Neutralization of Low Molecular Weight Heparin by Polybrene Prevents Thromboxane Release and Severe Pulmonary Hypertension in Awake Sheep

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Protamine reversal of heparin anticoagulation in patients is occasionally associated with life-threatening acute pulmonary hypertension. In a sheep model, we evaluated the effect on this adverse cardiopulmonary reaction of modifying the type of heparin (low molecular weight heparin compared with unfractionated heparin) and the type of heparin antagonist (polybrene compared with protamine). Protamine reversal of low molecular weight heparin (LMWH) and polybrene reversal of unfractionated heparin induced more than a 10-fold increase of plasma thromboxane B_2 levels, a threefold increase of pulmonary vascular resistance and pulmonary artery pressure, and a 25% decrease of Pao_2. A similar adverse reaction followed protamine reversal of conventional unfractionated heparin. However, with polybrene (1 mg/kg) reversal of LMWH (1 mg/kg), we measured neither pulmonary hypertension (pulmonary artery pressure was 22.6±3.6 mm Hg at 1 minute after polybrene reversal of LMWH compared with 47.9±4.2 mm Hg after protamine reversal of unfractionated heparin, p<0.005 groups differ), hypoxemia (Pao_2 was unchanged 2 minutes after polybrene compared with a decrease of 26 mm Hg 2 minutes after protamine, p<0.05), nor acute release of thromboxane into arterial plasma (thromboxane B_2 was 0.2±0.1 at 1 minute after polybrene compared with 3.7±1.7 ng/ml at 1 minute after protamine, p<0.005). The hemodynamic effects and mediator release were also benign after neutralization of larger doses of LMWH (3 mg/kg) by polybrene (3 mg/kg). The increases of activated clotting time and activated partial thromboplastin time due to both types of heparin were completely reversed with polybrene. Anti-Xa activity increased to more than 3 IU/ml 4 minutes after LMWH anticoagulation (p<0.01) but was only partially neutralized by polybrene. Various polyanion-polycaction complexes that are formed when heparin anticoagulation is reversed induce thromboxane release and acute pulmonary vasoconstriction in awake sheep. Reversal of LMWH anticoagulation with polybrene does not elicit this adverse reaction. (Circulation 1990;82:1754–1764)

Protamine is widely used to reverse heparin anticoagulation in patients after cardiopulmonary bypass, cardiac catheterization, and vascular surgery. Protamine causes occasionally cat-

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approximately 1.5%. There are as yet no identified risk factors for this reaction. Although the incidence markedly differs, the heparin-protamine reaction in awake sheep closely
mimics the pulmonary vasoconstrictive response to heparin-protamine neutralization in humans, producing similar levels of acute pulmonary hypertension and acute release of thromboxane into arterial plasma.1–4,6,7 In sheep and pig models, indomethacin pretreatment has been shown to block the synthesis of vasoconstrictor prostanoids as well as the acute pulmonary vasoconstriction consistently associated with protamine reversal of heparin anticoagulation.4–7 Unfortunately, the hemorrhagic and renal side effects of cyclooxygenase-inhibiting drugs prevent their routine prescription before cardiopulmonary bypass.8,9

We hypothesized that the adverse heparin-protamine reaction in awake sheep would be prevented by modifying the nature of either the polyanion (heparin) or the polycation (heparin antagonist) or both. We examined a low molecular weight heparin (enoxaparine) (LMWH) as a substitute for conventional unfractionated heparin and used a synthetic polymeric salt, hexadimethrine bromide (polybrene), as a substitute for protamine sulfate. LMWHs have provided anticoagulation for cardiopulmonary bypass in animals as well as patients.10–13 The quaternary ammonium salt polybrene is routinely used in biological tests to determine heparin activity in human plasma.14 Polybrene has been used after cardiopulmonary bypass and has been recommended as an alternative neutralizing agent for patients who are allergic to protamine.5,15–17

To achieve its anticoagulant effect, conventional unfractionated heparin binds to antithrombin III and inhibits thrombin and factor Xa. LMWH fails to inhibit thrombin but forms complexes with antithrombin III. The latter inhibits factor Xa to provide an antithrombotic effect. To evaluate the level of anticoagulation with LMWH and the effects of heparin antagonists, we measured plasma anti-Xa activity, the activated partial thromboplastin time (APTT), and the activated coagulation time (ACT).

