REVERSAL OF HEPARIN ANTICOAGULATION BY RECOMBINANT PLATELET FACTOR 4 AND PROTAMINE SULFATE IN BABOONS DURING CARDIOPULMONARY BYPASS

The ability of recombinant platelet factor 4 and protamine to neutralize heparin after cardiopulmonary bypass was compared in anesthetized baboons. Clotting titration curves of heparinized baboon blood demonstrate an anticoagulant effect of protamine that is not seen with recombinant platelet factor 4. Neither drug caused meaningful changes in central pressures or cardiac output within 30 minutes after injection. After 30 minutes of cardiopulmonary bypass, recombinant platelet factor 4 normalized thrombin times and activated partial thromboplastin times within 5 minutes of injection, but protamine did not. Neither drug altered bleeding times. Recombinant platelet factor 4 caused a species-specific leukopenia in baboons and significantly increased activated complement protein 3 (C3a) more than protamine. However, the increase in plasma C3a was small and neither drug caused a significant increase in plasma neutrophil elastase- α_1 proteinase inhibitor complex. We conclude that recombinant platelet factor 4 is effective and safe in baboons, does not have an anticoagulant effect with excess concentration, and reverses *in vivo* heparin more rapidly than protamine. The data support progression to a clinical trial. (J THORAC CARDIOVASC SURG 1995;109:765-71)

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Protamine sulfate is universally used to neutralize heparin after cardiopulmonary bypass (CPB), but it is associated with a high incidence of adverse reactions.¹⁻³ The protamine-heparin complex activates complement by the classic pathway^{4,5} and generates the anaphylatoxins C3a, C4a, and C5a.⁵ These vasoactive peptides or possible histamine release (or both)^{6,7} may cause transient hypotension

that occurs 30 to 150 seconds after injection in approximately half of patients.³ Other adverse reactions, which are occasionally life-threatening or fatal, involve immunoglobulin G and immunoglobulin E antibodies⁸ or synthesis and release of thromboxane A₂ by platelets and pulmonary macrophages.^{9,10}

Platelet factor 4 (PF4) is a protein stored in platelet α -granules. The protein contains 70 amino acids (molecular weight = 7800) and binds with high affinity to heparin (see reference 11 for references). In contrast to the ionic bond between positively charged protamine and negatively charged heparin, PF4 binds and neutralizes heparin by means of a specific binding site at the C-terminus of PF4.¹¹ PF4-heparin complex is cleared by liver and kidney.¹² Recombinant PF4 (rPF4) is expressed as a fusion protein in *Escherichia coli* and has the same amino acid composition and terminal sequence as native PF4 after chemical cleavage and purification.¹³ Cook and associates¹⁴ demonstrated that rPF4 reverses heparin anticoagulation in rats without the adverse effects of protamine sulfate. Both rPF4 and protamine sulfate reverse heparin anticoagulation in the rat and affect heparin plasma clearance similarly; however, in contrast to rPF4, elevated levels

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of protamine sulfate induce paradoxical anticoagulation.¹⁵

We compared the efficacy, safety, and side-effects of rPF4 and protamine for neutralizing heparin in baboons with and without CPB.

Methods

Nine young adult female baboons (*Papio anubis*) were used in 24 studies. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (IACUC No. M-900618). After an overnight fast, the animals were sedated with ketamine (10 mg/kg intramuscularly), anesthesia was induced with thiopental (2.0 mg/kg intravenously), and the animals were intubated. Anesthesia was maintained with 1.5% to 2.0% isoflurane. Heart rate, arterial blood pressure, and rectal temperatures were continuously monitored by electrocardiogram (ES-1000, Gould Inc., Cleveland, Ohio), femoral arterial catheter, and rectal probe. A second femoral arterial catheter was placed for blood samples. Pulmonary arterial and central venous pressures were measured continuously (5F Swan-Ganz catheter; Baxter Healthcare Corp., Edwards Division, Santa Ana, Calif.); pulmonary capillary wedge pressure and thermodilution cardiac output (in triplicate) (Oximetrix 3 SO₂/CO computer, Abbott Laboratories, North Chicago, Ill.) were measured intermittently.

