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Platelet Factor 4 Injection Produces Acute Pulmonary Hypertension in the Awake Lamb

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Background: **Reversal of heparin anticoagulation by intravenous protamine sulfate consistently produces acute pulmonary vasoconstriction mediated by the release of thromboxane in the awake lamb. Recently, recombinant platelet factor 4 (rPF4) has been cloned, expressed in *Escherichia coli,* and infused to reverse heparin anticoagulation in the rat, without producing adverse hemodynamic or pulmonary morphologic effects. The authors sought to learn whether intravenous administration of PF4 is devoid of side effects in the pulmonary circulation of lambs.**

Methods: **The authors evaluated the hemodynamic response and plasma release rates of thromboxane during intravenous challenges with heparin-rPF4 (n = 2), rPF-free carrier (n = 5), rPF4 (n = 5), rPF4 after indomethacin (n = 5), protamine (n = 5) and heparin-protamine (n = 5) in 17 awake, hemodynamically monitored lambs. Each lamb underwent up to three random challenges with a 2-h recovery period between each challenge.**

Results: **In two lambs, systemic anticoagulation with heparin followed by reversal of anticoagulation with an intravenous bolus of rPF4 (4 mg/kg) led to acute pulmonary vasoconstriction and hypertension with the release of thromboxane (peak pulmonary artery pressure [Ppa] 40 and 33 mmHg and peak plasma thromboxane B_2 50 and 30 ng/ml, respectively). Intravenous administration of rPF4 (1.5 mg/kg) alone increased the Ppa from 17.2 ± 0.7 mmHg (mean ± SEM) at baseline to 31.2 ± 2 mmHg at 1 min (n = 5, $P < 0.05$). This was associated with an increase of plasma thromboxane B_2 from 0.06 ± 0.02 to 3.96 ± 1.21 ng/ml. Acute pulmonary vasoconstriction lasted approximately 5 min and was completely prevented by pretreatment with oral indomethacin (10 mg/kg). Intravenous bolus administration of rPF4 carrier (n = 5) or protamine (2 mg/kg) alone (n = 5) did not induce pulmonary hypertension or the release of thromboxane. In five lambs, intravenous heparin (200 U/kg) followed by protamine (2 mg/kg) consistently produced acute pulmonary vasoconstriction and hypertension.**

Conclusions: **Intravenous injection of human rPF4 into the awake lamb produces acute pulmonary vasoconstriction and hypertension associated with thromboxane release into circulating blood. The effects of rPF4 on the pulmonary vasculature should be evaluated in primates before rPF4 is substituted for protamine in reversing heparin anticoagulation in humans. (Key words: Heparin. PF4. Pulmonary hypertension. Thromboxane.)**

This article is accompanied by a Highlight. Please see this issue of ANESTHESIOLOGY, page 31A.

PROTAMINE reversal of heparin anticoagulation can produce acute pulmonary vasoconstriction, leading to a markedly elevated pulmonary artery pressure and cardiovascular collapse.[1] This adverse cardiopulmonary reaction occurs consistently in the lamb, is mediated by increased pulmonary thromboxane A_2 synthesis, and can be blocked by pretreatment with the cyclooxygenase inhibitor indomethacin or a specific TxA_2 receptor blocker.[2,3]

Platelet factor 4 (PF4) is a high-affinity, heparin-binding protein that is released during platelet aggregation. Recently, a purified form of human recombinant platelet factor 4 (rPF4) with the amino acid sequence of native PF4 has been synthesized.§[4] The DNA sequence was determined, cloned, and expressed in *E. coli.* Platelet factor 4 was then purified, and this cloned and purified rPF4 preparation was found to neutralize heparin efficiently *in vitro.*[5,6] Intravenous administration of PF4 to reverse heparin anticoagulation was without adverse effects in the rat.[7]

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§ Myers JA, Gray GS, Peters DJ, Grimaila RJ, Hunt AJ, Maione TE, Mueller WT: Expression and purification of active recombinant platelet factor 4 from a cleavable fusion protein. Protein Expression and Purification 2:136–143, 1992.

The purpose of the current study was to learn whether intravenous infusion of rPF4 in the awake, hemodynamically monitored lamb is free of adverse pulmonary and systemic hemodynamic effects.

