

## ORIENTAÇÃO DE RECURSOS

**BANCA: PSU-GO**

**ANO: 2024**

**QUESTÃO: 91**

Prezada banca,

Venho, respeitosamente, solicitar recurso na questão 91 da prova de acesso direto.

Segundo o artigo “Acute Pulmonary Vasoconstriction and Thromboxane Release During Protamine Reversal of Heparin Anticoagulation in Awake Sheep”, traduzindo, “Vasoconstrição pulmonar aguda e liberação de tromboxano durante reversão de protamina da anticoagulação com heparina em ovelhas acordadas”, publicado na Circulation Research (<https://doi.org/10.1161/01.RES.62.5.905>), na primeira página, em seu resumo, está escrito “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  $18.0 \pm 0.7$  to  $43.8 \pm 2.7$  mm Hg at 1 minute ( $x \pm SEM$ ;  $n = 10$ ).”, traduzindo, “Quando protamina (2 mg/kg) foi injetada por via intravenosa em ovelhas acordadas 5 minutos após a infusão de heparina (200 unidades/kg), houve vasoconstrição pulmonar difusa transitória com pressão arterial pulmonar média aumentando de  $18,0 \pm 0,7$  para  $43,8 \pm 2,7$  mm Hg em 1 minuto ( $x \pm SEM$ ;  $n = 10$ )”

Com isso, observamos que, segundo o artigo em questão, a infusão de protamina irá provocar vasoconstrição pulmonar direta, tornando a alternativa B correta.

Pelo exposto acima, solicito, respeitosamente, a mudança do gabarito para a alternativa B.

# 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  $18.0 \pm 0.7$  to  $43.8 \pm 2.7$  mm Hg at 1 minute ( $x \pm \text{SEM}$ ;  $n = 10$ ). In addition, there was profound leukopenia ( $36.9 \pm 7.7\%$  of baseline values at 2 minutes) with transpulmonary leukocyte sequestration and transiently elevated plasma concentrations of C3a (from  $420 \pm 146$  to  $1,599 \pm 249$  ng/ml;  $n = 3$ ,  $p < 0.01$ ) and thromboxane  $B_2$  (from  $0.30 \pm 0.05$  to  $6.3 \pm 2.8$  ng/ml;  $n = 10$ ,  $p < 0.0001$ ), without significant increases of plasma 6-keto-prostaglandin  $F_{1\alpha}$ , prostaglandin  $F_{2\alpha}$ , 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. (*Circulation Research* 1988;62:905-915)

Neutralization of heparin anticoagulation with protamine sulfate in humans is occasionally accompanied by severe acute pulmonary vasoconstriction, bronchoconstriction, and systemic hypotension.<sup>1,2</sup> 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,<sup>3</sup> recent studies suggest that a nonimmunological pathway via complement activation and eicosanoid generation, particularly thromboxane,<sup>4,5</sup> 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.<sup>6</sup> Recent clinical studies following cardiopulmonary bypass reported that the plasma levels of C3a and C4a are increased immediately after protamine administration for heparin neutralization,<sup>7,8</sup> thereby confirming in humans the occurrence of nonimmunological complement activation by heparin-protamine 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.<sup>9-11</sup> 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.<sup>12-14</sup> Moreover, chemically generated  $O_2$  metabolites have been shown to stimulate thromboxane  $A_2$  (TXA<sub>2</sub>) production and produce vasoconstriction in isolated perfused rabbit lungs.<sup>15</sup> 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

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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 ( $10^6$  units) was injected intramuscularly once each day.

#### *Hemodynamic Measurements*

Pa, Pv, Ppa, and Pw were monitored using calibrated Hewlett-Packard 1280C pressure transducers with the zero reference level at the midthoracic line in the standing position. The pressures were continuously recorded on an eight-channel recorder (model 7758B, Hewlett-Packard Co., Palo Alto, California), and mean values at the various time intervals were measured at end-expiration. Cardiac output was recorded as the mean of duplicate determinations by thermodilution using an Edwards Laboratories Model 9520A Cardiac Output Computer and injecting 5 ml of 0° C saline. Systemic vascular resistance (SVR) was calculated by subtracting Pv from mean Pa and dividing by cardiac output; pulmonary vascular resistance (PVR) was calculated by subtracting Pw from mean Ppa and dividing by cardiac output.

