Platelet Factor 4 Efficiently Reverses Heparin Anticoagulation in the Rat Without Adverse Effects of Heparin–Protamine Complexes

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Background. It has been observed that the reversal of heparin anticoagulation in humans by protamine sulfate (PS) results in various adverse reactions including leukopenia, thrombocytopenia, activation of complement, increased vascular permeability, systemic hypotension, pulmonary vasoconstriction, and pulmonary edema. The purpose of this study was to compare the efficacy and effects of native platelet factor 4 (PF4) and recombinant platelet factor 4 (rPF4) with those of PS in heparin neutralization in vivo, using a rat model.

Methods and Results. Sprague-Dawley rats were anesthetized with sodium pentobarbital, and the right femoral vein and carotid artery were cannulated. For determination of activated partial thromboplastin time, platelet count, white blood cell count, and complement titer, arterial blood samples were taken before and immediately after heparin (10 units/100 g) infusion and at several time points after the infusion of the neutralizing agent (PS, 0.1 mg/100 g; PF4, 0.5 mg/100 g). In separate groups of animals, mean arterial blood pressure was monitored throughout identical protocols and the lungs were prepared for histological examination. The anticoagulant activity of heparin was effectively reversed by all of the neutralizing agents (PS, PF4, and rPF4). Platelet count (48% of initial), white blood cell count (52% of initial), complement titer (60% of initial), and mean arterial pressure (20% decrease) decreased significantly in heparinized animals receiving PS but not in those receiving PF4 or rPF4. Lung interstitium appeared normal when heparin was followed by PF4; however, interstitial edema and hemorrhage were observed with heparin–PS.

Conclusions. These results suggest that PF4 efficiently reverses heparin anticoagulation in the rat without the adverse effects of heparin–protamine complexes. Therefore, rPF4 may be an appropriate substitute for PS in patients undergoing cardiovascular surgery and other procedures that require heparin anticoagulation. (Circulation 1992;85:1102-1109)

KEY WORDS • heparin anticoagulation • protamine sulfate • platelet factor 4 • complement activation • rat lung edema

Patients undergoing cardiopulmonary bypass, cardiac catheterization, and hemodialysis require heparinization followed by the neutralization of the heparin anticoagulant activity at the conclusion of the procedure. Protamine sulfate (PS), a highly basic protein obtained from fish sperm, is widely used for this purpose. However, the administration of PS may result in adverse reactions in heparinized patients. These reactions include urticaria, flushing, leukopenia, thrombocytopenia, bronchospasm, elevated pulmonary arterial pressure, pulmonary edema, and systemic hypotension, occasionally leading to cardiovascular collapse and death.1-5 The incidence of mild reactions to PS is as high as 16% and that of severe reactions is between 0.2% and 3.0%.1,4,6 In theory, when considering more than 350,000 open-heart operations performed each year in the United States,7 the lives of 400–6000 patients in this country are endangered each year by the use of protamine. In addition, subtle pathological effects of protamine go unnoticed and may contribute to postsurgical morbidity and mortality.

The adverse effects of PS could be mediated by an immune response, or heparin–protamine complexes could directly activate the complement pathway. That is, antiprotamine antibodies could be present in patients allergic to fish8,9 and in those who had previously received insulin–protamine.10 Heparin–protamine complexes can also activate the classic complement pathway in the presence of red cells,11,12 leading to the sequestration of neutrophils in the lungs, the formation of thrombocyanate A3, and pulmonary edema.13 Kirklin et all found that cardiopulmonary bypass itself activates
the complement system as indicated by an increase in C3a and C4a. It was reported that the administration of PS caused a significant increase of these complement components that was maximal at 10 minutes and remained elevated at 30 minutes. Cavarocchi et al. also found increased levels of C3a and C4a in 100 heparinized patients at the conclusion of cardiopulmonary bypass. These patients had received PS for heparin neutralization.

Polybrene, a highly basic compound effective in neutralizing heparin, appears to be even more toxic than PS. At present, alternatives to PS that do not elicit adverse effects are not available to the numerous patients in need of such a drug.

