Reverse Correlation between Urine Nitric Oxide Metabolites and Insulin Resistance in Patients with Type 2 Diabetes Mellitus

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Abstract. We studied the possible relationship between nitric oxide (NO) production and insulin resistance in patients with type 2 diabetes mellitus. Urine NO metabolites (NOx) were measured as an index for NO production by HPLC combined with a Cd column, Griess reaction and a spectrophotometer in 403 healthy control subjects and 102 hospitalized patients with type 2 diabetes. Glucose infusion rate (GIR) was measured as a reverse index for insulin resistance by euglycemic glucose clamp study using an artificial pancreas in 20 of 102 diabetic patients. Urine NOx was lower in the patients with type 2 diabetes than in healthy control subjects (mean $\pm$ SE: 3.18 ± 0.02 versus 3.25 ± 0.01 log [μ mol/gCr], $p < 0.01$). Urine NOx was correlated with body mass index (BMI) in 102 diabetic patients ($r = -0.372$, $p < 0.001$), but not related to either age, sex, fasting plasma glucose, HbA_{1c} or blood pressure. Urine NOx was correlated with GIR independent of BMI in 20 diabetic patients ($r = 0.774$, $P < 0.0001$). These findings suggest that NO production is closely related with insulin resistance in patients with type 2 diabetes.

Key words: Nitric oxide, Insulin resistance, Type 2 diabetes mellitus, Urine, Euglycemic glucose clamp
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INSULIN resistance is one of the major pathological conditions in type 2 diabetes mellitus. Hyperinsulinemia induced by sustained hyperglycemia deteriorates insulin-mediated glucose transport in the skeletal muscle. Hyperglycemia itself is considered a critical factor for insulin resistance. It is also suggested that insufficient blood flow in the skeletal muscle is associated with insulin resistance [1, 2]. Nitric oxide (NO) is known to be a physiological vasodilator generated in vascular endothelial cells [3]. Several lines of evidence suggest a close relationship between insulin resistance and hypertension [4–8]. It was reported that L-arginine, a natural precursor of NO, improved insulin resistance [9, 10]. These findings suggest that reduced NO production may

contribute, at least partly, to insulin resistance in type 2 diabetes. In the present study, we examined the possible relationship between urine NO metabolites (NOx) levels as an index for NO production and glucose infusion rate (GIR) under euglycemic clamp as a reverse index for insulin resistance in patients with type 2 diabetes.

Research Design and Methods

Subjects

We examined 403 healthy control subjects (164 males and 239 females) and 102 hospitalized patients with type 2 diabetes (71 males and 31 females). Mean ($\pm$ SD) age was 50 ± 15 yr and 58 ± 13 yr, respectively. The healthy control subjects did not show any abnormality in blood pressure, body mass index (BMI), serum indices for hepatic function (total protein, albumin, GOT, GPT and LDH) and

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renal function (BUN, creatinine, uric acid, Na, K and Cl) and urinalysis. Blood pressure and serum indices for hepatic and renal functions were normal in all the patients with type 2 diabetes. Mean ($\pm$ SD) values of fasting plasma glucose, HbA_{1c} and body mass index (BMI) were 6.2 ± 1.0 mmol/l, $6.5 \pm 1.0\%$ and 23.5 ± 4.3 kg/m², respectively.

NO metabolites (NOx) assay

NO production was estimated by measuring NO metabolites ($\text{NOx} = \text{NO}_2^- + \text{NO}_3^-$) in the urine. Urine samples were collected in the early morning after an overnight fast. Sodium azide was added to the samples, which were then stored at 4°C until assayed. Urine NOx was measured by NOx analyzing HPLC system (ENO-10, Eicom, Kyoto, Japan) as described previously [11], and corrected by creatinine concentration. All values were expressed on a logarithmic scale as described previously [11].

Euglycemic clamp study

Insulin resistance was evaluated by euglycemic clamp study performed according to the method of DeFronzo *et al.* [12], using the artificial pancreas (STG-22, Nikkiso Co., Tokyo, Japan) as reported previously [13]. The study was carried out in 20 patients with type 2 diabetes selected at random from 102 hospitalized diabetic patients. Informed consent was obtained from all patients who participated in the study. After overnight fasting, indwelling catheters were inserted into both antecubital veins at 9 am, Regular human insulin (Eli Lilly Co., Indianapolis, IN, U.S.A) was infused into an antecubital vein at a dose of 800 mU over 10 min as a priming. This was followed by a 120-min constant infusion of insulin at the flow rate of 40 mU/m²/min. Blood glucose levels were monitored and automatically maintained at the fasting level of 5.5 mmol/l by 10% glucose infusion at a flow rate which was corrected and recorded every minute. The steady-state glucose infusion rate was obtained between 90 and 120 min after the start of the constant insulin infusion. The mean infusion rate of glucose during the last 30 min was presented as the glucose infusion rate (GIR), a reverse index for insulin resistance.

Statistical analysis

Statistical differences between two groups were evaluated by unpaired Student's *t* test. Regression analysis was performed by the least square method. A probability of $P < 0.05$ was considered statistically significant.

