Introduction

Diabetic retinopathy (DR), a principal cause of blindness, is characterized by increased retinal vascular permeability and progressive retinal vascular closure, resulting in tissue hypoxia and neovascularization, but the precise mechanisms are not fully understood. Recently, those pathogenetic factors have been reported to be associated with the renin-angiotensin system and vascular endothelial growth factor, probably via nitric oxide (NO).

NO is a free radical gas that is synthesized from l-arginine by three different isoforms of NO synthase (NOS). NO plays an important role in homeostatic vasodilation and regulation of blood flow, but excess release induces tissue disorders because of increased oxidative stress, especially caused by the production of peroxynitrite. It has been reported that NO is responsible for various vascular complications such as coronary arteriosclerosis and diabetes. However, few studies have investigated whether plasma NO levels are associated with DR. Because NO is extremely unstable and easily oxidized, it is difficult to measure NO levels in vivo. Recently, evaluations of the stable NO end products nitrite (NO$_2^-$) and nitrate (NO$_3^-$), collectively NO$_x$, in biologic fluid, based on the Griess method, have been widely conducted to estimate NO production. Some investigators have reported increased NO levels in patients with diabetic retinopathy.

Relation Between Plasma Nitric Oxide Levels and Diabetic Retinopathy

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Abstract

Purpose: Nitric oxide (NO) plays an important role in homeostatic vasodilation and the regulation of blood flow. On the other hand, excess release of NO causes various vascular complications. There are only a few reports on the relationship between plasma NO levels and microvascular complications, especially diabetic retinopathy (DR) in patients with type 2 diabetes. The purpose of this study was to determine the relationship between plasma NO levels and DR.

Methods: In a prospective study, blood samples were obtained from 36 patients with diabetes and no diabetic retinopathy (NDR), 43 patients with nonproliferative diabetic retinopathy (NPDR), 18 patients with proliferative diabetic retinopathy (PDR), and 40 subjects without diabetes mellitus, who served as controls. The levels of plasma NO$_x$ (nitrite and nitrate), the stable metabolites of NO, were measured by high-performance liquid chromatography with the Griess method.

Results: The plasma NO$_x$ levels were 92.8 ± 16.0, 70.2 ± 6.8, 90.3 ± 9.1, and 53.8 ± 6.1 µmol/l in patients with NDR, NPDR, or PDR, and in the controls, respectively. The plasma NO$_x$ levels in the three diabetic groups were significantly higher than those in the control group ($P < 0.05$ in each case).

Conclusion: The increased plasma NO levels in patients with type 2 diabetes indicate that NO may be associated with the pathogenesis of DR.

Key Words: diabetic retinopathy, Griess method, nitric oxide, type 2 diabetes

Received: February 10, 2005 / Accepted: February 28, 2006

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levels in the vitreous of patients with proliferative DR, based on samples of vitreous fluid obtained during vitreous surgery.12,13 These results suggest that NO may be associated with the pathogenesis of DR, but it is difficult to evaluate the NO level in the vitreous in patients with no retinopathy or early-stage retinopathy during routine examinations. Therefore, we considered that it would be useful to determine the relationship between plasma NO levels, which can be easily determined, and the pathogenesis of DR. In the present study, we evaluated plasma NOx levels in patients with type 2 diabetes.

Subjects and Methods

This study was conducted according to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Asahikawa Medical College. After signed informed consent was obtained, blood samples were collected from 97 patients with type 2 diabetes and 40 healthy hospital staff volunteers without diabetes, who served as controls. The subjects were required to fast overnight before the samples were obtained. Type 2 diabetes was diagnosed according to the criteria of the World Health Organization.14 Patients were excluded if they had any history of certain vascular complications (i.e., cardiac, cerebral, or peripheral vascular diseases), congestive heart failure, renal dysfunction (serum creatinine concentration >1.5 mg/dl), malignancy, or hematological diseases, and if they had taken any antihypertensive/hyperlipidemic medications such as angiotensin converting enzyme inhibitors/ statins that might influence NO levels.15 The levels of DR were determined by fundus findings in accordance with the method of the Early Treatment of Diabetic Retinopathy Study.16

Patients with type 2 diabetes were classified into three groups: those with no DR were the NDR group (36 patients aged 61 ± 2 years), those with nonproliferative DR were the NPDR group (43 patients aged 63 ± 2 years), and those with proliferative DR were the PDR group (18 patients aged 59 ± 2 years). The percentages of hemoglobin A1c (HbA1c) and serum creatinine were measured using standard methods.

About 2 ml of whole blood was drawn from each subject into heparinized tubes, which were promptly chilled in an ice bath. Plasma was isolated by centrifugation (15 min at 3000 g) and then deproteinized by the addition of an equal volume of methanol and centrifugation (10 min at 10 000 g at 4°C). Samples were stored at −80°C until assayed because NO2− and NO3− in frozen plasma have been shown to be stable regardless of the time of storage.17 The samples were analyzed by high-performance liquid chromatography (ENO-20; Eicom, Kyoto, Japan), by the Griess method, to determine the NO2− and NO3− concentrations. Briefly, after injection of 10 µl of the pretreated sample into the system, NO2− and NO3− were separated using a reverse-phase column, after which NO2− was reduced to NO3− in a reduction column packed with copperized cadmium at 35°C. These NO3− were then mixed with the Griess reagent (5 g/l sulfanilamide, 0.25 g/l N-naphthylethenediamine, and 1.25% HCl) in a reaction coil, and the change in absorbance was monitored at 540 nm with a flow-through spectrophotometer. The flow rate of the mobile phase, which consisted of 10% methanol containing 0.15 mol/l BaCl2·2H2O and 0.5 g/l ethylenediaminetetraacetate·4Na, was 0.33 ml/min. The Griess reagent was delivered at a rate of 0.1 ml/min. We applied 10 µmol/l NaNO2 and NaNO3 to the mobile phase as standard solutions to quantify the basis of the area under the curve of their spectra with integration software (PowerChrom 2.2.2; AD Instruments, Colorado Springs, CO, USA). The detection limit of the assay was 0.1 µmol/l with 10 µl of loading.18

Since the quantity of NO2− in plasma is extremely small compared with that of NO3−, NO2− may not be a sensitive index of NO. In the study, we measured plasma NOx levels, which reflect NO3− substantially, as described before.10,19 Measurement of every sample was performed singly or in duplicate. The person who measured the plasma NOx levels was completely masked to the information on the disease status during the studies.

Data are expressed as the means ± standard error. Statistical evaluation was performed using the Mann-Whitney U test with StatView 5.0 software (SAS Institute, Cary, NC, USA). Differences were considered significant when P < 0.05.

Results

The clinical characteristics of the study groups are shown in Table 1. There were no differences in sex, age, or serum creatinine between patients with diabetes and normal controls. Among the three groups with diabetic mellitus, there were no differences in sex, age, time since diagnosis, or the per-

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics of the subjects</th>
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<tr>
<td>Group</td>
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<tr>
<td>No. of patients</td>
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<tr>
<td>Sex (female/male)</td>
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<tr>
<td>Age (years)</td>
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<td>Time since diagnosis (years)</td>
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<tr>
<td>Hemoglobin A1c (%)</td>
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<td>Serum creatinine (mg/dl)</td>
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Data represent median values (range).
NDR, no diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.