Materials and Methods

Seventeen Suffolk lambs weighing 25–35 kg were used for our studies. The studies were approved and all lambs were cared for in accordance with the rules of the Massachusetts General Hospital Subcommittee on Research Animal Care. Using sterile techniques, an 8-Fr introducer was placed percutaneously into the jugular vein of each lamb after 1% xylocaine infiltration anesthesia. The lamb was then anesthetized *via* mask with 1–3% halothane (Halocarbon Laboratories, Hackensack, NJ) in O_2 and the trachea was intubated. The right femoral artery was cannulated and the catheter tip was advanced to the midthoracic aorta to sample blood and monitor systemic blood pressure (Psa). After awakening, a sterile 7-Fr flow-directed thermodilution catheter (American Edwards model 93A-131H-7F, Santa Ana, CA) was positioned with the tip in the pulmonary artery to measure mean pulmonary artery pressure (Ppa), balloon occlusion pressure (Pw), central venous pressure (Pv), thermodilution cardiac output (CO), and core blood temperature (T). The jugular venous catheter was used for fluid and drug administration. During the study, the lambs were housed in a Babraham veterinary cage with free access to food and water.

Exclusion Criteria

Lambs were not studied if they exhibited one or more of the following baseline criteria indicating preexisting illness: 1) pulmonary artery temperature > 40.0°C, 2) arterial WBC concentration > 12,000 or <3,000/mm^3, or 3) mean Ppa > 20 mmHg.

The Psa, Pv, Ppa, and Pw were measured and recorded continuously using calibrated Hewlett-Packard model 1280C pressure transducers and a Hewlett-Packard model 7754B recorder (Hewlett-Packard, Palo Alto, CA). Transducers were zeroed at the level of the right atrium and mean values measured at end expiration. Cardiac output was determined by thermodilution (Edwards Laboratories model 9520A cardiac output computer, Irvine, CA) using an average of three right atrial injections of 5 ml of 0°C saline at each sample time (−6, −1, 1, 2, 3, 5, 10, and 20 min).

At selected times (−6, −1, 1, 2, 3, 5, 10, and 20 min), 4 ml of arterial blood were collected in glass test tubes containing 0.5 ml of 15% EDTA and 100 μg of indomethacin, and were immediately transferred to ice. After centrifugation (2,000*g* for 10 min at 4°C), plasma was aspirated and stored at −70°C in polypropylene tubes. Thromboxane B_2 analyses were performed by radioimmunoassay.[8]

Other Variables

Total blood white cell (WBC) counts were determined using a Coulter counter (model Zf; Coulter Electronics, Hialeah, FL) at −6, −1, 1, 2, 3, 5, and 10 min. Activated clotting time (ACT) was measured by a Hemochron 400 system (International Technidyne, Edison, NJ); heparin assay, prothrombin time, partial thromboplastin time, and fibrinogen were measured at −6, −1, and 5 min by standard tests.[9–12] Arterial blood gas tensions and *p*H were measured with a polarograph (model M238; Corning Medical and Scientific, Medfield, MA) at −6, −1, 2, 3, 10, and 20 min.

Experimental Protocol

Recombinant platelet factor 4 in a concentration of 4.56 mg/ml was supplied by the Repligen Corporation (Cambridge, MA). The preparation was free of endotoxin (less than 0.05 endotoxin units/mg by standard limulus assay). The carrier solution consisted of 10 mM sodium acetate with a *p*H of 5.0 and 150 mM sodium chloride. Porcine-derived heparin (1,000 U/ml; Elkins-Sinn, Cherry Hill, NJ) and protamine sulfate (10 mg/ml; Lilly, Indianapolis, IN) were obtained from commercial sources. Indomethacin capsules (25 mg/capsule; Merck Sharp Dohme, West Point, PA) were opened and the contents suspended in 50 ml of water before administration *via* an orogastric tube.

