

# Serum Extracellular Superoxide Dismutase in Patients With Type 2 Diabetes

Relationship to the development of micro- and macrovascular complications

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**OBJECTIVE** — The aim of this study was to determine the distribution of serum extracellular superoxide dismutase (EC-SOD) concentrations in patients with type 2 diabetes and to assess whether increased EC-SOD concentration is associated with the development of diabetic vascular complications.

**RESEARCH DESIGN AND METHODS** — Serum EC-SOD concentrations were determined in 222 patients with type 2 diabetes and 75 healthy control subjects by an enzyme-linked immunosorbent assay. All subjects had the EC-SOD domain genotyped.

**RESULTS** — The serum EC-SOD concentrations showed a distinct bimodal distribution in both patients with diabetes and control subjects. All subjects with the high-level phenotype carried the Arg213Gly mutation. The frequency of this variant was similar in the diabetes and control groups. Within the group of subjects with the common EC-SOD phenotype, the serum EC-SOD concentration (mean  $\pm$  SE) was significantly higher in patients with type 2 diabetes ( $99.3 \pm 1.3$  ng/ml) compared with the control subjects ( $68.4 \pm 2.3$  ng/ml,  $P < 0.01$ ). Stepwise multiple regression analysis of the data from the diabetic common phenotype group showed a significant relationship between serum EC-SOD concentration and duration of diabetes ( $F = 5.31$ ), carotid artery intimal-media thickness ( $F = 8.24$ ), and severity of nephropathy ( $F = 16.05$ ) and retinopathy ( $F = 4.43$ ).

**CONCLUSIONS** — We observed a strong relationship between the serum concentration of EC-SOD and the severity of both micro- and macrovascular diabetic complications. These findings suggest that serum EC-SOD concentration levels may be a marker of vascular injury, possibly reflecting hyperglycemia-induced oxidative injury to the vascular endothelium and decreased binding of EC-SOD to the vascular wall.

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**Abbreviations:** EC-SOD, extracellular superoxide dismutase; IMT, intimal-media thickness; NDR, no diabetic retinopathy; PDR, proliferative diabetic retinopathy; PPDR, preproliferative diabetic retinopathy; SDR, simple diabetic retinopathy; UACR, urinary albumin-to-creatinine ratio.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

**H**yperglycemia is a major factor in the development of diabetic complications, although the mechanisms of how increased glucose levels contribute to these changes have not been fully elucidated. Adverse biochemical changes associated with hyperglycemia include increased flux of glucose through the polyol pathway, enhanced nonenzymatic glycation, and activation of the diacylglycerol–protein kinase C pathway. Hyperglycemia may also result in increased production of the reactive oxygen species within numerous biochemical pathways that have the potential to initiate adverse changes in endothelial function (1).

Extracellular superoxide dismutase (EC-SOD) is a secretory glycoprotein with an affinity for heparan-like substances (2–4), and it is the principal enzymatic scavenger of superoxide in the extracellular space (5). It has been shown that >99% of the enzyme is bound to heparan sulfate proteoglycans in vascular walls and to a lesser extent within the interstitium, and <1% is contained within the circulation in equilibrium between the plasma phase and the glycocalyx of the endothelium (6–8). Molecular genetic studies have shown that a single-base substitution causing exchange of glycine for arginine-213 (Arg213Gly) in the heparin binding domain of EC-SOD is associated with markedly increased plasma concentrations of the enzyme (9–11). This high-level EC-SOD phenotype is found in ~2–6% of healthy Japanese, Swedish, and Australian populations (9,11–14). It has also been shown that the frequency of the Arg213Gly mutation and the plasma levels of EC-SOD are higher in hemodialysis patients compared with healthy subjects (11,15). Based on this apparent relationship between EC-SOD and vascular disease, we undertook a study to determine the distribution of serum EC-SOD levels in patients with type 2 di-

abetes and healthy control subjects and to investigate the relationship between serum EC-SOD concentrations and the prevalence of diabetic vasculopathies.