Methods

Animal Preparation

Suffolk sheep weighing 25–35 kg were anesthetized by mask with 1–2% halothane (Halocarbon Laboratories, Hackensack, N.J.) in pure oxygen and surgically instrumented using sterile techniques. The right femoral artery was cannulated with a polyvinyl chloride catheter advanced to the midthoracic aorta to sample blood and monitor systemic blood pressure (Psa). The following day, the sheep was placed in a Babraham veterinary cage with a suspension harness to maintain the sheep in a standing position during the study. The experiments were performed on awake sheep having free access to food and water. With 1% xylocaine local anesthesia and aseptic technique, a thermal dilution Swan-Ganz catheter (model 93A-131H-7F, Edwards Laboratories, Santa Ana, Calif.) was passed through an 8F introducer (Cordis Corp., Miami, Fla.) through the right external jugular vein into the pulmonary artery to measure mean pulmonary artery pressure (Ppa), balloon occlusion pressure (Pw), and central venous pressure (Pv). A venous line was used for fluid and drug administration.

Hemodynamic Measurements

Psa, Pv, Ppa, and Pw were monitored using calibrated Hewlett-Packard model 1280C pressure transducers and were continuously recorded on a Hewlett-Packard model 7758B recorder (Hewlett-Packard Co., Palo Alto, Calif.). The zero reference level was measured at the midthoracic line in the standing position. Mean values were measured at end expiration. Thermodilution cardiac output was determined by injecting 5 ml iced saline into the right atrium (model 9520A Cardiac Output Computer, Edwards Laboratories, Santa Ana, Calif.). We used the mean value of triplicate determinations except at 1 and 2 minutes after protamine when time only allowed duplicate measurements. Pulmonary vascular resistance was calculated as (Ppa minus Pw) divided by CO, and systemic vascular resistance was calculated as (Ppa minus Pw) divided by CO.

Thromboxane B2 Plasma Levels

Plasma levels of thromboxane B2 (TxB2), the stable metabolite of thromboxane A2 (TxA2), were determined at each time period. Four milliliters of arterial blood was collected in glass test tubes containing 0.05 ml 15% EDTA and 100 mg indomethacin, and samples were immediately placed on ice. After centrifugation at 2,000g for 10 minutes at 4°C, plasma was aspirated and stored in polypropylene tubes at –70°C. TxB2 analyses were performed on unextracted plasma by radioimmunoassay using specific antisera obtained from Dr. L. Levine as described elsewhere.4,18 Determinations were reproducible within ±10% in a given assay. Therefore, the assay was considered sufficiently reproducible that each analysis reported was the result of a single determination on each plasma sample. In previous experiments, we documented that ovine plasma TxB2 immunoreactivity was extracted and found to chromatograph coincident with a TxB2 standard by reverse-phase high-performance liquid chromatography.

Anticoagulation Tests

Activated clotting time (ACT) in 2 ml whole blood was measured with a Hemochron 400D system (International Technidyne, Edison, N.J.).19 To measure anti-Xa activity and APTT, blood was collected in a 0.13-M sodium citrate solution. These samples were centrifuged at 1,500g for 15 minutes, and plasma was aspirated and stored in polypropylene tubes at –20°C for later analysis.

The potentiating effect of heparin on sheep plasma anti-Xa activity was determined by an amidolytic assay using the method of Teien and Lie.20 Briefly, sheep plasma anticoagulated with sodium citrate (0.13 M) was mixed with an equal volume of purified bovine antithrombin III (100 ml), and the mixture was diluted 10-fold with Tris-EDTA buffer, pH 8.4. An aliquot of test plasma or a heparin standard was
incubated at 37°C for 2 minutes followed by addition of purified bovine Xa (0.1 ml) for 1 minute. At the end of this incubation period, the chromogenic substrate (CH3SO2-D-Leu-Gly-Arg-pNA, AcOH, American Bioproducts, New York, N.Y.) was added for precisely 30 seconds, and the reaction was stopped by adding acetic acid (0.1N). The optical density of each test sample was read in a MicroElisa reader (Dynatech, Alexandria, Va.) at 405 nm against a blank obtained by mixing acetic acid (0.1 ml), factor Xa (0.1 ml), plasma (diluted, 0.1 ml), and chromogenic substrate (0.1 ml).

A calibration curve using heparin in concentrations ranging from 0.05 to 0.8 IU/ml plasma was drawn on a linear graph, and a straight line was obtained for heparin levels between 0.1 and 0.7 IU/ml. The heparin levels in the test samples were read from the heparin standard curve, thereby converting optical density into units of heparin per milliliter.

Activated partial thromboplastin time was performed in plasma by the method of Proctor and Rapaport in a coagulation profiler model CP-7A (BioData, Hatboro, Penn.) using micronized silica and platelet Factor 3 as reagents (General Diagnostics, DIZ Warner-Lambert Co., Morris, N.J.).

Other Variables

Total white blood cell (WBC) count was determined with a Coulter counter (model ZF, Coulter Electronics, Hialeah, Fla.), and hematocrit level was determined with an Adams Autocrit centrifuge (Clay Adams, Parsippany, N.J.). Arterial blood gas tensions and pH were measured with a polarograph (model 175, Corning Medical and Scientific, Medfield, Mass.).

