Role of Hyperglycemia in Nitrotyrosine Postprandial Generation

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OBJECTIVE — Recently, much attention has been paid to the possibility that postprandial hyperglycemia may be a cardiovascular risk factor in diabetes. Oxidative stress has been involved in the pathogenesis of diabetic complications, and increased plasma levels of nitrotyrosine, a product of peroxynitrite action, have been found in the plasma of diabetic subjects. The aim of the present study was to evaluate whether postprandial hyperglycemia is accompanied by nitrotyrosine generation and, if so, to explore a possible direct role of hyperglycemia in such a phenomenon.

RESEARCH DESIGN AND METHODS — A total of 23 type 2 diabetic patients and 15 matched normal healthy subjects were recruited for this study. Two different tests were performed in diabetic patients: a standard meal preceded by regular insulin (0.15 units/kg body wt) or insulin aspart (0.15 units/kg body wt) to achieve different levels of postprandial hyperglycemia. The meal test was also performed in healthy control subjects. At 0 min and 1, 2, 4, and 6 h after each meal, blood glucose, triglyceride, and nitrotyrosine levels were measured.

RESULTS — Fasting nitrotyrosine was significantly increased in diabetic patients and was further increased during both meal tests in diabetic subjects but not normal subjects. As compared with regular insulin, aspart administration significantly reduced the area under the curve of both glycemia (P < 0.04) and nitrotyrosine (P < 0.03), whereas that of triglycerides was not significantly affected by the treatment.

CONCLUSIONS — This study shows a direct correlation between postprandial hyperglycemia and the production of nitrotyrosine, a marker of oxidative stress, in patients with type 2 diabetes.

P atients with diabetes have an increased risk of cardiovascular morbidity and mortality (1). In recent years, much attention has been paid to the causal relationship between the postprandial state and atherogenesis (2,3), even in diabetes (4,5), and it has been suggested that postprandial hyperglycemia may be an independent risk factor for cardiovascular disease (CVD) (6).

There is growing evidence that oxidative stress, i.e., the imbalance between free-radical production and antioxidant defense, is involved in the pathogenesis of CVD in diabetes (7). The postprandial generation of an oxidative stress, with consumption of antioxidant defenses, has been demonstrated in type 2 diabetic patients (8).

It has been shown that hyperglycemia is associated with a simultaneous increase in the generation of superoxide anion (O2–) and nitric oxide (NO) (9). This increase is harmful because NO and O2– react to produce peroxynitrite, a potent oxidant that lives for a long time (10). The peroxynitrite anion is cytotoxic because it inhibits mitochondrial electron transport, oxidizes sulfydryl groups in protein, initiates lipid peroxidation without the requirement for transition metals, and nitrates amino acids such as tyrosine, which in turn affects many signal transduction pathways (10). The production of peroxynitrite can be indirectly inferred by the presence of nitrotyrosine (NT) residues (11). Increased NT has been found in the plasma of diabetic patients (12), and there is evidence that an acute increase of glycemia induces an increase of NT (13). However, no data concerning the production of NT in the postprandial state after a normal meal and the possible contribution of hyperglycemia to that phenomenon are available as of yet.

The aim of the present study was to evaluate whether postprandial hyperglycemia is accompanied by NT generation and to explore the direct role of hyperglycemia on this phenomenon. We measured plasma NT levels in the postprandial state in insulin-treated type 2 diabetic patients and in healthy control subjects. Moreover, to point out the role of postprandial hyperglycemia in type 2 diabetic subjects, NT was assayed in blood samples drawn after a meal preceded by either regular insulin or insulin aspart administration. Insulin aspart is a modified form of human insulin, in which a single amino acid (proline B28) has been changed to an aspartic acid residue (14). This change reduces the tendency of the molecule to aggregate in solution and, as a result, greatly increases its speed of absorption after subcutaneous injection (14). It has been shown that in type 2 diabetic subjects, the control of postpran-
Meals and nitrotyrosine in diabetes

