Increased plasma concentration of nitric oxide in type 2 diabetes but not in nondiabetic individuals with insulin resistance

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SUMMARY

Objective: Insulin resistance (IR) is a key element in the pathogenesis of type 2 diabetes. The results of recent experiments on insulin-mediated vasodilatation have suggested that vascular insensitivity is a component of IR. However, it is still controversial that patients with type 2 diabetes have a decreased ability of insulin to increase endothelial nitric oxide (NO) release.

Method: Plasma concentration of NO was examined in 26 patients with type 2 diabetes and 78 nondiabetic volunteers during an insulin suppression test. The test measured the efficacy of insulin in promoting disposal of the infused glucose load, in which the steady state plasma glucose (SSPG) during the 150-180 min of the test was used as an index of IR. Plasma NO levels were assessed by measurement of the stable end products of their metabolism. Comparison of plasma NO levels between groups were performed by Mann-Whitney test and relationships between SSPG and different variables were analyzed by partial correlations.

Results: Our results showed that the plasma NO levels were significantly higher in the diabetic group. When the nondiabetic subjects were analyzed according to their SSPG levels, there was no difference of plasma NO levels between those with SSPG > 160 mg/dl and those with SSPG < 160 mg/dl. There were also no difference of NO levels between those with a family history of type 2 diabetes and those without. In the nondiabetic group, SSPG correlated with BMI, fasting insulin, triglyceride and HDL-cholesterol, but neither with plasma NO levels nor fasting plasma glucose.

Conclusion: Our data suggests that the impairment of NO activity in patients with type 2 diabetes is due to an impaired effect rather than its production. This altered NO signaling pathway is not an early event in insulin resistant individuals. Any such changes will not be apparent until type 2 diabetes with overt hyperglycemia develops.

Key-words: Nitric oxide · Type 2 diabetes · Insulin resistance.


RÉSUMÉ

Augmentation des concentrations plasmatiques de monoxyde d’azote chez le diabétique de type 2, mais non chez le sujet non diabétique insulinorésistant

Objectif : L’insulino-résistance (IR) est un élément clé de la pathogénie du diabète de type 2. Les résultats d’expérimentations récentes sur la vasodilatation médiane par l’insuline ont suggéré que l’insensibilité vasculaire est un composant de l’IR. Cependant, la notion d’une réduction de la capacité de l’insuline à augmenter la libération endothéliale de monoxyde d’azote (NO) chez le diabétique de type 2 est controversée.

Méthodes : Les concentrations plasmatiques de NO ont été déterminées chez 26 diabétiques de type 2 et 78 volontaires non diabétiques lors d’un test de suppression insulinique. Le test mesure l’efficacité de l’insuline à promouvoir l’utilisation d’une charge en glucose perfusée, la glycémie en état stable (steady state plasma glucose SSPG) au cours des 150-180 min du test étant choisie comme index d’IR. Les concentrations plasmatiques de NO ont été déterminées par mesure des produits finaux stables de son métabolisme. La comparaison des concentrations plasmatiques de NO entre les groupes a été effectuée par test de Mann-Whitney test et les relations entre SSPG et les différentes variables analysées par corrélations partielle.

Résultats : Nos résultats montrent que les concentrations plasmatiques de NO sont significativement plus élevées chez les diabétiques. Lorsque les non diabétiques ont été analyses selon leur niveau de SSPG, il n’y avait pas de différence pour le NO entre ceux avec SSPG > 160 mg/dl et ceux avec SSPG < 160 mg/dl. Il n’y avait pas non plus de différence pour le NO entre ceux avec antécédents familiaux de diabète de type 2 et les autres. Dans le groupe non diabétique, SSPG est corrélé avec le BMI, l’insulinémie à jeun, les triglycérides et le HDL-cholestérol, mais non avec les concentrations plasmatiques de NO ni avec la glycémie à jeun.

