Evidence for a hyperglycaemia-dependent decrease of antithrombin III-thrombin complex formation in humans

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Summary. In the presence of increased levels of fibrinopeptide A, decreased antithrombin III biological activity, and thrombin-antithrombin III complex levels are seen in diabetic patients. Induced-hyperglycaemia in diabetic and normal subjects decreased antithrombin III activity and thrombin-antithrombin III levels, and increased fibrinopeptide A plasma levels, while antithrombin III concentration did not change; heparin was shown to reduced these phenomena. In diabetic patients, euglycaemia induced by insulin infusion restored antithrombin III activity, thrombin-anti-

thrombin III complex and fibrinopeptide A concentrations; heparin administration had the same effects. These data stress the role of a hyperglycaemia-dependent decrease of antithrombin III activity in precipitating thrombin hyperactivity in diabetes mellitus.

Key words: Antithrombin III activity, thrombin-antithrombin III complex, fibrinopeptide A, hyperglycaemia, thrombin hyperactivity.

The key event in the coagulation of blood is the conversion of prothrombin to thrombin. Once generated, this enzyme converts fibrinogen to fibrin with the release of fibrinopeptide A and B [1]. Alternatively, thrombin may be inhibited by antithrombin III (ATIII), the most relevant physiological inhibitor of this serine protease [2]. Inhibition by ATIII leads to the formation of an inactive protease-inhibitor complex [3], and this reaction is catalysed very effectively by heparin [4].

ATIII plays an important role in the control of the coagulation system, as it can inhibit the enzymes of the clotting pathway. Determination of the thrombin-antithrombin III complex (TAT) should reflect the functional state of the clotting system and represents a diagnostic tool for the detection of coagulation disorders [3]; at the same time, the measurement of fibrinopeptide A (FPA) is considered a sensitive test of *in vivo* thrombin formation [1].

Several studies of the fluid phase of coagulation suggest that diabetes is associated with a hypercoagulable state [5, 6]. However, the existence of a direct link between abnormal glucose levels and the increased activity of coagulation system remains unclear [5, 6].

In this study we measured TAT, FPA and ATIII activity and concentrations, in both diabetic and normal subjects, and evaluated the effect of hyperglycaemia on these parameters.

Subjects and methods

Thirty Type 1 (insulin-dependent) diabetic subjects, selected according to the National Diabetes Data Group (NDDG) criteria [7], 15 males and 15 females (age 30 ± 1.23 years, mean $\pm$ SEM; body mass index 23.4 ± 1.42 kg/m²; duration of diabetes 8.51 ± 1.22 years; insulin regimen 18–70 IU/day, mean 42.8 ± 3.24 IU/day) gave informed consent to this study.

Thirty healthy normal subjects, without personal or family history of diabetes, with normal glucose tolerance according to NDDG criteria [7], matched for sex (14 males and 16 females), age (31 ± 1.28 years) and body mass index (22.4 ± 1.25 kg/m²), served as control subjects.

All subjects consumed weight maintaining diets with at least 250 g carbohydrate/day for three days before the study. The women were not taking oral contraceptives. This experiment was approved by the ethical committees of our institutions.

Basal study

Plasma glucose, TAT, FPA and ATIII activity and concentrations were measured in the fasting state, in both the diabetic and normal subjects.

Hyperglycaemic studies

In five diabetic subjects (fasting glycaemia 6.5–9.0 mmol/l), and in five normal subjects three different studies were performed in randomized order on different days. In the morning, after a 12 to 14 h

Table 1. Basal fasting values in diabetic and normal subjects

	Diabetic subjects <i>n</i> = 30	<i>p</i> -value	Control subjects <i>n</i> = 30
Fibrinopeptide A ng/ml	3.9 ± 0.43	< 0.01	1.7 ± 0.21
Thrombin-antithrombin III complex µg/l	1.0 ± 0.04	< 0.01	1.9 ± 0.07
Antithrombin III activity %	89.6 ± 4.12	< 0.01	103.9 ± 2.22
Antithrombin III concentration µmol/l	0.58 ± 0.12	NS	0.58 ± 0.12

Data are reported as mean ± SEM

fast an i.v. cannula was introduced into an antecubital vein in both arms, and kept patent with slow infusion of 0.9% NaCl. Venous samples for laboratory analysis were obtained from one cannula; the other cannula was used for administration of the various substances (glucose, insulin and heparin).