Experimental Protocols

To avoid studying infected or sick animals, we considered as exclusion criteria a Ppa greater than 20 mm Hg, a temperature above 40.2°C, or a WBC concentration lower than 4,000/mm³ or greater than 12,000/mm³.

A timed protocol was used to induce the heparin-protamine reaction. Baseline measurements were obtained at –6 minutes and then heparin (unfractionated heparin or LMWH) was administered intravenously as a bolus (–5 minutes). After another set of measurements was obtained at –1 minute, either protamine sulfate or polybrene was administered as an intravenous bolus at 0 minutes. All sheep were then monitored for 20 minutes. All hemodynamic variables, WBC, and TxB₂ plasma levels were measured at each sampling point (–6, –1, 1, 2, 3, 5, 10, and 20 minutes). We previously noticed that the nadir of PaO₂ occurs between 2 and 3 minutes, and arterial blood for gas analysis was withdrawn only at –1, 2, 3, and 10 minutes. Hematocrit level was measured at –6 and 20 minutes. Samples for anticoagulation tests were taken at –6, –1 and 5 minutes.

In separate studies of five sheep, we found that three heparin-protamine challenges can be repeated at 90-minute intervals without altering either the peak level of thromboxane release into plasma or the pulmonary hypertensive response (Figure 1). Fourteen sheep were monitored for the present study. Each animal underwent a maximum of three challenges on the same day with a 2-hour recovery period between reactions. Each sheep underwent a control heparin-protamine reaction; the effects of 200 units/kg unfractionated beef lung heparin (approximate molecular weight, 17,000 daltons; Lyphomed, Melrose Park, Ill.) was reversed by 2 mg/kg protamine sulfate (approximate molecular weight, 4,000 daltons; Eli Lilly, Indianapolis, Ind.).

Polybrene (approximate molecular weight, 5,000 daltons; Aldrich Chemical Co., Milwaukee, Wis.) was drawn as aliquots before each study into plastic test tubes to avoid surface adsorption of polybrene to glass. After reviewing previous reports, we chose a dose of 0.7 mg polybrene to neutralize 1 mg unfractionated heparin. Doses of LMWH (enoxaparine; approximate molecular weight 4,500 daltons; Pharmak, Gennevilliers, France) and appropriate doses of protamine sulfate to neutralize this LMWH were chosen in accordance with the amounts administered to sheep and patients in previous studies. The amount of polybrene necessary to reverse LMWH anticoagulation was calculated assuming polybrene has 70% of the neutralizing potency of protamine.

Three treatment groups were used in the study. Group 1, heparin-polybrene (five sheep): the effects of unfractionated heparin (2 mg/kg) were reversed by polybrene (1.4 mg/kg). Group 2, LMWH-protamine (five sheep): the effects of LMWH (1 mg/kg) were reversed by protamine sulfate (1.5 mg/kg). Group 3, LMWH-polybrene (nine sheep): the effects of LMWH (1 mg/kg) were reversed by polybrene (1.05 mg/kg). In addition, to determine whether higher doses may change the results, we induced challenges after higher doses (the effects of 3 mg/kg LMWH were reversed by 3.15 mg/kg polybrene, four sheep).

The results obtained with each treatment were compared with those of the control heparin-protamine challenges for the same animals (the effects of 200 units/kg unfractionated heparin were reversed by 2 mg/kg protamine). The order of experimental treatments and control reactions was randomized.

Statistical Analysis

All data are expressed as mean±SEM. Changes of variables over time within and between study groups were evaluated with multivariate analysis of variance for repeated measures as implemented in the SAS statistical programs (Version 6.0, SAS Institute Inc., Cary, N.C.). Planned contrasts of the results in the control group with the results in the treatment group were performed with an F test and two-tailed t tests. Baseline values of each variable in both groups were compared using a one-way analysis of variance and a t test. Planned comparisons were tested individually with significance declared at an alpha level of 0.05.
The variable TxB₂ was logarithmically transformed before analysis to maintain the assumption of homogeneity of variance. Association between variables was determined by linear regression analysis, and a correlation coefficient was calculated between selected biochemical and hemodynamic variables.