Conclusion : Nos résultats suggèrent que l’altération de l’activité NO chez les diabétiques de type 2 est due à une altération de l’effet plutôt que de sa production. Cette altération de la signalisation NO n’est pas un événement précoce chez le sujet insulino-résistant. De tels changements ne sont pas apparents avant la révélation d’un authentique diabète de type 2 avec hyperglycémie.

Mots-clés : Monoxyde d’azote · Diabète de type 2 · Insulino-résistance.

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Insulin resistance (IR) is a key element in the pathogenesis of type 2 diabetes mellitus [1]. The link between IR and cardiovascular disease is well documented [2]. Recent experimental findings on insulin-mediated vasodilation have suggested that vascular insensitivity is also a component of IR [3-5]. Therefore both reduced insulin-stimulated glucose uptake and vascular endothelial dysfunction are important features of insulin resistance syndrome [6-9]. Moreover, association between the metabolic and vascular effects of insulin has been shown [10-13]. There is evidence that endothelial nitric oxide synthase (eNOS) polymorphism is associated with type 2 diabetes and the insulin resistance syndrome [14, 15]. A novel concept is evolving that impairment of nitric oxide (NO) activity may represent a central defect causing many of the metabolic, vascular and sympathetic abnormalities associated with IR [16].

Impairment of NO activity in IR can be caused by impaired production, uncoupling of receptor-mediated signal transduction or nullification of NO before it is able to activate the signaling cascade [17, 18]. However, not all published data are consistent and it is not possible to reach a consensus at present. Either decrease [19] or increase [20-25] in the ability of insulin to stimulate endothelial NO release has been reported. Furthermore, how early the alteration in the NO signaling cascade occurs in the pathogenesis of the insulin resistance syndrome is still not known. In this study, we enrolled 26 patients with type 2 diabetes and 78 nondiabetic volunteers with or without a family history of type 2 diabetes. Their degree of IR was quantified by an insulin suppression test and plasma NO concentrations were monitored during the test. The relationship of IR and NO concentration and the clinical parameters of metabolic syndrome were examined.

Subjects and methods

The study population consisted of 26 patients with type 2 diabetes and 78 nondiabetic volunteers. Diabetic patients (16 women and 10 men; aged 52.4 ± 7.4) were recruited from our metabolic clinic; 4 of these were treated by diet control and 22 with oral hypoglycemic agents. Their diabetic history ranged from 3 months to 10 years and the concurrent HbA1c was 7.4 ± 0.8%. None of the 26 patients had microalbuminuria and 2 had background retinopathy. None of the patients were taking medication during the study. Volunteers (42 women and 36 men, aged 47.3 ± 6.1) were recruited from individuals attending a health screening center as well as medical center employees. Volunteers were in good general health, without a history of diabetes or hypertension, nonsmokers, and no medication that would affect carbohydrate or lipid metabolism. The study protocol was approved by the ethics committee of our institute and the subjects consented to the procedure after full explanation. Overnight fasting blood samples were obtained for measurement of fasting plasma glucose, insulin, and lipoprotein. In addition, liver and renal function tests were performed, and only individuals with normal liver and renal function were enrolled. Finally, measurements were made of blood pressure, body mass index (BMI), and ratio of waist-to-hip (WHR) girth. Volunteers were excluded if they had a fasting plasma glucose (FPG) concentration greater than 126 mg/dl (FPG < 110 mg/dl in 70 controls; FPG 111-125 mg/dl in 8 controls), a BMI greater than 30 kg/m², or a blood pressure greater than 160/90 mmHg. Insulin-stimulated glucose disposal was determined as described previously [26, 27]. Briefly, after an overnight fast, intravenous catheters were placed in each arm. Blood was sampled from one arm for plasma glucose and insulin concentration, and the contralateral arm was used for administration of test substances. Each subject was given a continuous infusion of octreotide acetate at 30 µg/h following a bolus of 25 µg, insulin at 25 mU/m²/min, and glucose at 240 mg/m²/min for 180 minutes. Blood was sampled hourly until 2 hours into the study, and then every 10 minutes at 150, 160, 170 and 180 minutes. These last 4 values were averaged and used as an estimate of the steady-state plasma glucose (SSPG) and steady-state plasma insulin (SSPI) concentrations. Because octreotide inhibits endogenous insulin secretion, the infusion of exogenous insulin resulted in similar SSPI concentration, providing a measurement of the efficacy of insulin in promoting disposal of the infused glucose load. After the insulin suppression test, the nondiabetic volunteers were divided into insulin-sensitive (SSPG < 160 mg/dl) and insulin-resistant (SSPG > 160 mg/dl) groups. Table I presents the demographic and metabolic characteristics of the 26 type 2 diabetic patients and the 78 normal controls. NO levels were assayed by measurement of the end products of its metabolism NO₂⁻ (NO₂⁻ + NO₃⁻) by chemiluminescence method as previously described [28, 29]. All subjects fasted for 12 hours overnight and had no food intake during examination. We used a NO analyzer (NOA 280; Sievers Inc., Denver, USA) to detect the plasma NO₃⁻. For measurement of the NOₓ levels, the samples were converted into NO by a strong reducing agent, VCl₃ (0.4%, purchased from Boehringer Mannheim, Germany), in the presence of 2N HCl. The NO analyzer allows the interaction of NO with ozone to elicit chemiluminescence. The chemiluminescence reaction is shown as