After the subjects had rested for 90 min, two basal samples were obtained (−30 and 0 min), before the start of the study. In the diabetic subjects, acute elevation of glycaemia was induced with a bolus of 0.33 g glucose · kg^{−1} body weight; then plasma glucose was clamped between 17 and 22 mmol/l for 1 h, with bedside monitoring of the plasma glucose concentration which served to adjust the rate of a 50% glucose infusion.

In the normal subjects, hyperglycaemia was induced according to the method of Cerasi and Luft [8]. Each subject received a bolus of 0.5 g glucose · kg^{−1} body weight in less than 2 min; glucose (20 mg · kg^{−1} · min^{−1}) was then infused via a peristaltic pump for 1 h. Blood samples were collected every 10 min in the first h and at 90, 120 and 180 min. As in the control study, each subject received an equivalent infusion of an isovolumic-isotonic NaCl solution.

The studies described above were repeated in both the normal and diabetic subjects, with the sole exception that heparin (5000 U as i.v. bolus, followed by an i.v. infusion at the rate of 750 U/h) accompanied the hyperglycaemic clamp.

Normoglycaemic studies

In five diabetic subjects (fasting glycaemia 14.2–17 mmol/l), after a 30 min baseline period, normoglycaemia was achieved with a 1 mU · kg^{−1} · min^{−1} insulin infusion for 2 h. Samples were obtained at −30, 0, 30, 60, 70, 80, 90, 100, 110, 120 and 180 min.

Heparin studies

After a 30 min baseline period i.v. heparin (5000 U as a bolus plus 750 U/h) was infused for 1 h in five diabetic subjects; blood samples were obtained at −30, 0 and every 10 min in the first h, and at 90, 120 and 180 min.

Analytical methods

Venous blood was drawn in plastic syringes containing 3.8% sodium citrate 9:1 volume/volume). Subsequently, samples were centrifuged for 15 min at 2000 × *g* at 4°C and the plasma obtained was used to test glycaemia, TAT levels, ATIII activity and concentration. Plasma glucose was measured by the glucose-oxidase method. ATIII biological activity was measured according to Abildgaard [9]. ATIII concentration was evaluated by radial immunodiffusion, according to Mancini [10].

TAT concentrations were quantitated by a solid phase ELISA, according to Pelzer [11], with the commercially available kit from Behringwerke AG (Marburg, FRG).

FPA estimation was performed by RIA, using a commercially obtained kit (Mallinkrodt, St. Louis, Mo., USA). Samples were drawn directly into a vacutainer containing the anticoagulant diluent supplied with the kit.

For each time point of the study the ratio of the mean basal period haematocrit to the observed haematocrit was used to correct the dilutional error introduced by blood volume expansion.

Statistical analysis

Statistical significance was determined by the analysis of variance. Data are reported as mean ± SEM.

Results

Basal study

ATIII functional activity and TAT concentrations were significantly reduced in the diabetic patients (Table 1 *p* < 0.01). FPA levels were increased in diabetic subjects, while the ATIII concentration was not different between the diabetic and control subjects (Table 1).

Hyperglycaemic studies

All subjects, both diabetic and normal, had sustained elevation of plasma glucose levels, as the result of glucose infusion. No difference between mean glycaemic incremental areas (from 0 to 120 min) existed in the two experiments (with or without heparin) in the two groups of normal (13.5 ± 1.2 vs 13.6 ± 1.3 mmol/l) or diabetic (11.6 ± 1.4 vs 11.4 ± 1.3 mmol/l) subjects.

