Introduction

Since the discovery of the endothelium relaxing factor (EDRF) (FURCHGOTT and ZAWADZKI, 1980) and the identification of it as nitric oxide (NO) (MONCADA and HIGGS, 1993; IGNARO, et al., 1987), numerous papers concerning NO have been published because it has a very important role in cardiovascular control and others (VANHOUTTE and MOMBOULI, 1996; CELERMAJER, 1997; COOKE and DZAU, 1997). However, most of them are indirect findings, such as response to the

Plasma Nitrate/Nitrite Concentration in Healthy Population and Patients with Diabetes Mellitus - Relationships with Gender, Aging and Diabetic Complications -

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ABSTRACT

Although there are numerous papers concerning nitrite/nitrate (NOx), most of them are indirect evaluations with acetylcholine or arginine analogue. Measuring the plasma concentration of NOx ([NOx]) may be a useful predictor of the vascular endothelial function. We analyzed [NOx] in healthy mass population and investigated the relationship with gender, aging and some diabetic complications. The subjects were 738 healthy volunteers (512 males and 226 females), who received annual medical check and were considered healthy based on physical and laboratory findings, and 289 patients with type 2 diabetes mellitus attending our hospital, a total of 1,027 subjects. [NOx] was measured by chemiluminescence assay (Sivers Inc. Model 280). The results at the fasting stage were as follows: the mean value of [NOx] in the healthy subjects was 47.5 ± 26 (M/L), and the ratio of nitrite to nitrate was approximately 1:6. After an intake of celery containing rich nitric compounds, [NOx] markedly increased to 2.5 times, mainly because of an increase in nitrate. In the male subjects, [NOx] gradually increased with aging, whereas in the females, it tended to decrease until the menopausal stage but turned upward after that. [NOx] in the group without any diabetic complication was much lower than that in the control group (P < 0.01), but the group with coronary artery disease showed a higher value (P < 0.001). In conclusion, [NOx] was influenced by exogenous factors, aging, and difference of gender, and showed some correlations with hyperglycemic vascular impairments.

Key Words: Nitric Oxide, Endothelial Function, Aging, Gender, Diabetes Mellitus
inhibitor of NO synthesis - arginine analogue - or to the stimulators of NO release - acetylcholine and bradykinin (EGASHIRA et al., 1993; OTSUJI et al., 1995; KUGA et al., 1997). These situations may be due to the difficulty in direct NO measurement (ARCHER, 1993); specifically, NO is a gaseous and unstable substance and consequently reacts readily with oxygen, yielding nitrite (NO$_2^-$) or nitrate (NO$_3^-$) called nitric oxides (NOx). The direct measurement of the value of plasma NOx ([NOx]) in human beings would enable us to evaluate the basic endothelial function as NO release.

We attempted to clarify the following concerns in this paper: (1) the establishment of a standard [NOx] level in healthy mass subjects and its relationships with gender and aging; (2) influence on [NOx] of the intake of green vegetables containing rich organic nitric compounds, and of cigarette smoking causing endothelial cell impairment; and (3) the relationship between [NOx] and several kinds of diabetic complications.

Methods

Subjects
The total number of subjects was 1,027: 738 consecutive, healthy volunteers (512 males and 226 females) without any disease, and 289 diabetic patients (177 males and 112 females) regularly attending our hospital. This study was approved by the ethical committee of Osaka Medical College, and all of them gave a written informed consent for this study.

Measurement of [NOx]
A blood sample was taken from all of the subjects in the fasting state. [NOx] was measured by chemiluminescence assay (NO analyzer, Sivers Inc., COX RD, 1980). In brief, after centrifugation, plasma was incubated in an airtight tube containing Aspergillus nitrate reductase to reduce nitrate to nitrite, then changed into NO by adding hydrochloric acid. The produced NO was combined with ozone to produce photons, and [NOx] was determined (µM/L).

Influence of celery intake on [NOx]
To observe the influence of taking exogenous nitric compounds on [NOx], ten male volunteers (mean age: 32.3 years) took 150 grams of celery, a kind of green vegetables, and a blood sample was taken from each of them in the fasting state and two hours later.

