Relationship of plasma extracellular-superoxide dismutase level with insulin resistance in type 2 diabetic patients

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Abstract

Extracellular-superoxide dismutase (EC-SOD) is a secretory glycoprotein located in blood vessel walls at high levels and may be important in the antioxidant capability of vascular walls. The aim of this study was to assess plasma levels of EC-SOD and to evaluate the relationship of the EC-SOD level with insulin resistance in type 2 diabetic patients. We determined plasma EC-SOD in 122 patients and found for the first time that the EC-SOD level was strongly and positively related to adiponectin ($r=0.503$, $P<0.001$), and significantly and inversely related to fasting plasma glucose (FPG) ($r=-0.209$, $P=0.022$), body-mass index (BMI) ($r=-0.187$, $P=0.040$) and homeostasis model assessment–insulin resistance index (HOMA-R) ($r=-0.190$, $P=0.039$). Stepwise-multiple regression

analysis also showed a significant influence of adiponectin ($F=33.27$) on the EC-SOD level. Administration of pioglitazone to 19 diabetic patients significantly increased the plasma levels of EC-SOD (69.9 ± 19.3 ng/ml to 97.4 ± 25.9 ng/ml; $P<0.0001$) and adiponectin, while it decreased tumor necrosis factor- α (TNF- α). The present observations suggest that factors related to the pathogenesis of insulin resistance play an important role in the regulation of the plasma EC-SOD concentration. It is possible that the increase in the EC-SOD level by pioglitazone administration in diabetic patients is due to a decline of TNF- α , which is known to suppress EC-SOD expression.

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Introduction

Insulin resistance, which is characterized by a reduced sensitivity of target tissues to insulin, is an important risk factor associated with atherosclerosis (Matsuzawa *et al.* 1999). Abnormalities in lipid and glucose metabolism are thought to contribute to atherosclerotic vascular damage in the pathologic condition of insulin resistance (Pieper *et al.* 1995). Diabetic hyperglycemia has been reported to promote the overproduction of reactive oxygen species such as superoxide, causing hyperglycemic vascular damage *in vivo* (Faure *et al.* 1999) and *in vitro* (Nishikawa *et al.* 2000). Superoxide is secreted from various cells in the vascular system (Ohara *et al.* 1993) and is implicated in the insulin-resistant state (Bertelsen *et al.* 2001). The increase in superoxide may contribute to oxidative processes in the vessel wall, such as induction and enhancement of cell membrane lipid peroxidation (Jain 1989) and oxidation of low-density lipoprotein (LDL) (Heinecke *et al.* 1986). Moreover, superoxide reacts extremely rapidly with nitric oxide (NO), which modulates vasomotor tone, inhibits platelet- and leukocyte-aggregation, and produces peroxynitrite (Koppenol *et al.* 1992). An increase in superoxide

dismutase (SOD) has been reported to attenuate diabetic vascular dysfunction (Craven *et al.* 2001).

The vascular wall contains large amounts of extracellular-superoxide dismutase (EC-SOD), one of the SOD isozymes produced and secreted to the extracellular space by fibroblasts and smooth muscle cells (Marklund 1990). EC-SOD has an affinity for heparin-like substances (Adachi & Marklund 1989) and glycosaminoglycans on the endothelial cell surface (Adachi *et al.* 1996). After secretion, the EC-SOD probably diffuses slowly in the vascular wall and distributes itself according to the amount and affinity of the heparin-like glycosaminoglycans present in the vessel wall (Strålin *et al.* 1995). The presence of EC-SOD on the endothelial cell surface at a high level might have an important protective effect against superoxide in the vascular wall, supporting the antioxidant role of NO (Wink *et al.* 1993), and a role in prevention of LDL oxidation (Takatsu *et al.* 2001). EC-SOD expression in cultured cell lines is known to be regulated by numerous substances such as cytokines. For example, tumor necrosis factor (TNF)- α is one of the suppressive factors of EC-SOD expression in vascular cells (Marklund 1992, Strålin & Marklund 2000). On the other hand, TNF- α is