Clotting time titration curves were performed by means of activated partial thromboplastin times (Organon Teknika Corp., Durham N.C.) to determine the effects of various doses of protamine (Elkins-Sinn Corp., Cherry Hill, N.J.) and rPF4 (Repligen Corp., Kendall Square, Mass.) to neutralize heparin in baboon plasma.

In 13 trials without CPB, heparin (100 U/kg intravenously; porcine intestinal, Elkins-Sinn Corp.) was given, followed after 5 to 10 minutes by either protamine (1 mg/kg intravenously, $n = 7$) or rPF4 (2 mg/kg intravenously, $n = 6$). Hemodynamic measurements and blood samples for hematocrit value, white count, platelet count, platelet aggregation, and activated clotting time were obtained before heparin, after heparin, and 5 and 30 minutes after protamine or rPF4. Template bleeding times were measured before heparin, after heparin, and after neutralization.

In 12 additional trials under sterile conditions, anesthetized baboons received heparin (300 U/kg, intravenously) and were cannulated for partial CPB. Incisions were made over the right jugular vein and a femoral artery. Under direct vision, a catheter (8F to 10F, Medtronic Bio-Medicus, Eden Prairie, Minn.) was inserted over a wire into the right atrium. A similar 8F catheter was inserted into the femoral artery. The perfusion circuit had a surface area of 0.9 m² and consisted of a roller pump, 0.8 m² spiral coil membrane oxygenator (Avecor Inc., Plymouth, Minn.), polyvinyl chloride venous reservoir, polycarbonate connectors, and 1/4-inch inner diameter silicone rubber tubing. The oxygenator was ventilated with 95% oxygen and 5% carbon dioxide at a rate of 2 L/min. The circuit was primed with 350 ml Normosol-R solution. Flow rate was 50 ml/kg per minute at 37° C for 30 minutes. Normal saline solution (3:1 volume) was given to replace

blood samples and shed blood. At the end of CPB either protamine sulfate (3 mg/kg) or rPF4 (6 mg/kg) was given to reverse the anticoagulant effect of heparin.

The electrocardiogram, systemic and pulmonary arterial pressures, central venous pressure, and rectal temperature were monitored continuously as described earlier. Pulmonary capillary wedge pressure and triplicate thermodilution cardiac output measurements were made before heparin, just before stopping CPB, 10 minutes before heparin neutralization, and 5 and 30 minutes after neutralization of heparin. Template bleeding times and blood samples for hematocrit value, white cell count, platelet count, platelet aggregation, plasma β -thromboglobulin (BTG), activated clotting time, thrombin time, partial thromboplastin time, C3a des-Arg, and neutrophil elastase- α_1 proteinase inhibitor complex were obtained at the same times.

Hematocrit value was determined by centrifugation. Platelet and white cell counts were performed within a hemocytometer under phase microscopy. The percentages of circulating leukocyte subpopulations were determined by performing a differential cell count of 100 cells and a Wright-stained peripheral blood smear. Activated clotting time was measured in a chronometer (Chrono-Log Inc., Havertown, Pa.). Activated partial thromboplastin time was measured with a Coag-a-mate X2 and automated activated partial thromboplastin time reagent (Organon Teknika Corp.). Thrombin time was measured with Thromboquik reagent (Organon Teknika Corp.) and a fibrometer, according to the manufacturer's instructions. Template bleeding times (Simplate II, Organon Teknika Corp.) in duplicate were measured using the shaved ventral surface of the forearm and a blood pressure cuff at 40 mm Hg. Complement activation was determined by measuring plasma levels of C3a des-Arg, a stable metabolite of C3a, by radioimmunoassay (Amersham Corp., Arlington Heights, Ill.). Plasma neutrophil elastase- α_1 antitrypsin complex was measured by enzyme-linked immunosorbent assay (Merck immunoassay, EM Sciences, Gibbstown, N.J.). Plasma BTG was measured with recombinant neutrophil activating peptide 2, a derivative of BTG, with tyrosine inserted at the C-terminal end (Repligen Corp.) as a tracer. The tracer had the same antigenic reactivity as original BTG.