Relationship with [NOx] between gender and aging
The 728 healthy subjects were divided into the following subgroups according to their ages: twenties (from 20 to 29 years old, n = 79, 28 males and 51 females); thirties (from 30 to 39 years old, n = 134, 71 males and 63 females); forties (from 40 to 49 years old, n = 197, 128 males and 69 females); fifties (from 50 to 59 years old, n = 229, 192 males and 37 females); and sixties (from 60 to 69 years old, n = 89, 83 males and six females).

Cigarette smoking and [NOx]
To elucidate the relationship between cigarette smoking and [NOx], 440 healthy males were divided into a smoker group (SM (+), smoking more than ten cigarettes a day for three years or longer, n = 180) and a nonsmoker group (SM (-), n = 260).

Diabetic complications and [NOx]
Blood sampling from diabetic patients was performed with an interruption of any cardiovascular regimen, especially long-acting nitrates for 24 hours.

The diabetic patients were divided into the following subgroups: Group 1: patients with albuminuria of more than 100 mg a day (Prot., n = 69); Group 2: patients suffered from hypertension (HT, n = 139) with a systolic blood pressure higher than 150 mmHg or a diastolic blood pressure higher than 95 mmHg despite regularly taking antihypertensive agents; Group 3: patients suffered from coronary artery disease (CAD, n = 55) who had coronary events in the past and showed significant stenosis on coronary arteriograms; and Group 4: patients without any kind of complications mentioned above (None, n = 121). The patients having two or more of these complications were included in each subgroup, and to perform an age-matching comparison, 318 healthy subjects were selected as a control group.

Statistical analysis
Data are presented as mean ± SD. An unpaired t test was used to compare the two groups (smoker and nonsmoker), and a paired t test was used for celery intake. Variance analysis (ANOVA) was used to make a comparison between the ages and genders. P < 0.05 was considered statistically significant.
Results

(1) The ratio of nitrite (NO\textsubscript{2}⁻) to nitrate (NO\textsubscript{3}⁻) in [NO\textsubscript{x}] and the influence of celery intake (Table 1) [NO\textsubscript{x}] in ten healthy males was 43.0 ± 18 (\textmu M/L), consisting of 6.4 ± 2.1 of nitrite and 36.6 ± 18.5 of nitrate in the fasting status. [NO\textsubscript{x}] markedly increased to 92.0 ± 14.9 ( \textmu M/L, \textit{P} < 0.01) two hours after an intake of 150 grams of celery, and the main factor contributing to it was the increase in nitrate (\textit{P} < 0.001).

(2) Standard value of [NO\textsubscript{x}] in healthy subjects and its relationship with gender and aging (Table 2)

The mean value of [NO\textsubscript{x}] in all healthy subjects was 47.5 ± 26 ( \textmu M/L). In males, it gradually increased with aging, and the values of the subjects in fifties and sixties were much higher than the values of those in twenties and forties. In females, on the other hand, [NO\textsubscript{x}] gradually decreased from twenties to forties, which showed the lowest level among all age groups, but it showed an upward tendency in the subjects in fifties and sixties. Additionally, [NO\textsubscript{x}] in the females in forties was much lower than that in the males of the same age group.

(3) Cigarette smoking and [NO\textsubscript{x}]

[NO\textsubscript{x}] in the smoker group was 42.1 ± 22 ( \textmu M/L, \textit{P} < 0.001 vs. non-smoker).

### Table 1 The ratio of nitrite (NO\textsubscript{2}⁻) and nitrate (NO\textsubscript{3}⁻) in the value plasma of nitric oxides and its change after celery intake

<table>
<thead>
<tr>
<th></th>
<th>µM/L</th>
<th>NO\textsubscript{x}</th>
<th>Nitrite (NO\textsubscript{2}⁻)</th>
<th>Nitrate (NO\textsubscript{3}⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fasting</td>
<td>n=10</td>
<td>43.0±18.0</td>
<td>6.4±2.2</td>
<td>36.6±18.5</td>
</tr>
<tr>
<td>after</td>
<td>n=10</td>
<td>92.0±14.9</td>
<td>#</td>
<td>85.2±15.8</td>
</tr>
</tbody>
</table>

\* \textit{p}<0.01 vs fasting
\# \textit{p}<0.001 vs fasting

\textit{Note:} Mean±SD.