Table 1 Clinical characteristics of the study population

	(A) Plasma concentration		(B) Relation vs EC-SOD					
	Male	Female	Male		Female		Total	
			<i>r</i>	<i>F</i>	<i>r</i>	<i>F</i>	<i>r</i>	<i>F</i>
EC-SOD (ng/ml)	81.9 ± 20.8	104.4 ± 33.2**	−0.072	—	−0.390†	6.41	−0.209†	4.59
FPG (mM)	8.2 ± 2.0	8.0 ± 2.0	−0.073	—	−0.210	—	−0.099	—
HbA _{1c} (%)	7.0 ± 1.1	7.1 ± 1.3	0.019	—	−0.445††	7.63	−0.133	—
IRI (mU/ml)	8.4 ± 6.6	10.2 ± 7.4	−0.174	—	−0.370†	5.73	−0.187†	—
BMI (kg/m)	23.3 ± 2.2	24.7 ± 4.6	−0.072	—	−0.506††	10.95	−0.190†	—
HOMA-R	3.1 ± 3.1	3.6 ± 2.7	0.380††	13.3	0.490††	9.55	0.503††	33.27
Adiponectin (μg/ml)	6.5 ± 3.2	9.1 ± 5.1**	−0.117	—	−0.163	—	−0.048	—
TC (mg/dl)	196.7 ± 30.7	210.7 ± 36.3*	−0.020	—	0.152	—	−0.107	—
HDL-C (mg/dl)	51.8 ± 10.5	54.9 ± 12.6	−0.135	—	−0.250	—	−0.129	—
LDL-C (mg/dl)	124.2 ± 27.6	131.6 ± 34.0	0.118	—	0.148	—	0.026	—
TG (mg/dl)	111.7 ± 54.4	123.3 ± 66.6						

(A) Data are presented as mean ± s.d. Significant differences between males and females (* $P < 0.05$, ** $P < 0.01$) were analyzed with Student's *t*-test or Welch's *t*-test. (B) Data are correlation coefficients (*r*) between EC-SOD and other characteristics, and significant relationships are shown with symbols († $P < 0.05$, †† $P < 0.01$). Stepwise-multiple regression analysis was performed, and significant relationships ($F > 4.0$) are shown.

a proposed mediator of insulin resistance, overexpressed in adipose tissue and skeletal muscle of obese and type 2 diabetic subjects (Iwata *et al.* 2001).

The present study was undertaken to clarify the relationship between the plasma EC-SOD level and indices of insulin-resistance, and the change in the EC-SOD level in insulin-resistant patients by administration of pioglitazone, a thiazolidinedione (TZD) insulin-sensitizing drug,

described in our previous reports (Adachi *et al.* 1994) with minor modifications. The plasma adiponectin concentration was assayed with an ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan). The plasma TNF- α concentration was assayed with a Quantikine HS immunoassay kit (R & D Systems, Minneapolis, MN, USA). Other characteristics were measured by standard clinical laboratory methods.

Materials and methods

Study population

One hundred and twenty-two diabetic patients (82 men, average age 59.9 ± 8.8 years, and 40 women, average age 58.4 ± 12.2 years) who showed a normal plasma creatinine level of below 1.1 mg/dl and did not receive insulin or pioglitazone treatment were eligible for this study on the relationship between the plasma EC-SOD level and characteristics of insulin resistance. The study on the change in the plasma EC-SOD level by pioglitazone treatment included 19 diabetic patients (10 men and nine women). They showed higher diabetes/insulin resistance-parameters: for example, fasting plasma glucose (FPG) of 8.88 ± 2.79 mM; hemoglobin A_{1c} (HbA_{1c}) of $8.5 \pm 1.2\%$ and homeostasis model assessment-insulin resistance index (HOMA-R) of 6.7 ± 8.7 . The subjects were administered 15 mg (female patients) or 30 mg (male patients) of pioglitazone once daily. All the study subjects provided written informed consent according to the institutional guidelines.

