6.2 years (9; 2:7)

Total 730 diseases or disease combinations in 465 patients: 64.4±1.0 years (n = 207 in male) and 68.0±1.0 years (n = 258 in female). Abbreviations: CRP: C-reactive protein, HB: hepatitis B, HCV: hepatitis C virus, COPD: chronic obstructive pulmonary disease

regression analysis were performed to determine the relationship between the data in a group. The $p < 0.05$ was considered statistically significant.

RESULTS

Healthy control group: The average NO_x level in the serum of the 49 healthy controls was 55.0±2.9 (Range: 23.3-110.5) µmol L⁻¹.

Disease groups: Serum NO_x levels in the 465 patients were measured and compared among the different disease groups (Table 1) and were also compared to the healthy control group. As shown in Fig. 1, patients in the following groups consisted of enough numbers to compare between the groups (more than around 40) showed significantly higher serum NO_x values than healthy control subjects: cardiovascular diseases (n = 39), hyperlipaemia (n = 113), gastrointestinal diseases (n = 41), hepatitis and liver cirrhosis (n = 91), diabetes (n = 62) and hypertension (n = 175). On the other hand, the patients with immune diseases, respiratory diseases showed no significant difference compared to the healthy control subjects.

Cardiovascular disease group: This group consisted of three subgroups that included recovery from myocardial infarction, angina pectoris and atherosclerosis-associated cardiovascular diseases such as heart failure (n = 3) and

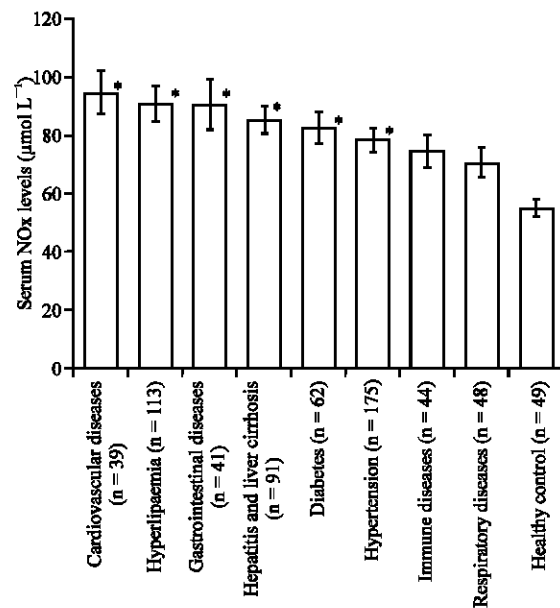


Fig. 1: Comparison of serum NO_x levels in the disease groups consist of enough numbers to compare with healthy control volunteers. Numbers under each column represent the number of cases. *Significant differences of $p < 0.05$ between the levels of each group and healthy control subjects

aortic aneurysm (n = 1) (Fig. 2). In patients with ischemic heart disease, myocardial infarction recovery patients

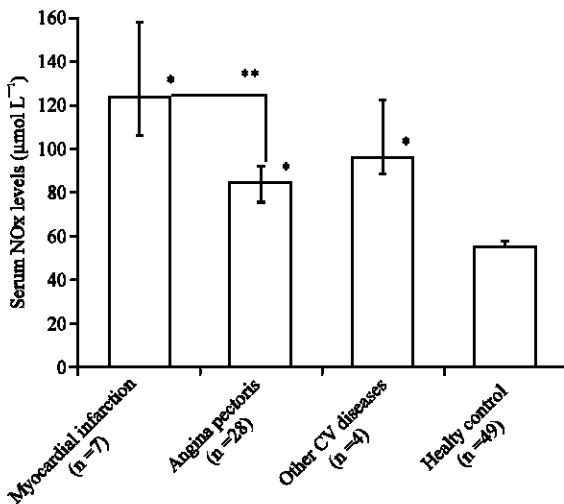


Fig. 2: Comparison of serum NOx levels in cardiovascular disease subgroups and healthy control volunteers. Numbers under each column represent the number of cases. * and ** represent significant differences of $p < 0.05$ between the levels of each group and the healthy control group and between myocardial infarction and angina pectoris, respectively