#### *Measurement of Eicosanoids*

Plasma levels of thromboxane B<sub>2</sub> (TXB<sub>2</sub>), 6-keto-PGF<sub>1 $\alpha$</sub> , and PGF<sub>2 $\alpha$</sub>  were determined by radioimmunoassay as described previously,<sup>16</sup> using antisera provided by Dr. L. Levine, Brandeis University, Waltham, Massachusetts. The assays were standardized with TXB<sub>2</sub>, 6-keto-PGF<sub>1 $\alpha$</sub> , and PGF<sub>2 $\alpha$</sub>  obtained from Upjohn Diagnostics, Kalamazoo, Michigan. Further evidence for the identification of TXB<sub>2</sub> was obtained by reverse phase high-performance liquid chromatography (HPLC), demonstrating that plasma TXB<sub>2</sub> immunoreactivity had the chromatographic mobility of standard TXB<sub>2</sub>. Radioimmunoassays were also used to detect plasma levels of the sulfidopeptide leukotrienes C<sub>4</sub>, D<sub>4</sub>, and E<sub>4</sub>, as well as leukotriene B<sub>4</sub>.

#### *Plasma Dimethylsulfoxide and Dimethylthiourea Levels*

Plasma concentrations of dimethylsulfoxide (DMSO) and dimethylthiourea (DMTU) were assessed by gas chromatography as previously described.<sup>17-19</sup> Proteins were precipitated with perchloric acid before injection.

#### *Plasma C3a 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.<sup>20</sup> 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.<sup>21</sup>

Rabbit anti-sheep C3a antisera were obtained after initial immunization of New Zealand red rabbits with 50  $\mu$ g purified sheep C3a des Arg emulsified with complete Freund's adjuvant and repetitive boosting with 50  $\mu$ g antigen in incomplete adjuvant. Radioimmunoassay procedures similar to those developed for the human anaphylatoxins<sup>22</sup> 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.<sup>23</sup>

Total WBC count was determined with a Coulter cell counter (model Z<sub>F</sub>, 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).<sup>24</sup>

#### *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:  $134 \pm 19$  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.<sup>4</sup> 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<sup>25</sup> and Fiser<sup>26</sup> 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 38-485, 10 mg/kg,  $n = 5$ ); 4) pretreatment with an  $\cdot\text{OH}$  scavenger (DMSO, 1 g/kg,  $n = 4$ ); 5) pretreatment with a  $\text{H}_2\text{O}_2$  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/mm<sup>3</sup>, 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 38-485 (3-(1*H*-imidazol-1-yl methyl)-2-methyl-1*H*-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  $\mu\text{g}$  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 TXB<sub>2</sub> 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  $\mu\text{l}$  90% ethanol, diluted in sterile saline to a final concentration of 150  $\mu\text{g}/\text{ml}$ , and infused intravenously at a constant rate (100  $\mu\text{g}/\text{kg}/\text{min}$ ) for 5 minutes.

#### Statistical Analysis

All data were stored in a DEC LSI-11/73 computer. Mean  $\pm$  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  $p < 0.05$ .<sup>27</sup> 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

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, Pw, or Pw (Figure 1). However, when protamine was injected after heparin, it induced acute pulmonary hypertension (mean Ppa increasing from  $18.0 \pm 0.7$  to  $43.8 \pm 2.7$  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 B<sub>2</sub>*

When protamine was injected in sheep not given

heparin, TXB<sub>2</sub> levels were minimal and unchanged (Figure 2). In contrast, administering protamine to animals after heparin increased plasma TXB<sub>2</sub> levels from  $0.30 \pm 0.05$  to  $6.3 \pm 2.8$  ng/ml at 1 minute ( $p < 0.0001$ ). Plasma TXB<sub>2</sub> 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 TXB<sub>2</sub> levels after protamine administration. Pretreatment with DMSO did not prevent the major elevation of TXB<sub>2</sub> while administration of either 0.5 or 1.0 g/kg of DMTU completely abolished the TXB<sub>2</sub> increase and the associated pulmonary vasoconstriction and acute pulmonary hypertension. No significant elevation of the arterial plasma levels of 6-keto-PGF<sub>1 $\alpha$</sub>  or PGF<sub>2 $\alpha$</sub>  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-PGF<sub>1 $\alpha$</sub>  ( $248 \pm 12$  to  $476 \pm 84$  pg/ml;  $p < 0.05$ ) and PGF<sub>2 $\alpha$</sub>  ( $30 \pm 19$  to  $300 \pm 69$  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 TXB<sub>2</sub> level at 1 minute. A 10-fold increase of

