Meal-Generated Oxidative Stress in Type 2 Diabetic Patients

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OBJECTIVE — Free radical production has been reported to be increased in diabetic patients and to be involved in the pathogenesis of diabetic complications. In this study, a standardized meal was administered to 10 type 2 diabetic patients and 10 healthy matched normal subjects to evaluate its effects on plasma oxidative stress generation.

RESEARCH DESIGN AND METHODS — In diabetic patients, at baseline and after the meal, plasma malondialdehyde (MDA), vitamin C, protein SH groups, uric acid, vitamin E, and total plasma radical-trapping parameter, which evaluates plasma antioxidant capacity due to known and unknown antioxidants present in the plasma as well as their mutual cooperation, were measured.

RESULTS — After the meal, plasma MDA and vitamin C increased, while protein SH groups, uric acid, vitamin E, and total plasma radical-trapping parameter decreased more significantly in the diabetic subjects than in control subjects.

CONCLUSIONS — This finding shows that in the absorptive phase, free radicals are produced in diabetic patients. Since plasma glucose, but not insulin, rose significantly more in diabetic subjects than in control subjects, hyperglycemia may play an important role in the generation of postprandial oxidative stress in diabetic patients.

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Long-term complications are the main cause of morbidity and mortality in diabetic patients. While there is accumulating evidence of a relevant role of oxidative stress in the pathogenesis of both micro- and macroangiopathic diabetic complications (1,2), the relationship between hyperglycemia (3), hyperinsulinemia (4), hyperlipidemia (5), and oxidative stress generation is still unknown.

The hypothesis that hyperglycemia should be able to cause an oxidative stress is supported by in vitro studies (3), and particularly by evidence that several biochemical pathways activated during hyperglycemia can increase the production of free radicals (1–3). In vivo, some antioxidants have been shown to be able to counteract hyperglycemia-induced alterations (2), and some markers of oxidative stress have been found to be increased in diabetic patients (2,3,5), providing no more than indirect evidence of a causal role of hyperglycemia in oxidative stress generation. Similarly, hyperinsulinemia (4) and lipid alterations (5) have been hypothesized as contributory factors to oxidative stress generation in diabetes.

The life of type 2 diabetic patients is marked by very frequent rapid increases, particularly in the postprandial phase, in their blood glucose, insulin, and lipid (primarily triglyceride) levels. If blood glucose and/or insulin and/or lipid levels are important determinants of oxidative stress in diabetes, then their postprandial elevations should play a major role in the production of oxidative stress.

To test this hypothesis, we evaluated the impact of a meal on the antioxidant status of diabetic and nondiabetic subjects by measuring plasma levels of malondialdehyde (MDA), total radical-trapping antioxidant parameter (TRAP), and several circulating antioxidants.

RESEARCH DESIGN AND METHODS

Subjects

Informed consent to participate in the present study was obtained from 10 type 2 diabetic patients (six men and four women aged 54.1 ± 1.5 years [mean ± SEM], duration of diabetes 10.0 ± 1.2 years, BMI 25.6 ± 1.1 kg/m^2) and from 10 healthy normal subjects (six men and four women) matched for age (55.1 ± 1.7 years) and BMI (25.9 ± 1.5 kg/m^2) after a clear explanation of its experimental nature. The study protocol was approved by the ethics committee of our institution.

The diabetic patients were in satisfactory metabolic control, as judged by glycated hemoglobin (7.6 ± 0.3%) levels. They were treated by diet and the hypoglycemic drug glybenclamide (range of daily dose 2.5–7.5 mg). All subjects were nonsmokers. Diabetic and control subjects were also well matched for diet habit, in terms of consumption of fruit and fresh vegetables. Dietary information was obtained by trained interviewers through the administration of a previously validated questionnaire (6).

None of the subjects were on antioxidant supplementation or had clinically symptomatic macroangiopathy, as judged by the absence of pathological changes in the resting electrocardiogram and history of cardiac angina, myocardial or cerebral infarction, and intermittent claudication. None had micro- or macroalbuminuria.