($123.0 \pm 34.7 \mu\text{mol L}^{-1}$, $n = 7$) showed much higher serum NOx levels ($p < 0.05$) than those with angina pectoris ($83.7 \pm 8.3 \mu\text{mol L}^{-1}$, $n = 28$). There was no significant difference in serum NOx levels between users of nitro-vasodilators (2/7 in myocardial infarction recovery and 9/28 in angina pectoris, $101.2 \pm 14.4 \mu\text{mol L}^{-1}$) and non-users (resting, $n = 24$, $87.0 \pm 12.6 \mu\text{mol L}^{-1}$). The other atherosclerosis-associated cardiovascular disease group ($n = 4$) also showed a higher value ($p < 0.05$) than the healthy control subjects.

Hyperlipaemia group: This group showed a significantly higher serum NOx level (Mean: 90.7 ± 6.1 , Range: $21.8-406.0 \mu\text{mol L}^{-1}$, $p < 0.05$, $n = 113$) compared to healthy control subjects. No significant correlation of NOx levels with triglycerides, T-cholesterol, or blood pressure was found.

Gastrointestinal disease group: This group consist of the diseases having chronic gastritis symptoms such as gastritis ($n = 18$), gastric ulcers ($n = 8$), duodenal ulcers ($n = 2$) and esophagitis ($n = 1$) and acute enteritis ($n = 12$) showed a higher serum NOx level ($90.2 \pm 8.7 \mu\text{mol L}^{-1}$, $n = 41$, $p < 0.05$) than control subjects. The patients with acute enteritis caused by various bacterial infections in this group showed remarkably higher NOx levels ($122.6 \pm 22.5 \mu\text{mol L}^{-1}$, $n = 12$, $p < 0.05$)

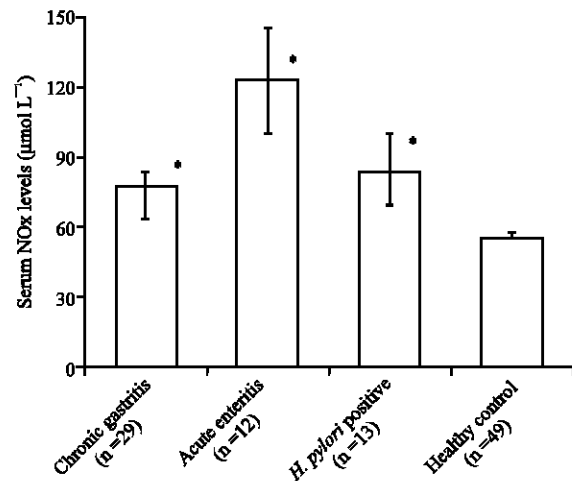


Fig. 3: Comparison of serum NOx levels in gastrointestinal disease subgroups and healthy control volunteers. Numbers under each column represent the number of cases. * Significant differences of $p < 0.05$ between the levels of each group and healthy control subjects

than the chronic gastritis group. Using a serum screening test, *Helicobacter (H.) pylori* antibody-positive patients without any gastric symptoms (13 cases among 41 examined patients) exhibited higher serum NOx levels ($83.4 \pm 16.2 \mu\text{mol L}^{-1}$, $p < 0.05$) than control subjects (Fig. 3).

Hepatitis and liver cirrhosis group: Patients in this group had chronic hepatitis B ($n = 10$), C ($n = 47$), or liver cirrhosis ($n = 18$) and showed a higher serum NOx level (82.9 ± 12.6 , 92.5 ± 7.4 and $78.0 \pm 7.8 \mu\text{mol L}^{-1}$, respectively, $p < 0.05$) than controls (Fig. 4). In the patients with liver cirrhosis, the disease origin was HBV, HCV, or unknown ($n = 2$, 11.1%; $n = 10$, 55.6% and $n = 6$, 33.3% of the 18 total cases, respectively).