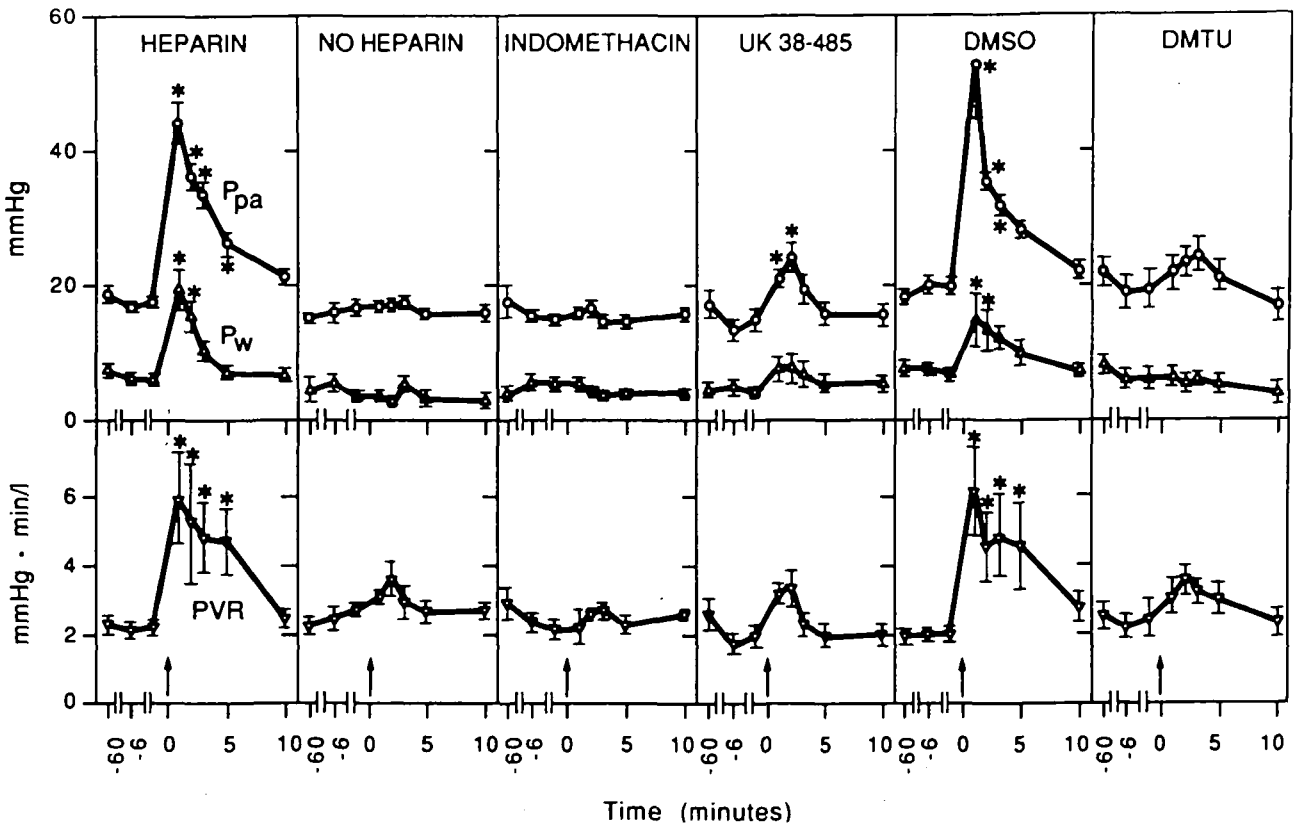


FIGURE 1. Effect of a bolus injection of protamine (arrow) on mean pulmonary arterial pressure (Ppa), pulmonary capillary wedge pressure (Pw), and pulmonary vascular resistance (PVR) in the six treatment groups. Heparinized controls, n=4; unheparinized controls, n=10 (except for times +1 and +2 minutes 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  $\pm$  SEM. \* $p < 0.05$  value differs from before protamine injection.

