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Adverse Cardiopulmonary Effects and Increased Plasma Thromboxane Concentrations Following the Neutralization of Heparin with Protamine in Awake Sheep Are Infusion Rate–dependent

Denis R. Morel, M.D., Pierre M. Mo Costabella, M.D.,† Jean-François Pittet, M.D.†*

The effect of the rate of intravenous infusion of protamine on the acute hemodynamic and pulmonary effects of heparin neutralization was investigated in six adult sheep surgically instrumented for chronic studies. Bovine lung heparin at a dose of 200 IU/kg was injected intravenously over 10 sec, 5 min before the start of protamine administration. On separate experimental days, each sheep received protamine at the same dose of 2 mg/kg, but it was infused over four different time periods: 3 s, 30 s, 300 s, or 30 min. At an additional session, protamine was administered over 3 s without prior heparinization to assess the effect of protamine alone. The sequence of the sessions was randomized and performed blindly. Injecting protamine in unheparinized sheep produced no change in any of the measured variables. In contrast, when protamine was injected over 3 s in heparinized sheep, it induced a transient and significant ($P < 0.001$) pulmonary hypertension (from 17.2 ± 1.5 to 45.6 ± 2.4 mmHg at 1 min) with an increased pulmonary (five-fold) and systemic (2.5-fold) vascular resistance; a decrease of cardiac output (from 3.85 ± 0.43 to 1.93 ± 0.29 l/min) without change in left atrial pressure (from 5.3 ± 1.3 to 6.0 ± 1.7 mmHg; P = NS); a significant ($P < 0.001$) increase of plasma thromboxane B_2 (TxB_2) concentrations (from 349 ± 131 to 974 ± 218 pg/ml); leukopenia (76 ± 4% of baseline white blood cell counts); and hypoxemia (Pa_{O_2} decreased from 81 ± 3 to 63 ± 4 mmHg at 2 min). Administering the same amount of protamine after heparin at a slower infusion rate significantly attenuated and delayed all components of the adverse response to protamine. This attenuation occurred in an infusion rate-dependent fashion, so that when protamine was infused over 30 min, no significant changes in any of the measured variables were noted. The time course of plasma heparin concentrations following protamine indicated that chemical heparin was completely neutralized over the time period of protamine infusion. These results demonstrate that the *rate* of generation of heparin–protamine complexes (as detected by changes of plasma concentrations of chemical heparin) during iv protamine infusion started 5 min after heparin administration is a factor involved in the generation of sufficient mediators required to initiate a characteristic physiologic response in sheep, including systemic and pulmonary vasoconstriction, TxB_2 generation, and leukopenia. Infusing a neutralizing dose of protamine over 30 min avoids these adverse reactions in sheep. (Key words: Anticoagulants, blood: heparin; protamine. Heart: vascular pressures. Hormones, prostaglandin: thromboxane A_2. Drug interactions. Lung: vascular resistance.)

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NEUTRALIZATION of heparin anticoagulation with protamine is occasionally accompanied by major acute hemodynamic and pulmonary effects characterized by pulmonary vaso- and bronchoconstriction and systemic vascular collapse requiring prompt intervention.[1,2] Some protamine reactions seem to be independent of the dosage and rate of administration, since even minimal doses and slow infusion rates of protamine have produced this syndrome.[1,2] Apart from rare true anaphylactic immunoglobulin-mediated reactions to protamine alone, recent animal and human data suggest that a nonimmunologic pathway *via* complement activation and eicosanoid generation, particularly thromboxane, may be responsible for these acute manifestations observed during protamine reversal of heparin anticoagulation.[3–5]

Recently, we identified a similar pattern in three of 49 patients investigated prospectively during the neutralization of heparin with protamine.[6] All three patients had increased plasma C_{5a} and thromboxane B_2 (TxB_2) concentrations; however, in the other subjects, these mediators and pulmonary hemodynamics did not change. The reason why some patients have these acute unpredictable reactions and in others heparin neutralization is without clinical effect is still unclear. However, studies performed both *in vitro* and in animals, indicating the requirement of a narrow heparin–protamine ratio to produce effective

* Research Associate in Anesthesia.
† Research Fellow in Anesthesia.

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Address reprint requests to Dr. Morel: Département d'Anesthésiologie, Hôpital Cantonal Universitaire, 1211 Genève 4, Switzerland.

complement activation, may explain the absence of significant reactions by the lack of sufficient anaphylatoxin generation in a clinical situation where this optimal ratio is rarely attained.[7,8]

To increase the likelihood of occurrence of this effective heparin–protamine ratio *in vivo* and, thus, to better document the pathophysiologic mechanism leading to this situation, we recently administered a neutralizing dose of protamine by bolus intravenous injection only 5 min after heparin was administered intravenously in awake, chronically instrumented sheep.[9] This mode of protamine administration resulted in a consistent and reproducible transient biologic and physiologic reaction, including complement activation, circulating leukopenia, increased plasma TxB_2 concentrations, and pulmonary vasoconstriction. The described animal model enabled us to elucidate the mediator profile underlying this syndrome, but the factors that occur occasionally in a clinical situation leading to this excessive complement activation and resulting in increased C_{5a} concentrations, granulocyte activation, and subsequent thromboxane release are still not clear.

