Reversal of Low-Molecular-Weight Heparin Anticoagulation by Synthetic Protamine Analogues

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INTRODUCTION

Protamine sulfate reversal of standard unfractionated heparin and low-molecular-weight heparin (LMWH) anticoagulation causes a number of non-immunologic-mediated adverse side effects including hypotension, bradycardia, diminished oxygen utilization, pulmonary artery hypertension, and declines in platelet and white blood cell counts. We have recently demonstrated that the efficacy and toxicity of protamine reversal of standard heparin anticoagulation is dependent on the charge of the compound and that alternatives to protamine for heparin anticoagulation reversal exist [1]. The purpose of the present investigation was to test the hypothesis that a similar relationship exists for the reversal of LMWH with protamine-like variants. Additionally, we attempted to modify protamine-like variant structure to prevent in vivo compound degradation and decrease the occurrence of heparin rebound, a phenomenon that may be dependent on degradation of the reversal agents.

METHODS

Three protamine-like peptides, with different cationic charges, were synthesized on preloaded Wang resins or on RINK resin with 9-fluorenylmethoxycarbonyl amino acid derivatives using an Applied Biosystems Model 431 peptide synthesizer (Applied Biosystems, Foster City, CA). The hydroxybenzotriazol esters of the 9-fluorenylmethoxycarbonyl amino acids were formed using 2-(1H-benzotiazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as an activation agent.

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Coupling and deprotection of the nascent peptide chains were accomplished under standard conditions for the synthesizer (FastMOC cycles). Cleavage and final deprotection were in 90% trifluoroacetic acid containing 5% ethanethiol, 2.5% thioanisole, and 2.5% anisole for 2 hr at room temperature. The peptides were precipitated from the trifluoroacetic acid by 20 vol of diethyl ether at −20°C. Once synthesized, these peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) on a 2-in. × 25-cm preparative reversed-phase column (Rainin, Dyanamax). The flow rate was 17 ml/min and the gradient was from 5 to 60% acetonitrile in 90 min. In some instances the peptides were subsequently desalted on Sephadex (Pharmacia, Piscataway, NJ) G-15 gel filtration columns equilibrated with 1 N acetic acid. Each purified peptide was characterized by amino acid analysis, analytical reversed-phase HPLC, and mass spectroscopy to confirm purity before its use. Inclusion of norleucine as an internal standard allowed accurate assessment of peptide concentration.

Peptides were synthesized so that the number of lysine (K) residues determined the total peptide cationic charge ranging from [+16] to [+18] with protamine's primary charge being [+21]. Constructs tested were: [+16] P(AK₂A₂K₂)₄ and [+18] PK(K₆A₆K₂)₄K₆AK₆, compared to protamine, [+21] PR₆S₃RPVR₃PRVSR₆G₃R₆. Lysine, like arginine (R) in native protamine, carries a single positive charge at physiologic pH and is of similar size, yet its use in peptide synthesis avoids certain technical difficulties associated with automated production of peptides containing large numbers of arginine residues. Assignment of amino acid and peptide charge was based on the known pKₑ of 9.7 for lysine and 12.5 for arginine. At physiologic pH 7.4, it is expected that more than 99% of the lysine and arginine residues carry a charge of [+1]. In these compounds, the glycine connecting amino acids of our previously reported variants [1] were replaced by alanines (A) in order to increase the propensity for α-helix formation on binding to LMWH [2]. Peptide length was maintained constant at 29 amino acids. In order to prevent in vivo degradation by aminopeptidases at the amino terminus and carboxypeptidases at the carboxyl terminus, the [+18] compound was acetylated and amidated and called [+18B]: acetyl-PA(K₆A₆K₂)₄K₆-amide. This also has the effect of increasing the dipole moment of an α-helix formed by this peptide. This should result in an increased α-helix forming propensity for [+18B] on binding to low-molecular-weight heparin. This compound has an amino acid length of 32 residues, reflecting the necessary addition of three amino acid residues in order to maintain optimal spacing for α-helix formation on binding to heparin. In these variants, the terminal proline of protamine was retained for consistency.

Each of the variant peptides plus standard protamine was tested in 7 female mongrel dogs, having a mean weight of 12.3 kg. Animals were anesthetized with 15 mg/kg sodium pentobarbital, intubated, and maintained on positive pressure ventilation with 4 liters/min supplemental oxygen during the study. Hydration was maintained with an intravenous infusion of lactated Ringer's solution, 22 ml/kg/hr. All animals were housed and cared for in the University of Michigan Unit for Laboratory Animal Medicine under the direction of a veterinarian according to the guidelines of the "Principles of Laboratory Animal Care" (National Society for Medical Research) and "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1985).

