Acute Pulmonary Vasoconstriction and Thromboxane Release During Protamine Reversal of Heparin Anticoagulation in Awake Sheep
Evidence for the Role of Reactive Oxygen Metabolites Following Nonimmunological Complement Activation

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When protamine (2 mg/kg) was injected intravenously into awake sheep 5 minutes after infusing heparin (200 units/kg), there was transient diffuse pulmonary vasoconstriction with mean pulmonary arterial pressure increasing from 18.0 ± 0.7 to 43.8 ± 2.7 mm Hg at 1 minute (x ± SEM; n = 10). In addition, there was profound leukopenia (36.9 ± 7.7% of baseline values at 2 minutes) with transpulmonary leukocyte sequestration and transiently elevated plasma concentrations of C3a (from 420 ± 146 to 1,599 ± 249 ng/ml; n = 3, p < 0.01) and thromboxane B2 (from 0.30 ± 0.05 to 6.3 ± 2.8 ng/ml; n = 10, p < 0.0001), without significant increases of plasma 6-keto-prostaglandin F1α, prostaglandin F2α, leukotrienes, or histamine. Intravenous injection of protamine alone produced no hemodynamic effects and did not increase plasma levels of vasoconstrictor eicosanoids. Intravenous pretreatment with either a cyclooxygenase inhibitor or a hydrogen peroxide scavenger (dimethylthiourea) blocked both the increases of thromboxane levels and the pulmonary vasoconstriction.

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Neutralization of heparin anticoagulation with protamine sulfate in humans is occasionally accompanied by severe acute pulmonary vasoconstriction, bronchoconstriction, and systemic hypotension. These reactions occur sporadically and the underlying biochemical mechanisms leading to this syndrome remain unclear. Apart from rare anaphylactic immunoglobulin G (IgG)-mediated reactions to protamine, recent studies suggest that a nonimmunological pathway via complement activation and eicosanoid generation, particularly thromboxane, may be responsible for many of the acute reactions observed during protamine reversal of heparin anticoagulation.

In vitro studies have demonstrated that, similar to antibody-antigen binding, the interaction of polyanions (e.g., heparin) and polycations (e.g., protamine) will activate the complement system via the classical pathway without involving immunoglobulins. Recent clinical studies following cardiopulmonary bypass reported that the plasma levels of C3a and C4a are increased immediately after protamine administration for heparin neutralization, thereby confirming in humans the occurrence of nonimmunological complement activation by heparin-protein interaction.

Infusing zymosan-activated complement into animals causes an acute profound leukopenia with pulmonary white blood cell (WBC) sequestration and the release of thromboxane by cells within the lung, resulting in transient, severe pulmonary hypertension and hypoxemia. Furthermore, a number of authors have noted an association between complement activation, pulmonary sequestration, and activation of leukocytes with the release of toxic oxygen products. Moreover, chemically generated O2 metabolites have been shown to stimulate thromboxane A2 (TXA2) production and produce vasoconstriction in isolated perfused rabbit lungs. These recent studies led us to examine if protamine neutralization of heparin would cause a similar cascade of reactions leading to thromboxane release and pulmonary vasoconstriction in an intact sheep model.

Materials and Methods

Sheep Preparation
After endotracheal intubation and mechanical ventilation during halothane anesthesia, adult Suffolk
sheep weighing 25–35 kg were surgically instrumented using sterile techniques. Both femoral arteries were cannulated with polyvinyl chloride catheters advanced to the midthoracic aorta for intermittent blood sampling and continuous systemic blood pressure (Pa) monitoring. The left femoral vein was cannulated for fluid and drug administration. Two Swan-Ganz flow-directed thermodilution catheters (model 93A-131H-7F, Edwards Laboratories, Santa Ana, California) were inserted into the right internal jugular vein and advanced to the pulmonary artery for measurement of pulmonary artery (Ppa) and occlusion pressure (Pw) as well as for mixed venous blood sampling. Central venous pressure (Pv) was measured via a side hole of this catheter situated in the right atrium. The catheters were filled with normal saline containing heparin (200 units/ml). Penicillin G (10^4 units) was injected intramuscularly once each day.