The aim of the current study was to determine the dependency of the observed reaction on the rate of infusion of protamine administered intravenously and its relationship to the pharmacokinetic pattern of chemical and biologic heparin neutralization. Our hypothesis was that a slower infusion rate would decrease the instantaneous circulating protamine concentration able to interact with 'free' heparin molecules, therefore yielding to a lesser degree of acute complement activation and anaphylatoxin generation. Alternatively, if maximal complement activation depends rather on the total amount of generated heparin–protamine complexes, a slower infusion rate would, in turn, not significantly modify the biologic and physiologic pattern of the reaction. Since the response is transient and without further sequelae, we planned to test this hypothesis in awake, instrumented sheep by varying the infusion rate of a fixed protamine dosage on different days in the same animal. In addition to the measurement of plasma heparin concentrations, we documented and correlated the effects of heparin neutralization on circulating granulocytes, plasma TxB_2 concentrations, lung mechanics, and systemic and pulmonary hemodynamics to provide a better understanding and a more precise illustration of this nonimmunologic intravascular complement activation.

Materials and Methods

Sheep Preparation

The experimental protocol conformed to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiologic Society and the institutional Animal Care Committee. Six adult sheep of either sex weighing 30–40 kg were surgically instrumented with sterile techniques during halothane anesthesia, tracheal intubation, and mechanical ventilation of the lungs. Two 4.1-mm OD polyvinyl chloride catheters were introduced into the mid-thoracic aorta, one through the left carotid artery for intermittent arterial blood sampling and one through the right femoral artery for continuous measurement of systemic arterial blood pressure. Through a left-sided thoracotomy (fourth intercostal space), a transit-time ultrasonic blood flow probe (16S-Sil flow probe, Transonic Systems Inc., Ithaca, NY) was placed around the main pulmonary artery for continuous cardiac output measurement. A polyvinyl chloride catheter was inserted into the left atrium for left atrial pressure measurement. A compliant, balloon-tipped, air-filled catheter was inserted into the pleural space at the level of the right atrium to measure pleural pressure. The thorax was closed after complete re-expansion of the lungs by several large sighs. The operative wound was infiltrated with 20 ml of 0.5% bupivacaine for postoperative pain relief. The vascular catheters were filled with a heparinized solution (5,000 IU/ml), and a local antibiotic (2% sodium fusidate cream) was applied on the wounds daily.

After a recovery period of at least 4 days following thoracotomy, the sheep underwent a second induction with halothane anesthesia during which a chronic tracheostomy was surgically prepared. In addition, a 7-Fr, flow-directed, thermodilution catheter was introduced through the right jugular vein into the pulmonary artery for continuous measurement of right atrial pressure and pulmonary artery pressure (PAP), and the measurement of central body temperature; another catheter was placed into the right atrium through the left jugular vein for the infusion of fluids and drugs. The animals were then allowed to recover from anesthesia for at least 24 h. During this recovery period, a 12-mm OD Silastic™ T-cannula was placed into the trachea through the tracheostomy to allow breathing through the natural airways and to maintain the tracheostomy patent.

Measurements of Hemodynamics and Lung Mechanics

Aortic, central venous, left atrial, and pulmonary arterial pressures were continuously measured using calibrated pressure transducers (Honeywell, Zürich, Switzerland) positioned at the level of one-third of the distance from the brisket to the top of the back. Cardiac output was measured with a transit-time flowmeter (T101CDS, Transonic System, Inc., Ithaca, NY) using a 16S-Sil transit-time ultrasonic flow probe. Vascular pressures and blood flow were recorded on a six-channel recorder (Gould

Electronics, Zürich, Switzerland). Systemic vascular resistance (SVR) was calculated by dividing the difference between mean aortic pressure and central venous pressure measured at end-expiration by mean pulmonary blood flow; pulmonary vascular resistance (PVR) was calculated by dividing the difference between mean PAP and left atrial pressure measured at end-expiration by mean pulmonary blood flow. A standard three-lead electrocardiogram was continuously displayed on a Hewlett-Packard monitor with digital readout of heart rate by means of chronically implanted subcutaneous electrodes.

After topical anesthesia of the tracheal mucosa, a low-pressure, cuffed, tracheostomy cannula (no. 10) was inserted into the trachea in place of the T-cannula 1 h before the start of the experiment. Intratracheal pressure was measured by a catheter introduced into and positioned 1 cm distal of the extremity of the tracheostomy cannula. Transpulmonary pressure was determined by a differential pressure transducer (Hewlett-Packard 267B) taking the difference between tracheal and pleural pressure. Tidal volume was determined by integration of the respiratory flow signal measured with a pneumotachograph (Gould Godart, model 17212) by means of a heated Fleisch flowtransducer (no. 2) connected to the tracheostomy cannula. Transpulmonary pressure, tidal volume, and airflow were continuously recorded on a four-channel recorder (Hewlett-Packard, 7754B). Total airflow resistance across the lungs was determined by dividing the difference in transpulmonary pressure by inspiratory plus expiratory flow at mid-tidal volume. Dynamic pulmonary compliance was obtained by dividing tidal volume by the difference in transpulmonary pressure at points of zero flow. Respiratory parameters were averaged for five successive tidal volumes.

