Zinc as a Cofactor for Heparin Neutralization by Histidine-rich Glycoprotein*

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We have studied the ability of histidine-rich glycoprotein (HRG) to neutralize the anticoagulant activity of heparin in plasma and in a purified component clotting assay. Addition of HRG to plasma or to the purified component assay did not neutralize the anticoagulant activity of heparin unless micromolar concentrations of zinc were present. Higher zinc concentrations were required for citrated than for heparinized plasmas due to competition of citrate with HRG for zinc binding. Zinc concentrations as low as 1.25 μM revealed HRG to be a powerful competitor of antithrombin for heparin in the purified component assays. HRG binding of heparin also was shown by affinity chromatography of HRG from immobilized heparin in the presence and absence of zinc. In the absence of zinc, HRG was eluted by 0.1 M NaCl but, in the presence of zinc, elution of HRG required 1.0 M NaCl. Investigation of other divalent cations (copper and magnesium) indicated that augmentation of heparin binding by HRG in the presence of antithrombin was restricted to zinc. The HRG-Zn complex effectively competes with antithrombin for heparin, which restricts the availability of heparin to bind antithrombin and allows thrombin-mediated fibrinogenesis to proceed unimpeded. This could be initiated by zinc released from activated platelets.

Since its discovery in 1972 (1), histidine-rich glycoprotein (HRG)1 has been reported to interact with heparin (2–7), plasminogen (3, 8, 9), divalent metals (10–12), autorosette inhibition factor (13), fibrinogen and fibrin (14), monocytcs (15), T lymphocytes (16), and various components of the complement pathway (17). Although fibrinogen, heparin cofactor II, complement factors H and I, apolipoprotein B, fibronectin, vitronectin, von Willebrand factor, thrombospondin, α-2-macroglobulin, inter-α-trypsin inhibitor, and transferrin bind heparin (18), HRG appears to be the only significant physiological competitor for heparin with antithrombin in plasma (19). Despite extensive studies, little is known regarding the modulation of heparin binding by HRG.

Our research has focused on the reported heparin-neutralizing function of HRG and its potential importance in hemostasis by studying the effects of thrombin, HRG, antithrombin, heparin, and divalent cations on fibrin generation in plasma and in a purified component clotting assay. Our initial experiments with plasma confirmed previous findings that micromolar concentrations of zinc significantly increased heparin binding by HRG (4), and competition experiments performed by using the purified component clotting assay revealed that heparin binding by HRG in the presence of zinc was greater than heparin binding by antithrombin.

This research has shown that zinc concentrations in normal plasmas are not sufficient to serve a cofactor function for HRG, thus allowing heparin to serve its cofactor function for antithrombin. However, micromolar concentrations of zinc, compatible with concentrations released from activated platelets, are sufficient to serve as a cofactor function for HRG to bind heparin more effectively than antithrombin and thereby promote fibrinogenesis. These data prompt us to propose that this paradigm could function to control the locality and amount of fibrin deposited in tissues.

EXPERIMENTAL PROCEDURES

Proteins and Reagents—Human HRG was purchased from Celsus Laboratories (Cincinnati, OH) as a 100 μg/ml solution with a mean molecular mass of 60 kDa. We also prepared HRG from fresh human plasma (see below), and this preparation is referred to as HRGk75. Human antithrombin (AT) was obtained from Celsus Laboratories (58 kDa, 150 μg/ml), and from Enzyme Research Laboratories, South Bend, IN (58 kDa, 1.59 mg/ml). Bovine thrombin was purchased from Parke-Davis (Morris Plains, NJ) as USP Thrombostat (100 units/ml), and human thrombin (1.05 mg/ml) was purchased from Enzyme Research Laboratories. Heparin (12–15 kDa, 10,000 USP units/ml) was obtained from Elkins-Sinn (Cherry Hill, NJ). Aprotinin was purchased from ICN Biomedicals (Aurora, OH). Phosphocellulose (P11) was purchased from Whatman International (Fairfield, NJ). Mouse IgG1 monoclonal antibody (MO35) to an epitope in the first 229 amino acids of the N-terminal region of HRG was purchased from PanVera Corporation (Madison, WI), and monoclonal antibody to high molecular weight kininogen was from Dr. J. A. McIntyre of this center. Glycerol was purchased from Fisher Scientific (Pittsburgh, PA). Human fibrinogen (type I), epichlorohydrin-activated heparin-scarose resin, EDTA, DTT, 6-amino-n-hexanoic acid, benzamide hydrochloride, and ultra pure grades of zinc acetate and the chloride salts of zinc, copper, and magnesium were purchased from Sigma.