All animals received intravenous LMWH (Logiparin; Novo, Denmark) 150 IU/kg factor Xa activity followed 30 min later by administration of protamine variant peptides or standard protamine (Eli Lilly, Indianapolis, IN), 1.5 mg/kg (100 IU/mg). The protamine or protamine-like variants were given intravenously into a femoral vein over 10 sec to maximize hemodynamic effects. Changes in LMWH anticoagulation occurring from its metabolism alone were determined in a separate group of five dogs not given any reversal agent.

Hemodynamic monitoring included measurement of systemic mean arterial blood pressure (MAP) and heart rate (HR) by means of a carotid artery catheter, pulmonary artery systolic and diastolic pressures (PAS/PAD) and mixed venous oxygen saturation (SᵥO₂) by means of an oximetric Swan–Ganz catheter (Abbott Laboratories, North Chicago, IL), and systemic arterial oxygen saturation (S₂O₂) by a catheter placed into the femoral artery. Pulmonary artery flow as a measure of cardiac output (CO) was determined with an electromagnetic square-wave flow probe (Narcomatic, Houston, TX). These measurements allowed for calculation of systemic oxygen consumption (VO₂) by Fick equation relationships (i.e., oxygen consumption = flow × hemoglobin × 1.34 [SᵥO₂ − S₂O₂]. All hemodynamic data were collected and assessed with an on-line computer program (Workbench; Strawberry Tree, Sunnyvale, CA) that allowed continuous monitoring of both measured and calculated hemodynamic parameters. Measurements and calculations were made at baseline, before LMWH administration, 3 min before reversal, every 10 sec for 5 min after reversal, and at 10, 20, and 30 min after reversal. Although measurements were made every 10 sec, only the values every 30 sec were recorded for purposes of this communication. A total toxicity score (TTS) was calculated for each peptide, which included the maximum change in MAP, CO, VO₂, and HR during the first 5 min after reversal. The maximum changes that occurred in the individual dog were divided by the standard deviation derived from the entire group of animals in all groups combined and then the scores were added to compute the TTS for each individual dog. In each group, the mean of the scores was then calculated in order to obtain a TTS for each peptide studied. The more nega-
tive the value, the more toxic the compound during LMWH reversal.

Coagulation and hematologic studies were performed in venous blood samples obtained before LMWH administration, 3 min before reversal, and at 3, 10, and 30 min after variant or protamine administration. These studies included determinations of activated clotting time (ACT), heparin antifactor Xa activity, thrombin clotting time (TCT), heparin antifactor IIa activity, activated partial thromboplastin time (aPTT), platelet count, and white blood cell count. ACT measurements were made immediately on blood withdrawn with 2 ml whole blood and celite-containing tubes (Hemochron; International Technidyne, Edison, NJ). The remainder of the blood was placed into standard citrated tubes and either stored on ice for heparin antifactor Xa or antifactor IIa determinations or spun down at 900 rpm for 10 min to obtain platelet-rich plasma (FRP). TCT studies used 0.2 ml PRP and 0.1 ml TCT reagent (American Dade, Miami, FL) and were run on a fibrometer (Baxter, Miami, FL), while aPTT tests were made on 0.1 ml PRP with commercially available GPC cartridges (Hemotec, Englewood, CO) activated by addition of 0.1 ml rabbit cephaloplastin (Baxter). Thrombin clotting times and aPTT measurements were all run in duplicate and the values averaged. Platelet and white blood cell counts were analyzed by hand hemocytometry methods with a diluted red blood cell lysis method (Unopette; Becton-Dickenson, Rutherford, NJ).

Heparin antifactor Xa (FXa) and antifactor IIa (FIIa) assays were performed after cold centrifugation (2900 rpm × 20 min at 4 °C) of citrated blood previously stored on ice, resulting in a platelet-poor plasma (PPP) super-

FIG. 1. Total toxicity scores for [+16], [+18], [+18B] variants, as well as protamine [+21].