Biochemical Analyses

Plasma concentrations of TxB_2 were determined by standard radioimmunoassay using a commercial TxB_2 kit (Amersham International plc, Buckinghamshire, United Kingdom) with a sensitivity of <2 pg/tube, *i.e.*, <2 pg/100 μl plasma and with a working range of 30–4,000 pg/ml plasma (undiluted). The average slope of the six-double point standard curve for five consecutive TxB_2 RIA kits is 0.0209 ± 0.0005 $\log_{10}$pg/percent of tracer binding B/BO change (mean ± SE), with a variation coefficient of $6.5 \pm 0.4\%$, and the average linear regression analysis coefficient is $r = 0.980 \pm 0.002$ (mean ± SE). Percent cross reactivity is <0.3% for other prostaglandins, except for prostaglandin D_2 (PGD_2) where it is 2.5%. Duplicate determinations of each sample differed by <5%. Thromboxane B_2 levels measured in the same animal on each study day presented a variation coefficient ranging from 5–63%.

Plasma heparin concentrations were determined by the colorimetric assay for chemical heparin based on the metachromasia of the biologic dye azure A.[10] Standard curves were made from baseline venous plasma of each individual sheep taken on the day of arrival from the animal farm. Congruently, the biologic (*i.e.*, anticoagulant activity of heparin) was detected by measuring the activated clotting time (ACT) of whole blood with a Hemocron 400D system (International Technidyne, Edison, NJ).

Arterial Blood Gas Analysis and Circulating White Blood Cell Count

Circulating leukocytes were measured by phase microscopy and total hemoglobin concentration in blood by spectrophotometry. Blood gas tensions, oxygen hemoglobin saturation, and *p*H were analyzed by an automated AVL (AVL Biomedical Instruments, Schaffhausen, Switzerland) 940 oximeter. Alveolar-arterial oxygen gradient was calculated by a standard formula.

Experimental Protocol

The studies were carried out with the animals in a documented, stable, baseline respiratory and hemodynamic state (± 10%). If the animals showed signs of infection (blood leukocyte concentration >12,000/mm^3, core temperature >40° C) or pulmonary hypertension (mean PAP >25 mmHg) at the beginning of the experiment, the study was postponed. The experiments were started in awake sheep standing in a specially adapted cage for chronic studies 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. In the beginning of the experiment, a tracheostomy cannula (no. 10) was introduced after topical anesthesia into the trachea in place of the T-tube and connected to the pneumotachograph. Intravenous infusion of a sodium chloride solution was started at a rate of 10 ml · kg^{-1} · h^{-1} to compensate for the subsequent intravascular volume loss from blood sampling. The intravascular catheters were aspirated before each study to remove the remaining heparin solution inside the catheters and then continuously flushed with a standard technique (Intraflo®, 4 ml/h) using pressure bags containing a normosaline solution (without heparin).

After connecting the different catheters to the measuring instruments, the animals were allowed to rest for a period of at least 1 h. 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 s, additional time was allowed for the ACT to return to normal (normal values for sheep: 134 ± 19 s; range, 89–160 s).

On each experimental day, the sheep received 200 IU/kg of bovine lung heparin (Liquémine®, Hoffmann-La Roche & Co., AG, Basel, Switzerland) as an intravenous bolus through the right atrial catheter. Five minutes later, protamine hydrochloride (Protamine 1000, Hoffmann-La Roche & Co. AG, Basel, Switzerland) was infused through the right atrial catheter at the same dose of 2 mg/kg on each experimental day over four different time periods: 3 s, 30 s, 300 s, and 30 min. At an additional session, protamine was administered over 3 s without prior administration of heparin to assess the effect of protamine alone. The sequence of the protocol was randomized. The sampling of arterial blood was done at the following times: −6 min and −1 min, *i.e.*, immediately before the administration of heparin and protamine, respectively, and thereafter at 1, 2, 3, 5, 10, 20, and 30 min after the start of protamine infusion. The total volume of arterial blood sampling was approximately 70 ml for each experimental day, which corresponds to about 3.5% of the animal's estimated total blood volume. Samples for the determination of hematocrit and white blood cell (WBC) count, arterial blood gases, and the ACT were processed immediately according to standard techniques; the samples for thromboxane and heparin concentrations were transferred to chilled glass test tubes containing 0.05 ml of 15% EDTA and 100 μg of indomethacin. The samples were immediately centrifuged at 2500 × *g* for 10 min, and the plasma was aspirated and stored in polypropylene tubes at −70° C for further analysis.

Pharmacokinetics of Heparin

On the day of arrival from the animal farm to the laboratory, the sheep were given a single dose of heparin (200 IU/kg) *via* a central venous catheter inserted through a jugular vein to determine the pharmacokinetics of heparin in sheep. A second central venous catheter inserted into the contralateral jugular vein was used to take venous blood samples at the following times: just before heparin (time = 0), and 1, 2, 3, 5, 10, 15, 20, 30, 45, 60, 75, and 90 min following heparin administration.

Statistical Analysis

Mean ± SE values of data at the different time intervals were calculated and are reported in figures 1–7. Statistical comparison over time and between the different experimental days was conducted by analysis of variance for repeated measures, followed by Duncan's multiple comparison test if the analysis of variance resulted in $P < 0.05$.[11] The TxB_2 data were statistically analyzed after being subjected to logarithmic transformation; this provided a better approximation to a normal distribution at the various time intervals.