Preparation of HRG from Fresh Human Plasma—Human HRG was prepared according to the method of Rylatt et al. (20). Briefly, phosphocellulose P11 was prewashed in 1 × HCl and 1 × NaOH, and prepared in a column (2.5 × 20 cm) in equilibration buffer (pH 6.8) containing 10 mM sodium phosphate, 10 mM EDTA, 10 mM DTT, 1 mM 6-amino-n-hexanoic acid, 1 mM benzamide hydrochloride, and 0.5 M NaCl. Blood from two normal adult male donors was collected into Vacutainer tubes containing acid-citrate-dextrose (71 mM citric acid, 85 mM sodium citrate, and 111 mM dextrose) and immediately centrifuged at 1000 × g for 15 min. The pooled plasmas (40 ml) containing 10 mM EDTA, 10 mM DTT, 1 mM phenylmethysulfonyl fluoride, 1 mM benzamide hydrochloride, 1 mM 6-amino-n-hexanoic acid, 50 units/ml aprotinin, and 0.5 M NaCl was applied to the column. The column then was washed with 10 bed volumes of washing buffer...
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containing 0.8 M NaCl in equilibration buffer, and HRG was eluted with 5 bed volumes of elution buffer containing 2.0 M NaCl in equilibration buffer at a flow rate of 1 ml/min. The eluted HRG was concentrated, equilibrated with Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4), aliquoted (100 μg/ml), and lyophilized. The protein concentration of HRG was determined by either sodium SDS-polyacrylamide (8%) gels (21), by silver staining of the gels (22), and by Western blotting (23) with anti-HRG monoclonal antibody. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma) was used to detect reaction of anti-HRG antibody to HRG.

Plasma and Serum Collection—Blood samples (20–40 ml) for thrombin time measurements were drawn from six healthy males (23 to 44 years) and six healthy females (25 to 31 years) into 20-ml plastic syringes containing 2 ml of 0.1 M sodium citrate, or 10, 20, and 40 units of heparin, or no anticoagulant. Final concentrations thus were 0.01 M sodium citrate or 0.5, 1.0, or 2.0 units/ml heparin. The bloods were centrifuged (15 min, 1000 × g), plasmas or sera were pooled according to the type and amount of anticoagulant, and each pool was aliquoted into 0.5-ml samples and stored at -80 °C. Monthly determinations of HRG in these pools by single radial immunodiffusion (24) revealed a mean concentration of 100 ± 10 μg/ml, which is consistent with reported values in human plasma (1). All experiments were performed on fresh aliquots from these pools, so repeat freezing/thawing was avoided.

Measurement of Zinc by Atomic Absorption Spectrophotometry—Zinc in plasma and serum was measured according to the method of Buttrill and Purkiss (25) by using an atomic absorption spectrophotometer equipped with a single element hollow cathode lamp and autosampler (Perkin-Elmer 5100 PC and AS-51). A computer interface was used to measure zinc at 213.9 nm with an air-acetylene flame. Plasma and serum samples were obtained as described above and diluted 1:5 with 1% (v/v) HCl. Zinc standard solutions were prepared with 1% (v/v) HCl containing 5% (v/v) glycerol.

Thrombin Time Measurements of Plasma—These experiments were performed in triplicate in disposable Coa Screener cuvettes (American Labor Corp., Durham, NC) to which were added 50 μl of plasma and 100 μl of TBS, as above. Following incubation for 2 min at 37 °C, in a Coa Screener (American Labor Corp.), thrombin times were determined by adding 50 μl of thrombin (0.18 units in TBS) and recording the time required for formation of a fibrin clot. Parke-Davis thrombin was used in these experiments unless stated otherwise. The concentration of all reagents used in thrombin time experiments are expressed as the final concentration present in the Coa Screener cuvettes.