**Plasma Dimethylsulfoxide and Dimethylthiourea**

Plasmas Dimethylsulfoxide and Dimethylthiourea (DMSO) and dimethylthiourea (DMTU) were ascribed by gas chromatography as previously described.15 Proteins were precipitated with perchloric acid before injection.

**Plasma Levels**

Plasma levels of sheep C3a (des Arg) antigen were determined by radioimmunoassay methods that were specifically developed to detect the ovine complement-derived anaphylatoxin. Briefly, sheep C3a des Arg was purified to homogeneity from zymosan-activated sheep serum by methods that were similar to those previously employed to purify human C3a.20 Sheep C3a des Arg differed from the human polypeptide in that it was considerably less cationic; that is, it eluted from the SP-Sephadex column with application of 0.2 M ammonium formate at pH 5.0 rather than with 0.7 M salt. Additionally, final purification of the ovine anaphylatoxin was achieved by HPLC performed on a Pharmacia Mono S column as previously described.21

Rabbit anti-sheep C3a antisera were obtained after initial immunization of New Zealand red rabbits with 50 µg purified sheep C3a des Arg emulsified with complete Freund's adjuvant and repetitive boosting with 50 µg antigen in incomplete adjuvant. Radioimmunoassay procedures similar to those developed for the human anaphylatoxins22 and now available commercially (Upjohn Diagnostics) were utilized to quantitate circulating levels of sheep C3a antigen.

**Plasma Histamine Levels and Additional Measurements**

Plasma histamine levels were determined with a radioenzymatic assay.23 Total WBC count was determined with a Coulter cell counter (model Z3, Coulter Electronics, Hialeah, Florida), platelet concentration with phase microscopy, and pH and arterial blood gas tensions with a polarograph (model 175, Corning Medical and Scientific, Medfield, Massachusetts). Activated clotting time (ACT) of whole blood was measured by a Hemocron 400D system (International Technidyne, Edison, New Jersey).24

**Experimental Protocols**

Animals were allowed 24 hours to recover from anesthesia and cannulation. Catheters were aspirated before each study and then continuously flushed (2 ml/hr) with saline solution without heparin on the day of the experiment. Studies were carried out on awake sheep standing in cages with a loosely fitting sling placed under the animal to prevent it from sitting during the study period; the sheep had free access to food and water. An intravenous infusion of a Ringer's lactate solution was given at 10 ml/kg/hr throughout each study period to compensate for intravascular volume loss by blood sampling. The animals were allowed to acclimate to the experimental environment for 2 hours. Repeated determinations of the ACT of whole blood were performed to ensure the absence of residual anticoagulant effect from the heparin solution placed in the intravascular catheters between the experimental sessions. If the ACT exceeded 160 seconds, additional time was allowed for the ACT to return to normal (normal values for sheep: 134 ± 19 seconds, range 89–160).
In a preliminary study in sheep, we observed that the pulmonary vascular response following a protamine sulfate injection given over 1 hour after systemic heparinization was highly variable. The degree of pulmonary vasoconstriction appeared proportional to the plasma level of heparin, which was neutralized by protamine. To produce a consistent animal model of the interaction of injected protamine with circulating heparin, Stefaniszyn and Fiser intravenously administered protamine (3 mg/kg) 15 minutes after infusing heparin (300 units/kg) in pigs and were able to reliably induce a transient but consistent pulmonary hypertension due to an elevated pulmonary vascular resistance. We also employed a timed protocol to induce this reaction and investigated its mechanism in the awake sheep, an animal commonly used for studies of the pulmonary circulation allowing chronic cannulation.

A total of 12 sheep were studied. Initially, four sheep were investigated during protamine reversal of heparin without drug pretreatment to confirm the consistency of the transient pulmonary and biological reaction produced during this interaction. Two animals were studied on 3 days and showed an identical pattern of pulmonary vasoconstriction on each day. Therefore, we decided to investigate the effect of various pretreatments on six sheep by repeated experiments allowing 48 hours between treatments. None of our sheep ever developed acute respiratory failure.

