Non-enzymatic glycation reduces heparin cofactor II anti-thrombin activity

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Summary. The effects of non-enzymatic glycation on heparin cofactor II activity, at glucose concentrations which might be expected in physiological or diabetic conditions have been evaluated in this study. Radiolabelled glucose incorporation was associated with a loss of heparin cofactor anti-thrombin activity. The heparin cofactor heparin and dermatan sulfate-dependent inhibition of thrombin was significantly reduced, showing a remarkable decrease of the maximum second order rate constant. This study shows that heparin cofactor

can be glyated at glucose concentrations found in the blood, and that this phenomenon produces a loss of heparin cofactor-antithrombin activity. These data suggest, furthermore, a possible link between heparin cofactor glycation and the pathogenesis of thrombosis in diabetes mellitus.

Key words: Heparin cofactor II, glycation, anti-thrombin activity, heparin, dermatan-sulfate.

Heparin-cofactor II (HCII) is a plasma protease inhibitor which inhibits thrombin by forming a stable 1:1 molar complex [1–5]. It is well known that the thrombin inhibitory activity of HCII is dramatically accelerated by some sulfated polysaccharides such as heparin and dermatan sulfate [6].

In vivo studies indicate both increased fibrinogen turnover and fibrinopeptide A levels in diabetic patients [7–8], and it has been suggested that an abnormal plasma or vascular environment is a more likely basis for this phenomenon. It is interesting that heparin infusion restores fibrinogen survival [7] and reduces the hyperglycaemia-dependent increase of fibrinopeptide A [8] in diabetic subjects. Correction by heparin suggests that such abnormality may be related to the action of thrombin or its inhibitors. This hypothesis is supported by the evidence that antithrombin III, a physiological thrombin-inhibitor, shows decreased activity in diabetes, due to non-enzymatic glycation [9–11].

Since HCII is another thrombin inhibitor, the possibility that non-enzymatic glycation may influence its activity, as well as antithrombin III, has been evaluated in this study.

In our studies, in vitro non-enzymatic glycation of HCII has been conducted at a physiological concentration of HCII (1.2 $\mu\text{mol/l}$) and glucose (5 mmol/l). In addition, a glucose concentration was used that closely approximates the condition that might be expected in severe diabetes

(15 mmol/l). We have carefully evaluated the inhibition kinetics of thrombin by glyated HCII and have compared the results with non-glyated HCII.

Materials and methods

HCII (chemical purity assured at greater than 99% by the manufacturers), human serum albumin (HSA), and thrombin were supplied by Boehringer Mannheim (Mannheim, FRG). Porcine mucosa heparin (168 units/mg, M_r 7000–20000) and porcine skin dermatan sulfate (M_r 20000–40000) were purchased from Sigma (St. Louis, Miss, USA).

D-(U-¹⁴C)-glucose was purchased from Amersham (Arlington Heights, Ill., USA). This was initially purified to remove unwanted contaminants that are known to modify proteins faster than glucose itself [12]. To accomplish this, 150 μCi D-(U-¹⁴C)-glucose was incubated with 1.5 mg bovine serum albumin in 1 ml 0.2 mol/l sodium phosphate, pH 8, for 2 days at 37°C. Free glucose was separated from the protein-bound glucose by chromatography in Sephadex G-25. This fraction of free glucose was used for subsequent non-enzymatic glycation of HCII. Non-enzymatic glycation of HCII was carried out as follows. Six 3 ml solutions of HCII (1.2 $\mu\text{mol/l}$) were incubated at 37°C in 0.15 mol/l NaCl, 0.02 mol/l tris-HCl, pH 7.4. The first contained 5 mmol/l glucose, the second 5 mmol/l glucose plus 0.6 mmol/l HSA, the third 15 mmol/l glucose, the fourth 15 mmol/l glucose plus 0.6 mmol/l HSA, the fifth was used as control and contained no glucose or HSA, the sixth contained only HSA at 0.6 mmol/l. Sodium azide (0.02%) was added to prevent bacterial growth, and the mixtures were incubated for 15 days. Fifty microcuries of D-(U-¹⁴C)-glucose (230 $\mu\text{Ci/mol}$) was added as a tracer.