Study design

All medication was withheld for 3 days before the study, when the subjects under-

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Abbreviations: ABAP, 2,2′-azobis-(2-amidinopropane) dihydrochloride; MDA, malondialdehyde; NEFA, nonesterified fatty acid; R-PE, R-Phycoerythrin; TRAP, total radical-trapping antioxidant parameter.

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

went a standard meal test. Even liquid formula diet is not a natural approach to eating; a formula food was chosen to achieve a degree of reproducibility that could not be guaranteed by handmade meals: Ensure Plus (Abbott, Zwolle, The Netherlands) (total energy: 600 kcal, 53.3% carbohydrate, 30% fat, and 16.7% protein). The standardized meal was administered between 8:00 and 9:00 A.M., after a 12- to 14-h fast. All subjects were at rest, in a seated position, throughout the test. Blood samples, obtained in absence of venous stasis, were collected at baseline and 60, 120, and 180 min after the meal. In every sample, plasma glucose, insulin, cholesterol, triglycerides, nonesterified fatty acids (NEFAs), MDA, uric acid, protein-bound SH groups, vitamin C, vitamin E, and TRAP were measured.

Assays

Plasma glucose was assayed by the glucose-oxidase method and insulin by a radioimmunologic method. Glycated hemoglobin (HbA_{1c}) was measured by aminophenylboronic acid affinity chromatography (7). Cholesterol and triglyceride concentrations were assayed enzymatically on a clinical chemistry analyzer (Monarch; Instrumentation Laboratory, Lexington, MA). NEFAs were measured by a commercially available kit (NEFA Quick; Boehringer Mannheim, Tokyo).

MDA was evaluated by a highly sensitive fluorometric method according to Conti et al. (8). The intra- and interassay coefficients of variation for this method were 6.5 and 8.8%, respectively.

Measurement of TRAP. 2,2′-azobis-(2-amidinopropane) dihydrochloride (ABAP), R-Phycoerythrin (R-PE), Trolox, and all other chemicals were purchased from Sigma (St. Louis, MO).

TRAP was evaluated according to Ghiselli et al. (9). In this method, the production of peroxyl radicals obtained by thermal decomposition of ABAP leads to a linear decrease in R-PE fluorescence emission over 1 h. When plasma is added to the reaction mixture, a period of complete protection of R-PE is observed. The length of this lag-phase (*T*) is here taken to be directly related to total plasma antioxidant capacity. To quantify the TRAP, the *T* produced by plasma is compared with the *T* produced by a known amount of Trolox. The values of *T* are calculated by extrapolating the slope of maximal R-PE decay to intersect with the slope of plasma and Trolox protection. The projection of these intersection points on the *x*-axis gives the *T* values, which represent the time required to achieve the maximal R-PE peroxidation rates. By comparing the *T* of plasma with the *T* of Trolox, taking into account the concentration (Conc) of Trolox, the TRAP value of a plasma sample is obtained according to the following proportion: Conc Trolox:*T* Trolox = *x*:*T* plasma (9).

The resulting value of *x* is then multiplied by 2.0 (the stoichiometric factor of Trolox) and by the dilution factor of plasma (250); values are expressed as μmol/l.

Sample preparation. According to the procedure of Ghiselli et al. (9) the reaction mixture consisted of 1.5×10^{-8} mol/l R-PE in 75 mmol/l phosphate buffer, pH 7. A volume of 8 μl plasma or 30 μl of 120 μmol/l Trolox was added to the 2.0 ml final volume, and the resulting solution was maintained at 37°C for 5 min in 10 mm quartz fluorometer cells. The oxidation reaction was started by adding ABAP to a final concentration of 4.0 mmol/l, and the decay of R-PE fluorescence was monitored every 5 min on a Perkin-Elmer LS-50 Luminescence Spectrometer (Norwalk, CT) equipped with a thermostatically controlled cell-holder; monochromators were operating at excitation wavelength 495 nm/5 nm slit width and emission wavelength 575 nm/5 nm slit width. TRAP values were calculated as described above. Intra- and interassay coefficients of variation for this method were 10 and 12%, respectively.