On each experimental day, the sheep received 200 units/kg of bovine lung heparin (Lyphomed, Melrose Park, Illinois) as an intravenous bolus through the right atrial catheter. Five minutes later, protamine sulfate (Eli Lilly, Indianapolis, Indiana) was infused through the right atrial catheter in a dose of 2 mg/kg over a period of 10 seconds. The sheep underwent the following treatment sessions: 1) no pretreatment (controls, n = 6); 2) pretreatment with a cyclooxygenase inhibitor (indomethacin, 10 mg/kg, n = 3); 3) pretreatment with a thromboxane synthetase inhibitor (UK 38-458, 10 mg/kg, n = 5); 4) pretreatment with an -OH scavenger (DMSO, 1 g/kg, n = 4); 5) pretreatment with a H2O2 scavenger (DMTU, 0.5 g/kg, n = 3); and 6) pretreatment with DMTU at 1.0 g/kg, n = 3.

To determine the effect of protamine administration alone, in four sheep protamine (2 mg/kg) was administered after a normal saline injection (no heparin). Two additional sheep were studied twice after pretreatment with DMTU 0.5 or 1.0 g/kg and received only a protamine injection (no heparin). The order of the experimental treatments was randomized with the exception of DMTU, which was always given last because it produced lethargy and anorexia persisting for several days. For this reason we studied three sheep at 0.5 g/kg DMTU, a dose that produced less lethargy. Since we found no differences in any of the responses at both treatment doses, we pooled the data of these two treatment groups. Many of the sheep did not complete the full study protocol due to clotting of cannulae or infection. We considered sheep with a core temperature above 40° C, a blood leukocyte concentration above 12,000/mm³, or a mean Ppa above 25 mm Hg infected and unsuitable for study.

All pretreatment drugs were infused intravenously over 30 minutes from 45 to 15 minutes before protamine injection. Indomethacin (Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania) was dissolved in normal saline (1 mg/ml) and neutralized to pH 7.4 with sodium bicarbonate; UK 38-458 (3-(1H-imidazol-1-yl methyl)-2-methyl-1H-indole-1-propanoic acid, Pfizer, Inc., Groton, Connecticut) was dissolved in normal saline (1 mg/ml); DMSO (Sigma Chemical, St. Louis, Missouri) was infused as a 15% solution in normal saline; and DMTU (Alfa Products, Danvers, Massachusetts) was dissolved in normal saline at a concentration of 100 mg/ml.

An arterial blood sample was withdrawn 1 hour before protamine injection. Seven milliliters of arterial blood and 4 ml of venous blood were withdrawn simultaneously from the femoral arterial and the pulmonary arterial catheters at the following times: immediately before the administration of heparin and protamine, and thereafter at 1, 2, 3, 5, 10, 15, and 20 minutes after protamine injection. Cardiac output and Pw were measured at similar intervals. Hematocrit, WBC count, and arterial blood gas tensions were also measured. Blood was collected in plastic syringes and transferred to ice-cold heparinized glass test tubes to measure histamine, and to glass test tubes containing 0.05 ml of 15% EDTA and 100 μg of indomethacin to assay eicosanoids and C3a. The samples were immediately centrifuged at 2,000g for 10 minutes at 4° C. Plasma was aspirated and stored in polypropylene tubes at -70° C for later analysis.

To determine whether the oxygen metabolite scavengers exerted their effects via inhibition of cyclooxygenase metabolism, we measured the pulmonary vascular response and plasma TXB2 levels during a brief infusion of arachidonic acid in five sheep that had received DMTU (two given 1.0 g/kg and three given 0.5 g/kg). Arachidonic acid (Sigma) was freshly dissolved in 300 μl 90% ethanol, diluted in sterile saline to a final concentration of 150 μg/ml, and infused intravenously at a constant rate (100 μg/kg/min) for 5 minutes.