### Table 2 The trends of aging and disparity in gender of plasma nitric oxides

<table>
<thead>
<tr>
<th>age</th>
<th>gender</th>
<th>20s'</th>
<th>30s'</th>
<th>40s'</th>
<th>50s'</th>
<th>60s'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>male</td>
<td>38.5±25 (n=28)</td>
<td>49.7±29 (n=71)</td>
<td>46.6±24 (n=128)</td>
<td>53.3±29 (n=192)</td>
<td>55.9±29 (n=83)</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>42.7±25 (n=51)</td>
<td>41.1±25 (n=63)</td>
<td>33.5±24 (n=69)</td>
<td>46.9±22 (n=37)</td>
<td>52.3±27 (n=6)</td>
</tr>
<tr>
<td>total</td>
<td></td>
<td>41.8±24 (n=79)</td>
<td>45.6±27 (n=134)</td>
<td>42.0±23 (n=197)</td>
<td>52.5±27 (n=229)</td>
<td>55.6±28 (n=89)</td>
</tr>
</tbody>
</table>

\* \textit{p}<0.05 vs 20s'
\*\* \textit{p}<0.01
\# \textit{p}<0.05 vs 40s'
\#\# \textit{p}<0.01
\*\*\* \textit{p}<0.001 vs male

\textit{Note:} 20s': a generation from 20 to 29 years old, and so are the following generations.

### Table 3 The value of plasma nitric oxides in patients with diabetic complications

<table>
<thead>
<tr>
<th>Complications</th>
<th>Control</th>
<th>None</th>
<th>Proteinuria</th>
<th>Hypertension</th>
<th>CAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO\textsubscript{x} (µM/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µM/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=318)</td>
<td>51.0±29</td>
<td></td>
<td>35.4±16</td>
<td>43.2±23</td>
<td>45.6±23</td>
</tr>
<tr>
<td>(n=121)</td>
<td>(n=69)</td>
<td>(n=139)</td>
<td>(n=55)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* \textit{p}<0.01 vs Control
\# \textit{p}<0.001 vs None

\textit{Note:} None: a group of patients without any complications; CAD: Coronary artery disease.

The data are indicated as mean ± SD.
M/L, and the value in the nonsmoker group was 41.7 ± 18 (M/L). There was no significant difference between the two groups.

4) Diabetic complications and [NOx] (Table 3) The mean age and the ratio of males to females were not different between the "Control" and diabetic groups and between the subgroups.

[NOx] in the "None" group subjects, who did not have any complications, was much lower than that of the "Control" group (P < 0.01). In addition, [NOx] of both groups with Prot. and HT tended to be higher than that of the "None" group, and the value of the CAD group in particular was much higher than that of the "None" group (P < 0.001).

Discussion

One of the most important aims of this paper is to determine whether or not the measurement of plasma nitric oxides can be used as an index of endothelial NO production. In fact, the evaluation of its measurement is complicated because its value represents the flux of production, degradation and excretion of NOx (WENNMALM et al., 1993). Particularly, the sources of plasma NOx consist of endothelial production, cytokotic injury (AKIYAMA et al., 1998) via i-NOS and the intake of exogenous nitric oxides, such as green vegetables, meat preservatives and so on (ZEBALLOS et al., 1995). Additionally, the plasma NOx level is influenced by its distribution to extracellular fluid, plasma half-life and urinary excretion (WENNMALM et al., 1993). In spite of the presence of these complicated factors, we attempted to investigate the usefulness of the measurement of plasma nitric oxides.

The quantitation of NO can be achieved with the following approaches: firstly the spectrophotometrical method that is reacted NO with oxyhemoglobin to yield nitrate anion and methemoglobin, and secondly the colorimetric assay that is measured nitrate and nitrite anion using a diazotization assay (Griess method). These are indirect methods, having an enzymatic and chemical process, and in this study, we employed the chemiluminescence assay because it is direct measurement of NOx with a reaction by ozone, especially at the low concentrations in biological fluids (FAN et al., 1997).