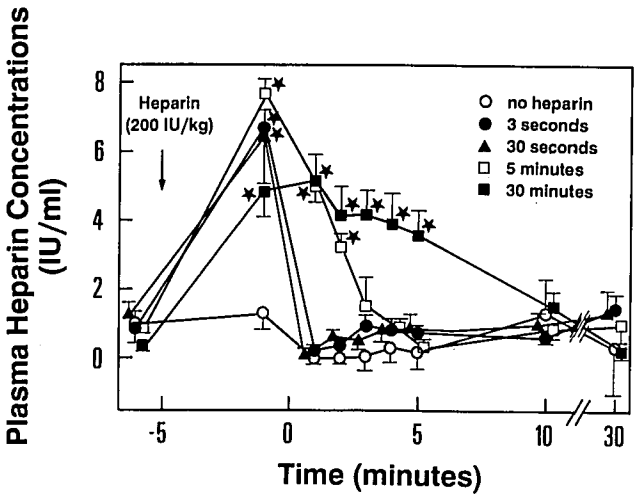

FIG. 1. Plasma concentrations of heparin measured by its metachromatic activity before and after administration of protamine hydrochloride infused over 3 s, 30 s, 300 s, and 30 min. Heparin is injected at time −5 min; protamine infusion is started at 0 min. Data points represent mean ± SE values; n = 6 in each group. *$P < 0.05$ from unheparinized group.

Results

Plasma concentrations of heparin measured by its metachromatic activity increased to more than 4 IU/ml in all animals injected with the 200 IU/kg dose, whereas in unheparinized sheep, heparin concentrations remained at baseline values (fig. 1). Administration of protamine at the different infusion rates completely neutralized the metachromatic activity of heparin in plasma over the various periods of protamine infusion, *i.e.*, complete heparin neutralization was always achieved at the end of the protamine infusion period. The ACT was below 140 s in all groups at time −6, *i.e.*, before heparin administration. In sheep given heparin, ACT increased above 580 s at time −1, whereas it was 139 ± 4 s in the unheparinized group. At 10 min, ACT values were 124 ± 10 s in unheparinized animals, 104 ± 4 s in sheep given protamine over 3 s, 129 ± 12 s when given over 30 s, 114 ± 11 s when given over 300 s, and 412 ± 87 s when given over 30 min; in the latter group, ACT returned to baseline values at 30 min (123 ± 8 s).

The effect of protamine on pulmonary hemodynamics is depicted in figure 2. In unheparinized sheep, bolus protamine injection produced no change in mean PAP. In contrast, when protamine was injected 5 min after heparin over 3 s, it elicited acute pulmonary hypertension (mean PAP increasing from 17.2 ± 1.5 to 45.6 ± 2.4 mmHg at 1 min; $P < 0.001$ from baseline value) that was associated with a five-fold increased PVR ($P = 0.006$ from baseline), indicating intense pulmonary vasoconstriction. This vasoconstrictor response was accompanied by re-

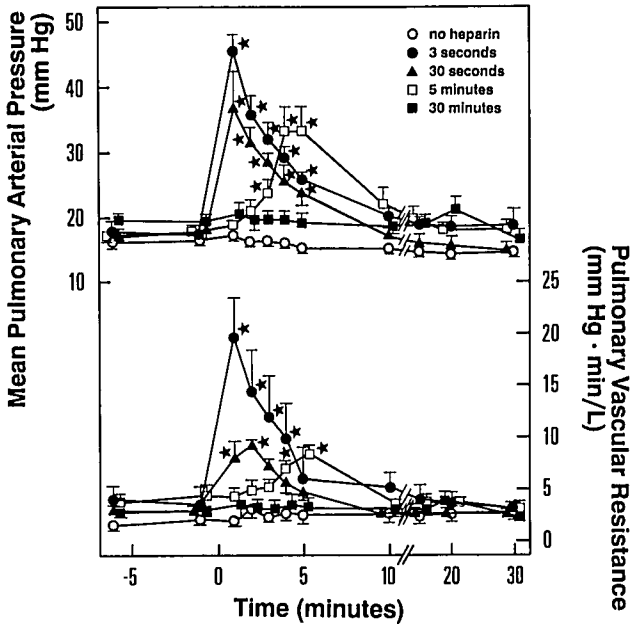

FIG. 2. Pulmonary hemodynamics before and after administration of protamine infused over 3 s, 30 s, 300 s, and 30 min. Heparin is injected at time −5 min; protamine infusion is started at 0 min. Data points represent mean ± SE values; n = 6 in each group. *$P < 0.05$ from unheparinized group.

duced cardiac output, reduced stroke volume, and increased SVR but was without significant change of left atrial pressure and mean systemic arterial pressure (figs.