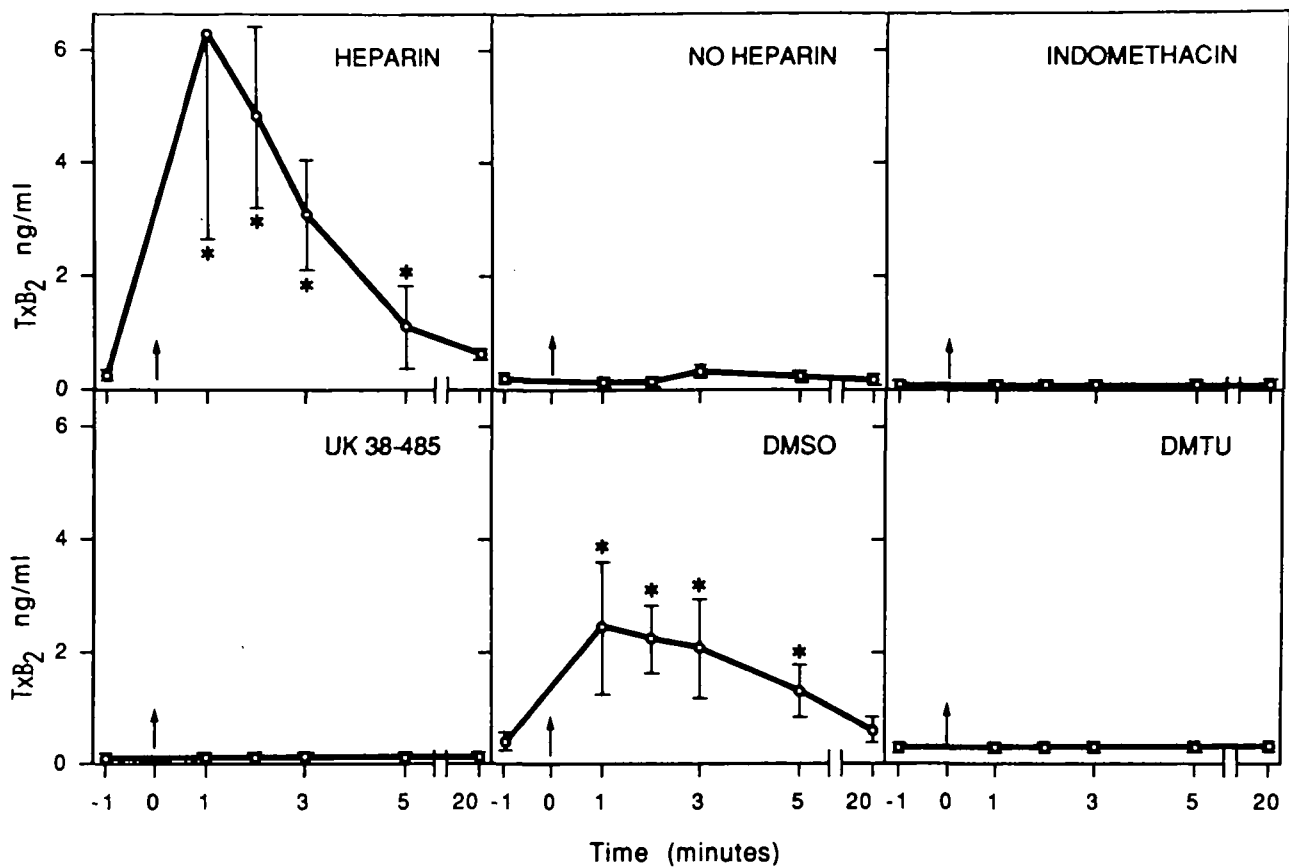


FIGURE 2. Effect of a bolus injection of protamine (arrow) on arterial plasma thromboxane B<sub>2</sub> (TXB<sub>2</sub>) levels (ng/ml). Treatment groups are the same as in Figure 1. Values are the mean  $\pm$  SEM. \* $p < 0.05$  value differs from before protamine injection. Before protamine injection the TXB<sub>2</sub> levels were significantly decreased by pretreatment with indomethacin and UK 38-485.

arterial TXB<sub>2</sub> 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<sub>4</sub> 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 lipoxygenase 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<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub> 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  $\mu$ g/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<sub>2</sub> or pulmonary vasoconstriction), intravenous infusion of arachidonic acid resulted in a 10-fold increase of arterial plasma TXB<sub>2</sub> 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 ( $63.1 \pm 7.7\%$  reduction from the baseline value). The circulating leukocyte concentration remained significantly decreased for over 5 minutes. Pretreatment with either