Treatment Groups

Each lamb was allowed to recover after surgery for 2 h before intravenous challenge. Two lambs were studied by anticoagulation with heparin (200 U/kg) intravenously at −5 min, followed by reversal with rPF4 (4 mg/kg) intravenously at 0 min. Subsequent intravenous doses of PF4 were reduced to 1.5 mg/kg because of the limited supply of rPF4. The remaining challenges were divided into five groups and administered at random:

- Group 1 (saline/rPF4, n = 5 challenges): normal saline (10 ml intravenously) at time −5 min, followed by rPF4 (1.5 mg/kg intravenously) at time 0 min.
- Group 2 (saline/carrier, n = 5 challenges): normal saline (10 ml intravenously) at time −5 min, fol-

lowed by rPF4 diluent buffer (carrier without rPF4, 10 ml intravenously) at time 0 min.

- Group 3 (saline/protamine, n = 5 challenges): normal saline (10 ml intravenously) at time −5 min, followed by protamine (2 mg/kg intravenously) at 0 min.
- Group 4 (indomethacin/saline/rPF4, n = 5 challenges): indomethacin pretreatment (10 mg/kg *via* nasogastric tube 2 h before the experiment), saline (10 ml intravenously) at time −5 min, followed by rPF4 (1.5 mg/kg intravenously) at 0 min.
- Group 5 (heparin/protamine, n = 5 challenges): heparin (200 U/kg intravenously) at time −5 min, followed by protamine (2 mg/kg intravenously) at 0 min.

Each lamb underwent a maximum of three challenges with a 2-h recovery period between each challenge. The order of the challenges was determined at random. Indomethacin-pretreated lambs underwent only one challenge.

Statistical Analysis

All data are expressed as mean ± SEM. Differences over time within or among study groups were evaluated by ANOVA for repeated measures as implemented in the SAS statistical program (Version 6.0; SAS Institute Inc., Cary, NC). Depending on the variable, different sets of planned comparisons were tested individually with significance declared at an α level of 0.05 after a Bonferroni correction.

Results

Pulmonary Hemodynamics

In the two initial studies, reversal of heparin anticoagulation with rPF4 led to severe acute pulmonary hypertension (peak Ppa 40 and 33 mmHg).

Intravenous administration of 1.5 mg/kg rPF4 alone (group 1) increased the Ppa from 17.2 ± 0.7 mmHg (mean ± SEM) at baseline to 31.2 ± 2 mmHg at 1 min ($P < 0.05$, fig. 1). The degree of peak Ppa hypertension was comparable with the magnitude of the acute pulmonary hypertension in the heparin-protamine reversal reaction in group 5 (fig. 1, P = NS peak values differ). Neither injection of the carrier alone (group 2) nor intravenous injection of protamine alone without heparin pretreatment (group 3) produced pulmonary hypertension. Pretreatment with indomethacin (group 4) completely blocked the pulmonary vasoconstriction after the injection of PF4 alone (fig. 1).

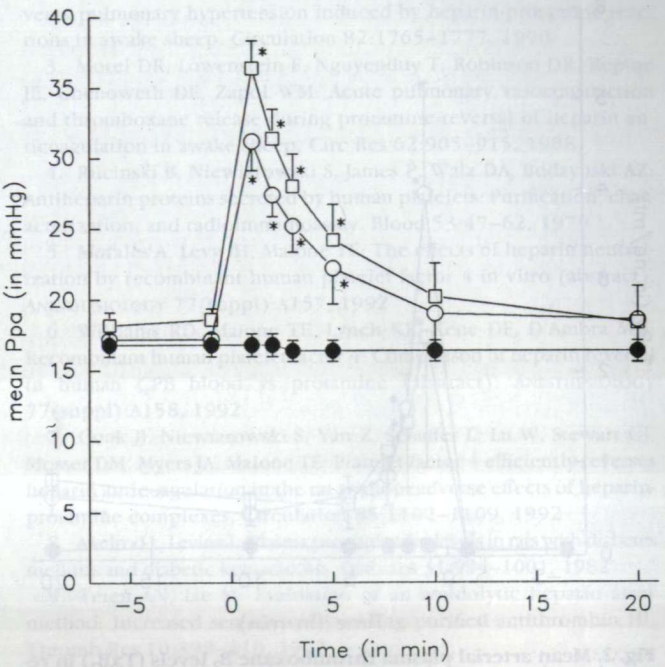

Fig. 1. Mean pulmonary artery pressure (Ppa) response to a bolus injection of 1.5 mg/kg rPF4 (at time 0 min, open circles), 2 mg/kg protamine (at time 0 min, open squares) in heparinized lambs (2 mg/kg given at time −5 min), or 1.5 mg/kg rPF4 (at time 0 min, filled circles) in lambs pretreated with 10 mg/kg indomethacin. Values are mean ± SEM, n = 5. *$P < 0.05$ value differs from baseline.