Thrombin Time Measurements of Plasmas Supplemented with HRG—These experiments were performed in triplicate in disposable Coa Screener cuvettes to which were added 50 μl of plasma and 100 μl of TBS, as above. The plasmas used were from the citrated and the heparinized plasma pools, and the amount of HRG added to each aliquot of plasma was 0.0, 0.13 (2.5% increase), 0.25 (5% increase), 0.50 (10% increase), 1.0 (20% increase), or 2.0 μg (40% increase). All cuvettes contained 150 μl of solution consisting of 50 μl of plasma and the above defined amounts of HRG in 100 μl of TBS. The cuvettes were incubated 2 min at 37 °C in the Coa Screener, and thrombin times were determined by adding 50 μl of thrombin (0.18 units in TBS) and recording the time required for formation of a fibrin clot.

Thrombin Time Measurements of Plasmas Supplemented with Zinc—These experiments were performed in triplicate in disposable Coa Screener cuvettes to which were added 50 μl of plasma and 100 μl of TBS, as above. The plasmas used were from the citrated pool and aliquots from the citrated pool that were heparinized with 0.018 units of heparin in TBS. Following incubation for 2 min at 37 °C, in a Coa Screener, and thrombin times were determined by adding 50 μl of thrombin (0.18 units in TBS) and recording the time required for formation of a fibrin clot.

Thrombin Time Measurements of Plasmas Supplemented with HRG and Zinc—These experiments utilized aliquots from the citrated plasma pool supplemented with a constant amount of zinc and increasing amounts of HRG, and each preparation was studied for its ability to neutralize the anticoagulant activity of 0.089, 0.108, and 0.130 units/ml heparin by using thrombin times. The experiments were performed in triplicate in disposable Coa Screener cuvettes. Each cuvette contained 150 μl of solution consisting of 50 μl of citrated plasma containing 150 μg/ml zinc and 0.0, 0.25, or 2.0 μg of HRG and the above amounts of heparin in TBS. The cuvettes were incubated for 2 min at 37 °C in the Coa Screener, and thrombin times were determined by adding 50 μl of thrombin (0.18 units in TBS) and recording the time required for formation of a fibrin clot.

Measurement of the Effect of Citrate on the Availability of Zinc—Thrombin times were performed as above to measure the effect of citrate on the availability of zinc. The experiments utilized aliquots of heparinized (0.125 units/ml) and heparinized/citrated (2.5 mM sodium citrate) plasmas containing 0.0–800 μM supplemental zinc. Plots of the thrombin time results for heparinized and heparinized/citrated plasmas were used to display the effect of citrate on the availability of zinc. A second set of thrombin time experiments was performed with aliquots of heparinized (0.025 units/ml) TBS containing one of the following: TBS only; AT (3.2 μg); AT (3.2 μg) plus heparin (0.02 units); AT (3.2 μg) plus heparin (0.02 units) plus HRG (2.0 μg).

Zinc concentrations in the cuvettes for each of the above four solutions were 0.0, 0.63, 1.25, 2.5, or 5.0 μM. The cuvettes were incubated 2 min at 37 °C in the Coa Screener, and thrombin times were determined by adding 50 μl of fibrinogen (125 μg/ml in TBS) and recording the time required for formation of a fibrin clot. The thrombin and the AT that were used in these experiments were from Enzyme Research, and the HRG was HRGk75 shown by Western blotting and silver staining of polyacrylamide gels to consist predominantly of native (i.e. 75 kDa) molecule.