Statistical Analysis

All data were stored in a DEC LSI-11/73 computer. Mean ± SEM values of data at the various blood sample time intervals were calculated and are reported in the figures. Statistical comparisons over time as well as between treatment groups were conducted by analysis of variance for repeated measures, followed by Duncan's multiple comparison test if the analysis of variance resulted in a value of p < 0.05. The eicosanoid data were statistically analyzed after being subjected to logarithmic transformation; this provided a better approximation to a normal distribution at the various time intervals. Association between variables was determined by linear regression analysis, and the Pearson product-moment correlation coefficient was used to detect any significant association.

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between changes of hemodynamic and biological variables.

**Results**

**Effect of Protamine Injection on Pulmonary Hemodynamics**

Protamine injection (2 mg/kg) alone produced no change of Ppa, PVR, or Pw (Figure 1). However, when protamine was injected after heparin, it induced acute pulmonary hypertension (mean Ppa increasing from 18.0 ± 0.7 to 43.8 ± 2.7 mm Hg at 1 minute) due to a threefold increase of both the PVR and the Pw (Figure 1). Pulmonary vasoconstriction was transient with Ppa and Pw returning to baseline values by 10 minutes. Sheep gave no evidence of labored respiration and tachypnea. Pretreatment with indomethacin completely blocked increases of Ppa, Pw, and PVR; pretreatment with UK 38-485 significantly blocked increases of Pw and PVR, and markedly reduced the increase of Ppa. In contrast, pretreatment with either 0.5 or 1.0 g/kg DMTU (but not DMSO) blocked the acute pulmonary hypertension, the pulmonary vasoconstrictor response, and the increase of Pw seen in heparinized sheep given protamine.

**Effects of Protamine Injection on Arterial Plasma Levels of Thromboxane B₂**

When protamine was injected in sheep not given heparin, TXB₂ levels were minimal and unchanged (Figure 2). In contrast, administering protamine to animals after heparin increased plasma TXB₂ levels from 0.30 ± 0.05 to 6.3 ± 2.8 ng/ml at 1 minute ($p<0.0001$). Plasma TXB₂ levels remained significantly elevated for more than 5 minutes. Pretreatment with the cyclooxygenase inhibitor indomethacin or the thromboxane synthetase inhibitor UK 38-485 completely abolished increases in plasma TXB₂ levels after protamine administration. Pretreatment with DMSO did not prevent the major elevation of TXB₂ while administration of either 0.5 or 1.0 g/kg of DMTU completely abolished the TXB₂ increase and the associated pulmonary vasoconstriction and acute pulmonary hypertension. No significant elevation of the arterial plasma levels of 6-keto-PGF₁₀ or PGF₂α were measured after protamine infusion with the exception of those sheep pretreated with UK 38-485, in which the heparin-protamine interaction was associated with a transient and significant increase of both 6-keto-PGF₁₀ (248 ± 12 to 476 ± 84 pg/ml; $p<0.05$) and PGF₂α (30 ± 19 to 300 ± 69 pg/ml; $p<0.02$) at 3 minutes after protamine infusion. Figure 3 presents the individual Ppa changes occurring 1 minute after protamine injection in all sheep, whether drug treated or untreated, in relation to the logarithmic value of changes of the plasma TXB₂ level at 1 minute. A 10-fold increase of

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Effect of a bolus injection of protamine (arrow) on mean pulmonary arterial pressure (Ppa), pulmonary capillary wedge pressure (Pw), and pulmonary vascular resistance (PVR) in the six treatment groups. Heparinized controls, $n=4$; unheparinized controls, $n=10$ (except for times +1 and +2 minutes for PVR values, where $n=6$ and $n=7$, respectively); indomethacin-pretreated heparinized sheep, $n=3$; UK 38-485-pretreated heparinized sheep, $n=5$; DMSO-pretreated heparinized sheep, $n=4$; DMTU-pretreated heparinized sheep, $n=6$. Values are the mean ± SEM. *$p<0.05$ value differs from before protamine injection.
FIGURE 2. Effect of a bolus injection of protamine (arrow) on arterial plasma thromboxane B, (TXB$_2$) levels (ng/ml). Treatment groups are the same as in Figure 1. Values are the mean ± SEM. *p < 0.05 value differs from before protamine injection. Before protamine injection the TXB$_2$ levels were significantly decreased by pretreatment with indomethacin and UK 38-485.