Laboratory measurements

Fasting blood samples were obtained. The EC-SOD concentrations in the plasma were determined by ELISA

Statistical analysis

Data were analyzed with the *t*-test (Student's *t*-test or Welch's *t*-test) and the Wilcoxon signed-rank test. A stepwise multiple-regression analysis was performed to assess the influence of variables on EC-SOD concentrations. Variables were deleted from the regression analysis if the *F* value was not significant (< 4.0) at any step of the calculations. A *P* value less than 0.05 was considered significant.

Results

Plasma EC-SOD level in diabetic patients

We assayed the plasma EC-SOD level in 122 diabetic patients (average ± s.d. of EC-SOD level: 89.5 ± 27.5 ng/ml) and found the level was higher ($P < 0.001$) in females (104.4 ± 33.2 ng/ml) than in males (81.9 ± 20.8 ng/ml). The plasma adiponectin level was lower in males than in females, consistent with a previous report (Nishizawa *et al.* 2002), whereas there were no significant gender differences in FPG, HbA_{1c}, immunoreactive insulin (IRI), body-mass index (BMI) and HOMA-R, as shown in Table 1. The plasma EC-SOD levels were significantly and inversely related to FPG ($r = -0.209$, $P = 0.022$),

Figure 1 Relationship between the plasma EC-SOD and adiponectin levels. White circles and black circles show female and male patients respectively. The regression lines are presented as a solid line for the male group and a dotted line for the female group.

BMI ($r = -0.187$, $P = 0.040$) and HOMA-R ($r = -0.190$, $P = 0.039$), whereas they were strongly and positively related to adiponectin ($r = 0.503$, $P < 0.001$). Stepwise multiple-regression analysis showed a significant relationship between EC-SOD and adiponectin ($F = 33.27$), and FPG ($F = 4.59$). There were no significant correlations between EC-SOD and HbA_{1c} ($r = -0.099$) or IRI ($r = -0.133$). In men, there was a significant positive correlation between EC-SOD and adiponectin ($r = 0.380$, $P < 0.001$, $F = 13.03$) (Fig. 1). On the other hand, EC-SOD in women showed a significant positive correlation with adiponectin ($r = 0.490$, $P = 0.001$, $F = 9.55$) (Fig. 1).

and negative correlations with FPG ($r = -0.390$, $P = 0.013$, $F = 6.41$), IRI ($r = -0.445$, $P = 0.005$, $F = 7.63$), BMI ($r = -0.370$, $P = 0.019$, $F = 5.73$) and HOMA-R ($r = -0.506$, $P = 0.001$, $F = 10.95$). There were no significant correlations between EC-SOD and lipid parameters such as total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C) and triglyceride (TG).

Change in the plasma EC-SOD level by pioglitazone treatment

We investigated the change in the plasma EC-SOD level by treatment with the TZD antidiabetic agent pioglitazone. As shown in Fig. 2A, daily administration of pioglitazone to 19 patients for about 3 months significantly increased their plasma EC-SOD concentration (69.9 ± 19.3 ng/ml to 97.4 ± 25.9 ng/ml, $P < 0.0001$). Furthermore, we observed a significant increase in the plasma adiponectin concentration (5.1 ± 1.9 μg/ml to 15.8 ± 7.8 μg/ml, $P < 0.0001$) (Fig. 2B), as consistent with a previous report (Iwata *et al.* 2001). Moreover, the plasma TNF-α level was significantly decreased by the pioglitazone treatment (2.4 ± 0.7 μg/ml to 1.9 ± 0.4 μg/ml; $P < 0.0001$), as shown in Fig. 2C. On the other hand, the subjects ($n = 7$) in the nonpioglitazone-treatment group did not show any significant changes in their plasma EC-SOD, adiponectin and TNF-α levels (Fig. 2A–C).

Discussion

The present study demonstrates for the first time that plasma EC-SOD in patients with type 2 diabetes were significantly positively correlated with adiponectin and inversely related to indices of insulin resistance.