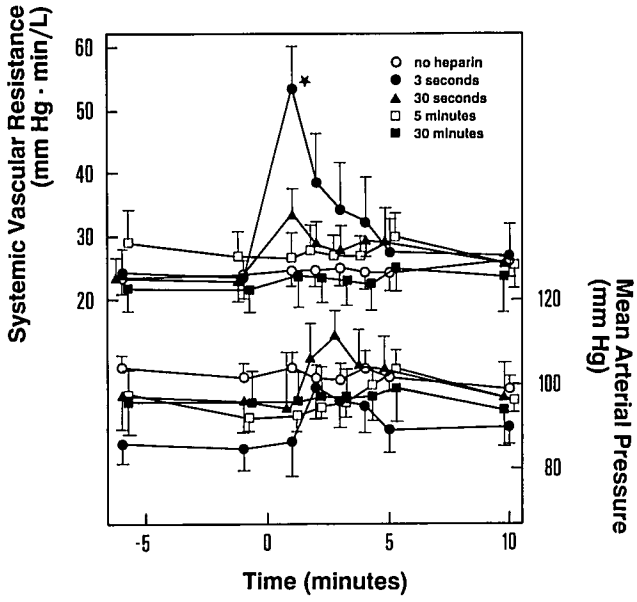

FIG. 3. Systemic vascular resistance and mean arterial pressure before and after administration of protamine infused over 3 s, 30 s, 300 s, and 30 min. Heparin is injected at time −5 min; protamine infusion is started at 0 min. Data points represent mean ± SE values; n = 6 in each group. *$P < 0.05$ from unheparinized group.

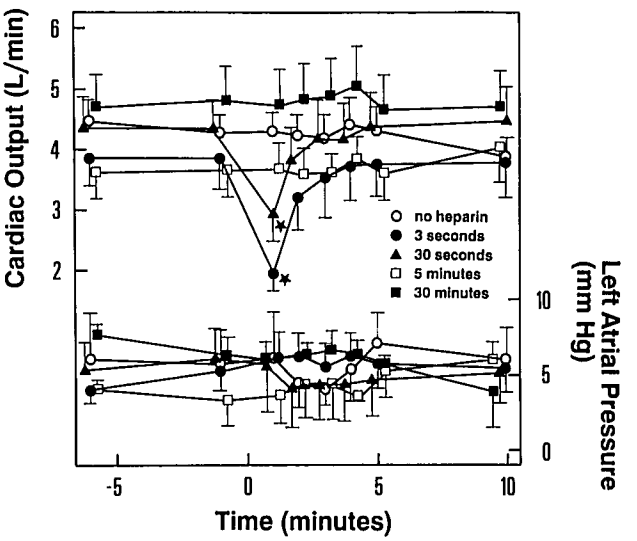

FIG. 4. Cardiac output and left atrial pressure before and after administration of protamine infused over 3 s, 30 s, 300 s and 30 min. Heparin is injected at time −5 min; protamine infusion is started at 0 min. Data points represent mean ± SE values; n = 6 in each group. *$P < 0.05$ from unheparinized group.

3 and 4). Pulmonary vasoconstriction was transient, with mean PAP and cardiac output returning to baseline values by 10 to 15 min following protamine that was injected over 3 s.

Administering the same amount of protamine after heparin at a slower infusion rate significantly attenuated and delayed the vasoconstrictor response to bolus protamine administration, and this was done in an infusion rate-dependent fashion. When protamine was injected in sheep not given heparin, TxB_2 concentrations were min-

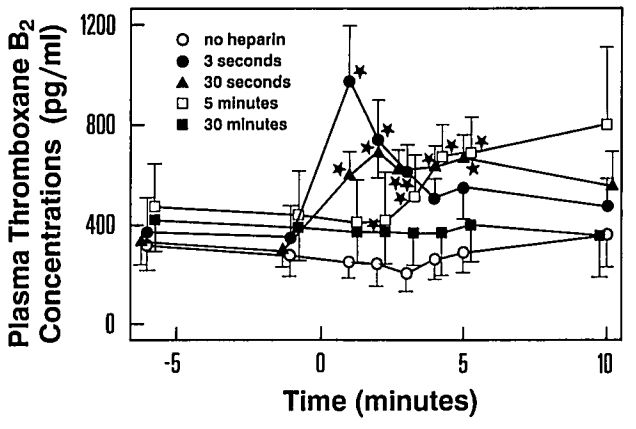

FIG. 5. Plasma thromboxane B_2 concentrations before and after administration of protamine infused over 3 s, 30 s, 300 s, and 30 min. Heparin is injected at time −5 min; protamine infusion is started at 0 min. Data points represent mean ± SE values; n = 6 in each group. *$P < 0.05$ from unheparinized group.

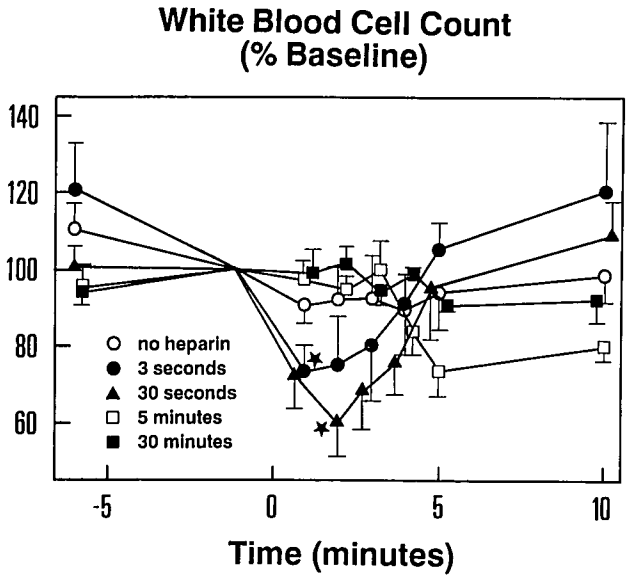

FIG. 6. Arterial white blood cell concentration (percent of baseline) before and after administration of protamine infused over 3 s, 30 s, 300 s, and 30 min. Heparin is injected at time −5 min; protamine infusion is started at 0 min. Data points represent mean ± SE values; n = 6 in each group. *$P < 0.05$ from unheparinized group.