Glucose interference on TRAP assay. Glucose is not supposed to interfere with the TRAP assay (A. Ghiselli, personal communication). However, to exclude a possible direct interference of glucose on the TRAP assay, glucose solutions of 5.5, 11, and 22 mmol/l were directly tested in the TRAP system assay after 60 min of preincubation. Moreover, glucose was added to 10 normal plasma samples to reach the final concentration of 16.6 mmol/l, and TRAP was measured before and after glucose addition.

Plasma antioxidant assay. Vitamin C was evaluated spectrophotometrically according to Tsan et al. (10). Vitamin E levels were measured by high-performance liquid chromatography (11). Protein SH groups were measured by the method described by Koster et al. (12). Uric acid assay was performed by a commercially available enzymatic colorimetric test (Instrumentation Laboratory).

Statistical analysis

Statistical analysis of data was accomplished by means of the BMDP statistical software package (12a). Baseline differences between diabetic and normal subjects were evaluated by unpaired Student's *t* test. The distribution of values of the various parameters in the two populations studied (patients with diabetes and control subjects) did not show a significant departure from normality at the Shapiro-Wilk *W* test. Therefore, analysis of variance for repeated measures was performed to identify the existence of a significant difference in mean concentration of the various parameters, both at the different times of a specified experiment and among the two different groups of subjects. All values are expressed as means ± SEM.

RESULTS — The baseline characteristics of the two groups of subjects are reported in Table 1. In diabetic patients, plasma glucose and MDA levels were significantly higher, while protein SH groups, uric acid, vitamin C, vitamin E, and TRAP were significantly lower. Adding glucose did not interfere with the plasma TRAP assay (before: 827.5 ± 45.2 μmol/l; after: 836.4 ± 51.7 μmol/l), and glucose solutions tested in the TRAP assay system did not show any interference. Figures 1 and 2 describe the variations of the various parameters after the standardized meal.

The results of the analysis of variance for repeated measures demonstrate that plasma glucose ($P = 0.0001$), insulin ($P = 0.0001$), triglycerides ($P = 0.0001$), NEFA ($P = 0.0001$), MDA ($P = 0.0001$), uric acid ($P = 0.0001$), SH groups ($P = 0.01$), vitamin C ($P = 0.0001$), vitamin E ($P = 0.001$) (corrected for cholesterol plus triglycerides values at any time), and TRAP ($P = 0.0001$) significantly varied with time in each group. The extent of the variations of each parameter was significantly different between the two groups, except for insulin and NEFA: plasma glucose ($P = 0.0001$), triglycerides ($P = 0.02$), MDA ($P = 0.0001$), uric acid ($P = 0.02$), SH groups ($P = 0.0003$), vitamin C ($P = 0.0001$), vitamin E ($P = 0.0001$), and TRAP ($P = 0.0001$) .

CONCLUSIONS — Oxidative damage by reactive oxygen species appears to be involved in the pathogenesis of diabetic complications (1–5).

In the present study, we evaluated the production of an oxidative stress after a meal by means of several parameters, i.e., MDA, various single antioxidant compounds, and TRAP.

The measurement of oxidative stress in vivo is a very complex question. Standard-

ization for oxidative stress measures is not available, and the same is true even for the reference ranges of several antioxidants. Variations in plasma levels of single antioxidants are not good markers of oxidative stress generation, and MDA evaluation is not a very specific method. However, it is the method most frequently used in the literature (13,14). We reported data on MDA to facilitate comparisons with other reports, but we stress that a good marker of oxidative stress is the evaluation of TRAP, a recent but very useful tool to measure total plasma antioxidant capacity. Indeed, TRAP is a global measure of the antioxidant capacity of plasma, taking into account all known and unknown antioxidant activities present in plasma, as well as their synergisms (9). In fact, the overall antioxidant capacity of plasma is determined not only by the absolute concentrations of the various antioxidant compounds, but also by their interactions (15).