**Results**

**Group 1, Polybrene Reversal of Unfractionated Heparin**

Polybrene administration induced marked acute pulmonary hypertension (Figure 2) (Ppa increased from 16.2±0.7 of baseline to 49.6±4.1 mm Hg at 1 minute, *p*=0.0003), which is similar to that observed after protamine neutralization of unfractionated heparin (control heparin-protamine reaction: from 19.4±0.4 to 50±5.2 mm Hg at 1 minute, *p*=0.001). Pw, Pv, Psa, cardiac output, and systemic vascular resistance did not significantly differ between these groups (Table 1). The threefold increase of pulmonary vascular resistance was similar after either polybrene or protamine reversal of unfractionated heparin anticoagulation. Arterial plasma TxB₂ levels increased significantly from baseline values after either polybrene or protamine reversal of unfractionated heparin (Figure 3). We measured a significant reduction of PaO₂ after either protamine (from 99±4 at −1 minute to 73±11 mm Hg at 2 minutes after protamine) or polybrene (Figure 4). There was no change of PaCO₂ in either heparin-protamine reactions (43±6 at −1 minute to 42±4 mm Hg 2 minutes after protamine) or heparin-polybrene reactions (39±6 at −1 minute to 41±6 mm Hg 2 minutes after polybrene). Similarly, pHa remained stable in both groups (7.40±0.02 at −1 minute to 7.39±0.03, 2 minutes after protamine; 7.44±0.01 at −1 minute to 7.43±0.02, 2 minutes after polybrene). The nadir of WBC concentration occurred at 2 minutes (*p*<0.05). The lowest value differs from baseline in both heparin-polybrene and control reactions (Figure 5). In all sheep, the ACT increased to more than 300 seconds at −1 minute (4 minutes after heparin injection) and returned to baseline values 5 minutes after either polybrene or protamine administration (Table 2).

**Group 2, Protamine Sulfate Reversal of LMWH**

Protamine reversal of both LMWH and unfractionated heparin caused similarly significant increases of Ppa (Figure 2), Pw, and pulmonary vascular resistance (Table 1). Hemodynamic variables did not differ between LMWH-protamine reactions and control heparin-protamine reactions (Table 1). The elevated plasma TxB₂ levels in control challenges (*p*<0.05 compared with baseline at 1–3 minutes) did not differ from the elevated plasma TxB₂ levels in LMWH-protamine reactions from 1–3 min-
utes (Figure 3). Protamine neutralization of LMWH induced a 23% decrease of PaO₂ (p=0.02 compared with baseline at 2 minutes) that is similar to the decrease in control reactions (Figure 4). There was no difference of PaCO₂ (at 2 minutes: 44±2 mm Hg in control challenges compared with 46±5 mm Hg in LMWH-protamine challenges) or pHa (at 2 minutes: 7.40±0.02 in control challenges compared with 7.39±0.01 in LMWH-protamine challenges). A similar degree of leukopenia occurred after either type of heparin was neutralized with protamine (Figure 5).

ACT in control challenges increased to more than 300 seconds at -1 minute in all animals; 5 minutes after protamine injection, the ACT was 124±5 seconds (+5 minutes) and did not differ from the baseline value (135±4 seconds at -6 minutes). Four minutes after LMWH administration (-1 minute), the APTT increased significantly from baseline but remained much lower than after anticoagulation with unfractionated heparin (50±4 compared with 127±14 seconds, respectively, p<0.001) (Table 2). After either type of heparin, protamine administration completely reversed the APTT and ACT to baseline levels. Anti-Xa activity increased significantly after both types of heparin anticoagulation and returned promptly to baseline after protamine administration.

**Group 3, Polybrene Reversal of LMWH**

Neutralization of LMWH with polybrene (1 mg/kg) did not produce a significant change of Ppa (from 17.6±0.7 at baseline to 22.6±3.6 mm Hg at 1 minute after polybrene), whereas severe acute pulmonary hypertension occurred in control heparin-protamine reactions (from 17.1±0.8 to 47.9±4.2 mm Hg at 1 minute, p<0.0001). Ppa significantly differed between both groups for more than 10 minutes (p<0.005 at 1 minute, Figure 2). Increases of pulmonary vascular resistance (from 190±17 at baseline to 621±91 dynes·sec/cm² at 1 minute in controls, p<0.001, compared with 193±10 at baseline to 223±37 dynes·sec/cm² at 1 minute after polybrene reversal of LMWH, p=NS) between control reactions and LMWH-polybrene reactions were highly significant (p<0.005 from 1 to 10 minutes). Increases of Pw (p<0.001 at 1 minute) and Pv (p<0.01 at 1 minute) occurred only in control reactions (Table 1). Plasma TxB₂ levels increased significantly from baseline values in control heparin-protamine reactions but were not affected by polybrene reversal of LMWH (p<0.01 TxB₂ values differ between controls and LMWH-polybrene reactions during the 3 minutes after heparin antagonist administration, Figure 3). Consistent hypoxemia occurred when sheep received unfractionated heparin and protamine sulfate (ΔPao₂ at 2 minutes, -26 mm Hg, p<0.01) but did not occur after receiving LMWH and polybrene (ΔPao₂ at 2 minutes, -2 mm Hg, p=NS). Thus, PaO₂ differed significantly between LMWH-polybrene and control reactions for 10 minutes (Figure 4). There was no significant change of PaCO₂ (from 42±5 at baseline to 40±4 mm Hg at 2 minutes) or pHa (7.40±0.02 at baseline to 7.43±0.03 at 2 minutes) in control reactions. Similarly, PaCO₂ (from 43±2 at baseline to 44±3 mm Hg at 2 minutes) and pHa (7.42±0.01 at baseline to 7.39±0.01 at 2 minutes) remained stable in LMWH-polybrene reactions. LMWH-polybrene and control reactions induced a similar and significant transient leukopenia (Figure 5).