Table 1—Baseline characteristics at the screening of normal and diabetic subjects

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Diabetic subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>8/7</td>
<td>13/10</td>
</tr>
<tr>
<td>Age</td>
<td>57.1 ± 4.7</td>
<td>58.1 ± 4.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.9 ± 3.5</td>
<td>26.6 ± 3.1</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.8 ± 0.2</td>
<td>8.2 ± 1.1*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.8 ± 0.2</td>
<td>7.3 ± 0.5*</td>
</tr>
<tr>
<td>Resting systolic blood pressure (mmHg)</td>
<td>118.3 ± 7.5</td>
<td>122.4 ± 6.5</td>
</tr>
<tr>
<td>Resting diastolic blood pressure (mmHg)</td>
<td>78.4 ± 2.1</td>
<td>82.2 ± 3.1</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.5 ± 0.6</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.9 ± 0.2</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.4 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>NT (µmol/l)</td>
<td>0.22 ± 0.1</td>
<td>0.57 ± 0.1*</td>
</tr>
</tbody>
</table>

Data are means ± SD. *P < 0.001 vs. control subjects.

dial glucose levels with insulin aspart, dosed immediately before a meal, is better than that achieved with regular human insulin dosed at 30 min before a meal as recommended (15).

**RESEARCH DESIGN AND METHODS** — A total of 23 type 2 diabetic patients on insulin therapy and 15 matched healthy subjects were recruited for this study. The clinical characteristics of the subjects are shown in Table 1. The mean duration of insulin treatment was 3.0 ± 0.9 years. The mean usual daily insulin dose was 28.4 ± 5.6 units, administered in two or three daily injections. The diabetic patients were in satisfactory metabolic control, as judged by means of their glycated hemoglobin levels (7.3 ± 0.5%). All subjects were nontobie healthy, nonsmokers. Each subject had a normal resting electrocardiogram and no personal history of vascular disease.

None of the subjects were taking aspirin, lipid-lowering agents, or supplemental vitamins. All subjects were recommended to consume their usual diet during the entire period of the study (1 week) and to not inject intermediate insulin the night before each test. Written consent was obtained from all subjects, with local ethics committee approval.

**Study design**

After visit 1 (screening and informed consent), patients were randomized (visit 2) on the basis of a computer-generated random table list to the first of the two treatment sequences. On the day of the study, a blood sample was drawn for fasting glycemia, and each patient started an intravenous infusion of regular insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) to achieve a premeal glucose level ranging from 7.2 and 8.3 mmol/l.

The intravenous insulin infusion was then stopped, and after 30 min, the experiment was started. Actrapid (0.15 units/kg body wt) was administered 30 min before the meal, whereas insulin aspart (NovoRapid; Novo Nordisk) (0.15 units/kg body wt) was injected subcutaneously at the beginning of the meal. The dose of 0.15 units/kg body wt was selected according to the recommendations by Rosenfalk et al. (15). The two tests were performed in each subject within 3 days of each other. The meal test was also performed in healthy control subjects. The cooked meal contained 600 kcal (50% carbohydrate, 30% fat, 20% protein) (16). At 0 min and 1, 2, 4, and 6 h after each meal, glycemia, triglyceride, and NT levels were evaluated.

**Biochemical measurements**

Plasma glucose was measured by the glucose oxidase method, triglycerides were measured enzymatically by a commercially available method (Roche Diagnostics, Basel), and HbA1c was measured by high-performance liquid chromatography (Menarini Diagnostics, Pisa, Italy).

**NT measurement**

NT plasma concentration was assayed by enzyme-linked immunosorbent assay (ELISA), developed according to Ter Steege et al. (17) and recently validated by our laboratory (12).

BSA, tetramethylmethane (TNM), IgG secondary antibody, and tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO). Maxisorp ELISA plates were from NUNC, Life Technologies (Grand Island, NY), and IgG monoclonal anti-nitrotyrosine antibody was from Upstate (Lake Placid, NY).