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NO + O₃ \rightarrow NO₂ + O₂ + hv
\]

Results were calculated under the curve of the photomultiplier tube (PMT) current for each determination paralleling the amount of NO. For experiments, the NO₃⁻ and NO₂⁻ levels in each sample were determined by an interpolation of a standard curve made from a series of well-known concentrations of sodium nitrate. A tiny amount of the plasma (50 µl) was subjected...
to measurement by the NO analyzer, after alcohol precipitation of protein in the samples at 1:2 (volume/volume) ratio.

Plasma glucose concentration was determined on an autoanalyzer (Olympus Reply; Olympus Optical, Mishima, Japan). HbA1c was measured by affinity HPLC (PRIMUS CLC 385, PRIMUS Corporation, Kansas City, MO, USA). For other laboratory tests, aliquots of plasma and serum were immediately frozen and stored at -70°C until analysis. Insulin was measured by radioimmunoassay (Insulin radioimmunoassay kit; CIS Bio International, France), total cholesterol with a standard enzymatic method (T-CHOL(S), Seiken, Tokyo, Japan), triglyceride with the standard GPO (glycerol-3-phosphate oxidase method, Seiken), HDL-cholesterol by a selective inhibition assay (HDL-C Auto “DAICHI”; Daiichi Pure Chemical, Tokyo, Japan), and LDL-cholesterol by a 2-step detergent assay (CHOLESTEROL LDL; Daiichi Pure Chemical).

Comparisons of the plasma NO levels between groups were performed by Wilcoxon-Mann-Whitney test, between serial observations over time by Friedman statistics. Relationships between SSPG and different variables after adjustment for age, sex and BMI were analyzed by partial correlations. Data evaluation was conducted using SPSS 9.0, 1998, for Windows (SPSS, Inc, Chicago, IL). The level of significance was determined as p less than 0.05.

### Results

#### Comparison of NO levels between diabetic and nondiabetic subjects

In the insulin suppression test, under similar SSPI concentration, the plasma NOx levels at basal (80.5 ± 58.0 μM vs 57.5 ± 45.5 μM), 30 min (72.6 ± 53.6 μM vs 51.1 ± 41.2 μM), 60 min (69.9 ± 48.3 μM vs 49.2 ± 38.2 μM), 120 min (66.0 ± 43.2 μM vs 46.5 ± 35.0 μM), and 150 min (64.9 ± 47.6 μM vs 45.7 ± 34.1 μM) after insulin infusion were significantly higher in the diabetic group as compared to the nondiabetic controls (Tab II).