Plasma Levels of Thromboxane B_2

Reversal of heparin anticoagulation with rPF4 produced a marked elevation of plasma thromboxane B_2 levels concomitant with the episode of transient acute pulmonary vasoconstriction. Plasma TxB_2 levels increased from <0.25 ng/ml to 50 and 30 ng/ml in the two lambs studied. The injection of rPF4 alone (group 1) was associated with increased plasma TxB_2 levels from 0.06 ± 0.02 ng/ml to 3.96 ± 1.21 ng/ml at 1 min (fig. 2). This was comparable with plasma TxB_2 levels at 1 min in the heparin protamine neutralization animals (group 5, fig. 2, P = NS values differ). Pretreatment with indomethacin (group 4) completely prevented the increase of arterial plasma TxB_2 levels (fig. 2). Injection of carrier (group 2) or protamine alone (group 3) did not increase plasma TxB_2 levels.

Systemic Hemodynamics

Injection of rPF4 (group 1) led to transient systemic vasoconstriction (a 75% increase of SVR from the base-

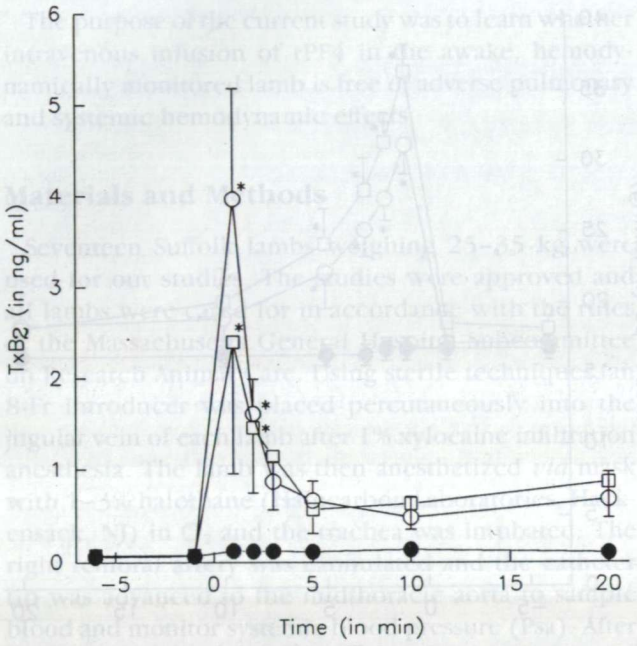

Fig. 2. Mean arterial plasma thromboxane B_2 levels (TxB_2) in response to a bolus injection of 1.5 mg/kg rPF4 (given at time 0 min, open circles), or 2 mg/kg protamine (given at time 0 min, open squares) in heparinized sheep (2 mg/kg given at time −5 min), or 1.5 mg/kg rPF4 (given at time 0 min, filled circles) in lambs pretreated with 10 mg/kg indomethacin. Values are mean ± SEM, n = 5. *$P < 0.05$ value differs from baseline.

line level at 1 min) with brief, mild systemic hypertension (10% at 1 min, data not shown). The cardiac output was transiently decreased by about 40% at 1 min. These systemic hemodynamic changes were completely prevented by pretreatment with indomethacin (group 4). The systemic hemodynamic measurements in all the other groups did not change significantly during challenge (data not shown).

Other Variables

The circulating leukocyte concentration and Pa_{O_2} did not change in animals given intravenous rPF4 alone (data not shown). In both studies of intravenous PF4 reversal of heparin anticoagulation, PF4 was found to rapidly and completely reverse heparin's prolonging effect on the activated clotting time, prothrombin, and partial thromboplastin time, without any change of plasma fibrinogen levels (data not shown).