**Preparation of nitrated protein.** Nitrated protein solution was carried out by incubating 1 mg/ml BSA in 50 mmol/l KH₂PO₄ at pH 7.4 for 30 min at 37°C with 1 mmol/l TNM, an efficient nitrating agent (18). After adjusting the pH to 10 with 3 mol/l NaOH, the amount of NT present in the TNM-treated BSA solution was measured at 430 nm (18).

**ELISA.** A standard curve was constructed by incubating in the wells serial dilutions of nitro-BSA in 0.1 mol/l Na₂CO₃-NaHCO₃ coating buffer at a pH of 9.6. Plasma samples assayed for NT were diluted up to five times. Standard and plasma samples were applied to Maxisorp ELISA plates and allowed to bind overnight at 4°C. Afterward, nonspecific binding sites were blocked with 1% BSA in PBS. The wells were incubated at room temperature for 2 h with a mouse IgG monoclonal anti-nitrotyrosine (3 µg/ml) with a peroxidase-conjugated goat anti-mouse IgG secondary antibody diluted 1:4,000. After washing the plates, the peroxidase reaction product was generated using a TMB microwell peroxidase substrate. Plates were incubated for 10 min at room temperature, and the reaction was stopped with 50 µl per well of stopping reagent and read at 492 nm in a microplate reader (19). The concentration of nitrated proteins that inhibited anti-nitrotyrosine antibody binding was estimated from the standard curve and expressed as micromoles per liter NT (17).

Glucose interference was excluded when performing the ELISA of standard solution in the presence of various glucose concentrations (20, 40, and 100 mmol/l). The limit of detection was 10 mmol/l, whereas the intra- and interassay coefficients of variation were 4.5 and 8%, respectively.

**Statistical analysis**

All measurements are presented as means ± SD. Analyses of the difference in clinical characteristics between diabetic and nondiabetic subjects were made using unpaired and two-tailed t tests. The changes in variables during each test were assessed by two-way ANOVA with re-
Effect (i.e., the real efficacy of treatment interaction, or of a period-treatment interaction), of a period-tendency for patients to do better in one of the periods of the trial), of a period-effect, a two-sample Student t's test. Comparing d1 with d2 (20). Therefore, the results of the serial measurements of glucose, triglycerides, and NT were first summarized by calculating for each parameter the area under the curve (AUC) in those patients first treated with regular insulin (AUCA) or regular insulin (AUCR). To verify the possibility of a period-effect, a two-sample Student’s t test (two-tailed) was performed, comparing d1 (mean of individual differences AUCA − AUCP in those patients first treated with insulin aspart) with d2 (mean of individual differences AUCR − AUCA, changed of sign, in those patients first treated with regular insulin) (20). The possibility of a period-treatment interaction was evaluated by a second two-sample Student’s t test (two-tailed), comparing the average of the individual observations [(AUCA + AUCR)/2] in the aforementioned two groups of patients (20). The treatment effect was tested by performing a third two-sample Student’s t test (two-tailed), taking into consideration the average effect of treatment in the two periods, i.e., comparing d1 with d2 (20).

Statistical significance was defined as $P < 0.05$. All statistical tests were performed using the SPSS statistical software package.

**RESULTS** — As reported in Table 1, at the screening evaluation, fasting NT concentration was significantly higher in diabetic patients than in healthy control subjects. On the testing day, because patients were instructed to not take intermediate insulin the night before, fasting glycemia (although higher than at the screening evaluation in diabetic subjects) was not different in the patients taking regular insulin compared with the patients taking insulin aspart (10.8 ± 2.4 and 11.3 ± 2.3 mmol/l, respectively). Figure 1 shows the changes in the plasma concentrations of glucose, triglycerides, and NT after meals in normal subjects and diabetic patients given either regular or aspart insulin. Glycemia ($F = 18.5, P = 0.001$), by ANOVA for repeated measures), triglycerides ($F = 12.1, P = 0.001$), and NT ($F = 26.2, P = 0.001$) significantly changed over time during the two tests. Compared with regular insulin, insulin aspart administration significantly reduced the area under the curve of both glycemia (58.3 ± 17.6 vs. 68.1 ± 17.7 mmol/l; $P < 0.04$) and NT (3.60 ± 2.04 vs. 4.49 ± 2.06 μmol/l; $P < 0.03$), whereas there was no difference in the area under the curve of triglycerides between the two treatments (8.2 ± 4.7 vs. 8.1 ± 4.4 mmol/l; $P < 0.8$). Glycemia ($F = 0.4$), triglycerides ($F = 1.5$), and NT ($F = 0.8$) postprandial variations did not reach significance in normal subjects.