The changes of the NOx concentrations during the insulin suppression test in both groups were similar (Tab II). A decrease, instead of increase, of the NOx levels was noted under the infusion of octreotide (30 μg/h) and insulin (25 mU/m2/min) during the test. In this test, simultaneous infusion of octreotide and insulin may interfere with the action of insulin on NOx release.

#### Comparison of the NO levels within nondiabetic volunteers

When the nondiabetic subjects were analyzed according to their SSPG concentration, there was no difference of plasma NOx levels between those with SSPG > 160 mg/dl and those with SSPG < 160 mg/dl at basal and all time points during the test (Tab III). There was also no differ-

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**Table I**

Clinical characteristic of the controls and type 2 diabetes patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Type 2 diabetes (N = 26)</th>
<th>Control SSPG &gt; 160 mg/dl (N = 34)</th>
<th>Control SSPG &lt; 160 mg/dl (N = 44)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>52.4 ± 7.4</td>
<td>47.3 ± 5.8</td>
<td>47.2 ± 6.3</td>
<td>0.003</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>16/10</td>
<td>19/15</td>
<td>23/21</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>121 ± 14</td>
<td>120 ± 18</td>
<td>115 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>78 ± 8</td>
<td>81 ± 9</td>
<td>77 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>WHR (cm)</td>
<td>0.93 ± 0.06</td>
<td>0.88 ± 0.05</td>
<td>0.87 ± 0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.1 ± 3.3</td>
<td>24.8 ± 2.3</td>
<td>23.1 ± 1.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (mU/ml)</td>
<td>13.6 ± 6.0</td>
<td>10.8 ± 5.9</td>
<td>8.0 ± 6.4</td>
<td>0.001</td>
</tr>
<tr>
<td>FPG (mg/dl)</td>
<td>162 ± 31</td>
<td>97 ± 10</td>
<td>97 ± 9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FPG &lt; 110/FPG 111-125</td>
<td>29/5</td>
<td>41/3</td>
<td>41/3</td>
<td>NS</td>
</tr>
<tr>
<td>T-Chol (mg/dl)</td>
<td>194 ± 39</td>
<td>173 ± 25</td>
<td>171 ± 32</td>
<td>0.017</td>
</tr>
<tr>
<td>HDL-Chol (mg/dl)</td>
<td>45 ± 9</td>
<td>40 ± 11</td>
<td>44 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-Chol (mg/dl)</td>
<td>117 ± 32</td>
<td>104 ± 25</td>
<td>106 ± 27</td>
<td>NS</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>161 ± 75</td>
<td>147 ± 105</td>
<td>98 ± 55</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Continuous data were expressed as the mean ± SD. Comparison among the 3 groups was performed by one-way ANOVA for continuous data and generalized Fisher’s exact statistics for categorical data. Abbreviation: BMI, body mass index; WHR, waist-to-hip ratio; BP, blood pressure; SSPG, ateady-state plasma glucose; FPG, fasting plasma glucose; T-chol, total cholesterol; HDL-Chol, high-density lipoprotein; LDL-Chol, low-density cholesterol; NS, not significant.
ence of plasma NO\textsubscript{x} levels between those with FPG < 110 mg/dl and those with FPG between 111 and 125 mg/dl at basal and all time points during the test (Tab IV). Furthermore, when nondiabetic volunteers were analyzed according to family history of type 2 diabetes (parent or sibling), there was no difference of plasma NO\textsubscript{x} levels between those with a family history and those without (Tab V).