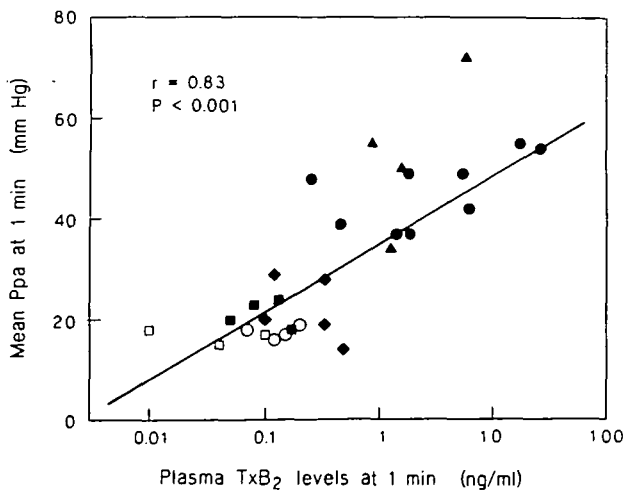


FIGURE 3. Relation between individual changes of the arterial plasma thromboxane  $B_2$  levels and simultaneous changes of mean Ppa values at 1 minute after 29 protamine injections. ●, heparinized controls; ○, unheparinized controls; □, indomethacin-pretreated heparinized sheep; ■, UK 38-485-pretreated heparinized sheep; ▲, DMSO-pretreated heparinized sheep; ◆, DMTU-pretreated heparinized sheep.

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 ( $29.0 \pm 7.9\%$  and  $26.9 \pm 10.7\%$ , 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  $134 \pm 19$  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  $5.5 \pm 0.5$  to  $3.7 \pm 0.6$  l/min at 1 minute after protamine), and stroke volume decreased (from  $44 \pm 4$  to  $28 \pm 6$  ml), accompanied by a significant increase of SVR (from  $18.2 \pm 1.8$  to  $23.2 \pm 4.3$  mm Hg min/l), whereas Pa (from  $98 \pm 5$  to  $86 \pm 8$  mm Hg), Pv (from  $1.6 \pm 0.7$  to  $2.3 \pm 1.0$  mm Hg), and heart rate (from  $124 \pm 5$  to  $137 \pm 9$  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  $420 \pm 146$  to  $1,599 \pm 249$  ng/ml at 3 minutes ( $p < 0.01$ ) and  $1,456 \pm 325$  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 TXB<sub>2</sub> concentration that was concomitant with a marked elevation of PVR, Ppa, and Pw (Figures 1–3). Inhibiting the production of TXA<sub>2</sub> by pretreating the sheep with either the cyclooxygenase inhibitor indomethacin or a thromboxane synthetase inhibitor prevented both the increase of plasma TXB<sub>2</sub> and the pulmonary vasoconstriction. TXA<sub>2</sub> is a potent vasoconstrictor that is rapidly hydrolyzed to TXB<sub>2</sub>.<sup>28</sup> TXA<sub>2</sub> has been shown to be released and cause pulmonary hypertension in sheep after injecting zymosan-activated plasma,<sup>9–11</sup> endotoxin,<sup>29</sup> or phorbol myristate acetate,<sup>30</sup> and is increased at the onset of cardiopulmonary bypass.<sup>31</sup> Isolated sheep blood neutrophils and lymphocytes can produce thromboxane when stimulated in vitro by zymosan-activated plasma.<sup>32</sup> Other sheep pulmonary interstitial cells can also produce thromboxane in large quantities<sup>9</sup>; platelets do not appear to be a major source of thromboxane release in sheep.<sup>10,33</sup>

In UK 38-485-pretreated sheep, but not in untreated animals, PGF<sub>2a</sub> and 6-keto-PGF<sub>1a</sub> were significantly increased during the pulmonary vasoconstriction 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 H<sub>2</sub> was available for the generation of other prostaglandins. Although PGF<sub>2a</sub> is a 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.<sup>5</sup> 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<sub>2</sub> 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<sup>25</sup> and Fiser<sup>26</sup> 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