Effect of Zinc on Binding of HRG to Immobilized Heparin—Heparin coupled to epichlorohydrin-activated 4% bead agarose (Sigma, H0402, washed to 4.0% bead) was loaded to 3.75 mg/ml onto a 5-cm glass column and washed with 10 bed volumes of 0.02 M Tris-HCl, 3.0 M NaCl buffer (pH 7.2) containing 1.0 mM EDTA and 0.05% Na2EDTA. The column was equilibrated with 10 bed volumes of 0.02 M Tris-HCl, 0.01 M NaCl buffer (pH 7.2) containing 0.1 mM zinc (equilibration buffer), was loaded with 0.5 mg HRGk75 in 1.0 ml of equilibration buffer, and equilibrated with equilibration buffer at a flow rate of 0.5 ml/min. The effect of zinc on the bound heparin-HRG to immobilized heparin immunoadsorbent was determined by how much HRG was removed from the heparin-agarose by 0.1, 1.0, and 3.0 M NaCl in the presence of the zinc or the zinc chelator, EDTA. Elution profiles of HRG from the immobilized heparin matrix were studied by the using the two following methods. Method A consisted of 0.1 M NaCl, 0.1 mM zinc acetate; 1.0 mM NaCl, 0.1 mM zinc acetate; 3.0 M NaCl, 0.1 mM zinc acetate; 0.1 M NaCl, 1.0 mM EDTA; 1.0 M NaCl, 0.1 mM zinc acetate; 1.0 M NaCl, 1.0 mM EDTA; and 3.0 M NaCl, 0.1 mM zinc acetate. All the buffers used in both methods contained 0.02% Tris-HCl, 0.05% Na2EDTA, and were pH 7.2.

Comparison of Zinc, Copper, and Magnesium for Heparin Neutralization in the Purified Component Clotting Assay—The purified component clotting assay was performed as described above. In these experiments, the Coa Screener was used to measure the rates of thrombin-mediated conversion of fibrinogen to fibrin as an index of the availability of heparin to serve its cofactor function for AT to inhibit thrombin. All experiments were performed in triplicate in disposable Coa Screener cuvettes to which were added 50 μl of plasma and 100 μl of TBS, as above. The concentration of all reagents performed in triplicate in disposable Coa Screener cuvettes to which were added 50 μl of plasma and 100 μl of TBS, as above. The plasmas used were from the citrated pool and aliquots from the citrated pool that were heparinized with 0.018 units of heparin/ml. Each cuvette was supplemented with 6.2, 12.5, 25, 50, 100, or 200 μM zinc in TBS. Initial experiments used both zinc chloride and zinc acetate but no differences were found, so all zinc experiments employed zinc acetate unless stated otherwise. All cuvettes contained 150 μl of solution consisting of 50 μl of either citrated or citrated/ heparinized plasma and the above amounts of zinc in TBS. The cuvettes were incubated 2 min at 37 °C in the Coa Screener, and thrombin times were determined by adding 50 μl of thrombin (0.18 units in TBS) and recording the time required for formation of a fibrin clot.

Results

Biochemical Characterizations—SDS-polyacrylamide gel electrophoresis and silver staining of Parke-Davis thrombin
revealed a major band of 37 kDa and several minor bands at ≥ 50 kDa, and thrombin from Enzyme Research produced one major band of 37 kDa (data not shown). These thrombins were identical in their functional capacities to convert fibrinogen to fibrin. SDS-polyacrylamide gel electrophoresis and silver staining of the fibrinogen showed three major bands between 50 and 60 kDa, corresponding to the α, β, and γ chains (26), and AT from Celsus and Enzyme Research produced one major 58-kDa band (data not shown). The above proteins were found to be free of HRG by Western blotting with the mouse monoclonal antibody to HRG (data not shown). Silver staining of SDS-polyacrylamide gels of HRGk75 revealed mostly 75-kDa protein and small amounts of 50-kDa material, while analysis of Celsus HRG revealed much less 75-kDa material and bands at 67, 50, 48, and 40 kDa (Fig. 1A), which are consistent with earlier biochemical studies of this molecule (27). Western blotting with monoclonal antibody to HRG revealed reactivity with the 75-, 67-, 50-, 48-, and 40-kDa bands (Fig. 1B), and Western blots of our normal sera and plasma pools reacted with monoclonal antibody to HRG in almost identical qualitative and quantitative patterns as with HRGk75. In addition, Western blots of HRG75k, Celsus HRG, and AT did not react (data not shown) with monoclonal antibody to high molecular weight kininogen, which is another histidine-rich glycoprotein (28).