Platelet-rich plasma and platelet-poor plasma were prepared by differential centrifugation at 150 g for 10 minutes and 13,600 g for 5 minutes, respectively. Platelet aggregation studies in platelet-rich plasma (platelet count was adjusted to 150,000 μL^{-1} by dilution with platelet-poor plasma) were performed in an aggregometer (model 340, Chrono-Log Inc.) The threshold dose of adenosine diphosphate (i.e., the lowest dose of agonist able to produce irreversible aggregation of at least 60% light transmission of platelet-poor plasma in 5 minutes) was determined. The same dose of adenosine diphosphate was used to determine percent aggregation of baboon platelets in all subsequent samples. Results are reported as a percentage of the normalized 100% control sample in arbitrary light transmission units.

For calcium mobilization studies baboon and human neutrophils were isolated and loaded with fura 2.¹⁶ Mo-

Fig. 1. Clotting time titration curves (partial thromboplastin times) after reversal of heparin anticoagulation in vitro (A) or heparinized baboon blood ex vivo (B). A, Protamine or rPF4 was added in the doses indicated to citrated baboon plasma containing a heparin concentration of 0.6 units/ml. B, A heparin dose of 300 units/kg body weight was injected intravenously into a baboon; five minutes later blood was drawn into citrate and neutralized with indicated doses of each drug.

bilization of cytosolic calcium was tested by the method of Gryniewicz, Poenie, and Tsien.¹⁷

Statistics. Data from both trials were compared by two-way analysis of variance with the Bonferroni correction and paired Student's *t* statistic for within- and between-group comparisons after CPB and at 5 and 30 minutes after neutralization.

Results

Baboon plasma appears to be at least two times more sensitive to heparin than human or rat plasma (data not shown). Fig. 1, A shows that baboon plasma containing a heparin concentration of 0.6 units/ml clots in 220 seconds. Reversal of heparin anticoagulation (clotting time below 50 seconds) occurs at protamine sulfate concentrations between 62 and 125 $\mu\text{g/ml}$ or rPF4 concentrations between 125 and 1000 $\mu\text{g/ml}$. These data are consistent with our previous observations in human and rat plasma,^{12,13} neutralizing concentrations of rPF4 are twice those of protamine sulfate. In contrast to protamine sulfate, however, an excess of rPF4 does not produce any anticoagulant effect.

Fig. 1, B shows the effect of protamine sulfate and rPF4 on the partial thromboplastin time of baboon plasma after the animal received a heparin dose of 300 units/kg. Clotting times are plotted against various concentrations of protamine and rPF4. The

calculated heparin concentration is 37.5 $\mu\text{g/ml}$; this concentration of protamine completely neutralizes this amount of heparin, but higher concentrations produce an anticoagulant effect. Considerably higher concentrations of PF4 are needed to neutralize heparin, but rPF4 does not show any anticoagulant effect at high concentrations.

Data from initial studies without CPB show only a few differences between drugs. Both drugs restore activated clotting times to the normal range within 5 minutes. Protamine causes significant ($p < 0.05$) decreases in mean systemic (73.9 ± 5.0 to 64.5 ± 4.0 mm Hg) and pulmonary arterial (15.0 ± 1.7 to 13.6 ± 1.0 mm Hg) pressures and pulmonary capillary wedge (10.4 ± 1.6 to 9.0 ± 1.0 mm Hg) pressures 5 minutes after injection. Systemic hypotension after protamine persists for at least 30 minutes and is significantly different from the results after rPF4 ($p < 0.01$). Both drugs cause a temporary decrease in leukocyte count at 5 minutes, and the decrease after rPF4 (9833 ± 1127 to 2791 ± 395) is significantly greater than after protamine. Other changes and differences are not significant within or between groups.