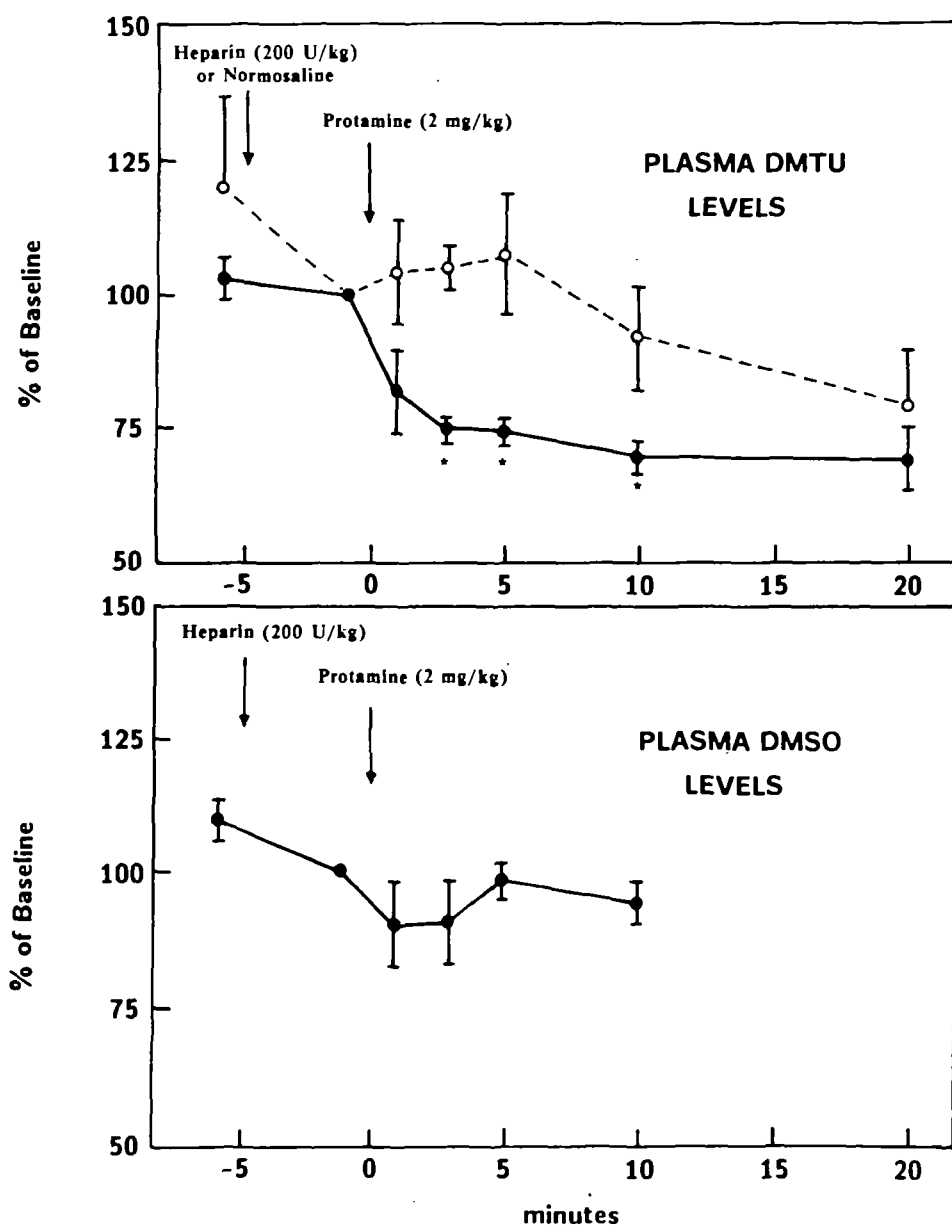


FIGURE 4. Effect of a bolus injection of protamine on arterial plasma DMTU and DMSO levels. Values are the mean  $\pm$  SEM. \* $p < 0.05$  value differs from before protamine injection. Open circles, unheparinized sheep given DMTU; closed circles, heparinized sheep given DMTU.