A lower plasma TRAP has been reported in both type 1 and type 2 diabetic patients in the fasting state (6,16,17), while to our knowledge, no data are available so far on TRAP in the postprandial phase. The basal (fasting) antioxidant status of our subjects is comparable to what has been previously reported (1–3,5).

In our diabetic patients, the meal was followed by a substantial decrease of plasma TRAP and an increase of MDA levels. Thus, it appears that meals in diabetic patients induce an oxidative stress that leads on one side to increased production of lipid peroxides and on the other to consumption of antioxidant capacity. The latter event was documented by a reduction of plasma TRAP, as well as decreasing levels of most of the measurable circulating antioxidants. This had previously been reported for uric acid, which was negatively correlated with postprandial glucose (18).

In our study, only one antioxidant behaved differently, i.e., vitamin C. Our standardized meal contained 100 mg of ascorbic acid, while other antioxidants were not so abundant. This may explain the postprandial rise in vitamin C. In our opinion, the formula food we used is the best choice to standardize the impact of antioxidants contained in the food on postprandial oxidative stress generation. Unfortunately, this commercially available formula food is fairly rich in vitamin C. Indeed, as vitamin C increased in plasma after our test meals, probably more vitamin C was absorbed than consumed. Since ascorbic acid would

Table 1—*Baseline values, in diabetic and normal subjects, of the parameters evaluated in the study*

	Diabetic patients	*P*	Normal control subjects
Plasma glucose (mmol/l)	11.4 ± 1.7	0.0001	5.2 ± 0.1
Insulin (pmol/l)	54.6 ± 11.4	NS	41.4 ± 6.8
Cholesterol (mmol/l)	5.6 ± 0.1	NS	5.7 ± 0.2
Triglycerides (mmol/l)	2.14 ± 0.5	NS	1.12 ± 0.1
NEFAs (g/l)	12.5 ± 1.5	NS	13.5 ± 2.3
Uric acid (μmol/l)	249.8 ± 5.9	0.02	315.2 ± 23.7
MDA (μmol/l)	1.3 ± 0.2	0.02	0.5 ± 0.2
SH groups (μmol/l)	484.5 ± 18.4	0.02	545.0 ± 14.9
Vitamin C (μmol/l)	18.9 ± 1.7	0.001	38.0 ± 2.2
Vitamin E (μmol/l)	20.2 ± 1.8	0.03	26.9 ± 2.0
Vitamin E (μmol/l)/cholesterol (mmol/l) + triglycerides (mmol/l)	3.8 ± 0.1	0.03	2.7 ± 0.1
TRAP (μmol/l)	642.4 ± 22.7	0.005	770.0 ± 32.8

Data are means ± SEM.