In vitro neutrophil calcium mobilization tests^{16,17} were done to explain the leukopenia after injection of rPF4 in baboons. rPF4 slightly, but significantly,

Fig. 2. In vitro calcium mobilization study for baboon and human neutrophils. Recombinant human neutrophil activating peptide 2 (NAP_2) (Repligen Corp.) has potent neutrophil activating properties^{15, 17} and served as a control. rPF4 significantly increases baboon neutrophil calcium, but does not change human neutrophil calcium. rNAP-2 dramatically increases leukocyte calcium in both species. Open bars, Baboon neutrophils; shaded bars, human neutrophils. Values are mean $\pm$ standard error. * $p < 0.05$ between protamine and rPF4. ** $p < 0.05$ within groups as compared with control values.

mobilizes calcium in baboon, but not human, neutrophils (Fig. 2). Neutrophils of both species respond similarly to recombinant neutrophil activating peptide 2.¹⁸

After CPB, both drugs caused systemic hypotension at borderline significance ($p = 0.06$), but this hypotension persisted and reached statistical significance in protamine-treated animals (Table I). At 5 minutes, both drugs normalized activated clotting times, but only rPF4 normalized partial thromboplastin and thrombin times (Fig. 3). The inability of protamine to completely neutralize heparin immediately was due to its anticoagulant effect, as demonstrated by protamine and rPF4 titration of heparinized baboon plasma (Fig. 1, A and B). Both drugs caused a transient leukopenia at 5 minutes, and both drugs significantly increased plasma C3a. The increase after rPF4 was slightly greater than the increase after protamine at both times. However, release of neutrophil elastase- α_1 proteinase inhibitor complex was the same in both groups and did not increase after either drug. Neither drug had any effect on platelet count. Baboon platelets were less

responsive to thrombin, adenosine diphosphate, and collagen than were human platelets (data not shown). Unlike human beings, baboons did not have an increase in plasma BTG during CPB. Bleeding times increased after CPB but were not affected by either drug.

Discussion

This study demonstrates the efficacy and safety of rPF4 for neutralizing heparin after CPB in baboons. rPF4 more rapidly reverses thrombin and partial thromboplastin times than does protamine and in contrast to protamine does not have an anticoagulant effect in excess doses. As observed in rats,¹⁵ the slower antiheparin effect of protamine may be due to the anticoagulant effect of the drugs or to slower inhibition of the anti-factor Xa effect of heparin.¹⁹ Neither drug affects platelets; therefore, bleeding times remain elevated after CPB with both.

In baboons both drugs cause a transient leukopenia that is more profound with rPF4. This leukopenia does not occur in the rat.¹⁴ To explain the rPF4-induced neutropenia in baboons, we examined