Diabetes group: The diabetes group had a higher serum NOx level ($82.5 \pm 5.7 \mu\text{mol L}^{-1}$, $n = 62$, $p < 0.05$) than the control group (Fig. 1). We examined 33 out of 62 cases in which HbA1c levels were available and observed a significant correlation between serum NOx and HbA1c levels in male patients ($r = 0.513$, $p < 0.05$, $n = 19$ in Fig. 5), but not in female patients ($r = 0.324$, $p > 0.25$, $n = 14$). However, there was no correlation between serum NOx and glucose levels.

Hypertension group: The hypertension group ($n = 175$) showed a significantly higher serum NOx level compared with healthy controls. However, hypertensive patients ($n = 13$) with no complications such as diabetes mellitus,

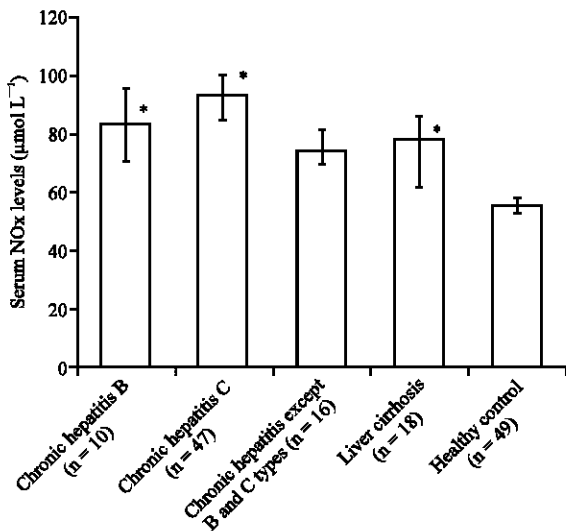


Fig. 4: Comparison of serum NOx levels in groups with hepatitis and liver cirrhosis and healthy control volunteers. Numbers under each column represent the number of cases. *Significant differences of $p < 0.05$ between the levels of each group and the healthy control subjects

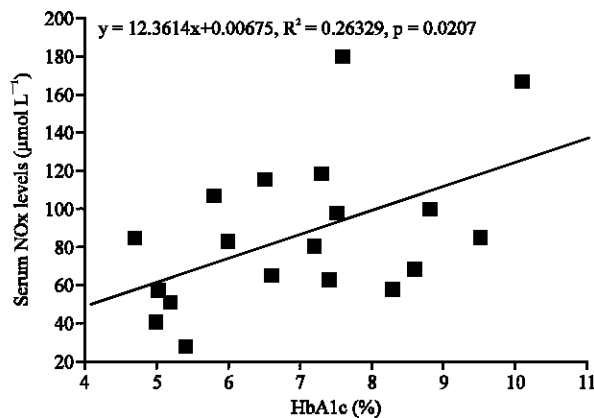


Fig. 5: Relationship between serum NOx and HbA1c levels in male ($n = 19$) patients with diabetes mellitus. The straight line is the regression line of the two variables. A significant correlation was observed between the two variables in the male group at $p < 0.05$

hyperlipaemia, or renal disorder showed no difference in serum NOx levels when compared with the healthy group (Higashino *et al.*, 2006).

Immune disease group: Serum NOx levels in the immune disease group ($n = 44$) showed no significant difference compared with the healthy controls. Among the three

subgroups consisting of patients with rheumatoid arthritis and SLE ($n = 22$, Group A), bronchial asthma ($n = 13$, Group B) and skin or food allergies ($n = 4$, Group C), Group A showed significantly higher serum NOx levels compared with Groups B and C (85.9 ± 9.7 vs. 65.6 ± 6.9 and 55.7 ± 14.4 $\mu\text{mol L}^{-1}$, $p < 0.05$, respectively).