## RESEARCH DESIGN AND METHODS

### Subjects and clinical investigations

The study protocol was approved by the Kyoto Prefectural University of Medicine institutional review board, and informed consent was obtained from all subjects. A total of 222 patients with type 2 diabetes (95 men and 127 women; age  $60.0 \pm 0.6$  years [mean  $\pm$  SE], range 37–81 years) who fulfilled the World Health Organization criteria for diabetes were recruited from the outpatient clinics of Kyoto Prefectural University Hospital and its affiliated hospitals. A total of 75 healthy control subjects with similar age and sex proportionalities to the diabetes group were also selected from an annual health examination at a city clinic for the purpose of obtaining normal range data for the EC-SOD assay. The study participants were considered to be a current smoker if they had regularly smoked more than five cigarettes per day for the previous 6 months. Patients with diabetes with a serum creatinine concentration  $\geq 106$   $\mu\text{mol/l}$  and a clinical history and/or signs of cardiovascular disease, cerebrovascular disease, or peripheral arterial disease were excluded from the study.

Height and weight were measured in all study participants, and BMI was calculated. Blood pressure was measured using a standard mercury sphygmomanometer and recorded as mean value of three measurements taken in the sitting position after the subject had rested supine for 5 min.

The severity of diabetic nephropathy was assessed from the average of at least three measurements of the urinary albumin-to-creatinine ratio (UACR). The patients were classified as either normoalbuminuric (UACR  $< 30$  mg/gCr), microalbuminuric (UACR  $\geq 30$ – $300$  mg/gCr), or macroalbuminuric (UACR  $\geq 300$  mg/gCr).

The diagnosis of retinopathy was made by experienced ophthalmologist using ophthalmoscopy and fluorescein angiography, with retinopathy being classified as either no diabetic retinopathy (NDR), simple diabetic retinopathy (SDR), preproliferative diabetic retinopa-

thy (PPDR), or proliferative diabetic retinopathy (PDR).

Intimal-media thickness (IMT) of the common carotid artery was measured by B-mode ultrasound using a Logiq 500 (General Electric Yokogawa Medical System, Tokyo) according to minor modifications of the method of Pignoli et al. (16). A longitudinal two-dimensional ultrasound image of the common carotid artery was scanned by a 10-MHz linear array transducer with the patients in a supine position. Measurements were made at the site of the greatest IMT and at two other sites, 1 cm upstream and downstream from this point. The average IMT at these three points was determined on both sides of the carotid artery, and the highest value was considered.

Fasting plasma glucose, HbA<sub>1c</sub>, creatinine, total and HDL cholesterol, and triglyceride concentrations were determined by standard laboratory methods. Urinary albumin was measured by latex turbidimetric immunoassay system (LA-system; ACI, Tokyo).

### Measurement of serum EC-SOD concentrations and genotyping of the Arg213Gly mutation at the EC-SOD gene

Serum EC-SOD was assayed using a two-step enzyme-linked immunosorbent assay with a monoclonal antibody as described previously (12). The Arg213Gly genotype was analyzed by the PCR–restriction fragment–length polymorphism method described by Marklund et al. (13). Briefly, genomic DNA was extracted from peripheral blood with the DNA extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. The DNA fragment of the EC-SOD gene containing the mutant site was amplified by PCR with the primers EC3 and EC5 (9), followed by digestion with the restriction enzyme *Mwo*I (New England Biolabs, Hitchin, U.K.). The digestion products were then separated on a 12% polyacrylamide gel and visualized using a silver stain.

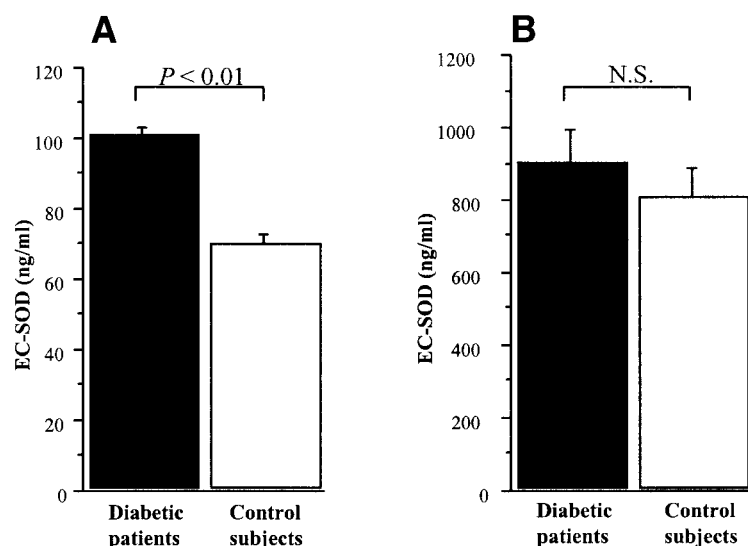
### Statistical analysis

All statistical analyses were performed using Statview version 5.0 for Macintosh (Abacus Concepts, Berkeley, CA), with data being expressed as means  $\pm$  SEM or as proportions. A *P* value  $< 0.05$  was considered statistically significant. Continu-