Marked anticoagulation was evident in controls and LMWH-polybrene reactions with a major increase of ACT, APTT, and anti-Xa activity 4 minutes after unfractionated heparin or LMWH administration (-1 minute). Heparin antagonists completely reversed the ACT and APTT in both
TABLE 1. Hemodynamic Data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pw (mm Hg)</th>
<th>Pp (mm Hg)</th>
<th>Psa (mm Hg)</th>
<th>CO (l/min)</th>
<th>PVR (dynes ⋅ sec/cm²)</th>
<th>SVR (dynes ⋅ sec/cm²)</th>
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</thead>
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<tr>
<td></td>
<td>−6 min</td>
<td>1 min</td>
<td>−6 min</td>
<td>1 min</td>
<td>−6 min</td>
<td>1 min</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Heparin-protamine</td>
<td>7.8±0.8</td>
<td>20.4±1.8*</td>
<td>2.0±0.4</td>
<td>5.4±1.4</td>
<td>97±7</td>
<td>119±13</td>
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<td>Heparin-polybrene</td>
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<td>20.2±4.2*</td>
<td>2.2±0.9</td>
<td>4.2±2.9</td>
<td>90±5</td>
<td>93±12</td>
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<td>Group 2</td>
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<td></td>
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<tr>
<td>Heparin-protamine</td>
<td>7.6±0.7</td>
<td>20.8±2.0*</td>
<td>2.0±0.4</td>
<td>5.2±1.5</td>
<td>95±7</td>
<td>106±8*</td>
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<td>LMWH-protamine</td>
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<td>18.2±0.9*</td>
<td>1.6±0.4</td>
<td>3.2±0.9</td>
<td>85±6</td>
<td>91±5</td>
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<td>Group 3</td>
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<tr>
<td>Heparin-protamine</td>
<td>6.5±0.5</td>
<td>17.1±2.0*</td>
<td>2.3±0.4</td>
<td>4.9±0.7*</td>
<td>89±3</td>
<td>107±9*</td>
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<tr>
<td>LMWH-polybrene (1 mg/kg)</td>
<td>7.3±0.3</td>
<td>10.2±1.9†</td>
<td>3.1±0.7</td>
<td>4.1±0.9</td>
<td>96±5</td>
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<tr>
<td>Heparin-protamine</td>
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<td>15.8±3.9*</td>
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<td>4.3±1.1</td>
<td>91±6</td>
<td>104±10</td>
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<td>LMWH-polybrene (3 mg/kg)</td>
<td>8.3±0.2</td>
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<td>1.8±0.2</td>
<td>1.8±0.5</td>
<td>98±5</td>
<td>101±10</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
Pw, pulmonary artery occlusion pressure; Pp, central venous pressure; Psa: systemic arterial blood pressure; LMWH, low molecular weight heparin; CO, cardiac output; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance.

*Value at 1 minute (peak of the reaction) significantly differs from −6 minute value (baseline, before giving heparin) within the same group; †Value at 1 minute in treatment group significantly differs from value in control heparin-protamine group at the same time period.

groups but incompletely reversed the effect of LMWH on anti-Xa activity at 5 minutes (Table 2).