Respiratory diseases: The respiratory disease group ($n = 48$), which consisted of patients with chronic bronchitis ($n = 25$) and bronchial asthma ($n = 23$), showed no difference in the serum NOx level (70.5 ± 5.0 $\mu\text{mol L}^{-1}$) compared to the healthy group.

Other disease groups might consist of insufficient numbers to compare accurately between the groups: The groups consisted of below 40 cases might be not enough to compare statistically in accurate between the groups. But, those findings might offer useful information for understanding the role of NOx.

Renal disorder group: The renal disorder group showed significantly higher NOx level (Mean: 100.6 ± 13.6 , Range: 28.7 - 216.4 $\mu\text{mol L}^{-1}$, $n = 15$, $p < 0.0001$) than the healthy control subjects. Six patients with renal failure showed much higher values (121.6 ± 23.2 $\mu\text{mol L}^{-1}$) in this group.

Hyperuricemia group: The hyperuricemia group ($n = 10$) had a serum uric acid level over 7.5 mg dL^{-1} upon diagnosis and showed a higher serum NOx level (89.6 ± 21.3 $\mu\text{mol L}^{-1}$, $n = 10$, $p < 0.05$) than healthy subjects.

Osteoporosis group: The osteoporosis group had calcium levels less than 0.5 g cm^{-2} in the radius as shown by a DXA exam, or had experienced vertebral bone fractures without trauma. This group showed a significantly higher serum NOx level (89.2 ± 11.9 $\mu\text{mol L}^{-1}$, $n = 26$, $p < 0.05$) compared with the healthy control group.

Cancer group: The cancer group ($n = 15$) as a whole showed a similar NOx level (81.6 ± 17.1 $\mu\text{mol L}^{-1}$) as the healthy controls. However, when comparing (a) the non-operative, untreated cancer group ($n = 8$; colon (2), breast (1), lung (1), liver (1), gallbladder (1), pancreas (1) and prostate cancers (1)) with (b) the post-operative, treated cancer group ($n = 7$; colon (4), liver (1), gallbladder (1) and bladder cancers (1)), the former group (a) showed a significantly higher NOx level than the latter group (b) (113.4 ± 27.4 vs. 45.3 ± 7.3 $\mu\text{mol L}^{-1}$, respectively). In particular, a patient with progressive breast cancer showed the highest value (287 $\mu\text{mol L}^{-1}$) in the cancer group.

Thyroiditis group: This group (n = 22) showed no significant difference in the serum NOx level compared with healthy controls.

Stroke group: The brain stroke group (n = 20), including patients with symptoms such as cerebral hemorrhage, thrombosis and embolism, showed no difference in the serum NOx level ($74.3 \pm 13.4 \mu\text{mol L}^{-1}$) compared to the healthy group.

Miscellaneous diseases: Nine patients with mild symptoms such as the common cold, headache, or fatigue without any severe symptoms showed a serum NOx level of $62.9 \pm 9.0 \mu\text{mol L}^{-1}$, which was almost the same as in the healthy control subjects.

DISCUSSION

Serum NOx levels were distributed widely from healthy normal to significantly higher levels depending on the disease groups in this study. In these differences, each reason why serum NOx elevate to higher levels should exist in each disease. From these above findings and related-references so far, therefore, the following speculations and presentations were introduced.

The role of serum NOx levels in patients with Coronary Artery Disease (CAD) is still controversial. The reports so far show: (1) In contrast to our data, there was no difference in the serum NOx levels between CAD subgroups (Ekmekçi *et al.*, 2006), (2) the CAD group had a significantly lower NO/endothelin-1 (ET-1) ratio and (3) monitoring the state and severity of the disease could be informative since they found increased NOx levels in patients with CAD, compared to control subjects (Akarasereenont *et al.*, 2001). Other types of heart disease such as myocardial ischemia (Node *et al.*, 1996) and dilated cardiomyopathy (Orús *et al.*, 2000) also showed higher serum NOx levels. In our findings, serum NOx levels in patients recovering from myocardial infarction were higher than in patients with angina pectoris, other cardiovascular diseases derived from heart failure, aortic aneurysm and healthy controls. In contrast to our data, when compared with the healthy controls, no difference or lower NOx levels in the CAD group overall were noted (Ekmekçi *et al.*, 2006; Kurita *et al.*, 2005). Thus, the NO release disorder may occur in the endothelium of patients with CAD. However, we obtained significantly higher values (over $70 \mu\text{mol L}^{-1}$) from the angina pectoris group, compared with the healthy control subjects. Therefore, myocardial infarction recovery, treated angina pectoris and other cardiovascular diseases may exhibit increased iNOS activity as a result of vascular inflammation,