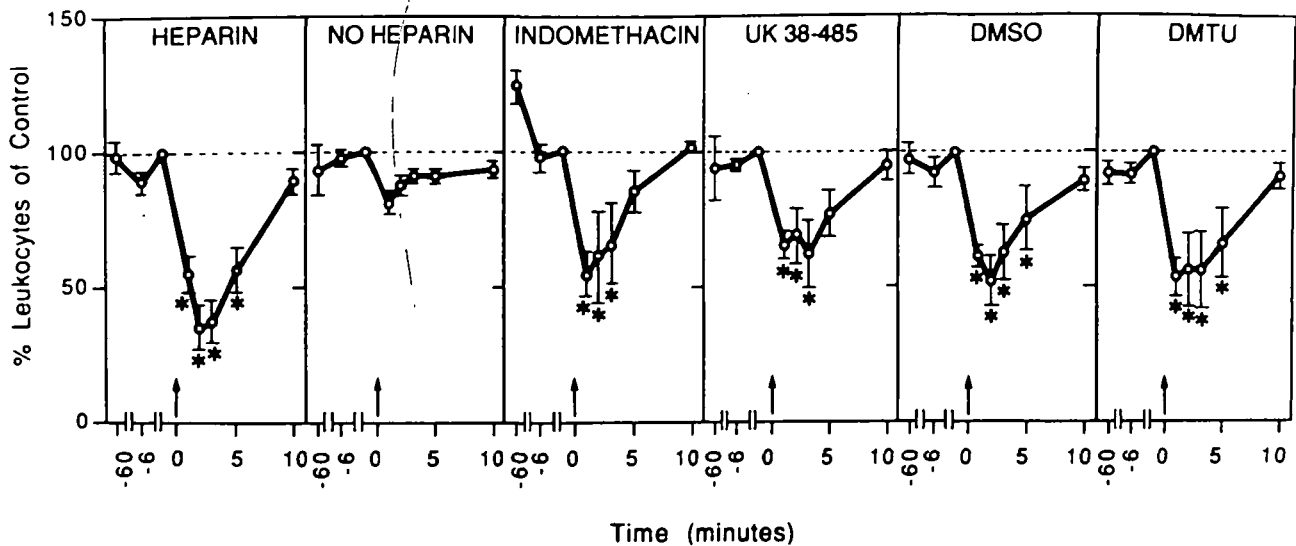


FIGURE 5. Effect of a bolus injection of protamine (arrow) on circulating arterial white blood cell concentration. Treatment groups are the same as in Figure 1. Values are the mean  $\pm$  SEM. \* $p < 0.05$  value differs from before protamine injection.

heparin administration, demonstrating that heparin is essential to combine with protamine to trigger the cascade of mediators producing the pulmonary vasoconstrictor 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,<sup>34</sup> including anaphylaxis in sheep,<sup>35,36</sup> suggests another mechanism causes the adverse reaction to protamine, which is not an IgG-mediated anaphylactic reaction.<sup>3,37</sup> The Pw increased after protamine neutralization with TXB<sub>2</sub> production, but Pw did not increase when thromboxane release was inhibited. Since we did not measure left atrial pressure (P<sub>LA</sub>) in these chronically instrumented sheep (no thoracotomy), we were uncertain whether the increased Pw was due to a rise of P<sub>LA</sub> or diffuse pulmonary vasoconstriction. 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.<sup>38</sup> Our recent unpublished studies show P<sub>LA</sub> does not change in sheep when protamine is infused after heparin. Our prior studies in man also showed unchanged or decreased P<sub>LA</sub> during episodes of pulmonary vasoconstriction due to protamine reversal of heparin anticoagulation.<sup>15</sup>

Rent et al<sup>6</sup> 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.<sup>5,7,8</sup> 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.<sup>39,40</sup> In our recent clinical study,<sup>5</sup> only those three patients developing a markedly increased plasma C5a concentration had a major elevation of plasma TXB<sub>2</sub> 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 vasoconstriction.