Fig. 1a-c. Effects of nonenzymatic glycation on heparin cofactor II (HCII).

a HCII-heparin activity and **b** HCII-dermatan-sulfate activity, as function of time. **c** Time-dependent incorporation of D-(U-¹⁴C)-glucose radioactivity in HCII. Control HCII with (○—○) or without (○—○) albumin. HCII at glucose concentration of 5 mmol/l with (●—●) or without (●—●) albumin. HCII at glucose concentration of 15 mmol/l glucose with (■—■) or without (■—■) albumin

Aliquots withdrawn at various times were passed through Bio-Gel P.10; HSA was separated by affinity chromatography on Affi-Gel Blue [13], followed by gel filtration on Bio-Gel P.150 (Bio Rad Lab., Richmond, Calif., USA). The protein fractions were subsequently dialysed. Radioactivity was determined in two ways: (1) counts were made directly on dialysed protein solution; (2) the exhaustively dialysed solutions were treated with cold 10% trichloroacetic acid (TCA) and filtered through a nitrocellulose filter followed by extensive washing. Radiolabelled glucose bound to the protein was held in the nitrocellulose filter, while free radiolabelled glucose was eliminated with filtrate. Excess TCA in the filter was decomposed by drying the filter over a heat lamp for 5 min, and the radioactivity in the filter was subsequently counted.

The dialysed proteins were assayed for HCII thrombin-inhibition activity in the presence of heparin or dermatan sulfate. Thrombin-inhibition reaction was carried out in triplicate in 1.5 ml polypropylene microcentrifuge tubes at 37°C. Thrombin (2.2 mmol/l), HCII or glycated HCII (1.2 μmol/l) plus heparin (0.5 U/ml) or dermatan sulfate (100 μg/ml) were mixed in a buffer containing 0.15 mol/l NaCl, 0.02 mol/l Tris-HCl, pH 7.4 and 1 mg/ml bovine serum albumin [14]. After 20 min, protease activity was determined by the addition of an equal volume of the appropriate chromogenic substrate (S-2238) in water. Hydrolysis of the substrate was terminated after 3 min by the addition of 1/10 volume of 50% (volume/volume) acetic acid, the precipitated glycosaminoglycan was removed by centrifugation and the absorbance of the supernatant solution was determined at 405 nm. In the absence of HCII, a standard curve of Δ405 thrombin dilutions was carried out, and the residual thrombin activity in the experiments expressed as a percentage of this reference curve.

The second-order rate constant (K_m) of the HCII-thrombin interaction was determined using a synthetic substrate S-2238 [15].

Results

Figures 1a and 1b show the effect of glycation on the HCII activity. Assays of aliquots withdrawn from the reaction mixtures (after chromatography and dialysis) revealed a time-dependent loss of activity. During incubation the control HCII (0.15 mol/l NaCl, 0.02 mol/l Tris-HCl, pH 7.4, no glucose) lost some HCII activity up to a maximum of 15% after 7 days. The presence of HSA did not interfere with this phenomenon. The incubation mixture of HCII in 5 mmol/l glucose lost 30% and HCII in 15 mmol/l glucose lost 50% of the activity in 7 days. After 15 days the same amount of activity was lost whether the incubation was done at 5 or 15 mmol/l glucose. Incubation in the presence of HSA did not significantly influence these results. When HCII was incubated in 5 and 15 mmol/l glucose containing a tracer amount of D-(U-¹⁴C)-glucose, significant radioactivity was gradually incorporated into the protein (Fig. 1c). This was demonstrated