FIG. 2. Mean arterial blood pressure (MAP), cardiac output (CO), oxygen consumption (VO₂), and heart rate (HR) changes for [+16], [+18], [+18B] variants, as well as protamine [+21].
natant. In the FXa assay (Coatest Heparin; Kabi Vitrum, Stockholm, Sweden), excess antithrombin III followed by factor Xa was added to the PPP, which was then reacted with the chromogenic substrate S-2222. The FIIa assay was performed in a similar manner by addition of thrombin (IIa), followed by the chromogenic substrate S-2238, to PPP previously incubated with excess antithrombin III. Heparin antifactor Xa or antifactor IIa activity was inversely proportional to absorbance at 405 nm, indicating the quantity of uninhibited factor Xa or IIa remaining.

Data in this report are expressed as mean ± SD. Statistical analysis included linear regression to determine correlation coefficients, analysis of variance (ANOVA), and unpaired two-tailed Student’s t test where appropriate (Statworks; Cricket Software, Philadelphia, PA). Coagulation results were corrected for the natural occurring metabolism of LMWH.

RESULTS

Protamine [+]21 reversal of LMWH was most toxic with a TTS −7.6 ± 4.8, compared to [+]16 −2.8 ± 2.0, [+]18B −1.8 ± 1.0, and [+]18B −0.7 ± 1.6 (P < 0.01, ANOVA; Fig. 1). Specifically, [+]16 vs protamine [+]21 (P < 0.05) and [+]18 vs protamine [+]21 (P < 0.01) were significantly different by t test, while [+]18B and protamine were dissimilar, but the difference did not achieve statistical significance (P = 0.084). Maximum mean declines in the first 5 min after reversal in MAP, CO, VO2, and HR, respectively, were: [+]16 −6 mm Hg, −8%, −10%, −7 beats/min; [+]18 −1 mm Hg, −3%, −4%, −1 beats/min; [+]18B −10 mm Hg, −18%, −12%, −9 beats/min; and protamine [+]21 −32 mm Hg, −32%, −26%, and −18 beats/min (Fig. 2). Pulmonary hypertensive responses were not included in the TTS calculations. Maximal mean increases in PAS and PAD for protamine [+]21 of +10 mm Hg and +10 mm Hg were inhibited for all variant compounds: [+]16 +1 mm Hg, +1 mm Hg; [+]18 +1 mm Hg, +1 mm Hg; and [+]18B 0 mm Hg, 0 mm Hg (Fig. 3).

Anticoagulation reversal revealed that [+]18 was more effective than [+]16. Anticoagulation reversal with [+]18B was more effective than that with either [+]16 or [+]18 and was as effective if not slightly more effective than protamine [+]21. Percentage reversal at 3 min for ACT, antifactor Xa, TCT, and antifactor IIa levels, respectively, were: [+]16 26, 25, 66, 43%; [+]18 49, 21, 91, 36%; [+]18B 87, 64, 99, 96%; and protamine [+]21 99, 63, 100, and 99% (Fig. 4). At 10 min after reversal, these same respective values were: [+]16 55, 19, 32, 7%; [+]18 52, 17, 67, 24%; [+]18B 93, 34, 95, 72%; and protamine [+]21 88, 45, 98%, and no value (Fig. 4). At 30 min after reversal, these same values were: [+]16 78, 29, 50, 44%; [+]18 61, 24, 64, 46%; [+]18B 102, 52, 96, 74%; and protamine [+]21 82, 44, 96, and 86% (Fig. 4). Concerning reversal as measured by aPTT values, little to no anticoagulation reversal was noted with [+]16 or [+]18 variants. In fact, both compounds produced a paradoxical increase in aPTT 3 min after administration. The reversal as noted by aPTT for [+]18B at 3, 10, and 30 min after administration was 64, 95, and 93%, respectively, while for protamine [+]21 these values were slightly less, being 50, 83, and 78%, respectively.

There was no decrease in thrombocytopenia for [+]18B, with a mean decline in platelet count of −56% as opposed to protamine [+]21 of −43%, although less thrombocytopenia was found with [+]16 −24% (P < 0.05) and [+]18 −8% (P < 0.01) compounds than [+]18B. The difference between the compounds was significant by ANOVA for thrombocytopenia (P < 0.01). WBC declines (no statistical difference by ANOVA) were found for [+]16, −4%; [+]18, −7%; [+]18B, −21%; and protamine [+]21, −3%.

DISCUSSION

Protamine sulfate may cause nonimmunologic adverse side effects. We have previously documented that in vivo
heparin reversal depends on the availability of positive charges and simultaneously, that the greater the net positive charge of the molecule, the more toxic the compound. Commercial protamine is a heterogeneous mixture of highly cationic polypeptides purified from salmon sperm. The major component is n-protamine, a 32-amino acid peptide with the sequence PR$_4$SRPVRRPVVRG$_2$R$_4$[3]. The positively charged arginines (R) account for 67% of the total sequence and for the charge of [+21]. Reversal occurs as protamine binds to negatively charged heparin, displaces the heparin from heparin-antithrombin complexes, and reverses the potentiation of antithrombin III for its effects against thrombin and many other components of the clotting cascade [4, 5].