although recovery of the release of endothelial NO does not occur. Thus, measurement of serum NOx may provide valuable information about the state or severity of the disease.

The hyperlipaemia group showed a significantly higher level of serum NOx compared with that of the healthy subjects. The following observations have been reported about the relation between serum NOx levels and hyperlipaemia: (1) hypercholesterolemic patients showed a lower ratio of plasma guanosine 3',5'-monophosphate (cGMP) to NOx and the ratio was maintained with treatment with a 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor (Ungvari *et al.*, 2004), (2) basal endothelium-derived NO synthesis may be decreased in patients with hypercholesterolemia with coronary artery diseases (Orlandi *et al.*, 2004), (3) patients with coronary artery disease and multiple comorbid diseases such as hyperlipaemia have higher serum NOx levels (Wang and Fitch, 2004) and (4) patients with familial hypercholesterolemia type II showed higher serum NOx levels, which decreased after treatment with an HMG-CoA reductase inhibitor (Duric *et al.*, 2003). However, these findings are still incompletely understood. In our previous report regarding hypertension and its complications (Higashino *et al.*, 2006), we speculated that the inflammatory changes in the endothelium of arteries associated with hypercholesterolemia or hyperlipaemia in middle-aged patients would elevate NO production via induction of iNOS, but not eNOS. Because of this observation, measuring serum NOx levels in hyperlipaemia patients with or without hypertension may assist in monitoring the state and severity of hyperlipaemia-induced coronary artery disease, which has been suggested by Akarasereenont *et al.* (2001).

Our results from patients with acute enteritis symptoms such as acute abdominal pain, diarrhea, fever, and/or pathological increases in leukocytes and C-reactive Protein (CRP) in the peripheral blood with or without pathogens in clinical tests are consistent with other reports also showing higher serum NOx levels in bacterial or non-bacterial enteritis (Kawashima *et al.*, 2004; Rees *et al.*, 1995; Parasher *et al.*, 2001; ter Steege *et al.*, 1998b; Ukawa *et al.*, 1998; Ikeda *et al.*, 1997). Thus, obtaining serum NOx levels from patients with abdominal symptoms due to acute enteritis would likely be informative. Slightly higher serum NOx levels than in healthy controls were observed in patients with chronic gastritis with frequent gastric symptoms such as gastric pain, nausea and an epigastric uncomfortable sensation, regardless of whether the patients were *Helicobacter Pylori* (*H. pylori*)-positive, negative, or unknown. Chronic gastritis is considered to be an inflammatory

disease, as reported by Yasuhiro *et al.* (1997) and Kamiya *et al.* (2005). Likewise, chronic *H. pylori* infection is known to be the most important factor causing stomach inflammation and our experiment showed higher serum NOx levels in *H. pylori*-positive patients (13 out of 41 cases of the group) even in the absence of any other gastric symptoms.

Patients with chronic viral hepatitis types B (n = 10) and C (n = 47) and liver cirrhosis caused by any types of hepatitis (n = 18) showed higher serum NOx levels compared with healthy controls. Several studies have reported that chronic hepatitis (Mihm *et al.*, 1997) and liver cirrhosis (Oekonomaki *et al.*, 2004; Mizumoto *et al.*, 1997) caused by Hepatitis C Virus (HCV) infection and lipopolysaccharide or drugs (acetoaminophen and carbon tetrachloride) (Kamanaka *et al.*, 2003) showed increases in both iNOS activity and serum NOx. In our study, chronic hepatitis other than HBV or HCV infection did not show higher serum NOx levels compared with healthy controls. Therefore, detection of serum NOx levels could be informative in determining the cause of chronic hepatitis.