Platelet factor 4 (PF4) is a high-affinity, heparin-binding, heparin-neutralizing protein that is secreted by blood platelets during the release of α-granule contents. Human PF4 contains 70 amino acid residues and has a molecular weight of 7,800. The mechanism of heparin neutralization by PF4 is much more specific than the simple neutralization of charge by PS. There is evidence that two factors are essential for high-affinity heparin binding: two pairs of lysines (lys 61, lys 62, lys 65, and lys 66) surrounding a pair of isoleucine residues at the C-terminus of PF4 as well as the secondary structure of the molecule determined by two S-S bridges (cis 10–cis 36 and cis 12–cis 52). Under physiological conditions, PF4 exists as a tetramer complexed with a high-molecular-weight carrier that is also secreted by the platelets. There is evidence that the tetrameric structure of PF4 is a requirement for the expression of its heparin-neutralizing activity. According to St. Charles et al., the PF4 tetramer contains two extended, six-stranded β-sheets, each formed by two subunits. The carboxyterminal α-helices, which contain the lysine residues that are thought to be involved in heparin binding, are arranged as antiparallel pairs on the surface of each extended β-sheet.

In this study, we compared the reversal of the heparin anticoagulant activity in the rat by PS and by native and recombinant human PF4. All agents effectively neutralized the anticoagulant activity of heparin. However, platelet count, neutrophil count, complement titer, and arterial blood pressure decreased significantly after PS injection to heparinized rats but remained normal after the injection of PF4. Microscopic examination of fixed lung slices showed pulmonary edema and interalveolar hemorrhage in heparinized rats infused with PS that were absent in rats infused with PF4 or recombinant PF4 (rPF4).

### Methods

**Reagents**

Protamine sulfate was purchased from DuPont Pharmaceuticals, Wilmington, Del. Heparin was from Elkins-Sinn, Cherry Hill, N.J. In one study, we used PS from Sigma Chemical Co., St. Louis, Mo., and heparin from The Upjohn Company, Kalamazoo, Mich.

**Isolation of Human Native Platelet Factor 4**

PF4 was prepared from human platelets by a modification of the method of Rucinski et al. In brief, outdated platelet concentrates were centrifuged and the pellet was discarded because the major part of PF4 had been released into the plasma during platelet storage (22°C). One liter of the supernatant (plasma) was mixed with 20 ml heparin-agarose and gently shaken. After centrifugation, the heparin-agarose slurry was extensively washed with 0.85% NaCl, and material rich in PF4 was eluted with 2.0 M NaCl. Further purification of the dialyzed and concentrated eluate was accomplished by reverse-phase high-performance liquid chromatography (HPLC). Crude PF4 dissolved in 0.1% trifluoroacetic acid was applied to a wide-pore Vydac C-18 silica matrix column (Separation Groups, Hesperia, Calif.). The gradient used for elution was 0.1% trifluoroacetic acid containing 0–40% acetonitrile. A single PF4 peak was identified by the enzyme-linked immunosorbent assay method using anti-human PF4 antibody raised in rabbits, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and N-terminal amino acid sequencing.

**Preparation and Characterization of Human Recombinant Platelet Factor 4**

A synthetic gene for human PF4 was expressed in *E. coli* as described elsewhere. The recombinant PF4 was expressed as a fusion protein containing an amino terminally linked 34–amino acid domain separated from the mature PF4 sequence by a unique internal methionine residue. The insoluble residue after cell lysis was extracted with guanidine, transferred to urea-containing buffer, and partially purified by carboxymethyl-heparin chromatography. Selected fractions were precipitated with ethanol and cleaved with cyanogen bromide in formic acid, separating the fusion product from the native sequence polypeptide. The dried, cleaved protein mixture was dissolved in 8 M urea buffer, and rPF4 was purified by S-Sepharose chromatography at pH 5.0. Traces of residual uncleaved fusion protein, other minor contaminants, and misfolded species were removed by reverse-phase HPLC. The refolded protein was retained on the heparin–agarose column and eluted with 1.2 M NaCl. As shown in Figure 1, rPF4 and native PF4 showed identical elution patterns from a Vydac C18 column in an acetonitrile gradient and identical mobility in SDS polyacrylamide gel electrophoresis using the PhastGel apparatus (Pharmacia, Uppsala, Sweden). The structure of rPF4 was also confirmed by analysis of amino acid composition and amino terminal sequencing. Formation of rPF4 tetramers was demonstrated by gel filtration. Figure 2 shows that both rPF4 and PS effectively reversed anticoagulation induced by heparin in human plasma. The specific heparin-neutralizing activity (expressed per 1 μg protein) of rPF4 was about two times less than with PS. In the radioimmunoassay, rPF4 and native PF4 displaced 125I-rPF4 from a complex with the anti-PF4 antibody in a similar, dose-dependent manner. Similar to native human PF4, we observed biphasic clearance of 125I-rPF4 from the rat circulation. Based on four experiments, the fast component of rPF4 clearance from the circulation (t1/2) was 1.7 minutes (range, 1.0–2.5 minutes) and the slow component (t1/2) was 28.5 minutes (range, 12–38 minutes). Previous studies have shown that the injection of heparin results in single-compartment clearance of native PF4 and significantly extends its half-life in the circulation. We also demonstrated that 125I-rPF4 injected into rats in combination with heparin disappeared from the circulation in a single compartment clearance with a half-life of 23 minutes (range, 12.0–39.5
minutes). As with native PF4, the liver was the major route of PF4 clearance from the circulation, and the injection of heparin shifted the distribution of PF4 from the liver to the kidney.