imal and unchanged (fig. 5). In contrast, administering protamine to animals after heparin significantly increased plasma TxB_2 concentrations. In sheep given the bolus injection of protamine, TxB_2 concentrations increased from 349 ± 131 to 974 ± 218 pg/ml at 1 min ($P = 0.028$ from baseline) and remained significantly elevated during 3 min; in animals given protamine with a slower infusion rate, the rise in TxB_2 concentrations was delayed and attenuated. When considering all six treatment groups together, individual hemodynamic responses to protamine administration at 1 min were significantly correlated to simultaneous plasma concentrations of TxB_2, *i.e.*, animals with the most elevated TxB_2 concentrations also had the lowest cardiac output ($r = -0.79$; $P = 0.0003$), highest SVR ($r = 0.87$; $P < 0.0001$), and highest PVR values ($r = 0.85$; $P < 0.0001$). Heart rate remained unchanged from baseline in either of the treatment groups.

The effect of heparin and protamine administration on arterial WBC concentration is shown in figure 6. Heparin infusion by itself did not alter the WBC concentration in any group. Although administration of protamine into unheparinized sheep did not produce a significant change of the circulating WBC concentration, in heparinized sheep, protamine induced a transient leukopenia only in sheep given protamine over 3 and 30 s, with the lowest circulating concentration measured at 2 min in sheep given protamine over 30 s (60.6 ± 8.7% reduction from baseline). The circulating WBC concentration recovered rapidly to baseline values by 5 min.

Although modest variations in pulmonary mechanics (*i.e.*, tidal volume, respiratory frequency, dynamic pulmonary compliance, and total airflow resistance across the lungs) occurred in individual sheep (especially after bolus protamine administration following heparin), there were no significant changes in the groups with either of the different infusion rates. For instance, total airflow resistance increased to a peak value of 145 ± 37% (range, 63–280%) of baseline at 5 min after protamine in the unheparinized group to 145 ± 60% (range, 75–554%) in heparinized sheep given protamine over 3 s, to 128 ± 29% (range, 73–210%) over 30 s, and to 122 ± 18% (range, 83–180%) over 3 min (all comparisons NS). In contrast, arterial blood oxygenation was significantly ($P < 0.05$) affected by protamine but only when infused over 3 s in heparinized sheep. The Pa_{O_2} decreased from 81 ± 3 to 63 ± 4 mmHg at 2 min after bolus injection of protamine and remained statistically below baseline values for 10 min (74 ± 6 mmHg). Similarly, alveolar-arterial oxygen tension difference ($A\text{-}aD_{O_2}$) increased in the same group from 20 ± 4 to 37 ± 3 mmHg at 2 min, and Pa_{CO_2} and *p*H were not significantly changed. All other measured variables were not significantly affected by either infusion rate of protamine injection.

Discussion

The results of this study demonstrate that the rate of generation of heparin–protamine complexes (as detected by changes of plasma concentrations of chemically active heparin) during intravenous protamine infusion is a determinant of the factors generating sufficient mediators

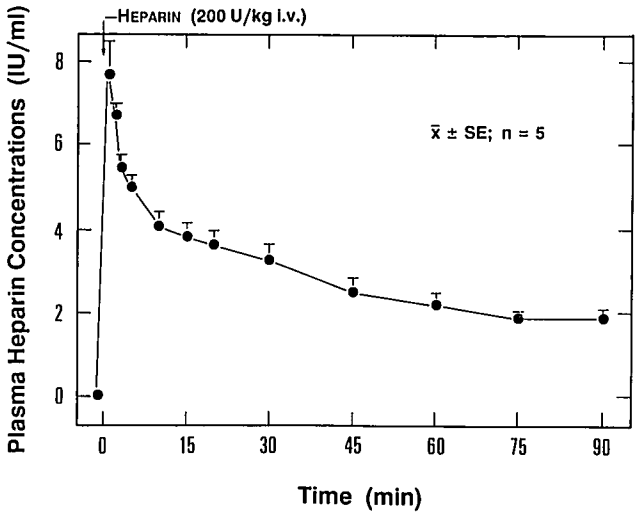

FIG. 7. Plasma concentrations of 200 IU/kg bovine lung heparin measured by its metachromatic activity in five normal awake sheep. Plasma β-half-life: 61.2 ± 11.5 (SD) min; total plasma clearance: 14 ± 5 ml/min; volume of distribution: 1209 ± 314 ml.

to initiate a characteristic physiologic response in sheep, including systemic and pulmonary vasoconstriction, TxB_2 generation, hypoxemia, and leukopenia. The degree of the response to protamine correlated with the rate of protamine administration, *i.e.*, the most rapid infusion induced the most severe adverse effects. Decreasing the rate of an identical amount of protamine infusion resulted in a proportional attenuation of the response.

