Hyperglycemia-Induced Thrombin Formation in Diabetes

The Possible Role of Oxidative Stress

Antonio Ceriello, Roberta Giacomello, Giuliana Stel, Enrico Motz, Claudio Taboga, Laura Tonutti, Mario Pirisi, Edmondo Falleti, and Ettore Bartoli

Diabetes is characterized by the existence of a thrombosis-prone condition, possibly related to hyperglycemia. However, the mechanism linking hyperglycemia to the activation of the coagulation cascade is still unclear. It has been recently suggested that diabetes is accompanied by increased oxidative stress. In this work, the possibility that oxidative stress may be involved in the hyperglycemia-induced coagulation activation has been evaluated. Prothrombin fragment 1 + 2 (F1+2), which represents a reliable marker of the amount of thrombin released in the circulation, has been chosen for studying thrombin formation in vivo. In nine type II diabetic patients and in seven healthy control subjects, matched for age and body mass index, three different experiments were performed: oral glucose tolerance test (OGTT), intravenous antioxidant glutathione (GSH) administration for 2 h, and OGTT plus intravenous GSH administration. Samples were drawn at −15 min and every 30 min from 0 to 180 min. During the OGTT, F1+2 significantly increased in both diabetic and healthy subjects. GSH administration during OGTT normalized this phenomenon. GSH administered alone significantly decreased F1+2 in diabetic patients, while no effect was observed in the normal subjects. These data suggest that hyperglycemia may induce thrombin activation, possibly inducing an oxidative stress, and that antioxidant GSH may counterbalance this effect. *Diabetes* 44:924–928, 1995

Several studies of the fluid phase of coagulation suggest that diabetes is associated with a hypercoagulable state (1). The existence of a direct link between abnormal glucose levels and the increased activity of coagulation system has been demonstrated (2). However, the mechanism by which hyperglycemia may produce a coagulation activation is still unclear. The presence of an oxidative stress has been established in diabetes (3–4), and glucose auto-oxidation and protein glycation have been claimed as possible sources of free radicals (5). The production of free radicals is correlated to metabolic control and more directly to hyperglycemia (6). Free radicals are also capable of activating coagulation (7).

Prothrombin fragment 1 + 2 (F1+2) represents a reliable marker of the amount of thrombin released in the circulation (8), and increased F1+2 plasma levels have been already demonstrated in diabetes (9).

In this study, we evaluated the effects of both hyperglycemia and the antioxidant glutathione (GSH) on F1+2 plasma levels in diabetic patients and normal subjects.

RESEARCH DESIGN AND METHODS

Nine type II diabetic patients (five men and four women; age 65.5 ± 1.16 years, mean ± SE; duration of disease 12 ± 1.84 years; body mass index [BMI] 26.2 ± 1.12 kg/m^2) and seven healthy normal subjects (four men and three women) matched for age (62.6 ± 1.55 years) and BMI (26.5 ± 1.75 kg/m^2) gave informed consent to participate in the present study after a clear explanation of its experimental nature. The study protocol was approved by the ethical committee of our institution.

The diabetic patients had satisfactory metabolic control as judged by fasting plasma glucose (8.8 ± 0.8 mmol/l), fructosamine (337.2 ± 23.7 μmol/l), and glycated hemoglobin (7.7 ± 0.3%) levels. No one had microalbuminuria. They were treated by diet and the hypoglycemic drug glibenclamide (range of daily dose 2.5–7.5 mg).

After a 3-day wash-out period, three different studies were performed on each diabetic and normal subject, on different days, in a randomized order: oral glucose tolerance test (OGTT) (75 g glucose); GSH (TATIONIL, Boehringer Mannheim, Mannheim, Germany) administration, 1 g/m^2 as bolus + 0.5 g · m^{-2} · h^{-1} i.v. for 2 h to obtain a stable high GSH plasma levels (10); and OGTT+GSH.

In the morning after a 12- to 14-h fast, an intravenous cannula was introduced into an antecubital vein of both arms with slow infusion of 0.9% NaCl. Venous samples for laboratory analysis were obtained from one cannula; the other cannula was used for the administration of saline plus GSH or saline alone when only OGTT was performed.

After the subjects had rested for 90 min, two baseline samples were obtained (−15 and 0 min) before the start of the study. Blood samples were then collected at 30, 60, 90, 120, and 180 min. In these samples, glycemia and F1+2 were assayed.