In summary, thrombin from Enzyme Research was electrophoretically more homogenous than thrombin from Parke-Davis, but they functionally were equivalent; AT from Enzyme Research and Celsus were electrophoretically equivalent, and Western blotting revealed that neither thrombin nor AT contained HRG and that neither thrombin nor AT nor HRG contained high molecular weight kininogen. The HRG from Celsus was less homogeneous and contained more degradation products than HRGk75, yet monoclonal antibody to HRG revealed that the fragments were of HRG origin, and our normal human plasmas were found to have the same distribution of fragments as those identified in HRGk75.

The Effect of HRG on Heparin Activity—Earlier investigators reported that HRG-depleted citrated plasmas less effectively resisted the anticoagulant effects of heparin (2, 4, 5, 29). After confirming these observations, we studied the role of HRG by supplementing rather than depleting plasmas of HRG. The plasmas used in these experiments were from the citrated and heparinized plasma pools, and the results of these experiments revealed no significant differences in thrombin times when plasma concentrations of HRG were increased from base line (i.e. 100 μg/ml) to 40% increase of base line. Each supplemented plasma was tested separately six times, and the results consistently failed to show any correlation between increasing plasma HRG concentrations and changes in the anticoagulant effects of heparin (data not shown).

Measurement of Zinc by Atomic Absorption Spectrophotometry—Measurements of zinc concentrations were performed on all reagents, plasmas and sera, and on solutions used in the thrombin time assays. The results showed negligible amounts of zinc (<0.01 μM) in the TBS, thrombin, and heparin. Undiluted plasma zinc concentrations were 8.6 ± 1.8 μM, and serum zinc concentrations were 11.6 ± 1.6 μM.

The Effect of Zinc on Heparin Activity—The interaction between heparin and HRG has been reported to require divalent cations (2, 5, 27). We studied the differential effects of zinc on thrombin-mediated fibrinogenesis in citrated and citrated/heparinized plasmas and found that the thrombin times of citrated plasmas were not changed by supplemental zinc but that the thrombin times of citrated/heparinized plasmas were shortened in a dose-dependent manner (Fig. 2). The zinc-induced reduction of the anticoagulant activity of heparin was found to be reversible, as demonstrated by the finding that the addition of heparin to citrated/heparinized plasmas containing ≥ 25 μM zinc increased the thrombin time yet again (Fig. 2, insert). In addition to illustrating a central role for zinc-induced modulation of HRG-heparin interactions, these results suggested that the zinc-catalyzed HRG binding of heparin was saturable, for titrations of heparin at 100 and 200 μM zinc produced identical thrombin times (Fig. 2, insert).

The Effect of HRG and Zinc on the Availability of Heparin—Citrated plasmas supplemented with a constant amount of zinc...
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FIG. 3. The effect of HRG and zinc on the availability of heparin. Citrated plasmas containing 150 μM zinc and the defined amount of HRG were analyzed for their ability to neutralize 0.098 (○), 0.108 (△), and 0.130 (□) units/ml heparin as measured by thrombin times. Each measurement was performed six times, and the results revealed striking correlations between plasma HRG concentrations and heparin neutralization in the presence of zinc.

Results of the above experiments indicated that supplementary zinc was required for heparin neutralization in plasma containing citrate, while heparinized plasmas required only 100 μM zinc. Neutralization of the catalytic effect of heparin on AT in heparinized/citrated plasmas containing 0.0–800 μM zinc (Fig. 6) due to the well established cofactor function of heparin for AT in the inhibition of thrombin-mediated conversion of fibrinogen to fibrin. Note a small increase in thrombin time by AT-heparin in the presence of 0.63 μM zinc, but no subsequent increases from 0.63 to 5.0 μM zinc. In striking contrast, when HRG was added, the rate of thrombin-mediated conversion of fibrinogen to fibrin was shortened progressively and returned to control values at ≥ 1.25 μM zinc (● in Fig. 6), again indicating that zinc served as cofactor for the neutralization of heparin by HRG. It should be pointed out that in this purified component clotting assay, the molar ratio of HRG to AT in the cuvette (i.e. 1.0:2.2) was the same as the ratio of HRG to AT in normal plasma (i.e. 1.0:2.3; see Ref. 29), and yet HRG disallowed heparin binding by AT in the presence of micromolar amounts of zinc.

