Inhibition of Heparin-Catalyzed Human Antithrombin III Activity by Nonenzymatic Glycosylation

Possible Role in Fibrin Deposition in Diabetes

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SUMMARY

The effect of nonenzymatic glycosylation on the biologic function of human antithrombin III was evaluated using a chromogenic thrombin substrate assay in the presence of catalytic amounts of heparin. Experimental conditions that increased the rate of nonenzymatic protein glycosylation were associated with decreases in the thrombin-inhibiting activity of antithrombin III. This glycosylation-induced inhibition of heparin-catalyzed antithrombin III activity was completely reversible by preassay incubation with excess sodium heparin.

These observations provide a biochemical explanation for the heparin-reversible, accelerated fibrinogen disappearance rate induced by hyperglycemia in diabetic patients. Defective inhibition of the coagulation cascade induced by excessive nonenzymatic glycosylation of antithrombin III in vivo could contribute to accumulation of fibrin in various diabetic tissues.

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Central to current understanding of the pathogenesis of diabetic microvascular disease, particularly in the retina, is the concept that regional ischemia at the capillary level is the initial factor underlying the characteristic functional, morphologic, and clinical features of diabetic microangiopathy.[1–4] A number of diabetes-associated hematologic abnormalities have been described that may contribute to capillary compromise and resultant hypoperfusion of critical regions.[5,6] Alterations in plasma viscosity,[7] platelet aggregability,[8] and erythrocyte deformability[9] may each interact adversely with an underlying diabetes-associated hypercoagulable state in which fibrinogen disappearance rates are significantly accelerated.[10] Currently, the sequence of events leading from abnormal glucose homeostasis to increased activity of the coagulation system is not known.

In the studies described in this report, we have evaluated the effect of nonenzymatic glycosylation on the biologic function of human antithrombin III. This protein, after binding catalytic amounts of heparin to critical lysine residues, functions as the major inhibitor of activated serine protease coagulation factors in serum.[11,12] Defective inhibition of the coagulation cascade induced by excessive nonenzymatic glycosylation of antithrombin III in vivo could contribute to accumulation of fibrin in various diabetic tissues.

MATERIALS AND METHODS

Antithrombin/thrombin inactivation studies. Antithrombin III from human plasma, thrombin from human plasma, and the chromogenic thrombin substrate tosyl-glycyl-prolyl-arginine-*p*-nitroanilide acetate were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Indiana). Antithrombin III was dissolved in 0.01 M EDTA, pH 9.5, to a final concentration of 1 U/ml. A thrombin stock solution was made by dissolving 1.5 U thrombin in 3 ml redistilled water. This was stored in plastic bottles at 4°C until use. At the time of assay, aliquots of thrombin were diluted, unless otherwise specified, to 0.1 U/ml in 100 mM Tris-HCl buffer, pH 8.1, containing 0.15 M NaCl, and 1.75 U/ml of beef lung sodium heparin (Upjohn Company, Kalamazoo, Michigan). All solutions were allowed to equilibrate to room temperature for 30 min before assay. To 1.0 ml of the thrombin solution in plastic micro-cuvettes, 0.1 ml antithrombin III was added with plastic pipettes. The mixture was allowed to stand at room temperature (25°C) for exactly 10 min. Chromogenic thrombin substrate (100 μl of a 1.9 mM solution) was added to start the reaction, and the change in absorbance at 405 nm was monitored at 1-min intervals. A molar extinction coefficient of 10.4 was used to determine the number of micromoles of thrombin substrate cleaved.

In vitro glycosylation of antithrombin III. Human antithrombin III was dissolved in 0.01 M EDTA, pH 9.5, contain-

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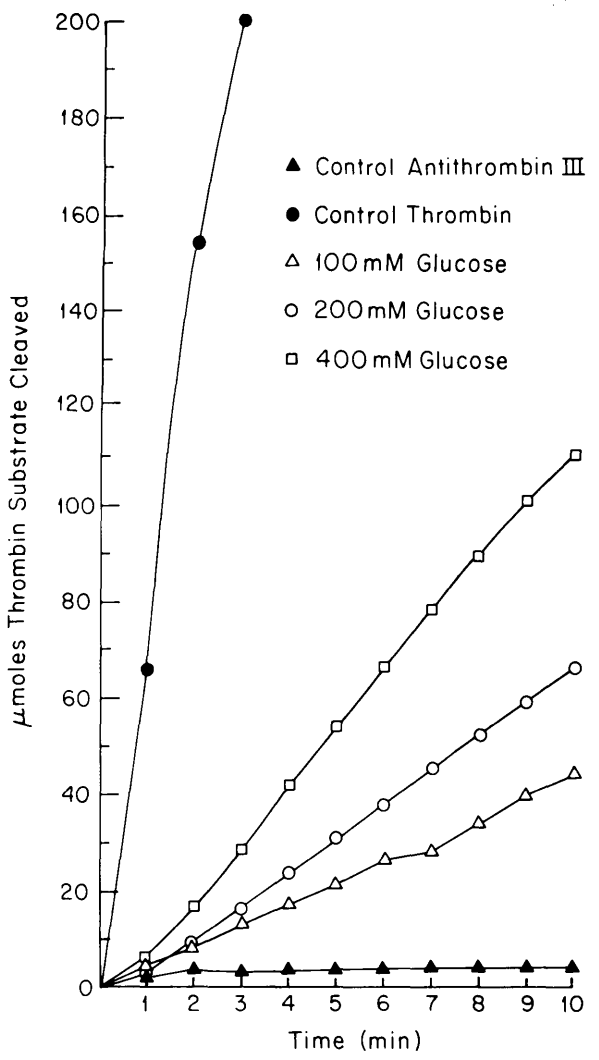