arterial TXB$_2$ concentration resulted in an elevation of mean Ppa by 13.3 mm Hg ($r = 0.83$, $p < 0.001$).

No consistent changes of the baseline plasma levels of immunoreactive sulfidopeptide leukotrienes or leukotriene B$_4$ were detected by radioimmunoassay. HPLC analysis of plasma samples (pooled 1-20 minutes after challenge) obtained from six heparinized protamine-injected control sheep did not evidence any increase of these lipooxygenase metabolites over control values.

Effect of Neutralization of Heparin by Protamine on Arterial Plasma Dimethylsulfoxide and Dimethylthiourea Levels

After DMTU infusion, protamine neutralization of heparin did not cause TXB$_2$ levels to increase and there was no acute pulmonary vasoconstriction. Injection of protamine was associated with a major reduction of arterial plasma DMTU levels within 1 minute, signifying consumption of DMTU by H$_2$O$_2$ production (Figure 4). No consistent transpulmonary arteriovenous gradient of DMTU was measured after protamine injection. In sheep given saline but not heparin, DMTU levels did not decrease after protamine injection (Figure 4). The acute increase of plasma TXB$_2$, levels and the pulmonary vasoconstriction measured in sheep pretreated with DMSO and given heparin and protamine was not associated with any change of plasma DMSO levels.

To assess whether DMTU blocked thromboxane generation by inhibiting the activity of cyclooxygenase or thromboxane synthetase, we infused arachidonic acid intravenously over 5 minutes at a rate of 100 µg/kg/min in five sheep after DMTU pretreatment (two sheep given 1.0 g/kg, three given 0.5 g/kg). In all five sheep (each had previously not responded to a protamine infusion after heparin administration with either an elevation of TXB$_2$ or pulmonary vasoconstriction), intravenous infusion of arachidonic acid resulted in a 10-fold increase of arterial plasma TXB$_2$ levels with concomitant pulmonary vasoconstriction and hypertension.

Changes in Circulating Arterial Leukocyte Concentrations During Heparin Followed by Protamine Administration

Heparin infusion did not alter the WBC concentration in any group (Figure 5). Bolus administration of protamine into unheparinized sheep did not produce a significant change of the circulating WBC concentration after protamine injection. In contrast, the same dose of protamine in heparinized sheep induced an acute and profound leukopenia with the lowest circulating concentration measured at 2 minutes (63.1 ± 7.7% reduction from the baseline value). The circulating leukocyte concentration remained significantly decreased for over 5 minutes. Pretreatment with either
UK 38-485, indomethacin, DMTU, or DMSO did not significantly attenuate the leukopenia. The acute leukopenia of control and DMSO-pretreated sheep was associated with a transient transpulmonary WBC gradient at 2 minutes (29.0 ± 7.9% and 26.9 ± 10.7%, respectively) indicating major leukocyte sequestration in the pulmonary circulation. Transpulmonary WBC gradients were not detected in the other pretreated sheep.

Mean hemodynamic and biochemical values before heparin and drug infusion did not differ between the treatment groups. The various pretreatment drug infusions themselves had no significant effect on the baseline WBC count or pulmonary hemodynamics (Figures 1 and 2).

**Effect of Intravenous Heparin on ACT Levels**

Intravenous heparin (200 units/kg) increased the ACT from 134 ± 19 seconds to greater than 600 seconds in all animals. The ACT always returned to the baseline range when measured at 10 minutes after protamine injection.