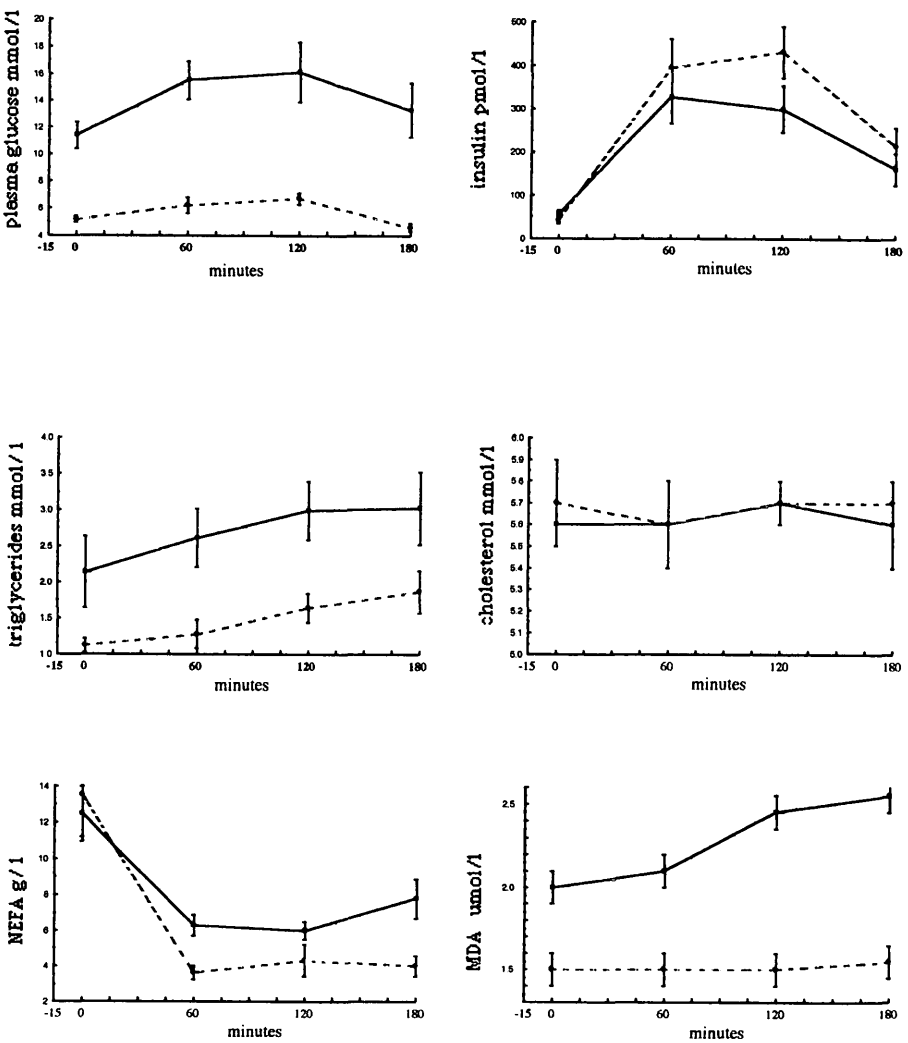

Figure 1—*Plasma glucose, insulin, triglycerides, cholesterol, NEFA, and MDA variations during a meal in normal (- - -) and diabetic (—) subjects.*