These results confirm the clinical impression of some surgeons and anesthesiologists that a slower protamine infusion rate may avoid adverse cardiovascular effects related to this drug. Indeed, the package insert accompanying protamine recommends that the infusion rate not exceed 50 mg in 10 min.[12] However, this premise is only supported by earlier studies in dogs,[13-15] whereas a number of clinical studies do not corroborate a higher incidence of systemic hypotension following protamine administration at higher infusion rates.[16,17] Conversely, very low doses and infusion rates of protamine have been reported to produce major cardiopulmonary effects in patients.[1] It seems likely that the discrepancy between the reported results on protamine infusion rate-related adverse effects to this drug is due to different underlying mechanisms involved in the different experimental or clinical conditions. True anaphylactic IgE-mediated reactions to protamine will not require large quantities of protamine to induce mediator release,[18] while isolated protamine-induced mast cell degranulation[19] or heparin–protamine-induced complement activation and thromboxane generation[6,9] will most probably depend on the amount of circulating protamine or heparin–protamine complexes. Although we do not know the quantity of heparin–protamine complexes required to initiate thromboxane generation, the rate of protamine infusion will almost certainly influence the amount of available heparin molecules that are instantaneously complexed and thus able to generate thromboxane.

Injecting protamine at the different infusion rates completely neutralized the metachromatic activity of heparin in plasma during the period of protamine infusion, indicating effective *chemical* neutralization of negatively charged heparin molecules in relation to the amount of positively charged protamine molecules. Measurement of plasma heparin concentrations allowed us to precisely quantify the disappearance of "free" circulating heparin, *i.e.*, molecules able to interact with the cationic protamine molecules. This method seems to be more adequate to determine the adverse effects of heparin–protamine complexes than measuring the anticoagulant activity of heparin. Anticoagulant activity of a given bolus dose of heparin measured by the ACT, activated partial thromboplastin time, or anti-Factor Xa concentrations persists much longer than plasma concentrations of circulating chemical heparin.[10,20-23] A lack of correlation between ACT and plasma heparin concentrations has been reported during cardiopulmonary bypass.[24] It has been suggested that heparin might cause a change in the proteins of the coagulation system that persists after the chemical heparin has disappeared.[10,22] Furthermore, there is no direct relationship between hemodynamic and hematologic effects of protamine reversal of heparin and anti-Factor Xa activity.[25]

These findings may explain the large variability of heparin–protamine-induced effects encountered in patients and the difference between the incidence of adverse response to protamine in human patients and experimental animal studies. Plasma half-life of heparin measured by its metachromatic activity in normal awake sheep was 61.2 ± 11.5 min (n = 5; fig. 7). This is similar to what has been reported by Estes in dogs[26] (56.6 ± 18.4 min) but shorter than that reported in human volunteers (87.5 ± 31.5 min).[26]

In contrast, plasma half-life of heparin measured by the Factor Xa inhibitor assay[27] or by the activated partial thromboplastin time[21] is remarkably variable among individual patients, ranging from 30 to 350 min for a single bolus injection (mean ± SD; 84 ± 71.5 min). This interindividual variability can be attributed to a variety of factors, including platelet number, platelet Factor IV availability, interference with fibrinogen or antithrombins, blood clotting factors, activation of fibrinolysis, renal clearance or hepatic metabolism, inactivation by antiheparin circulating substances, anesthesia, hypothermia, blood loss, and transfusion.

In patients, reversal of heparin anticoagulation is usually performed more than 1 h after the initial bolus injection of heparin. At this time, the plasma concentration of chemically active heparin is probably not high enough to induce a sufficient amount of heparin–protamine complexes able to lead to activation of the complement system and of the arachidonic acid cascade. This is true even if the anticoagulant activity of heparin measured by the ACT is sufficient to inhibit Factor Xa. This line of reasoning is supported by the results of our recent studies in sheep, demonstrating that a reproducible reaction to protamine in heparinized animals could be obtained provided protamine was injected a few minutes after initial bolus heparinization. When protamine was injected more than 1 h after heparin, the response was markedly attenuated and depended on residual anticoagulation.‡

The current study was not aimed at demonstrating the essential role of thromboxane during this situation since we already demonstrated that both indomethacin and a selective thromboxane synthetase inhibitor prevent the rise in plasma TxB_2 and the associated pulmonary hypertension in sheep.[9] Conversely, we did not measure the

‡ Unpublished data.

other vasoconstrictor prostanoids, PGD_2 or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), since these prostaglandins are much weaker vasoconstrictors than thromboxane A_2 (TxA_2); in similarly instrumented sheep in the previous study, plasma levels of these prostanoids were not increased after bolus injection of protamine in heparinized sheep.[9]

The source of thromboxane in this situation is not entirely clear. Platelets are a rich source of thromboxane and have been implicated in a large number of animal models of pulmonary hypertension. However, a recent study addressing the question of the role of platelets during heparin–protamine reaction has demonstrated that although sheep platelets *in vitro* will aggregate and synthesize and release large amounts of thromboxane when stimulated with thrombin, 99% depletion of circulating platelet concentration did not alter the magnitude and time course of acute pulmonary hypertension or the TxB_2 release in sheep.[28] Thus, at least in sheep, platelets seem not to be a major source of thromboxane following heparin–protamine interaction.