**Systemic Hemodynamics**

Administration of protamine alone did not alter systemic hemodynamics. In heparinized sheep, immediately following protamine injection there was a transient (lasting 2 minutes) but significant (p < 0.05) 30% decrease of cardiac output (from 5.5 ± 0.5 to 3.7 ± 0.6 l/min at 1 minute after protamine), and stroke volume decreased (from 44 ± 4 to 28 ± 6 ml), accompanied by a significant increase of SVR (from 18.2 ± 1.8 to 23.2 ± 4.3 mm Hg·min/ml), whereas Pa (from 98 ± 5 to 86 ± 8 mm Hg), Pv (from 1.6 ± 0.7 to 2.3 ± 1.0 mm Hg), and heart rate (from 124 ± 5 to 137 ± 9 beats/min) were unchanged after protamine injection. In all drug-pretreated groups receiving heparin and protamine, there was no significant change from baseline values of these systemic hemodynamic values. There were no consistent alterations of arterial blood gas tensions in the control group receiving heparin and protamine. No significant effect on circulating platelet concentrations was noted in any experimental group.

**Effect of Protamine Injection on Plasma Complement Anaphylatoxin and Histamine Levels**

To confirm that the observed cardiopulmonary reaction was associated with complement activation and was not mediated via histamine release, we measured the plasma levels of C3a and histamine in three control sheep during the heparin-protamine interaction. Following protamine injection, plasma C3a levels increased from 420 ± 146 to 1,599 ± 249 ng/ml at 3 minutes (p < 0.01) and 1,456 ± 325 ng/ml at 10 minutes (p < 0.01); in contrast, plasma histamine concentrations never increased above baseline values.

**Discussion**

The most important finding of our study was that the neutralization of circulating heparin by intravenous protamine injection in sheep was uniformly followed by a marked increase of arterial plasma TXB2 concentration that was concomitant with a marked elevation of PVR, Ppa, and Pw (Figures 1–3). Inhibiting the production of TXA2 by pretreating the sheep with either the cyclooxygenase inhibitor indomethacin or a thromboxane synthetase inhibitor prevented both the increase of plasma TXB2, and the pulmonary vasoconstriction. TXA2 is a potent vasoconstrictor that is rapidly hydrolyzed to TXB2. TXA2 has been shown to be released and cause pulmonary hypertension in sheep after injecting zymosan-activated plasma, endotoxin, or phorbol myristate acetate, and is increased at the onset of cardiopulmonary bypass. Isolated sheep blood neutrophils and lymphocytes can produce thromboxane when stimulated in vitro by zymosan-activated plasma. Other sheep pulmonary interstitial cells can also produce thromboxane in large quantities; platelets do not appear to be a major source of thromboxane release in sheep.

In UK 38-485–pretreated sheep, but not in untreated animals, PGF2α and 6-keto-PGF1α were significantly increased during the pulmonary vasocostriction elicited by the reversal of heparin anticoagulation. Since UK 38-485 inhibited thromboxane synthesis, it is most likely that an increased amount of the common precursor prostaglandin H2 was available for the generation of other prostaglandins. Although PGF2α is much less potent pulmonary vasoconstrictor than thromboxane, it is possible that this eicosanoid may have actively contributed to the slight but significant rise in Ppa observed in UK 38-485–pretreated sheep, a response that may have been attenuated by the concomitant release of the pulmonary vasodilator prostacyclin.

The neutralization of heparin by protamine produces a complex cascade of activated mediators, some of
which can in themselves cause pulmonary vasoconstriction, for example, complement fragments and free radicals. However, blocking thromboxane production abolished the pulmonary vasoconstriction and so these mediators alone are unlikely to be important pulmonary vasoconstrictors in this reaction.