When larger doses of LMWH were antagonized by polybrene (3 mg/kg, n=4), they produced the same benign effects that were measured at 1 mg/kg of each drug: Ppa increased from 18.3±0.9 at baseline to 25±1.6 mm Hg in LMWH-polybrene reactions compared with a severe increase in control reactions from 17.5±1.4 at baseline to 40.7±7.6 mm Hg (p<0.05 between groups). There was no significant change of Pw, Pp, and Psa when neutralizing high doses of LMWH with polybrene (3 mg/kg) (Table 1). Plasma thromboxane levels increased significantly in control reactions (from 0.07±0.01 at baseline to 0.57±0.26 ng/ml at 1 minute, p<0.05) but not in high dose LMWH-polybrene reactions (from 0.12±0.07 at baseline to 0.07±0.02 ng/ml at 1 minute, p=NS), and these groups significantly differed at 1 minute (p<0.05). The individual changes of TxB₂ are plotted against the changes of Ppa for control challenges and LMWH-polybrene challenges (Figure 6). A relation between the increase of TxB₂ and Ppa is evident (r=0.86, p<0.0001). Paco₂ decreased in control reactions (from 92±3 at baseline to 74±13 mm Hg at 2 minutes) but did not change after high dose LMWH-polybrene reactions (from 87±6 at baseline to 85±8 mm Hg at 2 minutes). A similar transient leukopenia occurred after both control reactions and polybrene reversal of 3 mg/kg LMWH (Figure 5). We measured a significant increase of anti-Xa activity, APTT, and ACT after the infusion of 3 mg/kg LMWH. Polybrene infusion partially reversed plasma anti-Xa activity but returned APTT and ACT levels to baseline (Table 2).

Discussion

The most important finding of our study is that reversal of LMWH anticoagulation by polybrene prevents the thromboxane release, acute pulmonary vasoconstriction, and hypoxemia associated with heparin neutralization in sheep (Figures 2−4). Furthermore, even extremely large doses of this combination were not associated with an untoward physiological response. Although this adverse cardiopulmonary reaction has been documented primarily with the conventional unfractionated heparin and protamine routinely used clinically, we also demonstrated that LMWH reversal by protamine and unfractionated heparin neutralization by polybrene are associated with adverse pulmonary hemodynamic effects. Thus, only the combination of LMWH and polybrene appears to avoid this adverse response. The prevention of this hemodynamic reaction occurred at doses of LMWH similar to those previously given for cardiopulmonary bypass in sheep (2 mg/kg enoxaparine).11 However like the other combinations, LMWH-polybrene complexes induced a transient leukopenia (Figure 5).

Acute and serious pulmonary hypertension due to the intravascular combination of polycations with polyanions in animal models has been previously studied using protamine sulfate to reverse unfractionated heparin anticoagulation.4,7 Fiser et al23 demonstrated in awake pigs that protamine reversal of porcine mucosal heparin, as well as protamine reversal of bovine lung heparin, induced severe pulmonary hypertension. Our results in awake sheep demonstrate that despite a major reduction of the size of the heparin molecule (approximately 4,500 daltons for LMWH compared with 17,000 daltons for unfractionated heparin), protamine reversal of LMWH caused an acute thromboxane release into arterial plasma with pulmonary vasoconstriction and hypoxemia. A similar increase of plasma TxB₂ levels has
been reported after neutralization of another type of LMWH by protamine in dogs.\textsuperscript{24}

Replacing protamine with the synthetic polycationic molecule polybrene has been suggested for eliminating adverse hemodynamic effects associated with the neutralization of protamine.\textsuperscript{5,15,17,25} In the awake sheep, polybrene reversal of unfractionated heparin induced a dramatic rise of Ppa, Pw, and pulmonary vascular resistance associated with elevated arterial plasma TxB\textsubscript{2} levels (Figures 2 and 3). Thus, polybrene does not obviate this nonallergic adverse neutralization reaction when anticoagulation is obtained with unfractionated heparin.

The increases of Ppa and plasma concentrations of TxB\textsubscript{2} in control heparin-protamine reactions and LMWH-protamine reactions were correlated (r=0.86, p<0.0001, Figure 6). A similar association (r=0.83) was previously reported in awake sheep given the same heparin-protamine protocol.\textsuperscript{4} However, we found marked individual differences in the levels of plasma thromboxane released after a standard heparin-protamine challenge. The level of plasma thromboxane may not always reflect the amount of thromboxane generated locally. Moreover, a limited increase of plasma thromboxane is sufficient to provoke acute pulmonary vasoconstriction. When sheep were challenged with heparin and protamine, they always developed an increase of plasma TxB\textsubscript{2} level greater than 300 pg/ml that was associated with
reaction because acute pulmonary vasoconstriction is inhibited by pretreating animals with either a cyclo-oxygenase inhibitor, a thromboxane synthetase inhibitor, or a specific thromboxone receptor blocker.\(^5\)\(^,\)\(^26\)\(^,\)\(^27\) The Pw increase that is concomitant with the rise of Ppa is believed to be due to diffuse arterial and venular vasoconstriction downstream from the tip of the catheter because neither left atrial nor left ventricular pressure is increased during this reaction in sheep.\(^4\)\(^,\)\(^7\) Thromboxone A\(_2\) is also a potent constrictor of bronchial smooth muscle, and bronchoconstriction may contribute to the hypoxemia of this reaction.\(^28\) In several sheep, the severe pulmonary vasoconstriction was associated with a decreased cardiac output, but mean cardiac output changes were not significantly different. We believe the thermodilution measurement technique is too slow to reliably measure the transient acute reduction of cardiac output in this reaction because of the time lag between measurements. A transiently decreased cardiac output can also contribute to the hypoxemia occurring during the acute phase of the heparin-protamine reaction.