Analytical methods. Plasma glucose was assayed by glucose-oxidase method. To study F1+2, blood was collected through a silicone-treated needle (diameter: 2 mm) and was allowed to flow freely into silicone-treated glass tubes, where it was mixed with 1/10 of its volume of 0.1 mol/l trisodium citrate. The blood was immediately centrifuged at 1,700 *g* for 20 min at 4°C and frozen at −80°C until assayed.

F1+2 plasma levels were evaluated by enzyme-linked immunosorbent assay according to Pelzer et al. (11). The intra- and interassay coefficients of variation were 5.1 and 6.0%, respectively.

Statistical analysis. F1+2 and glucose incremental areas were calculated by adding the areas under the curve between each pair of consecutive observations. The distribution of values of F1+2 and glucose incremental areas in the two populations studied (patients with diabetes and control subjects) did not show a significant departure from

From the Department of Clinical and Experimental Pathology and Medicine, Chair of Internal Medicine (A.C., E.M., M.P., E.B.) and Chair of Clinical Pathology (R.G., G.S., E.F.), University of Udine; and from the Diabetes Unit (C.T., L.T.), Udine General Hospital, Udine, Italy.

Address correspondence and reprint requests to Dr. Antonio Ceriello, Department of Clinical and Experimental Pathology and Medicine, University of Udine, Chair of Internal Medicine, P.le S. Maria della Misericordia, I-33100 Udine, Italy.

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ANOVA, analysis of variance; BMI, body mass index; GSH, glutathione; OGTT, oral glucose tolerance test; F1+2, prothrombin fragment 1 + 2.

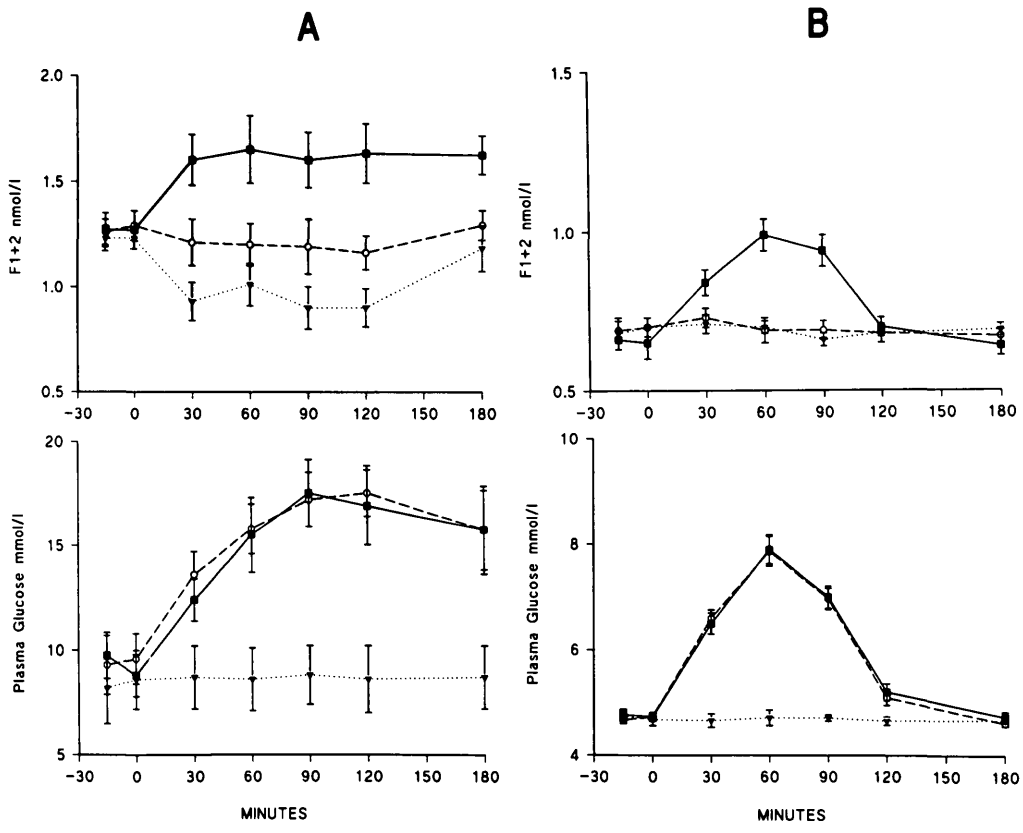

FIG 1. F1+2 and plasma glucose in diabetic (*A*) and normal (*B*) subjects during OGTT (■), OGTT+GSH (○), or GSH (▼). Values are expressed as means ± SE.