Table I. Changes in hemodynamics and blood constituents during CPB in baboons

	Before heparin	CPB	After CPB	Five minutes	Thirty minutes
Mean SAP (mm Hg)					
PS	71.2 ± 3.9		70.3 ± 5.8	63.6 ± 3.2	63.1 ± 3.3*
rPF4	69.6 ± 6.1		64.8 ± 4.2	59.8 ± 5.2	63.1 ± 4.1
Mean PAP (mm Hg)					
PS	12.8 ± 1		13.6 ± 1.8	13.6 ± 1.6	11.8 ± 1.1
rPF4	13 ± 0.8		13.4 ± 0.9	13.4 ± 1.1	12.9 ± 0.9
CVP (mm Hg)					
PS	4.7 ± 1.7		6.6 ± 2.3	5.9 ± 3.0	4.1 ± 2.0†
rPF4	5.2 ± 2.5		6.3 ± 3.2	6.0 ± 2.7	5.5 ± 2.7
PCWP (mm Hg)					
PS	10.4 ± 4.5		10.6 ± 4.1	10.3 ± 3.7	8.3 ± 2.6
rPF4	9.2 ± 2.3		11.0 ± 4.1	9.5 ± 2.4	9.7 ± 2.1
Cardiac output (L/min)					
PS	1.8 ± 0.2		1.5 ± 0.2	1.6 ± 0.2	1.4 ± 0.2
rPF4	1.7 ± 0.2		1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
Activated clotting time (sec)					
PS	96 ± 2	467 ± 12	409 ± 13	115 ± 3*	118 ± 6.2*
rPF4	99 ± 3	446 ± 18	394 ± 19	117 ± 6*	115 ± 7*
Thrombin time (sec)					
PS	11.2 ± 0.5	180 ± 0	180 ± 0	88 ± 25.2*†	13.5 ± 0.7*
rPF4	11.2 ± 0.3	180 ± 0	180 ± 0	12.6 ± 0.4*	12.6 ± 0.6*
PTT (sec)					
PS	35.4 ± 1.5	150 ± 0	150 ± 0	106.6 ± 11.6*†	45.4 ± 2.9*
rPF4	35.3 ± 2.4	150 ± 0	150 ± 0	42 ± 4.7*	41.3 ± 2.0*
Platelet count (Plts/ μ l) $\times$ 1000					
PS	254 ± 13.6	192.1 ± 20.8	187.7 ± 19.7	172.9 ± 12.3	183.3 ± 18.2
rPF4	273.8 ± 17.4	197.3 ± 17.5	196.5 ± 17.9	184.8 ± 17.4*	211.8 ± 19.4*
Platelet aggregation (% of maximum)					
PS	60 ± 3.4	65.9 ± 3.3	64.6 ± 1.6	59.1 ± 4	65.3 ± 3.2
rPF4	66.9 ± 4.6	62.1 ± 4.4	56.3 ± 4.3	53.5 ± 4.1	58.8 ± 2.9
BTG (ng/ml)					
PS	131 ± 16	134 ± 7.8	108 ± 13	105 ± 12	79 ± 10*‡
rPF4 ± 121 ± 19	121 ± 19	128 ± 13	108 ± 11	114 ± 13	131 ± 14
Bleeding time (min)					
PS	4.5 ± 0.3	12.1 ± 2.4		11.9 ± 2.4	
PF4	4.3 ± 0.2	15.2 ± 2.6		15.1 ± 1.4	
Hematocrit (%)					
PS	35.4 ± 0.7	26.4 ± 0.8	28.1 ± 0.9	28.4 ± 0.8	29.7 ± 1*
PF4	36.2 ± 1.5	27.5 ± 1.5	29.2 ± 2.1	28.7 ± 1.7	30.2 ± 2.2
C3a (ng/ml)					
PS	76 ± 5.7	104.9 ± 8.2	72 ± 4.2	99.4 ± 5.5*†	84 ± 6.2*†
PF4	89.7 ± 13.2	118.7 ± 8.5	91.3 ± 7.7	150 ± 3.5*	118.7 ± 3*
Elastase- α 1 proteinase inhibitor complex (μ g/ml)					
PS	5.45 ± 0.58	5.69 ± 0.76	5.28 ± 0.72	5.53 ± 0.86	5.51 ± 0.62
PF4	3.84 ± 0.46	5.43 ± 0.36	6.09 ± 0.68	6.27 ± 0.64	6.38 ± 0.97
White count (cells/ μ l) $\times$ 1000					
PS	6.7 ± 2.1	4.0 ± 1.0	6.7 ± 0.8	3.9 ± 0.9*	7.3 ± 0.6
PF4	4.6 ± 1.1	3.7 ± 0.9	5.6 ± 0.7	2.1 ± 0.3*	7.6 ± 0.6