ous variables were compared by Student's *t* test or a one-way ANOVA with Scheffé's multiple comparison test. Categorical data were assessed by  $\chi^2$  test. A stepwise multiple regression analysis was performed to assess the influence of the following independent variables on serum EC-SOD concentrations: sex, age, duration of diabetes, BMI, blood pressure, prevalence of nephropathy and retinopathy, IMT, smoking history, HbA<sub>1c</sub>, serum creatinine, total and HDL cholesterol, and triglyceride concentrations. Variables were excluded from the regression analyses if the *F* value was  $< 4.0$  at each step of the calculations.

**RESULTS**— The distribution of the serum EC-SOD concentrations in the healthy subjects was discontinuous and was clearly separated into a common phenotypic group (EC-SOD  $< 200$  ng/ml) and a high-level phenotypic group (range 668.9–1,016.8 ng/ml), as shown previously (12). Of the 75 healthy subjects, 71 (94.7%) had the common phenotype (range 40.8–126.1 ng/ml), whereas the remaining 4 subjects had the high-level phenotype (668.9–1,016.8 ng/ml). A similar bimodal distribution was observed in the patients with type 2 diabetes. Of the 222 patients with diabetes, 210 (94.6%) had the common phenotype (52.8–174.6 ng/ml) and 12 (5.4%) had the high-level EC-SOD phenotype (486.6–1,514.8 ng/ml). All 16 subjects with serum EC-SOD levels  $> 400$  ng/ml (4 control subjects and 12 with diabetes) carried the Arg213Gly mutation, with 1 subject being homozygous and the remaining 15 subjects being heterozygous. None of the subjects with serum EC-SOD levels  $< 200$  ng/ml had the Arg213Gly mutation.

The clinical characteristics of the 210 patients (90 men and 120 women) with the common EC-SOD phenotype were as follows: age  $60.7 \pm 0.7$  years; duration of diabetes  $10.2 \pm 0.4$  years; BMI  $23.1 \pm 0.3$  kg/m<sup>2</sup>; systolic blood pressure  $113.2 \pm 0.9$  mmHg; diastolic blood pressure  $76.8 \pm 0.6$  mmHg; smoker/nonsmoker 67/146; HbA<sub>1c</sub>  $7.7 \pm 0.1\%$ ; serum creatinine  $69.3 \pm 0.9$   $\mu\text{mol/l}$ ; total cholesterol  $5.35 \pm 0.05$  mmol/l; HDL cholesterol  $1.26 \pm 0.04$  mmol/l; triglycerides  $1.38 \pm 0.13$  mmol/l; IMT  $1.04 \pm 0.02$  mm; prevalence of nephropathy 62, 82, and 66 normoalbuminuric, microalbuminuric, and macroalbuminuric



**Figure 1**—Comparison of serum concentrations of the common phenotype EC-SOD (A) or high-level EC-SOD variant (B) in the type 2 diabetic patients and healthy control subjects. Data are means  $\pm$  SE. P values were evaluated by Student's t test.

subjects, respectively; and prevalence of retinopathy 59, 93, 48, and 10 for NDR, SDR, PPDR, and PDR, respectively. There were no significant differences between the patients with the common EC-SOD and high-level variant phenotype for all of the clinical factors recorded (data not shown).

As shown in Fig. 1, the mean serum EC-SOD concentration in the patients with diabetes with the common phenotype was significantly higher compared with the control subjects of this phenotypic group ( $99.3 \pm 1.3$  vs.  $68.4 \pm 2.3$  ng/ml,  $P < 0.01$ ). In contrast, the diabetic and control groups with the high-level variant had similar mean serum concentrations of EC-SOD ( $913.4 \pm 90.0$  vs.  $820.0 \pm 77.5$  ng/ml,  $P = 0.579$ ).