We recently implicated thromboxane production as a cause of pulmonary hypertension and bronchospasm in patients following protamine infusion. In a study of 49 cardiac surgical patients immediately after cardiopulmonary bypass, three patients developed acute pulmonary vasoconstriction and bronchoconstriction with systemic hypotension 1–3 minutes after 100 mg protamine was injected to neutralize heparin anticoagulation. This transient adverse cardiopulmonary response was treated with vasopressors or by resumption of cardiopulmonary bypass, and the patients recovered without developing respiratory failure or other apparent complications. These and five more recent patients treated in our hospital had a profound concomitant increase of plasma TXB, at the time of pulmonary artery hypertension, suggesting a cause and effect relation. Since the protamine reaction in humans is unpredictable and can be life threatening, we were motivated to examine an animal model to elucidate the cascade of mechanisms leading to this severe clinical cardiopulmonary reaction.

Studies in pigs by Stefaniszyn and Fiser reported that transient pulmonary hypertension is consistently produced when circulating heparin is reversed by injecting protamine. Pulmonary hypertension did not occur if protamine was injected alone without prior

![Figure 4. Effect of a bolus injection of protamine on arterial plasma DMTU and DMSO levels. Values are the mean ± SEM. *p < 0.05 value differs from before protamine injection. Open circles, unheparinized sheep given DMTU; closed circles, heparinized sheep given DMTU.](image-url)
heparin administration, demonstrating that heparin is essential to combine with protamine to trigger the cascade of mediators producing the pulmonary vasocostric response. These investigators did not report any mediator measurements. In our studies in sheep, we confirmed that thromboxane was not increased and pulmonary hypertension did not occur if protamine was injected alone (Figures 1–3). Arterial plasma histamine and leukotriene levels were not increased after protamine neutralized heparin (neither in this sheep study nor in the three patients who demonstrated this adverse cardiopulmonary reaction). The absence of a measurable increase of plasma histamine or leukotrienes, which are usually increased during anaphylactic reactions, including anaphylaxis in sheep, suggests another mechanism causes the adverse reaction to protamine, which is not an IgG-mediated anaphylactic reaction.3-37 The Pw increased after protamine neutralization with TXB2 production, but Pw did not increase when thromboxane release was inhibited. Since we did not measure left atrial pressure (PLa) in these chronically instrumented sheep (no thoracotomy), we were uncertain whether the increased Pw was due to a rise of PLa or diffuse pulmonary vasocostriction. Infusion of exogenous arachidonic acid or thromboxane analogues in perfused lungs has been shown to have a greater constrictor effect on the pulmonary veins than on pulmonary arteries.28 Our recent unpublished studies show PLa does not change in sheep when protamine is infused after heparin. Our prior studies in man also showed unchanged or decreased PLa during episodes of pulmonary vasocostriction due to protamine reversal of heparin anticoagulation.1,2

Rents et al8 reported that in vitro when the positively charged protamine molecule combines with the negatively charged heparin molecule, the complement cascade is activated via the classical pathway. Several human studies have demonstrated complement activation by protamine neutralization of heparin with an increase of plasma C3a and C4a (but not C5a), indicating classical pathway complement activation.2,3 In this sheep model, C3a levels were also increased after heparin was neutralized by protamine, confirming complement was activated and anaphylatoxins produced in this species as well as in man.

Despite universal evidence of complement activation and circulating leukopenia after protamine reversal of heparin anticoagulation, most patients do not develop an adverse cardiopulmonary reaction. C5a is known to be a potent leukoaggregating and leukoactivating mediator.39-40 In our recent clinical study, only those three patients developing a markedly increased plasma C5a concentration had a major elevation of plasma TXB2 and acute pulmonary hypertension. We believe each sheep receiving heparin and protamine developed a similar elevation of C5a, but we could not directly measure C5a because human C5a antibody did not cross-react with sheep C5a. Although it is possible, we do not know if humans first given heparin and then followed rapidly by a protamine injection would consistently raise their C5a levels, release thromboxane, and undergo pulmonary vasocostriction.

Exogenous substances that activate complement via the alternate pathway, such as cobra venom factor and zymosan, cause a sequence of leukoaggregation, free radical release, lipid peroxidation, thromboxane release, and transient pulmonary vasocostriction.9-15,32,33,41 A hypothetical sequence of events and mediators that are released when intravenously injected protamine neutralizes heparin is shown in Figure 6.