Experimental Rat Model

Female Sprague-Dawley rats weighing 200–300 g were anesthetized with 50 mg/kg of sodium pentobarbital (Nembutal) by an intraperitoneal injection and used for the measurement of arterial blood pressure or the study of hematological parameters. A heating pad and lamp were used to maintain body temperature at 37°C. The animal was intubated (PE 200), and the right femoral vein (PE 50) and right carotid artery (PE 50) were cannulated for supplemental anesthesia or administration of experimental agents and for measurement of blood pressure or removal of blood samples, respectively. Mean arterial blood pressure (MAP) was recorded for 15 minutes to ensure that the animal was in a stable physiological state. For the hemodynamic study, mammalian Ringer’s solution as a control (0.5 ml) or heparin (10 units/100 g in 0.5 ml) was injected intravenously (0.5 ml/min). Five minutes was allowed for the distribution of heparin in the circulation and to ensure that the MAP remained stable. The neutralizing agent (PS, PF4, or rPF4) was then injected intravenously, and MAP was recorded for an additional 40 minutes. After this procedure, the lungs were infused (under pressure of 23 cm H2O) with fixative (glutaraldehyde) through the endotracheal tube. After 30 minutes, the lungs were removed and processed (hematoxylin/eosin) for microscopic examination.

For the study of hematological parameters, a separate group of animals was surgically prepared and instrumented as described above. Arterial blood samples were withdrawn at four time points: before the injection of mammalian Ringer’s or heparin; 5 minutes after this injection; and 5 and 12.5 minutes after the injection of PS, rPF4, or PF4. Removal of a total of 3 ml of blood from these animals followed by the infusion of resuspended red blood cells did not produce circulatory distress. Samples for blood cell counts and the preparation of plasma for clotting time determination were drawn into a 1-ml syringe containing 0.1 ml of 3.8% sodium citrate. A third group of animals was subjected

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**Figure 1.** High-performance liquid chromatography tracking and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of native platelet factor 4 (PF4) and recombinant human PF4 (rPF4). Inset demonstrates identical electrophoretic mobility of PF4 and rPF4 in SDS-PAGE. Molecular weight standards: carbonic anhydrase, 31,000 d; soybean trypsin inhibitor, 21,500 d; lysozyme, 14,400 d.

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**Figure 2.** Graphs show in vitro reversal of heparin anticoagulation of human citrated plasma by protamine sulfate and recombinant platelet factor 4 (rPF4). Left panel: Increased concentrations of bovine lung heparin (Upjohn) were added to human plasma (1:10 citrate) and the clotting time was measured with the activated partial thromboplastin time (aPTT) reagent. Right panel: Effect of various concentrations of rPF4 (○–○) and protamine sulfate (Sigma) (●–●) on aPTT in heparinized plasma. aPTT without heparin equaled 31.7 seconds and with heparin was 195 seconds. Representative of two experiments.
to the same experimental protocol; however, blood samples were drawn for the complement hemolytic assay.