Stepwise multiple regression analysis of the data of diabetes patients with the common EC-SOD phenotype showed a significant relationship between serum EC-SOD concentrations and duration of diabetes ( $F = 5.31$ ), IMT ( $F = 8.24$ ), and severity of nephropathy ( $F = 16.05$ ) and retinopathy ( $F = 4.43$ ) (Table 1).

Figure 2 shows the serum EC-SOD concentrations for each group stratified by IMT measurements, urinary albumin excretion, or severity of retinopathy in the patients with the common EC-SOD phenotype. Mean serum EC-SOD in the patients with  $\geq 1.1$  mm IMT ( $111.2 \pm 3.1$  ng/ml) was significantly higher than in patients with  $< 1.1$  mm IMT ( $91.7 \pm 2.1$

ng/ml,  $P < 0.01$ ). Similarly, mean serum EC-SOD was significantly higher in the macroalbuminuric group ( $112.3 \pm 3.8$  ng/ml) compared with the microalbuminuric group ( $98.3 \pm 2.5$  ng/ml,  $P < 0.01$ ), which in turn had a higher mean level of the enzyme than the patients with normoalbuminuria ( $86.8 \pm 2.9$  ng/ml,  $P <$

$0.05$ ). The mean serum EC-SOD concentration was found to be significantly higher in patients with PDR/PPDR ( $117.1 \pm 5.3$  ng/ml) than in patients with SDR ( $98.8 \pm 3.6$  ng/ml,  $P < 0.01$ ) or in patients without diabetic retinopathy ( $93.0 \pm 2.1$  ng/ml,  $P < 0.01$ ). There was, however, no difference in enzyme between these latter two groups.

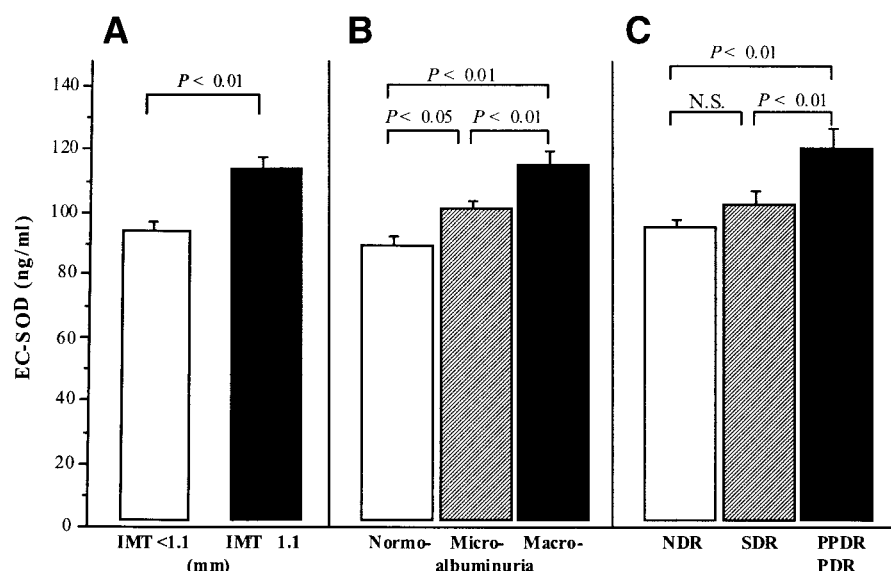
**CONCLUSIONS**— A major aim of this study was to assess whether serum EC-SOD concentrations were associated with the development of diabetic vascular complications. Within the common phenotypic group, we demonstrated a positive correlation between serum EC-SOD levels and the severity of microvascular complications, such as nephropathy and retinopathy, and macrovascular changes, measured as an increase in the IMT of the common carotid artery. Moreover, stepwise multiple regression analyses showed that severity of nephropathy and retinopathy, duration of diabetes, and IMT measurements were independently associated with the serum EC-SOD concentration.