LMWH, as well as unfractionated heparin, provided adequate anticoagulation as assessed by the high levels of anti-Xa activity. LMWH increased the APTT activity less than unfractionated heparin (Table 2). This difference in APTT activity after low and high molecular weight heparins has been reported by others.\(^10\)\(^,\)\(^11\)\(^,\)\(^29\) The anticoagulant effects of LMWH on the ACT and APTT were completely reversed at 5 minutes by protamine or polybrene administration, suggesting that neutralizing complexes are formed between LMWH and protamine or polybrene. Partial polybrene reversal of LMWH anti-Xa activity at 5 minutes has also been described after protamine reversal of LMWH. Several investigators have reported that protamine antagonizes the LMWH inhibition of factor Xa by 40–80\%,\(^13\)\(^,\)\(^29\)–\(^31\) The apparently complete reversal of LMWH-induced anti-Xa activity that we measured after protamine could be due to the lesser level of anticoagulation produced in these sheep (Table 2). The reason for the differential effect of heparin antagonists with regard to the anti-Xa activity of unfractionated heparin and LMWH remains uncertain. Differences of binding affinities,\(^31\) an effect of the size of the molecule,\(^13\) or the release of endogenous heparan sulfate\(^32\) may be responsible. Thus, our results demonstrate that LMWH provides effective anticoagulation and can be neutralized by polybrene as well as by protamine. Polybrene has greater neutralizing potency because it can be used at a 0.7 dose ratio of polybrene to protamine.

The in vitro interaction between polyanions and polycations activates the classic complement pathway.\(^33\) Our prior studies have demonstrated that in vivo neutralization of heparin anticoagulation by intravenous protamine sulfate administration increases arterial plasma anaphylatoxin C3a levels in sheep as well as in patients.\(^3\)\(^,\)\(^4\) In addition, patients who respond to protamine neutralization of heparin

**Figure 5.** Plots of white blood cell concentrations during polyanion-polycation reactions. There are no differences between groups. Data are mean±SEM. WBC, white blood cell concentration; LMWH, low molecular weight heparin.

acute pulmonary vasoconstriction. The same sheep were challenged 13 times with LMWH and polybrene at either 1 or 3 mg/kg, and all but one had no noteworthy increase of either TxB\(_2\) or Ppa (Figure 6). We cannot explain this single reaction to LMWH and polybrene. On average, the incidence and severity of the reaction were dramatically reduced by substituting polybrene and LMWH for protamine and heparin, respectively.

Previous studies demonstrated that thromboxane is the major mediator of the heparin-protamine

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TABLE 2. Anticoagulation Data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ACT (sec)</th>
<th>APTT (sec)</th>
<th>Anti-Xa activity (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-6 min</td>
<td>-1 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin-protamine</td>
<td>135±5</td>
<td>&gt;300*</td>
<td>121±6*</td>
</tr>
<tr>
<td>Heparin-polybrene</td>
<td>122±16</td>
<td>&gt;300*</td>
<td>114±7</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin-protamine</td>
<td>135±4</td>
<td>&gt;300*</td>
<td>125±5</td>
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<tr>
<td>LMWH-protamine</td>
<td>132±8</td>
<td>177±20†</td>
<td>119±9</td>
</tr>
<tr>
<td>Group 3</td>
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<td></td>
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<tr>
<td>Heparin-protamine</td>
<td>110±10</td>
<td>&gt;300*</td>
<td>104±8</td>
</tr>
<tr>
<td>LMWH-polybrene (1 mg/kg)</td>
<td>110±7</td>
<td>152±14†</td>
<td>118±5</td>
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<tr>
<td>Heparin-protamine</td>
<td>108±16</td>
<td>&gt;300*</td>
<td>103±15</td>
</tr>
<tr>
<td>LMWH-polybrene (3 mg/kg)</td>
<td>121±7</td>
<td>217±40†</td>
<td>130±12</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
ACT, activated clotting time; APTT, activated partial thromboplastin time. LMWH, low molecular weight heparin.