FIGURE 1. Effect of increasing levels of nonenzymatic glycosylation on antithrombin III inactivation of thrombin. Antithrombin III was incubated under the indicated conditions for 72 h as described in MATERIALS AND METHODS. Control antithrombin III was incubated in buffer alone. Each point represents the mean ± SEM of three determinations.

ing various concentrations of glucose. In all cases, the final concentration of antithrombin III was 1 U/ml. Antithrombin III glycosylated by incubation in 100 mM, 200 mM, or 400 mM glucose for 72 h at 37°C was assayed immediately. Aliquots of antithrombin III glycosylated in 400 mM glucose were preincubated with a 10-fold excess (165:1 molar ratio) of sodium heparin (17.5 U/ml) for 2 h before assay to assess the reversibility of glycosylation-induced inhibition of antithrombin III activity.

RESULTS

Effect of glycosylation on antithrombin III activity. Addition of thrombin substrate to thrombin in solution resulted in the rapid appearance of the chromogenic cleavage product *p*-nitroanilide. The presence of antithrombin III in this reaction mixture completely inhibited the ability of thrombin to hydrolyze its substrate. In contrast, identical quantities of antithrombin III that had been previously incubated with glucose showed a significant decrease in thrombin-inhibiting activity under the same experimental conditions. Higher incubation concentrations of glucose were associated with greater loss of antithrombin III activity (Figure 1). The glucose-induced decrease in antithrombin III's thrombin-inhibiting activity was dependent on time as well as on glucose concentration. No effect was observed at zero time, and modest yet significant decreases were observed at 24 h (data not shown). These observations are consistent with the fact that the rate and extent of nonenzymatic glycosylation of proteins are functions of both glucose concentration and duration of exposure to glucose.

Reversibility of antithrombin III inactivation. Incubation of antithrombin III in 400 mM glucose for 72 h resulted in a substantial degree of antithrombin III inactivation. This glucose-induced inhibition of antithrombin III activity was completely overcome, however, by addition of a 10-fold excess (165:1 molar ratio) of heparin to the glycosylated antithrombin III 2 h before assay (Figure 2). Since binding of catalytic amounts of heparin to antithrombin III is required for normal thrombin-inhibiting activity, these data suggest that nonenzymatic attachment of glucose moieties to lysine ϵ-amino groups of antithrombin III reduces the affinity of the antithrombin III for heparin, but does not eliminate its heparin-binding capacity.

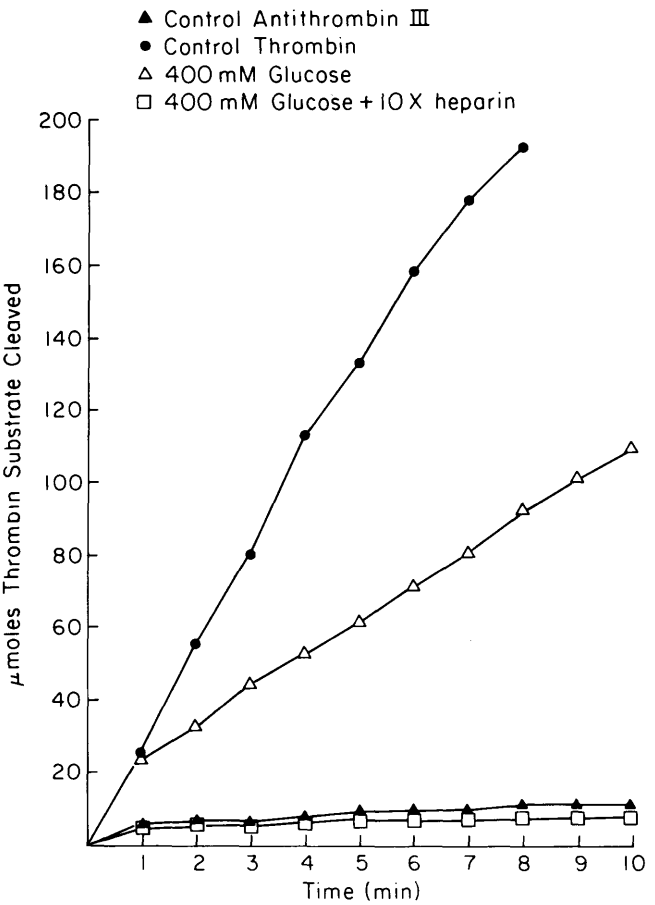

FIGURE 2. Reversibility of nonenzymatic glycosylation-induced inactivation of antithrombin III. Antithrombin III was glycosylated as described in MATERIALS AND METHODS. Control antithrombin III was incubated in buffer alone. Conditions of heparin incubation are given in the text. Each point represents the mean ± SEM of three determinations.