An intriguing possibility is that pulmonary intravascular macrophages (PIM) mediate or contribute to acute pulmonary arterial hypertension after heparin–protamine challenge. These PIM have been variably described in numerous cloven-hoofed animal species, including pigs and sheep.[29,30] They may play an important role in the pulmonary hemodynamic responses to infusion of foreign particles, including lipopolysaccharide and liposomes.[31] *In vitro* studies demonstrate that porcine PIM release numerous vasoactive prostanoids, including thromboxane.[32]

It is not entirely clear whether the complement system is a necessary pathway to produce increased TxB_2 concentrations following the neutralization of heparin with protamine. Infusion of activated complement in experimental animals is associated with the release of TxB_2; however, to our knowledge, the *in vivo* production of TxB_2 does not activate the complement system. Alternatively, heparin–protamine complexes may directly stimulate the arachidonic acid metabolism of PIM.

The applicability of the results of this study to the usual clinical situation may be limited by the animal model used. There are obvious differences in response to heparin neutralization by protamine between humans and sheep. Whereas in humans adverse reactions to neutralization of heparin anticoagulation with protamine sulfate consist of acute systemic hypotension associated with pulmonary hypertension and a decreased left atrial pressure, systemic vasodilation does not occur in sheep and pigs.[9,33] In dogs, protamine reversal of heparin anticoagulation causes both pulmonary artery hypertension and systemic vasodilation and hypotension documenting species-dependent variability of systemic hemodynamic responses to heparin–protamine challenge and indicating that systemic hemodynamic responses may be due in part to mechanisms other than those that cause pulmonary vasoconstriction. One explanation may be due to the large number of PIM in sheep that may indeed modify the mediator release during acute inflammatory reactions. In addition, the relative amount of mediators released from platelets (serotonin) and mast cells (histamine) in humans (as opposed to sheep) may also contribute to a different biologic and physiologic pattern of adverse response to protamine between these two species, particularly concerning the systemic vascular response.

The current study was also undertaken to document the response of left atrial pressure to the heparin–protamine challenge in sheep. The absence of changes in left atrial pressure in any of the infusion-rate protocols confirms that the increase in pulmonary capillary wedge pressure noted in sheep[9] does not indicate left heart decompensation but rather thromboxane-mediated pulmonary venoconstriction. Left atrial pressure did not decline during the vasoconstrictor response, which is different from reports of human studies,[1,17,34] showing decreased left atrial pressure on protamine-induced pulmonary vasoconstriction.

Using continuous measurement of pulmonary blood flow with a chronically implanted transonic flowmeter enabled us to precisely document and confirm the transient effect of the heparin–protamine challenge on cardiac output. Since systemic arterial blood pressure was conserved during the acute reaction, the transient reduction of cardiac output was compensated by a proportional increase in systemic vascular resistance, a finding that has also been shown in pigs,[34–37] but not in dogs[38,39] or humans.[17,34] The most likely reason for this species-related variation in systemic hemodynamics is attributable to the different systemic response to arachidonic acid metabolites in sheep (and pigs) compared to humans (and dogs). It seems that elevated plasma thromboxane concentrations in sheep are either more effective on the systemic vasculature or else not counteracted to the same degree in sheep as in humans by an associated production of the vasodilator prostacyclin, resulting in a better maintained systemic blood pressure and a better filling of the left ventricle and atrium in sheep. Another reason may be related to the kind and degree of anesthesia used in the reported patient studies that may alter the normal vascular responsiveness to mediator release.

Transient hypoxemia in the absence of significant changes in pulmonary mechanics suggests that this effect was most probably due to reversible alterations in adequate pulmonary vasomotor tone rather than to a decreased lung compliance, bronchoconstriction, or interstitial edema. These alterations of vasomotor tone could interfere with perfusion to well-ventilated areas, thereby inducing a mismatch of ventilation-perfusion ratio. In humans, adverse reactions to heparin–protamine interaction

are usually accompanied by transient bronchoconstriction, often the first clinical symptom to occur.[2,6,40] These clinical observations contrast with the findings of the present study and with the lack of reports on respiratory disturbances following protamine administration in animal experiments. The reasons for this discrepancy between animals and humans are not clear. Possible explanations include a different susceptibility of airway smooth muscle to TxA_2 or histamine, additional bronchoconstrictor mediators released in patients, or an increased incidence of true anaphylactic reactions in patients pre-exposed to protamine-containing insulin preparations.

Finally, our data clearly demonstrate that in sheep protamine administration on its own has no effects on any of the measured variables studied. This is similar to what has been reported in pigs,[33,35,36] cats, rabbits, guinea pigs,[13] and in human volunteers,[41] but not in dogs.[42,43]

In conclusion, the rate of intravenous protamine infusion in sheep is an important factor in the generation of sufficient mediators required to initiate a characteristic physiologic response, including systemic and pulmonary vasoconstriction, TxB_2 generation, hypoxemia, and leukopenia. Slowing the rate of protamine infusion results in a proportional attenuation of the response. This effect is most probably related to the amount of acutely formed circulating heparin–protamine complexes that secondarily initiate the generation of the vasoconstrictor TxA_2. Although sheep may be more reactive to the heparin–protamine challenge than humans, particularly because of the presence of large amounts of thromboxane generating pulmonary intravascular macrophages in this species, we remain convinced that the underlying mechanism leading to pulmonary hypertension in sheep is similar to that occurring in humans, although the physiologic pattern in humans may be modulated by additional factors. Additionally, in humans, the patient population usually has various cardiovascular diseases and protamine is injected immediately following cardiopulmonary bypass.

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