**CONCLUSIONS** — This study, for the first time, shows that the postprandial state is accompanied by a significant increase of NT in type 2 diabetic patients. The demonstration that lower postprandial glycemic levels, in the presence of the same level of postprandial triglycerides after insulin aspart administration, is associated with less production of NT suggests a specific and direct role of postprandial hyperglycemia in favoring NT formation.

The possibility that hyperglycemia may lead to NT formation is supported by studies showing the presence of NT in the placenta, kidney, heart, and plasma of diabetic patients (12,21,22) and in aortic tissue from diabetic cynomolgus monkeys (23). The direct role of high blood glucose levels has been substantiated by
showing that acute hyperglycemia induces NT overproduction, even in the plasma of healthy subjects and in working hearts from rats (13,24). Therefore, it is not surprising that the postprandial phase, which is accompanied in type 2 diabetic patients by a marked increase of glycemia, will show an acute increase in NT. It is also interesting to underline that NT did not increase after meals in normal subjects, whose postprandial triglyceride and blood glucose levels showed only a slight and nonsignificant increase.

Postprandial hypertriglyceridemia may represent an independent predictor of CVD in nondiabetic patients (25,26) and has also been a predictor of carotid intima-media thickness in patients with type 2 diabetes (27). However, in the multiple regression analysis in the same study, postprandial hyperglycemia was also found to be an independent risk factor (27).

This finding is not surprising because many epidemiological studies substantiate postprandial hyperglycemia as a risk factor for CVD (6,28). In our study, hyperglycemia seems to have more relevance in favoring NT formation than triglyceride variations, possibly because of an insufficient sample size, the composition of the mixed meal, and/or the outweighing role of the glycemic factor in the diabetic population.

The process through which postprandial hyperglycemia facilitates CVD has been suggested to be the production of oxidative stress (5). This hypothesis is supported by our finding that, in the postprandial phase, there is an increase in NT plasma levels, with NT being a marker of peroxynitrite production. The finding that peroxynitrite production in the postprandial phase appears to be increased may have important pathogenetic implications.

Peroxynitrite is a potent oxidant and nitrating agent that leads to a host of potentially harmful events, including VLDL peroxidation (29), depletion of antioxidant defenses (30), and inactivation of enzymes (31). In addition, it can be directly cytotoxic for endothelial cells (32). All these events may convincingly be involved in the pathogenesis of atherosclerosis and CVD. This hypothesis is strongly supported by the recent finding that the increased apoptosis of myocytes, endothelial cells, and fibroblasts in heart biopsies from diabetic patients (33) and in hearts from streptozotocin-induced diabetic rats (34) is selectively associated with the levels of NT found in those cells. Furthermore, the demonstration that NT can induce endothelial dysfunction by itself (35) and that it is present in atherosclerotic lesions in humans and diabetic cynomolgus monkeys (23,36) is additional evidence that peroxynitrite production may be strongly involved in atherogenesis.

In conclusion, the present study demonstrates a strong correlation between postprandial hyperglycemia and NT production in insulin-treated type 2 diabetic patients. Because NT is a marker of the production of peroxynitrite, which in turn is a putative determinant of oxidative stress and CVD, our data reinforce the hypothesis that the postprandial state, particularly in diabetic subjects because of their marked hyperglycemia, may have an important role in the pathogenesis of CVD through the production of oxidative stress.

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