Values are means $\pm$ standard errors before, during, and after cardiopulmonary bypass (CPB). Five minutes and thirty minutes refer to times after heparin neutralization. SAP, Systemic arterial pressure; PAP, pulmonary artery pressure; CVP, central venous pressure; PCWP, pulmonary capillary wedge pressure; PTT, partial thromboplastin time; BTG, β -thromboglobulin; PS, protamine sulfate ($n = 6$); rPF4, recombinant platelet factor 4 ($n = 6$).

* $p < 0.05$ within-group value compared with After CPB value.

† $p < 0.05$ for between-group values.

‡ $p < 0.01$ for between-group values.

the effect of rPF4 on the mobilization of cytosolic calcium in baboon and human neutrophils. rPF4 (3 μ mol/L) did not increase calcium mobilization in human neutrophils but caused a slight, but signifi-

cant, mobilization of calcium in baboon neutrophils. Neutrophils of both species showed the same sensitivity to neutrophil activating peptide 2 (Fig. 2). Because infusion of interleukin-8 (IL-8) in baboons

Fig. 3. Thrombin times and partial thromboplastin times before and after heparin neutralization by protamine (solid bars) and rPF4 (lighter bars) after CPB in baboons. **A**, Thrombin times. **B**, Partial thromboplastin times. Statistics appear in Table I.

causes a transient neutropenia²⁰ and because human rPF4 substituted with three amino acids (Glu-Leu-Arg) at its N-terminus activates neutrophils in a similar manner to IL-8,²¹ the neutropenia in baboons may result from the effect of human rPF4 on baboon IL-8 receptors. The data suggest that rPF4-induced leukopenia is species specific, acts via IL-8 neutrophil receptors, and is unlikely to cause leukopenia in human beings. rPF4 in baboons also increases plasma C3a more than does protamine. The actual amount of increase is not great and neutrophil elastase does not increase appreciably with either drug. The impact of rPF4 neutralization of heparinized human blood on C3a generation cannot be inferred from this study. In heparinized rats protamine causes leukopenia, thrombocytopenia, significant activation of complement, and

pulmonary interstitial edema, but rPF4 does not alter any of these parameters.¹⁴

Although this form of rPF4 has an amino acid sequence identical to that of the native peptide, the possibility of antibody formation and subsequent anaphylaxis with reexposure cannot be ruled out. Recently antibodies to heparin-PF4 complexes have been described in patients with heparin-associated thrombocytopenia.²² This concern and that of complement activation must be resolved in human trials.

From this study we conclude that rPF4 effectively neutralizes heparin after CPB and acts faster than does protamine. rPF4 does not have an anticoagulant effect with excess concentrations. The drug does not affect cardiac function or platelets, but does mildly activate complement. The data support a

clinical trial to resolve concerns raised by possible complement activation and antibody formation.

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Discussion

Dr. Andrew S. Wechsler (*Richmond, Va.*). Did you investigate C4a on the chance that the combination of PF4 and heparin would activate the extrinsic pathway?

Dr. Bernabei. No, we did not.

Dr. Wechsler. Why did you do this in the first place? And why does PF4 interact with heparin? What should we know about that?

Dr. Bernabei. It has been known for quite a while that PF4 neutralizes heparin and is possibly a more physiologic molecule. However, in the past, isolating PF4 was an economic burden and therefore was not clinically useful. However, recently PF4 was developed as an angiogenesis inhibitor and a possible powerful cancer oncologic agent. With our knowledge of the ability of PF4 to neutralize heparin, we speculated that it might be a better alternative to protamine.

Dr. Wechsler. Would it work with heparinoids as well, or is it specific for heparin?

Dr. Bernabei. It is known to neutralize all commercially available heparins including low molecular weight heparin.