Patients with diabetes showed higher serum NOx levels compared with healthy controls and a positive correlation between serum NOx and HbA1c levels in male patients was observed (Fig. 5). In our previous report (Higashino *et al.*, 2006), we showed significantly higher NOx levels in male hypertensive patients over 40 years with diabetes mellitus or hyperlipaemia compared with normotensive control subjects. Thus, it is possible that the aging effects in the circulatory system that are associated with diabetes will occur faster and be more readily apparent in males than in females. Insulin deficiency in diabetes may be related to the overproduction of NO, as shown by increased serum NOx levels in type 1 and type 2 diabetes patients complicated by proliferative diabetic retinopathy (Loukovaara *et al.*, 2005; Ozden *et al.*, 2003). More investigation of this phenomenon is needed for further conclusions.

As described in our previous report (Higashino *et al.*, 2006), serum NOx levels were not related to blood pressure levels. When hypertensive patients had complications such as diabetes, hyperlipaemia, or renal disorder, their serum NOx levels were elevated, especially in the male hypertensive patients. In those patients, the hypertensive group showed higher levels of serum NOx when compared with healthy subjects.

The patients in the immune disease group (n = 44) have diverse diseases and symptoms and showed no difference in serum NOx levels compared with healthy controls. However, patients in the subgroup of rheumatoid arthritis and SLE showed significantly higher serum NOx levels compared to those with bronchial

asthma, skin and food allergies and healthy subjects. As reported previously (Choi, 2003; Akarasereenont *et al.*, 2001), typically severe autoimmune diseases such as rheumatoid arthritis and SLE showed higher serum NOx levels, which is probably due to iNOS induction accompanied by several types of inflammatory cytokines.

The respiratory disease group with chronic bronchitis and COPD showed no differences in serum NOx levels compared with healthy controls. Therefore, neither severe immunological nor inflammatory changes enough to influence the serum NOx level might be linked to the course of this disease.

The following groups consisted of below 40 cases might be not enough to compare statistically in accurate between the groups. But, those findings may be useful for understanding the role of NOx as follows.

The higher NOx levels seen in the 14 disease groups studied were in patients with renal disorders such as chronic nephritis, nephrosis, renal sclerosis and renal failure. Our previous report (Higashino *et al.*, 2006) indicated that male hypertensive patients with renal disorders showed significantly higher NOx levels compared with hypertensive patients without renal disorders. Although, patients with puromycin aminonucleotide-induced nephritic syndrome or nephrectomized rats showed reduced iNOS, nNOS and eNOS protein and had reduced renal NOx excretion (Ni and Vaziri, 2003), the iNOS activity in glomeruli was increased in experimental glomerulonephritis (Datta *et al.*, 2006), as well as in patients with active proliferative lupus nephritis (Oates *et al.*, 1999). Cultured endothelial cells from the uremic plasma showed increased basal NO release (Thuraisingham and Yaqoob, 2003) and plasma NOx was increased in hepatorenal syndrome patients (Lluch *et al.*, 2006) and in Heymann nephritis rats (Koroliczuk *et al.*, 2001). A significant correlation between serum NOx and plasma creatinine levels was also reported (Mackenzie *et al.*, 1996); however, we found no such correlation in our data (n = 15, r = 0.264, p = 0.362). Consequently, patients with nephropathy and chronic renal disease were shown to have increased levels of iNOS, eNOS, or nNOS protein, producing a large amount of NO that reduces sodium sensitivity and the occurrence of proteinuria and also interferes with the progress of the disease (Konishi *et al.*, 2004; Matsuda *et al.*, 2003).