Figure 2 Comparison of the plasma EC-SOD (A), adiponectin (B) and TNF-α (C) levels before and after treatment with pioglitazone. The data are presented as mean $\pm$ S.D. for the pioglitazone-treated group (PIO+, $n = 19$) and pioglitazone-untreated group (PIO-, $n = 7$). Significant differences ($P < 0.0001$) compared with before treatment were analyzed by the Wilcoxon signed-rank test.

EC-SOD is the major SOD isozyme in extracellular fluids but is distributed mainly in the blood vessel wall (Strålin *et al.* 1995). In the vasculature, EC-SOD forms an equilibrium between the plasma phase and endothelial cell surface (Karlsson & Marklund 1988), and the change in the plasma EC-SOD level might reflect the changes in the expression of EC-SOD and/or EC-SOD-tissue binding. In this study, parameters associated with diabetes/insulin resistance showed more significant relationships to EC-SOD in female subjects than in male subjects (Table 1). It has been reported that cardiovascular risk factors, including male sex, high BMI and smoking, decreased the plasma EC-SOD level in an unselected middle-aged population (Marklund *et al.* 1997). The plasma levels of EC-SOD may be modulated by lifestyle and show a complex covariation with many of the conventional cardiovascular risk factors. From these observations, it is speculated that the cardiovascular risk factors obscure the relationship between EC-SOD and parameters associated with insulin resistance, especially in male subjects.

The major observation indicated in Table 1 is that plasma EC-SOD levels in diabetic patients were strongly positively correlated with adiponectin, whereas they were significantly inversely related to parameters associated with insulin resistance. Moreover, stepwise multiple-regression analyses showed that adiponectin especially highly affects EC-SOD level. The plasma level of adiponectin has been reported to relate inversely to BMI and HOMA-R (Hotta *et al.* 2000). The plasma EC-SOD level was also inversely related to the above parameters, suggesting that factors related to the pathogenesis of insulin resistance play an important role in the regulation of the plasma EC-SOD. Increases in the plasma levels of EC-SOD and adiponectin by the pioglitazone treatment indicated in Fig. 2 were consistent with the above observations. It has been reported that administration of pioglitazone normalized the overexpression of TNF- α and decreased the plasma TNF- α level in experimental animals (Hofmann *et al.* 1994, Murase *et al.* 1998). We also observed that the pioglitazone treatment significantly decreased the plasma TNF- α level in diabetic patients, as shown in Fig. 2C. The mechanism by which this occurs is unclear; however, TNF- α is known to suppress EC-SOD expression in cultured cells (Marklund 1992, Strålin & Marklund 2000). From these results, it is speculated that the decline in the TNF- α level by the pioglitazone treatment results in an increase in EC-SOD expression in the vascular system.

In insulin-resistant patients, the decline in the activities of antioxidative enzymes (Cavarape *et al.* 2001) and the increase in superoxide production (Yamagishi *et al.* 2001) may lead to the progression of atherosclerosis via the enhancement of LDL oxidation (Heinecke *et al.* 1986). It has been reported that exogenous addition of EC-SOD and overexpression of EC-SOD prevented endothelial cell-mediated oxidative modification of LDL (Laukkanen *et al.* 2000, Takatsu *et al.* 2001). Moreover, mice lacking

EC-SOD were reported to be more prone to develop diabetes than wild-type controls (Sentman *et al.* 1999). The plasma TNF- α level was reported to be higher in patients with insulin resistance (Nilsson *et al.* 1998, Mishima *et al.* 2001) and correlated well with the degree of atherosclerosis, as shown by factors such as LDL-cholesterol concentration (Skoog *et al.* 2002). In view of all these findings, it appears that both a decrease in EC-SOD expression and a decrease in the capability of the endothelial cell surface to possess EC-SOD in the diabetes/insulin-resistant state probably lead to a decrease in the capability of EC-SOD to protect the endothelial cell function, and may increase atherosclerotic development in insulin-resistant patients. Increasing the EC-SOD expression might be one approach to ameliorate the pathogenesis of atherosclerosis in these patients.

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