normality at the Shapiro-Wilk *W* test. Therefore, paired Student's *t* tests (two-tailed) were used for the comparison of the individual values of F1+2 and glucose incremental areas according to the different experimental conditions used. Finally, analysis of variance (ANOVA) for repeated measures was performed to identify the existence of a significant difference in mean F1+2 concentration both at the different times of a specified experiment and among the three different experimental conditions used. All values are expressed as means ± SE; all statistical tests were performed by means of the BMDP statistical software package.

RESULTS

Figure 1 presents the mean value ± SE of F1+2 in both diabetic patients and control subjects for each of the three experimental conditions. In diabetic patients, ANOVA for repeated measures demonstrated that the mean values of F1+2 at the different times considered differed significantly according to the experimental conditions used (OGTT, OGTT+GSH, and GSH alone: F within subjects = 14.11, P = 0.0001) and also that the mean values of F1+2 changed significantly in time in all experiments (F = 3.97, P = 0.001). The interaction between these two factors (experimental conditions and time) was also highly significant (F for interaction = 25.01, $P < 0.0001$). Similar results were obtained in control subjects (F = 4.15, P = 0.03; F = 12.51, P = 0.0001; and F = 11.06, $P < 0.0001$, respectively).

In diabetic patients, F1+2 plasma incremental areas during OGTT (Fig. 2) were significantly higher than those observed during OGTT+GSH (one-tailed paired Student's t test, 312.9 ± 16.4 vs. 232.1 ± 14.1 nmol · l^{-1} · min^{-1}, t = 9.45, $P < 0.0001$). Moreover, F1+2 plasma incremental areas during OGTT+GSH were significantly higher than during GSH alone (193.6 ± 8.3 nmol · l^{-1} · min^{-1}, t = 4.21, $P <$ 0.005). In control subjects, F1+2 plasma incremental areas during OGTT (Fig. 3) were significantly higher than those observed during OGTT+GSH (paired Student's t test, one-tail, 160.3 ± 6.4 vs. 131.4 ± 4.4 nmol · l^{-1} · min^{-1}, t = 4.10, $P < 0.01$). However, F1+2 plasma incremental areas during OGTT+GSH did not differ from those measured during GSH alone (134.8 ± 2.5 nmol · l^{-1} · min^{-1}).

Mean plasma glucose incremental areas during OGTT and OGTT+GSH were not significantly different in both diabetic patients (6.5 ± 0.9 vs. 6.9 ± 1.0 mmol · l^{-1} · min^{-1}) and healthy control subjects (3.6 ± 0.6 vs. 3.5 ± 0.7 mmol · l^{-1} · min^{-1}).

DISCUSSION

It has been demonstrated that the conversion of prothrombin to thrombin is associated with the prominent production of a cleavage product, namely F1+2 (8). It has been proposed that F1+2 plasma levels may be considered a very sensitive marker for hypercoagulable states in humans (8). This study, showing F1+2 increase during hyperglycemia, confirms previous data on the possibility that hyperglycemia may activate thrombin formation (12–15). However, it demonstrates for the first time that an antioxidant substance, GSH, may prevent this phenomenon.

A link between oxidative stress and coagulation activation in diabetes has been hypothesized (2). Some recent studies indirectly support this hypothesis, showing the existence of a direct correlation between the simultaneous increase of some coagulation parameters and some markers of oxidative stress in diabetic patients (16–17). Although the number of subjects examined in the present study was relatively small,