DISCUSSION

The studies described in this report demonstrate that nonenzymatic glycosylation of human antithrombin III reduces its ability to inhibit the activity of human thrombin in the presence of catalytic amounts of heparin. This inactivation of human antithrombin III by nonenzymatic glycosylation can be overcome by incubation with excess heparin. These data are consistent with current chemical concepts of the antithrombin III/thrombin reaction, in which the initial and rate-determining step appears to be binding of catalytic amounts of endogenous heparin to lysine residues of antithrombin III.[11,13] This step, which enhances the rate of inhibition of thrombin by antithrombin III nearly 1000-fold,[11] is independent of thrombin.[13] In vivo, heparin-like molecules occurring in vascular endothelium catalyze antithrombin interactions with thrombin at a rate similar to that of antithrombin III and heparin.[14]

The phenomena described in this communication are consistent with and provide an explanation for previously published observations concerning in vivo fibrinogen kinetics in diabetic patients.[10] Sustained hyperglycemia using a glucose-clamp technique resulted in an accelerated disappearance of radioiodinated autologous fibrinogen. Normalization of fibrinogen disappearance rates could be achieved by either reduction of maintained blood glucose levels to the normal range or by addition of heparin to the hyperglycemic glucose infusions. The effect of glucose on changes in fibrinogen disappearance rates suggests an underlying mechanism involving formation of a product between glucose and a protein important in the coagulation process. The independent effect of heparin further suggests that glycosylation of specific heparin-binding lysine residues on antithrombin III is the mechanism by which short-term elevations of glucose in the blood increase the rate of fibrinogen disappearance in diabetic patients.

Inhibition of antithrombin III activity by excessive nonenzymatic glycosylation would be expected to produce a transient functional deficiency of this protein in hyperglycemic patients. Clinical studies of antithrombin III biologic activity provide evidence for such a defect in both type II and type I diabetic patients.[15,16] In the one study presenting data concerning patients' glycemic status, the degree of reduction in antithrombin III activity is directly related to the level of both hemoglobin A_{1c} and fasting blood glucose.[16]

Antithrombin III is thought to account for more than 90% of coagulation-inhibiting activity of the plasma.[12] In addition to inhibiting thrombin, it reacts in a similar fashion with the other activated serine proteases generated in the coagulation cascade, factors XII_a, XI_a, IX_a, and X_a.[17] In vivo, glycosylation-induced inhibition of antithrombin III interactions with these other clotting components may result in more significant generation of coagulation activity than does direct interference with antithrombin III binding to thrombin, due to amplification by the protease cascade system.

It should be noted that large changes in antithrombin III activity would not be necessary for excessive long-term vascular fibrin deposition to occur. The amount of fibrin deposited in blood vessel walls reflects a finely balanced steady state between the coagulation system, antithrombin III, and the fibrinolytic system.[18] Slight decreases in antithrombin III activity would lead, over time, to equilibration at a new steady state with a greater constant amount of accumulated fibrin, unless a corresponding increase in fibrinolysis occurred, as well. In diabetes, however, additional increases in total fibrin accumulation would be anticipated at the new steady state in hyperglycemic individuals, since nonenzymatic glycosylation also reduces the susceptibility of fibrin to degradation by the specific fibrinolytic enzyme, plasmin.[19] Further amplification of a glucose-induced defect in antithrombin III/heparin interaction could well occur in vivo as a consequence of decreased tissue heparin sulphate content in diabetic tissues.[20]

In vivo, the rate of antithrombin III inactivation would be directly proportional to the number of critical lysine residues blocked by nonenzymatic glycosylation. This in turn is a function of the time-integrated level of blood glucose concentration.[6] Quantitative estimates of the degree to which clinically observed levels of hyperglycemia accelerate the deposition of fibrin in diabetic patients cannot be extrapolated from the data reported here, however, since the experimental conditions required higher-than-physiologic concentrations of all reactants. A rather slow rate of change in steady-state levels of accumulated fibrin would be most consistent with the long period of time known to be required for diabetic vascular complications to develop.

The observations described in this report provide a biochemical explanation for the heparin-reversible accelerated fibrinogen disappearance rate induced by hyperglycemia in diabetic patients. This consequence of nonenzymatic glycosylation may play a role in the abnormal accumulation of fibrin reported to occur in those diabetic tissues most affected by long-term complications.[21–24]

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