Basal plasma glucose levels, TAT, FPA, ATIII activity and concentrations were similar before the hyperglycaemic studies (with or without heparin) in each group of normal and diabetic subjects (Fig. 1). A significant decrease in both ATIII activity and TAT concentrations, and an increase of FPA occurred during hyperglycaemia in both of the groups (0–120 min; *p* < 0.01), while the ATIII concentration was unchanged (Fig. 1).

Heparin preserved ATIII activity and the TAT decrease, FPA showed a minor increase, while ATIII concentration was not affected (Fig. 1). The isovolumic-isotonic infusion produced only a slight and insignificant rise in FPA concentration (Fig. 1).

Normoglycaemic studies

Insulin-induced euglycaemia in diabetic patients increased both the ATIII activity and TAT concentrations, and decreased FPA concentrations (Fig. 2).

Heparin studies

The infusion of heparin in the diabetic subjects ameliorated ATIII activity and TAT plasma levels, decreased FPA concentration, while it did not affect the ATIII concentration (Fig. 3).

Fig. 1. Effect of induced-hyperglycaemia on diabetic and normal subjects. ○—○ glucose, ●—● glucose plus heparin, △—△ control. ATIII = antithrombin III, TAT = thrombin-antithrombin III complex, FPA = fibrinopeptide A

Discussion

In this study the presence in diabetes of an increased FPA concentration and decreased levels of TAT and ATIII activity, despite normal plasma concentrations, has been demonstrated.

The discrepancy between TAT and FPA levels in diabetes seems, apparently, surprising since both are considered a good index of hypercoagulability. Decreased ATIII activity, in the presence of its normal plasma concentration [12], and increased FPA levels [13] have already been demonstrated in diabetes, as well as the direct role of

hyperglycaemia in conditioning these phenomena both in diabetic [13, 14] and normal [15, 16] subjects. The FPA hyperglycaemia-mediated increase revealed the presence of a hyperglycaemia-dependent thrombin hyperactivation, however, a role for decreased ATIII activity in conditioning this phenomenon was hypothesized [5].

The presence of decreased TAT levels, correlated to ATIII decreased activity and FPA increase in diabetes, strengthens this hypothesis. The possibility of reproducing this situation during induced hyperglycaemia in healthy subjects, furthermore, shows that this phenomenon is clearly hyperglycaemia-dependent.

Fig. 2. Effect of normoglycaemia on diabetic patients. ATIII = antithrombin III, TAT = thrombin-antithrombin III complex, FPA = fibrinopeptide A

FPA levels and ATIII activity may be restored by heparin administration [13, 15, 17]. In this study heparin shows the ability to increase TAT levels, simultaneously increasing ATIII activity and decreasing the FPA concentration. This effect confirms that the level of ATIII activity plays an important role in conditioning hyperglycaemia-induced thrombin hyperactivity.

Our data suggest that the hyperglycaemia-related thrombin hyperactivation seems, very likely to be linked

Fig. 3. Effect of heparin administration on diabetic patients. ATIII = antithrombin III, TAT = thrombin-antithrombin III complex, FPA = fibrinopeptide A

to a hyperglycaemia-induced decrease of ATIII activity. The decrease of ATIII biological activity conditions a minor antithrombin effect of ATIII, as demonstrated by the reduced TAT levels. The reduced inhibition of thrombin may produce a relative hyperactivity of this enzyme, precipitating an increase in FPA.

The relevance of this study is the demonstration that the hyperglycaemia-induced decrease of ATIII activity may precipitate a decrement of TAT formation that per-

mits a thrombin hyperactivity, as demonstrated by the FPA increase. Thus, the hyperglycaemia-induced ATIII alterations seem to play a central role in the development of coagulation alterations in diabetes.

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Received: 24 July 1989
and in revised form: 16 October 1989

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