### Relationship between insulin resistance and various cardiovascular risk factors in nondiabetic volunteers

In the nondiabetic group, univariate analysis after adjustment for age, gender and BMI revealed that SSPG correlated with BMI (r = 0.36, p = 0.002), fasting insulin (r = 0.33, p = 0.004), triglyceride (r = 0.29, P = 0.013), and marginally with HDL-cholesterol (r = -0.28, p = 0.073), but not with the NO\textsubscript{x} levels (-0.10, p = 0.377), FPG, systolic and diastolic blood pressure, ratio of waist-to-hip girth, total-cholesterol, and LDL-cholesterol (Tab VI).

### Discussion

Diabetes is associated with early development of atherosclerosis. The endothelial cells under normal condition protect against the complication by producing vasoactive factors, e.g., NO, to keep the vascular tone, coagulation, and inflammation well balanced [30, 31]. Studies in diabetic patients have demonstrated impaired endothelium-derived NO activity with or without normal response to exogenous NO [4, 10, 32-34]. The current study shows that patients with overt diabetes had higher plasma NO concentrations than nondiabetic volunteers, no matter whether the latter were insulin sensitive or resistant. If NO concentration is
increased in diabetic patients, the endothelial dysfunction is probably due to a defect in the generation of its effector messenger or nullification of NO before it can activate the signaling cascade. This increase of NO levels could be an effort to compensate for the defect in subsequent signal transduction. In this way, even though the NO concentration is increased, its biological effect is still impaired. Reviewing existing literature, diabetes or hyperglycemia per se have both been reported to be associated with increased NO levels [20-23]. Furthermore, the mechanism of inactivation of NO through its reaction with reactive oxygen species may be of particular relevance for diabetic patients to determine the bioavailability of NO [20, 23, 35, 36].

However, in the nondiabetic insulin-resistant group, our study did not find the same phenomenon as in the diabetic group. There was no difference in plasma NO levels between those with SSPG > 160 mg/dl (insulin resistant) and those with SSPG < 160 mg/dl (insulin sensitive). There was also no difference between those with a family history of diabetes and those without. Furthermore, when the relationship between IR (SSPG) and plasma NO levels was assessed, there was no correlation demonstrated (Tab VI). Furthermore, although the cases with impaired fasting glucose (IFG) in this series were few, there was also no difference in plasma NO levels between normal fasting glucose individuals (FPG < 110 mg/dl) and those with IFG (FPG 111-125 mg/dl). Our data suggests that an altered NO pathway is not an early event in the pathogenesis of type 2 diabetes. Although most of the metabolic defects of diabetics, e.g., high fasting plasma insulin level, high triglyceride, low HDL, or increased BMI, were present in the insulin resistant nondiabetic individuals, it appears that the changes in the NO pathway will not be apparent until overt type 2 diabetes develops. In other words, the data did not support the hypothesis that an altered NO pathway is a central defect causing many of the metabolic and vascular disorders. Instead, alteration of the NO signaling cascade is rather a result of the hyperglycemia or oxidative stress caused by diabetes.

Finally, it has been proposed that insulin mediated vasodilation induced by NO release represents a physiological mechanism for postprandial glucose disposal [37-39]. Although other studies have reported that flow became an important modulator of glucose uptake only at supraphysiological concentrations of insulin [40]. In our study, there was a decrease of NO concentration observed during the insulin suppression test which had reached a plateau of SSPI around 100 uU/ml. It appears contrary to the hypothesis that the postprandial vascular effect of insulin is mediated by stimulating the production of NO. However, in our experiment, the use of somatostatin analog, which has a well-known inhibition effect on a variety of endogenous hormones, and has been demonstrated to modulate the release of NO by hepatic Kupffer cells from rats [41], probably might suppress the NO concentrations in our experiment. This needs to be carefully evaluated before drawing any definite conclusions.

In conclusion, our preliminary data suggests that the impairment of NO activity in patients with type 2 diabetes is due to an impaired effect rather than its production. This altered NO signaling pathway is not an early event in insulin resistant individuals. Any such changes will not be clinically apparent until type 2 diabetes with overt hyperglycemia develops.

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References

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