by analysis of HCII bound radioactivity after gel filtration and extensive dialysis of aliquots obtained from the reaction mixture at various times. Similarly, TCA precipitation of the protein in aliquots obtained at various times revealed radioactivity associated with the precipitated protein. The incorporation of radioactivity at 15 mmol/l glucose was essentially linear for at least 4 days, but it is not clear why apparent levelling off of radioactivity occurred after 4 days. This may be associated with the glucose-dependent cross-linking of the protein [16] known to occur during non-enzymatic glycation. Based on the specific activity of D-(U-¹⁴C)-glucose (1×10^3 cpm/nmol), the incorporation of 4×10^3 cpm in 8 nmol HCII represents an average of 0.5 mol glucose/mol protein. The presence of HSA did not promote HCII glucose incorporation, while, as previously reported [13], HSA incorporated also 0.5 mol glucose/mol albumin (Fig. 1c).

These results suggest that HCII can be glycated at normal physiological glucose concentrations but is more likely at a concentration that is close to that expected in severely diabetic patients.

Since plasma time of elimination of HCII in man is 2.5 days [17], the HCII solution that lost ~20% (corrected for control loss) after 2.5 days of incubation was used in the kinetics experiments, to determine its effectiveness in neutralizing the activity of thrombin. No attempt was made to isolate the pure glycated HCII because the goal was to investigate how the overall kinetic property of HCII may be affected in the blood of diabetic patients where the circulating HCII is assumed to be a mixture of

Fig. 2a and b. Kinetics of thrombin inhibition by HCII in the presence of heparin and dermatan-sulfate. Second-order rate constants (K_m) of inhibition (control HCII Δ – glycated HCII ▲) were plotted as a function of heparin and dermatan-sulfate concentrations. * $p < 0.01$

glycated and non-glycated HCII. The inactivation of thrombin by HCII in the presence of heparin or dermatan-sulfate was monitored by measuring the residual thrombin activity through the hydrolysis of chromogenic substrate S-2238 in 0.15 mol NaCl, 0.02 mol/l Tris-HCl, pH 7.4.

Effects of glycation of HCII on the heparin and dermatan sulfate-dependent inhibition of thrombin by HCII are shown in Figures 2a and 2b. The glycation of HCII resulted in a remarkable decrease of the maximum K_m of the reaction between HCII and thrombin. Unexpectedly, no decrease in K_m inhibition of glycated HCII was observed in the presence of excess amounts of heparin, while K_m of inhibition of HCII decreased at a heparin concentration above 50 $\mu\text{g/ml}$.

Discussion

These in vitro studies indicate that HCII can be glycated at a normal physiological concentration of glucose but even more so at a concentration expected to be encountered in severely diabetic patients. This property is clearly demonstrated by the radioactivity incorporation studies. The maximum loss of HCII activity and the maximum incorporation of radioactivity were observed between 7 and 10 days. These in vitro studies were kept as close to physiological conditions as possible, but obviously, glycation of proteins other than HCII occurs in vivo. However, although more blood proteins are glycated in circulating blood than just HCII, the HCII is, nevertheless, exposed to a steady-state concentration of glucose that is simulated in these studies. Thus, in vivo, glycation would not be expected to be less efficient than the in vitro glycation demonstrated. This affirmation, however, is supported by the experiments carried out in the presence of albumin at physiological concentration. The decrease of HCII activity and the HCII glucose incorporation were not significantly promoted by the presence of albumin.

Albumin and HCII incorporated the same amount of glucose/mol protein. Interestingly, a similar amount is incorporated by glycated antithrombin III [18]. These data support the hypothesis that the steady-state concentration of glucose may promote an equivalent glucose-protein linkage formation between various proteins.

Under the in vitro conditions in this investigation, the second order rate constant for the inhibition of thrombin by glycated HCII was found to be three times smaller than that of normal HCII. This result means that glycated HCII is three times weaker than normal HCII as an inhibitor of thrombin.

The relevance of our study is that HCII can be glycated at glucose concentrations found in the blood. However, the possible link, in vivo, between the glycation of HCII, antithrombin III or other clotting factors, and the coagulation alterations seen in diabetes, remains to be elucidated.

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