Activated partial thromboplastin time (aPTT) was measured using reagents purchased from Organon Teknika Corp. (Durham, N.C.), according to instructions of the manufacturer. Platelet count and total white blood cell count were determined using the Unopette Microcollection System (Becton-Dickinson, Rutherford, N.J.) with phase contrast microscopy. Complement titer (CH50) was measured in a classic hemolytic assay using IgM-coated red blood cells. The degree of hemolysis was determined by absorbance at 412 nm; 1 unit is defined as the reciprocal dilution giving 50% hemolysis.

Statistical Analysis

Paired t tests were used to compare preheparin control values with those after the infusion of heparin and the neutralizing agent for platelet count, white blood cell count, MAP, and complement activation. It was also confirmed that aPTT values after each neutralizing agent were not statistically different from preheparin clotting times.

Results

Preliminary experiments established that the injection of heparin into the rat (10 units/100 g) resulted in a significant prolongation of aPTT (clotting time >5 minutes) that lasted at least 30 minutes. Figure 3 shows the effect of various concentrations of PS and rPF4 on aPTT in plasma of rats that had been heparinized 2 minutes before the withdrawal of the blood sample. This demonstrates that both antagonists cause the reversal of heparin anticoagulation at a concentration of 20–60 μg/ml. Native PF4 neutralization activity was similar to that of rPF4 (not shown). Subsequently, we established that PS at a dose of 0.1 mg per 100 g body weight and PF4 at a dose of 0.5 mg/100 g body weight resulted in a rapid return of aPTT to preheparin control values (Table 1).

Figure 4 depicts the comparative effects of PS and PF4 on platelet count and white blood cell count. aPTT returned to the preheparin control value (average, 29 seconds) after the injection of each heparin-neutralizing protein. Samples taken after the injection of heparin but before PS or PF4 failed to clot within a 5-minute observation period. The injection of PS resulted in a 50% drop in platelet count and a 42% reduction in white blood cell count (Figure 4). On the other hand,

![Figure 3](image-url)  
**Figure 3.** Graph shows reversal of heparin anticoagulation ex vivo by protamine sulfate (□—□) and recombinant platelet factor 4 (rPF4) (▼—▼). Rat was injected with heparin 100 units/kg and blood was collected (3.8% sodium citrate) 5 minutes later. Aliquots of 0.4 ml whole blood were mixed with different concentrations of rPF4 or protamine sulfate. Samples were centrifuged for 5 minutes at 7,000g, supernatant was collected, and clotting times were measured (activated partial thromboplastin time, aPTT).

![Figure 4](image-url)  
**Figure 4.** Bar graphs show effect of injection of protamine sulfate and recombinant platelet factor 4 (rPF4) into heparinized rats on the number of circulating white blood cells (left panel) and platelets (right panel). Number of experiments in parentheses. *p<0.01, significantly different from preheparin platelet count. Values correspond to mean±SEM.
the injection of rPF4 (Figure 4) and native PF4 (data not shown) had no significant effect on these parameters. The injection of mammalian Ringer's solution into heparinized animals and of PS into animals that had not been pretreated with heparin were without effect, except that PS slightly decreased white blood cell count. Heparin alone had no effect on platelet and white blood cell count (data not shown). Reversal of heparin anticoagulation with PS resulted in a 40% decrease of complement titer, whereas complement titer was not altered after the neutralization of heparin with rPF4 (Figure 5).

Figure 6 presents mean values of blood pressure after the infusion of heparin followed by PS, native PF4, and rPF4. A significant decrease of MAP (20%) was observed in animals injected with heparin and PS but not in those receiving heparin and PF4. During these studies, irregular respiration was noted consistently in heparinized animals injected with protamine but not in the rPF4-treated group.

Histological examination of stained lung sections from these animals (Figure 7, panels A–E) revealed distinct hemorrhage, edema, and inflammatory cell infiltrates in the lungs of animals treated with sequential heparin and protamine (Figure 7E). No abnormalities were observed in the lungs of animals that had received intravenous heparin followed by rPF4 (Figure 7B), native PF4 (Figure 7C), or in lungs of animals that had received protamine but not heparin (Figure 7D).