*Value at 1 minute (peak of the reaction) significantly differs from -6 minute value (baseline, before giving heparin) within the same group; †value at 1 minute in treatment group significantly differs from value in control heparin-protamine group at the same time period.

with severe pulmonary hypertension demonstrate high plasma levels of C5a and TxB2, whereas the plasma levels of these mediators do not change in nonreacting patients.3 It remains uncertain whether complement activation stimulates thromboxane release or is merely an accompanying event during the neutralization reaction. The C5a fragment, a potent leuкоaggregating and leuкоactivating anaphylatoxin, is probably responsible for the consistent leukopenia measured in all the polyanion-polycation reactions (Figure 5).34 This was partially confirmed by Fehr and Rohr,35 who demonstrated that complement depletion of rabbits produced by administration of cobra venom factor prevented leukopenia during heparin-protamine challenges. Leukopenia is universal when reversing heparin activity with heparin antagonists; however, we found that leukopenia was not necessarily associated with an adverse hemodynamic response in sheep after polybrene reversal of LMWH. Morel et al3 reported leukopenia in patients who exhibited neither pulmonary hypertension nor thromboxane release after protamine reversal of heparin anticoagulation. Thus, complement activation and leukosequestration may be sensitive indicators of polyanion-polycation complex formation but may not be involved in triggering thromboxane production during the heparin-protamine reaction.

The precise cellular source of thromboxane release is unknown. We recently demonstrated that sheep platelets were not involved because both acute pulmonary hypertension and thromboxane release occurred with a similar magnitude in thrombocytopenic sheep (<10,000 platelets/mm3) when challenged with unfractionated heparin and protamine.22 Pulmonary hypertension has been reported after infusion of heparin-protamine complexes into isolated cat lungs perfused with an acellular dextran containing perfusate suggesting that blood elements may not be involved and that resident lung cells may be responsible for the release of thromboxane.36 The onset of pulmonary artery hypertension within the first minute after protamine injection suggests first-
pass activation of lung cells. Endothelial cells or the recently described pulmonary intravascular macrophages,57,38 which are abundant in certain species such as sheep and pig may be the source of thromboxane. Pulmonary intravascular macrophages were recently described in the human pulmonary circulation.39

A link between polyanion-polycation complexes and the stimulation of arachidonate metabolism in lung cells is possible. Protamine neutralization of low and high molecular weight heparins results from the interaction of opposite charges rather than by recognition of specific binding sites. In addition, size and solubility differences have been reported between various types of polyanion-polycation complexes.41 Such differences in physical structure and solubility may make the LMWH-polybrene complex less stimulatory to mediator-producing cells compared with other polyanion-polycation complexes. Furthermore, LMWH has a weaker affinity than unfractionated heparin for endothelial cells,42 suggesting reduced linkage of LMWH-polybrene complexes to vascular lining cells.

Our favorable results with polybrene reversal of LMWH indicate that this combination in humans may prevent the sporadic, but severe, adverse reaction occurring in heparinized patients after the administration of protamine. Clinical trials with various types of LMWH have reported its effectiveness for antithrombotic therapy,53,44 salutary effects in patients with heparin-induced thrombocytopenia,45 and possibly a lower risk of hemorrhage.10,44,46 Recent reports on the use of LMWH for anticoagulation during cardiopulmonary bypass in animal models10,11 confirms its effective antithrombotic activity and significantly lower postoperative blood loss.10 An early clinical trial conducted with enoxaparine in patients undergoing cardiopulmonary bypass demonstrated a lack of clotting in the circuit but excessive blood loss, probably because of an LMWH overdose (LMWH was initially given in large doses for safety).13 The problem appeared accentuated by the long biological half-life of LMWH.13,44 Subsequent studies must examine such problems before enoxaparine is accepted for use during cardiopulmonary bypass in humans.

Although polybrene is more potent at neutralizing heparin than is protamine, side effects can occur with both polycationic drugs (hemodynamic, hematologic, renal).15,25,47,48 Polybrene is not routinely used as a heparin antagonist, but its beneficial effect in patients with protamine hypersensitivity has been demonstrated.17,25 Polybrene has been successfully used in patients after cardiopulmonary bypass to neutralize heparin anticoagulation.15,16 Thus, both LMWH and polybrene have proven their separate efficacies for use during cardiopulmonary bypass. The absence of predictive factors to identify high-risk patients for the heparin-protamine reaction and the absence of a safe preventive treatment lead us to conclude that polybrene reversal of LMWH may be a practical alternative to protamine reversal of unfractionated heparin.

In conclusion, we have demonstrated in sheep that several polyanion-polycation interactions induce severe, acute pulmonary artery hypertension associated with the transient release of the potent vasoconstrictor thromboxane. We learned that anticoagulation with LMWH and reversal with polybrene does not increase plasma thromboxane levels nor cause severe pulmonary vasoconstriction. Further studies are now needed to confirm the usefulness of this technique of anticoagulation and neutralization in patients to prevent the occasional life-threatening cardiopulmonary reactions caused by protamine neutralization of heparin anticoagulation.

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\textbf{KEY WORDS} \ heparin \ hexadimethrine bromide \ protamine \ polybrene \ thromboxane
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