Effect of Zinc on Binding of Heparin to Immobilized Heparin—Results of the above experiments indicated that supplementary zinc shortened thrombin times of plasmas as well as purified component clotting assays, suggesting that the mechanism for this shortening of thrombin times was zinc-induced HRG binding of heparin. We thus asked if heparin-bound HRG was bound more tightly in the presence of zinc by studying the molarity of NaCl required to elute HRG from an affinity column of immobilized heparin in the presence and absence of zinc. The results of these experiments revealed that 0.1 mM NaCl removed a small amount of HRG in 0.1 mM zinc from the column and that 1.0 mM NaCl was required to remove most of the HRG from heparin in the presence of zinc (Fig. 7A). In contrast, 0.1 mM NaCl was sufficient to remove HRG from heparin in the absence of zinc, as demonstrated by elution in the presence of EDTA (Fig. 7B), which chelates and removes free zinc from solution. These data support the interpretation that zinc significantly increases the ability of HRG to bind heparin and that such binding deprives AT of its heparin cofactor for the inhibition of thrombin.

Comparison of Zinc, Copper, and Magnesium for Heparin
Neutralization in the Purified Component Clotting Assay—The specificity of zinc-induced HRG-binding of heparin was studied by using the purified component clotting assay to determine the possible role of other divalent cations reported to bind HRG (4, 5, 29). The thrombin times of heparinized and non-heparinized assays were determined with and without 5 μM zinc chloride, 5 μM copper chloride, or 5 μM magnesium chloride. The results of these experiments clearly showed that HRG effectively neutralized heparin only in the presence of zinc (Fig. 8), in which case it produced thrombin times that closely approximated those obtained from non-heparinized samples. In addition, control experiments performed by using the purified component clotting assay revealed that the small concentrations of divalent cations used in these experiments were not adequate to precipitate or denature any protein components of the assay (data not shown).

**DISCUSSION**

Understanding of the function of HRG was broadened by earlier observations that HRG-depleted plasmas were not coagulable in the presence of heparin, while thrombin times of normal plasmas increased only slightly with added heparin (2–5). We extended these observations in the presence of heparin and found that HRG-supplemented plasmas as compared with control plasmas were not distinguishable by measuring thrombin times, but the effects of increasing HRG concentrations on heparin neutralization became apparent in the pres-
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The cytoplasm and alpha-granules of human platelets contain zinc at 30–60-fold higher concentrations than plasma (35); both resting (36) and activated (37) platelets contain HRG. Indeed, serum zinc levels were experimentally shown to be higher than plasma zinc levels. We propose that platelets activated at the reaction site release zinc ions, which serve as a cofactor function by binding plasma and platelet HRG molecules that mediate high affinity binding of heparin. This promotes fibrinogen binding only at the reaction site and in amounts controlled by the microenvironmental concentration of zinc. The procoagulant effect of HRG would be limited by blood flow dilution of microenvironmental zinc and by plasmin cleavage of native HRG (27). Although HRG in purified systems has been shown convincingly to have a lower heparin dissociation constant than AT (4, 38), insolubilized unfunctionalized and low molecular weight heparins bind more AT than HRG from whole human plasma (18). This apparent discrepancy could relate to the availability of zinc as shown by the effects of citrate or EDTA on heparin binding by HRG (29).

Thus, our findings suggest that normally thromboresistant vessels can support site-directed fibrinogenesis when activated platelets release zinc ions that increase the hepatic binding of HRG versus AT, which focally and momentarily disallows thrombin inhibition.

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