Discussion

The results of this study confirm earlier and current reports that the reversal of heparin anticoagulation with PS elicits many undesirable effects. These experiments demonstrate that the injection of PS into heparinized rats caused a decrease in white blood cell and platelet count, the consumption of complement components, a reduction of MAP, respiratory distress and pulmonary edema, and interalveolar hemorrhage. Our observations are consistent with those of Morel et al, who used sheep as a model and demonstrated that heparin–protamine complexes activated the classical complement pathway leading to the formation of C5a, which causes leukoaggregation and leukoactivation, release of oxygen free radicals, lipid peroxidation, the formation of thromboxane A2 in lung tissue, and pulmonary vasoconstriction.

Similar events may take place in rat lungs, although the lungs of this species do not contain as many intravascular macrophages, which are abundant in sheep. It has been proposed that intravascular macrophages release thromboxane A2, which mediates vasoconstriction. Fairman et al reported that PS has a direct effect on isolated rat lungs, causing pulmonary edema and hypertension; however, in our study, PS injected into rats in the absence of heparin did not cause pulmonary abnormalities. It is known that polycations aggregate platelets directly; it has been reported that platelet aggregation by protamine in heparinized blood. A review of the literature suggests that protamine may induce hypotension via a direct cardiac depressant effect or histamine release. However, in our experimental system, the injection of protamine sulfate to nonheparinized rats affected neither platelet count nor MAP. It is conceivable that in the rat, the activation of the complement pathway by protamine–heparin complexes results in leukopenia, thrombocytopenia, decrease of blood pressure, and pulmonary hemorrhage. It is also possible that high doses of protamine may cause tissue injury in the absence of heparin. For instance, Koslow et al described pulmonary edema in 350–400-g rats injected with 10 mg protamine in the absence of heparin. The dose of protamine in the experiments of these authors was about 20 times higher than the dose used in the current study.

In agreement with Shanberge et al, PF4 was comparable in its ability to neutralize heparin; however, in contrast to protamine–heparin complexes, human PF4 injected into heparinized rats at the dose completely reversing anticoagulation did not cause complement consumption and other hematological and hemodynamic abnormalities. This is not surprising, because PF4 is not a polycation, and PF4 interaction with heparin is highly specific, determined by the presence of two pairs of lysines at the C-terminal helices of this tetrameric molecule. PF4, in contrast to protamine, does not cause platelets to aggregate. PF4–heparin complexes do not activate the complement pathway, and it is unlikely that the injection of rPF4 would cause an immunological response because an identical protein is naturally produced in the human body. To neutralize
5,000 units of heparin, a dose of about 250 mg rPF4 must be injected. This dose of rPF4 would correspond to about a 15- to 20-fold excess of the native PF4 normally present in the human body. Platelets circulating in the blood of an individual whose weight is 70 kg contain about 15-fold mg PF4 because the concentration of this protein is about 10 μg/10⁶ platelets. In addition, a fraction of extraplatelet PF4 (approximately 2.5 mg) associated with endothelial cells and hepatocytes can be released into the circulation after the injection of heparin. Therefore, neutralization of therapeutic doses of heparin could not occur through the release of endogenous PF4. It is unlikely that an excess of PF4 would cause thrombosis. In our hands, high concentrations (above 500 μg/ml) of rPF4 extended aPTT clotting time (unpublished observation). This observation is consistent with a recent study by Dumenco et al., who reported inhibition of the activation of Hageman factor (factor XII) by PF4.

Despite its obvious advantages, PF4 has a number of biological effects that might cause concern: stimulation of histamine release from human basophils, inhibition of megakaryocyte maturation, reversal of immunosuppression, and inhibition of angiogenesis. However, PF4 is rapidly cleared from the circulation, and its half-life is not related to the administered dose. Therefore, the potential adverse effects of this protein may never be realized, even if given at a very high dose.

Clearly, the clinical use of PF4 for heparin neutralization applications will require recombinant produc-
tion. The lack of covalently bound carbohydrate and methionine in PF4 have permitted its efficient expression as a fusion protein in E. coli and its subsequent chemical processing to a polypeptide of identical sequence to platelet-derived PF4. Scaling up of production of a modified version of this process to generate the material needed for this application would be possible, and the use of E. coli as the expression host should permit economical production.

Conclusions

We consider recombinant PF4 to be an advantageous substitute for PS to reverse heparin anticoagulation. Further research on rats and on other animal species aiming at the application of this protein to normalize hemostasis in patients undergoing cardiopulmonary bypass and other procedures is warranted.

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Cook et al  Heparin Neutralization by Protamine and PF4  1109


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