Discussion

This study demonstrates that rPF4, administered intravenously at a dose of 1.5 mg/kg into the awake lamb, produces an acute and marked elevation of pulmonary vascular resistance and pulmonary artery pressure. The magnitude of these increases was similar to that measured after protamine reversal of heparin anticoagulation in the lamb. These findings are surprising in view of the recently reported efficacy and hemodynamic safety of rPF4 reversal of heparin anticoagulation in the rat.[7]

Injection of rPF4 alone produced a marked and rapid increase of arterial plasma TxB_2 concentration that was concomitant with the increase of Ppa and PVR. This reaction appears similar to that occurring after heparin-protamine reversal in lambs. It appears that, in the case of heparin and protamine, it is the interaction of the polyanions (*e.g.,* heparin) and polycations (*e.g.,* protamine) to form complexes that activate complement *via* the classical pathway. In the case of rPF4, however, the reaction occurs without prior heparin induced anticoagulation and, therefore, is likely to be caused by the rPF4 molecule itself. The buffer solution used as a carrier for rPF4 did not provoke this adverse drug reaction in the lamb.

The degree of acute pulmonary vasoconstriction produced by rPF4 is comparable with that of the heparin-protamine reaction and is associated with a significant depression of cardiac output. However, hypoxemia and neutropenia did not occur after injecting 1.5 mg/kg rPF4, indicating that complement was not activated by the dose of rPF4 that we injected.

Inhibiting the production of thromboxane A_2 by pretreating the lambs with the cyclooxygenase inhibitor indomethacin prevented both the increase of plasma TxB_2 and pulmonary vasoconstriction after rPF4 injection. The precise cellular source of thromboxane in these drug reactions remains uncertain. Pulmonary artery hypertension induced by the heparin-protamine reaction has been documented in platelet depleted lambs,[13] as well as in cat lungs perfused with acellular dextran.[14] It has been suggested that the resident pulmonary intravascular macrophages produce thromboxane either directly or *via* stimulation of one or more other cell types.[15] There are large numbers of pulmonary intravascular macrophages in sheep.[16] These cells may release vasoactive mediators after acute stimulation with a foreign protein. Sheep and other *artyodactylae,* for example, are significantly more responsive to the intravascular infusion of liposomes than rats.[17] Rats and humans have far fewer pulmonary intravascular macrophages and, thus, may not be sensitive to intravenous rPF4 injection. However, some humans may have greater numbers of pulmonary intravascular macro-

phages, and they may well react adversely to the intravenous injection of PF4. Catastrophic pulmonary vasoconstriction after heparin-protamine neutralization in humans is a sporadic adverse response and, thus, many patients will have to be studied to reliably document whether this reaction occurs with heparin-rPF4 neutralization.

It is possible that the structural differences between human and lamb PF4 may be responsible for the marked stimulation of the arachidonic acid cascade and thromboxane release that we demonstrated in the lamb. Human rPF4 contains 70 amino acid residues and has a molecular weight of 7,800.[18] Complete amino acid sequences for human, rat, bovine, and rabbit PF4 have been described. The nonhuman PF4 proteins have additional amino acids at the N terminus that may make them differ immunologically.[19] Platelet factor 4 has considerable amino acid homology with several endogenous peptide cytokines, including connective tissue-activating peptide (Ctap)-III and their relatives IP-10 and interleukin 8.[19] Recombinant PF4 has been shown to bind to interleukin-8 receptors and activate neutrophils when its N-terminus is modified.[20] However, we did not find a leukopenia after rPF4 injection. It is possible that the sequence and structural differences between human and sheep PF4 may be responsible for the thromboxane release that we demonstrated in the lamb. Because we did not measure transient leukopenia after injection, it is unlikely that complement activation is directly involved in this ovine reaction.

This study demonstrates that intravenous injection of human rPF4 into the lamb causes the consistent release of large quantities of thromboxane into plasma and produces severe pulmonary vasoconstriction. The effects of rPF4 injection into other species, including primates and, especially, humans, should be carefully investigated in large numbers of patients before rPF4 is clinically adopted to reverse heparin anticoagulation.

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