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 vasoconstriction.<sup>9–15,32,33,41</sup> 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 O<sub>2</sub> 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.<sup>17–19</sup> DMSO is a good scavenger of ·OH *in vitro*, while DMTU can scavenge H<sub>2</sub>O<sub>2</sub> or ·OH *in vitro*.<sup>17</sup> In addition, it has been shown recently that reactions with DMTU causes DMTU disappear-

ance in isolated lungs and kidneys.<sup>19</sup> 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  $\cdot OH$  were probably not produced, although the latter is difficult to ascertain because  $H_2O_2$  can react to form  $\cdot OH$  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,<sup>4</sup> as well as the absence of pulmonary edema in patients reacting adversely to heparin reversal by protamine.<sup>5</sup>

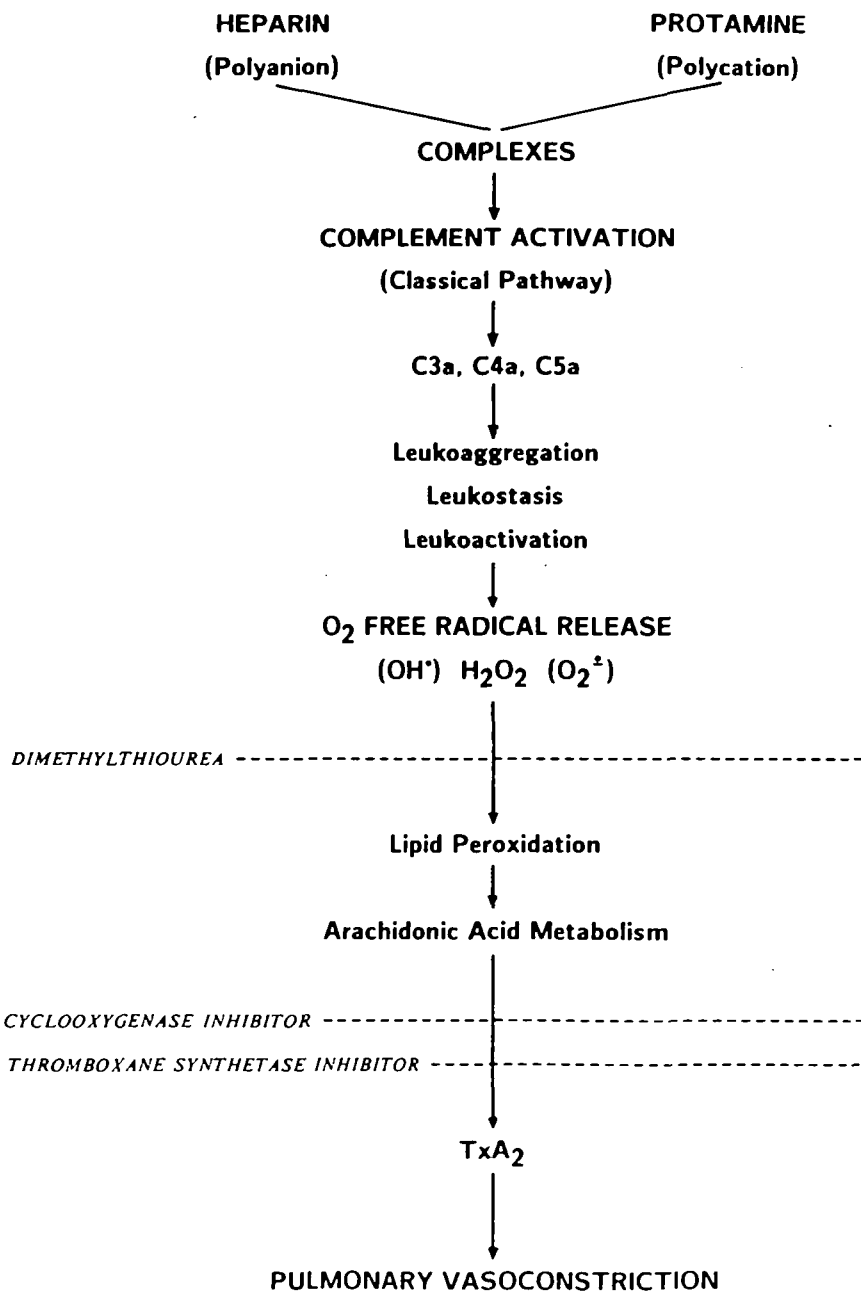


FIGURE 6. Proposed sequence of events leading to acute pulmonary vasoconstriction associated with the acute heparin-protamine interaction.

Although the adverse cardiopulmonary reaction following protamine injection in this study is not life-threatening to sheep, as reported in man,<sup>1,2</sup> 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,<sup>42</sup> 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 toxic to rats and can produce hepatic and renal failure<sup>43</sup>; 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.<sup>44</sup>

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KEY WORDS • protamine • pulmonary hypertension • thromboxane • oxygen radicals