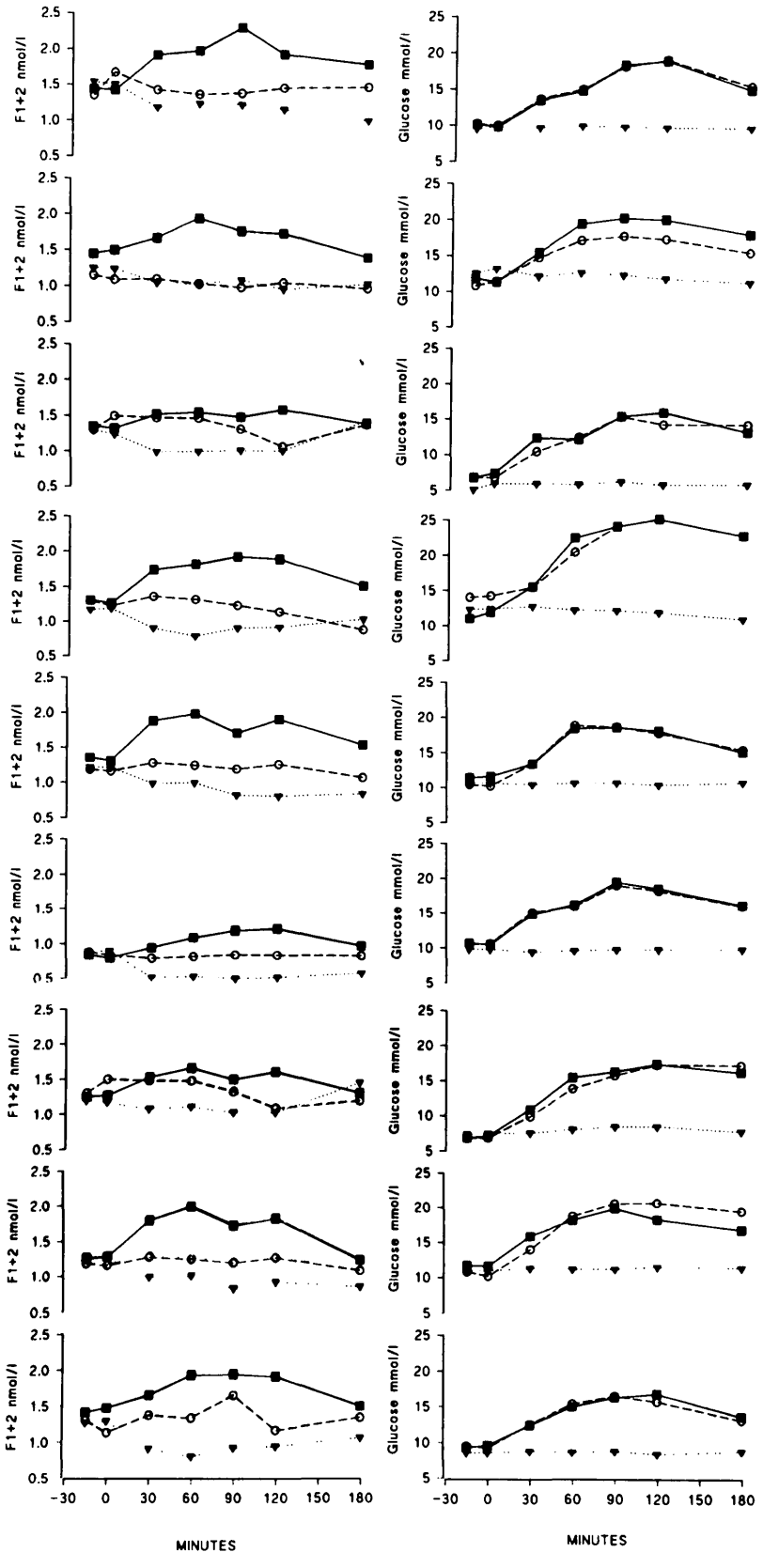

FIG. 2. F1+2 and plasma glucose in each of the nine diabetic patients during OGTT (■), OGTT+GSH (○), or GSH (▼).

which mandates caution in interpretation of the results, the data indicate that hyperglycemia-induced thrombin activation may be prevented by antioxidant GSH. This may be considered more directly evident of a possible link between hyperglycemia-induced coagulation activation and oxidative stress.

In our study, GSH reduces F1+2 plasma levels only in hyperglycemic subjects. In this view, since GSH is the only antioxidant tested, the possibility that its effect might be a primary one on glucose-induced F1+2 increase rather than on free radical production may be not discarded.