The hyperuricemia group (n = 10) showed higher serum NOx levels than healthy subjects. Uric Acid (UA) induces CRP expression, but inhibits NO release (Kang *et al.*, 2005) in human umbilical vein endothelial cells. Hyperuricemic rats show a decrease in serum NOx. Allopurinol, which is a xanthine oxidase inhibitor, reversed the pathophysiological changes of

hyperuricemia in these animals (Khosla *et al.*, 2005). These observations are in contrast to ours. Hyperuricemia has been thought to be associated with hypertension, systemic inflammation and cardiovascular disease in relation to endothelial NO dysfunction. We considered patients with hypertension (n = 3), hepatitis or fatty liver (n = 3), hyperlipaemia (n = 2), or gastritis (n = 2) as having complications, which may contribute to higher NOx levels through iNOS activation. More cases must be studied to provide more definite conclusions regarding this idea.

The osteoporosis group showed a significantly higher serum NOx level compared with the healthy control group. A few studies have reported serum NOx levels in osteoporosis, but they are controversial. The NO donors isosorbide mononitrate (Nabhan, 2006) and nitroglycerine (McFarlane *et al.*, 2004) may be useful for prevention of post-menopausal osteoporosis and the NO donor L-arginine appears to prevent glucocorticoid-induced osteoporosis in rats (Pennisi *et al.*, 2005). On the other hand, a negative correlation was observed between serum NOx and lumbar bone mineral densities and serum NOx levels were also not correlated with axial bone mineral densities (Sahin *et al.*, 2006). Most of the female patients in the osteoporosis group (24 females aged 75.2±2.2 years) had post-menopausal osteoporosis. Thus, it is likely that NO is overproduced through iNOS induction caused by inflammation occurring during the course of osteoporosis. Therefore, over-production of NO by iNOS as measured by serum NOx levels may reflect the degree of inflammation occurring during the course of osteoporosis.

The cancer patient group did not show higher serum NOx levels compared with healthy controls. However, when comparing the non-operative or untreated patient subgroup with the post-operative or treated patient subgroup, the former showed significantly higher NOx levels than the latter. It has been reported that patients with secondary pleural metastases (Timoshenko *et al.*, 2002), hepatocellular carcinoma (Moriyama *et al.*, 2000; Moussa *et al.*, 2000) and colorectal carcinoma (Szaleczky *et al.*, 2000) showed higher serum NOx levels and inter-individual differences in serum NOx levels were noted (Timoshenko *et al.*, 2002). Therefore, measuring serum NOx levels may to some extent be informative for the diagnosis and prognosis of cancer.

Neither Basedow's disease, hyperthyroiditis, nor Hashimoto's thyroiditis (n = 18, primarily hypothyroiditis), showed significantly different serum NOx levels compared with healthy controls. This finding is consistent with results showing that eNOS and iNOS levels do not change in thyroid disorders, even though NO is important

for the function and growth of thyroid epithelial cells (Kayser *et al.*, 2000; Colin *et al.*, 1997).

The stroke and other miscellaneous groups with different etiologies showed no differences in serum NOx levels compared with healthy controls. Therefore, neither severe immunological nor inflammatory changes enough to influence to the serum NOx level will be linked to the course of these diseases.

CONCLUSIONS

An increase in NO production during the course of diseases such as cardiovascular diseases, hyperlipaemia, gastrointestinal diseases, chronic viral hepatitis and liver cirrhosis, diabetes in male and hypertension may be accelerated through iNOS induction, probably due to a variety of cytokines, as described in several reports (Chou *et al.*, 1998; ter Steege *et al.*, 1998a, b; Van den Hove *et al.*, 2002). In addition, patient groups with renal disorders, hyperuricemia, osteoporosis, untreated cancers, autoimmune diseases such as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) were also higher, although the each number might be not enough to compare in accurate between the groups. Therefore, measuring serum NOx levels in patients with these particular diseases may be informative in understanding the causes, status and prognosis of the diseases.

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