Our results showed the ratio of nitrite to nitrate among NOx, which was approximately 1:6. This ratio is most equal to the values stated in a previous report (ZEBALLOS et al., 1995). Two hours after celery intake, [NOx] increased by approximately 2.5 times that of the basal level, with a very little change in nitrite but a significant increase in nitrate. Additionally, most of the volunteers were suffered from nausea and headache, probably due to venodilation following an abrupt increase in plasma nitrate. These results suggest that exogenous nitric oxides increased the NOx concentration, but most of them were oxidized to nitrate without changing the nitrite concentration. It is, therefore, necessary to take into consideration the considerable effects of exogenous nitric oxides in NO studies.

To evaluate various physiological conditions, we need a standard value of plasma nitric oxides in healthy mass population, but actually few reports are found. It was reported that plasma nitric oxides in eight healthy subjects were 42.5 ± 3.3 (M/L, mean ± SE) (AKIYAMA et al., 1998) and almost equal to our result of 47.5 ± 26 (mean ± SD) in the 728 subjects, so we think these are acceptable as a control value.

We also revealed the variation in plasma nitric oxides according to the age and the difference of gender. It is widely known that androgen (testosterone) is an atherogenic hormone that reduces HDL cholesterol and accentuates the coagulation pathway. Even healthy population cannot escape from lipid dysbolism, which is a cause of arteriosclerosis, and the blood pressure rise with aging. However, as made clear in a report using SHR rats (VAZIRI et al., 1998), the activity of e-NOS is thought to be up-regulated to protect blood vessels. In this study, our data showed that [NOx] gradually increased in the male subjects according to the age, and we think this result reflects the compensatory mechanism of e-NOS in vascular endothelial cells. On the other hand, estrogen, contrary to androgen, is a blood vessel protecting hormone that increases HDL cholesterol and stimulates endothelial NO release (CAULIN-GLASER et al., 1997; COLLINS et al., 1995; BEST et al., 1998), and its effect on the cardiovascular system in its supplementary therapy is also reported (STAMPFER et al., 1991). In the female subjects in our study, [NOx] gradually decreased to the age of forties, which is the menopausal stage, and turned upward after that. We presume that as its pathology, the decrease in estrogen secretion resulting from the impairment of the ovarian function caused the down-regulation of e-NOS and lowered the production and release of NO in vascular endothelial cells.

Cigarettes richly containing nicotine are well-
known as one of the major risk factors of atherosclerosis (HOLBROOK et al., 1984) through the impairment of vascular e-NOS (MAYHAN et al., 1999), but our result could not reveal the relationship between smoking and plasma nitric oxides. This result suggested that the measurement of nitric oxides in a basal state alone could not show the influence of smoking, and another procedure, for example, stimulation by acetylcholine, might be necessary.

It is widely recognized that hyperglycemia induces impairment of the endothelial function via increased antioxidant stress (AYDIN et al., 2001) or the activation of protein C (CALLES-ESCANDON and CIPOLLA, 2001). Some reviewers reported a significant decrease of plasma nitric oxides in patients with type 2 diabetes mellitus without any complications (16.8±11 μM/L vs. 28.8±11 μM/L) and some correlation with oxidant stress (VAMIZOR B et al., 2001). Another report proposed the decrease of NO bioavailability on smooth muscle cells in no complicated group (HINKU et al., 2001). Our results were coincided with these reports, and we presumed that the cascade of NO bioactivity and availability on smooth muscle cells in the early affected stage of diabetes mellitus and followed the decrease of endothelial NO production. Other reports revealed the elevation of the plasma nitrate (NO3⁻) level in patients with diabetes mellitus who had some advanced vascular complications, and they suggested a possibility of its impaired action and compensatory product of NO against increased oxidant stress (MAEJIMA et al., 2001). Also in our results, plasma nitric oxides was higher in the advanced stage, and these results may reflect the vascular injuries by increased oxidant stress and induced cytotoxic NO production via i-NOS activation.

In this study, we could not show direct evidence of to what extent plasma nitric oxides reflect endothelial NO production, because it is influenced by various factors as mentioned above and it also has an individual and circadian variation. However, we proposed the standard value of plasma nitric oxides in the largest ever number of healthy mass population, and revealed its trends with aging and the difference of gender and, moreover, showed some correlations with hyperglycemic vascular damage. In the near future, plasma nitric oxides may become a clinically useful indicator for evaluating not only the endothelial function but also vascular complications.

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