This study confirms earlier reports that have demonstrated that serum concentrations of the free radical scavenging enzyme EC-SOD are significantly higher in patients with type 2 diabetes compared

**Table 1**—Stepwise regression analysis between serum EC-SOD levels and 15 clinical variables in type 2 diabetic patients with the common EC-SOD phenotype

	Univariate correlation coefficient	$\beta$	F
Sex (M/F)*	0.136	—	—
Age (years)	0.290	—	—
Duration of diabetes (years)	0.407	0.170	5.31
BMI ( $\text{kg}/\text{m}^2$ )	-0.119	—	—
Systolic blood pressure (mmHg)	0.070	—	—
Diastolic blood pressure (mmHg)	0.060	—	—
Smoking (yes/no)†	-0.053	—	—
HbA <sub>1c</sub> (%)	0.138	—	—
Creatinine ( $\mu\text{mol}/\text{l}$ )	0.254	—	—
Total cholesterol (mmol/l)	0.033	—	—
Triglycerides (mmol/l)	0.138	—	—
HDL cholesterol (mmol/l)	-0.242	—	—
IMT (mm)	0.327	0.190	8.24
Severity of nephropathy (normo-, micro-, and macroalbuminuria)‡	0.415	0.277	16.05
Severity of retinopathy (NDR, SDR, PPDR, and PDR)§	0.351	0.145	4.43

Variables were deleted at each step in the regression analyses if F was not significant (i.e.,  $< 4.0$ ).  $\beta$  is the standard regression coefficient. \*Female = 0, male = 1; †no = 0, yes = 1; ‡normoalbuminuria = 0, microalbuminuria = 1, macroalbuminuria = 2; §NDR = 0, SDR = 1, PPDR = 2, PDR = 3.



**Figure 2**—Serum concentrations of EC-SOD stratified by IMT levels (A) and severity of nephropathy (B) or retinopathy (C). Data are means  $\pm$  SE. P values were evaluated by Student's t test (IMT) or one-way ANOVA with Scheffé's multiple comparison test (nephropathy and retinopathy).

with healthy control subjects (12,15). Genotypic analysis also confirmed that all subjects with serum EC-SOD concentrations  $>400$  ng/ml carried the Arg213Gly mutation. Serum levels of the enzyme were found to be similar in patients with this mutation, regardless of diabetic status. However, a larger investigation is required to validate this latter finding because the number of subjects with the high-level variant phenotype in our study was relatively small.

The exact mechanism that governs the physiological regulation of plasma EC-SOD levels is still unclear. Because the distribution of EC-SOD is known to be in equilibrium between the plasma phase and the heparan sulfate proteoglycans in the glycocalyx fraction of the vascular endothelium (6,8), the affinity of the enzyme to the cell surface may influence serum EC-SOD levels. Adachi et al. (17) demonstrated that nonenzymatic glycation of EC-SOD was associated with a reduction in heparin affinity and also that the proportion of glycated EC-SOD in the serum of patients with diabetes was significantly higher than in normal subjects. This finding suggests that the high serum concentrations of EC-SOD found in patients with diabetes with a common EC-SOD phenotype may result, in part, from chronic hyperglycemia causing excess nonenzymatic glycation of the enzyme.

The high level of EC-SOD found in

the serum of patients with diabetes with a normal genotype and vascular complications is probably the result of the combined effect of several factors. Evidence suggests that reduced tissue binding of EC-SOD may be a major cause of this increase. In diabetic nephropathy, it has been reported that heparan sulfate is reduced in glomerular basement membranes proportional to the degree of proteinuria (18). Similar findings have been observed in basement membranes of both small (19) and large vessels (20), and it has been suggested that injury to the glomerulus may reduce the ability of the basement membrane glycocalyx fraction to bind EC-SOD. This reduction in binding capacity may in turn lead to an increase in the plasma EC-SOD concentration (15,21). It has also been shown that there is a significant positive correlation between plasma homocysteine and EC-SOD concentrations (22,23), and in a recent publication, Yamamoto et al. (24) reported that homocysteine decreased the binding of EC-SOD to vascular endothelial cell surfaces by degrading endothelial heparan sulfate. This group also showed that nitric oxide and its decomposed reaction product, peroxynitrite, formed during hyperglycemia (25) resulted in a decrease in the binding of EC-SOD to endothelial cell surfaces (26).

In summary, our study demonstrated two distinct phenotypic groups for serum

EC-SOD activity in both patients with type 2 diabetes and normal healthy control subjects. We observed a strong relationship between the serum concentration of EC-SOD in type 2 diabetic patients with the common phenotype and the severity of both micro- and macrovascular diabetic complications. It has been suggested that the increase in serum EC-SOD concentration associated with diabetes may reflect decreased binding of the enzyme to the endothelium, resulting in the vascular wall being more vulnerable to oxidative damage. Our results suggest that serum EC-SOD concentration may be a marker of diabetic vascular injury, although a larger prospective follow-up study is required to validate this potential.

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