How hyperglycemia may stimulate thrombin activation

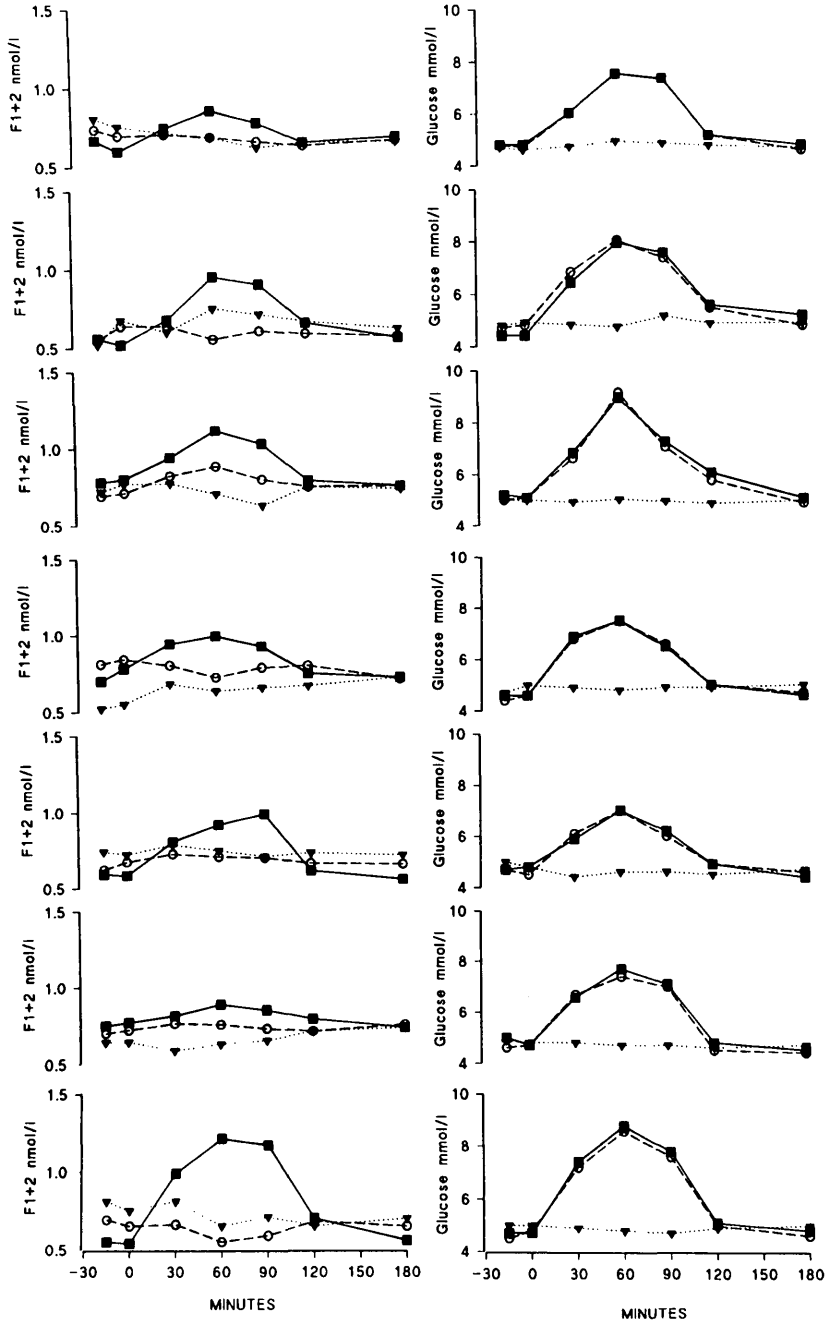

FIG. 3. F1+2 and plasma glucose in each of the seven control subjects during OGTT (■), OGTT+GSH (○), or GSH (▼).

remains hypothetical. We may put forward some hypotheses. It has been shown that hyperglycemia induces tissue-factor (18) and rapidly decreases NO production (19) in endothelial cells. The first one is a potent procoagulant factor (20), and the second one operates as antiaggregatory to platelets (21). Interestingly, decreased NO production in hyperglycemic conditions is restored by antioxidants (19), and this is consistent with the evidence showing that hyperglycemia may damage endothelial cells generating free radicals (22–23). An imbalance of these systems induced in vivo by hyperglycemia might be a sufficient stimulus to induce thrombin overproduction.

Our study shows that an antioxidant substance is capable of controlling hyperglycemia-mediated coagulation activation. These data may suggest the existence of a direct link between hyperglycemia, oxidative stress, and thrombophilia in diabetes.

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