Logiparin (LHN-1) is a LMWH produced from porcine intestinal mucosal heparin by enzymatic depolymerization using heparinase. Its molecular mass ranges from 600 to 20,000 Da, with the peak maximum molecular mass being approximately 5000 Da. Greater than 70% of its molecular mass ranges between 1500 and 10,000 Da. The biological activity is approximately 87 IU/mg of antifactor Xa activity with the 1st International Standard for LMWH as reference, and the antifactor Xa/antifactor IIa ratio is 1.7/1.0, making it intermediate in effect against factor Xa compared to other LMWH preparations. Little data concerning reversal of LMWH by protamine is available, but clearly the data that exists uniformly indicates that protamine incompletely reverses LMWH anticoagulation as measured by antifactor Xa assays [6–10]. However, the clinical significance of this partial inhibition of factor Xa inhibition has been debated in the literature [6].

In general, LMWH has an improved pharmacokinetic profile compared to standard unfractionated heparin, has less antiplatelet activity and thus potentially less bleeding potential, has less lyolytic effect, and has a half-life not dependent on the initial dose administered [11]. Recent studies have suggested that LMWH compounds can be used successfully in cardiovascular surgical applications [12–14]. In one study of patients undergoing aortofemoral bypass graft placement, LMWH produced more constant antifactor Xa inhibition, less protein C antigen decrease, less complement activation, and less inhibition of platelet aggregation (as assayed using collagen as agonist), when compared to standard unfractionated heparin [12]. These data suggest that LMWH may be preferable to standard unfractionated heparin for bolus injection during aortofemoral bypass.
surgery and others have suggested its use in cardiopulmonary bypass procedures [15]. In view of the possible and probable intraoperative use of LMWH compounds and their longer effective half-lives, an effective and safe agent for their reversal is clearly desirable.

In the present investigation, we found that protamine-like variant compounds with [+16] and [+18] charges reversed logiparin incompletely, but with less toxicity than standard protamine. Modification of the amino- and carboxyl-terminal of the [+18] variant, to prevent degradation and enhance α-helicity, not only improved upon the effectiveness over time but actually produced a compound that by all tests reversed logiparin anticoagulation as effectively as standard protamine. By aPTT measurements, the [+18B] variant was actually more effective than protamine. Associated with this improvement in anticoagulation reversal was greater toxicity. However, despite this, this compound was still much less toxic than standard protamine, although not at a statistically significant level (P = 0.084). The [+18B] variant did not lessen the thrombocytopenia normally associated with protamine reversal of logiparin and caused a nonsignificant decline in white blood cell count.

The curious finding about the paradoxic prolongation of aPTT values with [+16] and [+18] compounds may relate to a finding seen with standard protamine in high doses. In clotting assays, protamine sulfate has an inhibitory effect on thrombin in the conversion of fibrinogen to fibrin. This inhibition is concentration dependent, partial, and reversible [16]. In view of the reversal of anticoagulation noted by the other clotting assays, it is possible that the observed prolongations are most likely an artifact of the assay rather than actual prolongations of the clotting mechanism.

A number of mechanisms have been suggested to be responsible for protamine-related toxicity. These include complement activation [17–23], thromboxane generation [24–29], histamine release [30–32], inhibition of plasma carboxypeptidase N [33], direct actions on the peripheral vasculature and the heart [34], and various immunologic mechanisms including antibody-mediated and immediate anaphylactoid reactions without antibody involvement [35–43]. The most likely cause of systemic hypotension appears to be the elaboration of a vasoconstrictor factor, such as nitric oxide [44–46], as well as direct depression of myocardial function, including associated bradycardia [47]. Pulmonary artery hypertension, on the other hand, is thought to result from thromboxane release, primarily from nonplatelet sources within the pulmonary circulation [24, 26, 28, 48–53]. Lastly, thrombocytopenia and leukopenia are most likely the result of a direct toxic effect of protamine on phospholipid membranes of these blood elements [53–59]. The exact mechanisms that allow the variant protamine-like compounds, such as described in this investigation, to cause less adverse side effects are not known and are the subject of ongoing investigation.

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