To assess the contribution of O2 metabolites in our model, we infused DMSO or DMTU. These small molecules can diffuse intracellularly to reach the sites of free radical generation and their circulating blood levels can be measured.17-19 DMSO is a good scavenger of ‘OH in vitro, while DMTU can scavenge H2O2 and ‘OH in vitro.21 In addition, it has been shown recently that reactions with DMTU causes DMTU disappear-
In five sheep, we measured a rapid (1 minute) and consistent ($p<0.01$) reduction of arterial DMTU concentration after protamine reversed heparin anticoagulation (Figure 4). In contrast, in four control sheep, DMTU levels were not reduced when protamine was injected without heparin. Therefore, we believe the neutralization of heparin by protamine activates complement and releases anaphylatoxins causing profound leukopenia, white cell activation, and $H_2O_2$ release. The released $H_2O_2$ was scavenged by DMTU, preventing lipid peroxidation, arachidonate release, and the resulting thromboxane production and pulmonary vasoconstriction. DMTU did not block thromboxane synthesis or pulmonary vascular reactivity since an arachidonic acid infusion increased arterial $TXB_2$ levels and caused pulmonary vasoconstriction. DMSO at a dose of 1 g/kg did not alter the pulmonary vasoconstrictor response to heparin-protamine injection, and since blood levels of DMSO were not decreased, it suggests that large quantities of the injurious $-OH$ were probably not produced, although the latter is difficult to ascertain because $H_2O_2$ can react to form $-OH$ and no methods are available to directly measure these highly reactive toxins in vivo. This may account for the absence of any sustained increase of pulmonary microvascular permeability in this sheep model, as well as the absence of pulmonary edema in patients reacting adversely to heparin reversal by protamine.

**Figure 6.** Proposed sequence of events leading to acute pulmonary vasoconstriction associated with the acute heparin-protamine interaction.
Although the adverse cardiopulmonary reaction following protamine injection in this study is not life-threatening to sheep, as reported in man,\textsuperscript{1,2} we believe the underlying mechanism leading to acute pulmonary hypertension in both species is similar. It is possible that sheep are more responsive to acute intravascular complement activation than humans; since recent reports document the presence of large quantities of pulmonary intravascular macrophages in sheep,\textsuperscript{42} these cells may exacerbate mediator release during acute inflammatory stimulation. In addition, differing amounts of mediators released from human platelets (e.g., serotonin) and mast cells (e.g., histamine) may also contribute to a differing biological and physiological pattern of adverse response to heparin-protamine neutralization between sheep and man. Finally, the physiological pattern in clinical practice may be modified by both preexisting and/or acute cardiovascular and other diseases and the often unstable cardiovascular period after cardiopulmonary bypass when protamine is administered.

The knowledge we have gained of the pathways of this adverse cardiopulmonary reaction occurring after heparin is neutralized by a protamine bolus suggests that pharmacotherapy could prevent this reaction. Three pretreatment agents were proven effective at two strategic points. DMTU prevented release of thromboxane and pulmonary vasoconstriction. However, DMTU administration is too late to limit and can produce hepatic and renal failure\textsuperscript{43}; infused in sheep at 1 g/kg, DMTU produced central nervous system depression and anorexia, which was reduced at 0.5 g/kg. Indomethacin completely and UK 38-485 partially inhibited the thromboxane increase and prevented the pulmonary vasoconstriction and hypertension associated with this reaction. Our elucidation of the pathways of acute pulmonary hypertension when protamine neutralizes heparin may allow future therapy to prevent this untoward reaction by preventing the pulmonary hypertension. Since C5a is a probable key mediator, pretreatment by infusing anti-C5a antibodies may well be another site to inhibit this reaction. Anti-C5a IgG was recently shown to inhibit the mortality of bacteremic complement activation.\textsuperscript{44}

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References

KEY WORDS: protamine, pulmonary hypertension, thromboxane, oxygen radicals.


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