Results

Urine NO metabolites ($\text{NOx} = \text{NO}_2^- + \text{NO}_3^-$) were predominantly NO_3^- in both healthy control subjects and the patients with type 2 diabetes mellitus. The mean ($\pm$ SE) values of logarithmic urine NOx in 403 healthy control subjects were 1.55 ± 0.02 log [$\mu\text{mol/gCr}$] for NO_2^- , 3.23 ± 0.01 log [$\mu\text{mol/gCr}$] for NO_3^- and 3.25 ± 0.01 log [$\mu\text{mol/gCr}$] for NOx, respectively. Urine NOx was lower in 102 patients with type 2 diabetes than in the healthy control subjects (mean $\pm$ SE: 3.18 ± 0.02 log [$\mu\text{mol/gCr}$], $P < 0.01$) (Fig. 1). There was a reverse correlation be-

Fig. 1. Mean ($\pm$ SE) urine NOx levels in 403 healthy control subjects and 102 patients with type 2 diabetes mellitus. Urine NOx levels were expressed on a logarithmic scale. There was a significant difference ($P < 0.01$) between the two groups.

tween urine NO_x and BMI ($r = -0.372$, $P < 0.001$) (Fig. 2). Urine NO_x was not related to age, sex, fasting plasma glucose, HbA_{1c}, systolic blood pressure or diastolic blood pressure in these diabetic patients (Table 1).

Mean ($\pm$ SE) values of GIR were lower in 20 patients with type 2 diabetes than in 7 hospital control subjects (5.4 ± 0.4 versus 8.4 ± 0.5 mg/kg/min, $P < 0.001$). Urine NO_x was well correlated with GIR in these patients with type 2 diabetes ($r = 0.774$, $P < 0.0001$) independently of BMI (Fig. 3).

Table 1. Correlation between clinical characteristics and urine NO_x in 102 patients with type 2 diabetes mellitus.

	r	p
Sex	—	0.34
Age	0.137	0.17
FPG	0.032	0.75
HbA _{1c}	0.013	0.89
Systolic Blood Pressure	0.052	0.60
Diastolic Blood Pressure	0.048	0.63

Fig. 2. Correlation between urine NO_x levels and BMI in 102 patients with type 2 diabetes mellitus. There was a reverse correlation ($r = -0.372$, $P < 0.001$) between them.

Fig. 3. Correlation between urine NO_x levels and GIR in 20 patients with type 2 diabetes mellitus. There was a significant correlation ($r = 0.774$, $P < 0.0001$) between them. Open and closed squares indicate diabetic patients with BMI ≥ 26.0 ($n = 11$) and those with BMI < 26.0 ($n = 9$), respectively.

Discussion

The present study showed that the mean urine NOx levels were lower in the patients with type 2 diabetes than in healthy control subjects, and that urine NOx was correlated with GIR in these diabetic patients. Since GIR is a reverse index for insulin resistance, these findings suggest a reverse correlation between urine NOx and insulin resistance in the patients with type 2 diabetes.

Since NO is rapidly metabolized, direct measurement of NO *in vivo* is extremely difficult. NO is rapidly oxidized to nitrite and then to nitrate by oxygenated hemoglobin, molecular oxygen or superoxide anions, and excreted into the urine [14]. Therefore, we measured urine nitrite and nitrate excretion as an index for NO production in the present study.

NO is produced by constitutive NO synthase in vascular endothelium and neuronal cells, or by inducible NO synthase in macrophage, neutrophils and glia cells. The subjects were clinically free from infection and trauma in the present study, and it was assumed that NO was produced predominantly by constitutive NO synthase in the subjects examined. Our findings suggest that NO production is reduced in insulin resistant diabetic patients. The reduced NOx in these patients with type 2 diabetes was not explained by sex and age since these factors did not affect urine NOx levels [11].

Recently, it was reported that L-arginine, a natural precursor of NO, improved insulin resistance [9, 10]. These results suggest that NO may be involved in insulin resistance. NO plays an important role in regulating vascular tone, inhibiting platelet adhesion and aggregation, and protection from vasospasm and thrombosis [15, 16]. Acetylcholine is known to stimulate NO synthesis in the endothelium [3]. Since the stimulating effect of acetylcholine on forearm

blood flow is decreased in diabetic patients [17], it is speculated that NO production is impaired in diabetic patients.

As shown in the present study, urine NOx was reversely correlated with BMI that is reversely related with GIR [18]. It is noted that there was a closer correlation between urine NOx and GIR independently of BMI. These data suggest that reduced urine NOx is primarily associated with GIR in these diabetic patients. Furthermore, insulin resistance in essential hypertension was observed independently of obesity [5].

Since insulin has a trophic effect on vascular tissues [19], hyperinsulinemia is a critical factor for atherosclerosis that induces blood flow insufficiency in the skeletal muscle. It is suggested that NO synthesis is deteriorated in atherosclerotic vessels [20]. NO is reported to inhibit insulin secretion from pancreatic β -cells [21]. Therefore, the reduction of NO production may be involved in hyperinsulinemia. These data suggest the presence of a pernicious cycle of hyperinsulinemia – atherosclerosis – reduced NO production – decreased inhibitory effect of NO on insulin secretion. These findings taken together would indicate that NO plays a critical role in insulin resistance in the patients with type 2 diabetes. It remains to be clarified whether endogenous NO production is restored when insulin resistance is improved.

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