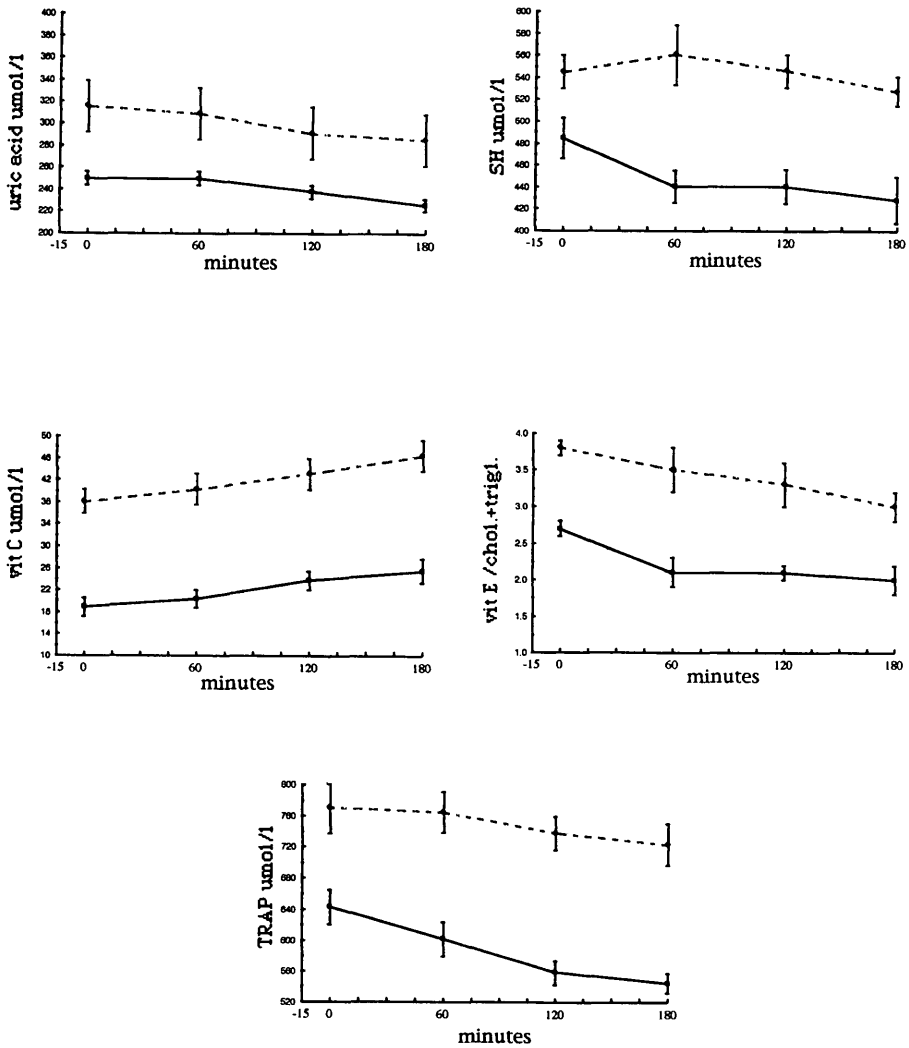

Figure 2—*Plasma uric acid, SH groups, vitamin C, vitamin E (corrected for cholesterol plus triglycerides values at any time), and TRAP variations during a meal in normal (- - - -) and diabetic (—) subjects.*

be expected to have a negative effect, if any, on the reduction in antioxidant power of plasma that we demonstrated, the observed TRAP decrease might have been greater in the absence of vitamin C supplementation. However, available data suggest that ascorbic acid contributes only ~5% to the total antioxidant power of plasma (9). On the other hand, another explanation could be that vitamin C circulates only in aqueous compartments, while the target of oxidative stress measured in this study is essentially linked to lipid compartments. By what biochemical mechanism eating a meal produces an oxidative stress in diabetic patients remains to be clarified. It has been suggested, however, that in diabetes the generation of an oxidative stress may be mostly due to hyperglycemia.

The production of free radicals associated with chronic hyperglycemia may result from nonenzymatic glycation (19) and glucose auto-oxidation (20). Superoxide radicals may also be generated inside the cells during exposure to hyperglycemia through an imbalance in the NADH-to-NAD^+ ratio linked to sorbitol pathway activation (21). Finally, it has recently been suggested that monocytes, which have a high potential for superoxide radical generation, may be a consistent source of free radicals in hyperglycemia (22).

During an acute rise of blood glucose level, the reactive oxygen species may derive mainly from the first step of nonenzymatic glycation (Schiff base formation) and by the imbalance of the NADH-to-NAD^+ ratio induced by glucose in cells, processes that have been shown to take place very rapidly (23,24).

The postprandial insulin surge might also be related to oxidative stress. It has been suggested that insulin, at least hyperinsulinemia, may directly induce intracellular production of free radicals (4). Vice versa, insulin resistance may be increased by oxidative stress (25).

In our study, there was no difference in the after-meal insulin variation between normal and diabetic subjects (the latter, being type 2, were insulin-resistant), so that insulin does not appear to play a causative role in the postprandial oxidative stress generation.

Lipids, particularly triglycerides, may also contribute to meal-related oxidative stress in diabetes. This hypothesis is supported by the different after-meal profile of triglycerides in diabetic and normal subjects and is convincingly confirmed by the amount of MDA produced. However, it should be kept in mind that triglyceride production itself is strictly dependent on plasma glucose level (26).

Clinical impact

There is evidence that poor hyperglycemia may play a significant role in the development of diabetic complications (27). In diabetes, however, the postprandial phase is characterized by a rapid and acute increase of glycemia, and the possibility that these postprandial hyperglycemic spikes may be relevant to the pathophysiology of late diabetic complications has been recently reviewed (28). Moreover, the possibility that an acute increase of glycemia is accompanied by an increase of free radical production is a well-recognized phenomenon (29). Because oxidative stress appears to be involved in the pathogenesis of diabetic complications, evidence that it is generated during the postprandial phase may contribute to the debate on the management of diabetes by underscoring the importance of controlling postprandial glycemic spikes, in addition to mean blood glucose levels as described by glycated hemoglobin.

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