Biochemistry of Serine Protease Inhibitors and Their Mechanisms of Action: A Review

Julie Wegner, PhD, CCP

Abstract: Cardiopulmonary bypass (CPB) activates and disrupts the hemostatic and inflammatory systems, which, in turn, makes an impact on the clinical outcome of patients. Postoperative bleeding is one common complication of CPB. Many techniques have been used to reduce post-operative bleeding, and pharmacological agents have demonstrated the greatest efficacy. In particular, the serine protease inhibitor, aprotinin, consistently reduces post-operative bleeding. The hemostatic mechanism of action of aprotinin; however, remains to be elucidated fully. The purpose of this review is to discuss the probable mechanisms of aprotinin action from the perspective of its interactions within the hemostatic and inflammatory pathways. Keywords: serine protease inhibitor, aprotinin, cardiopulmonary bypass, hemostasis, fibrinolysis, platelet. JECT. 2003;35:326–338

Cardiopulmonary bypass (CPB) has a significant impact on the function of the hemostatic and inflammatory systems, which, in turn, influences the clinical outcome of patients. Post-operative bleeding is one consequence associated with the disruption of these hemostatic and inflammatory pathways. Of the many techniques implemented during CPB to reduce post-operative bleeding, pharmacological agents have demonstrated the greatest efficacy. Such serine protease inhibitors as aprotinin and nafamostat mesilate, have been shown to reduce post-CPB bleeding and transfusion requirements consistently (1–5). Although the basic mechanism by which aprotinin inhibits a serine protease is known, this basic mechanism does not fully explain the efficacy of aprotinin. Hemostasis is an extremely complex system, thus an understanding of the full impact of aprotinin on CPB-induced post-operative bleeding requires an understanding of the pharmacological properties of aprotinin, the hemostatic process during CPB, and the sites within the hemostatic and inflammatory systems where aprotinin interacts. The purpose of this review is to discuss the probable mechanisms of aprotinin action from the perspective of its interactions within hemostatic and inflammatory pathways.

THE HEMOSTATIC SYSTEM AND CARDIOPULMONARY BYPASS

Hemostasis is a complex interaction between procoagu-
clot. Thus, the ability to re-establish hemostasis after CPB is determined in part by the magnitude of disruption generated between the coagulation, fibrinolytic, and inflammatory pathways during CPB.

The CPB-induced alterations of the hemostatic system have been characterized and associated with the extent of post-CPB bleeding. The most common explanations of post-CPB bleeding include transient platelet dysfunction and hyperfibrinolysis (6). Transient platelet dysfunction has been characterized as decreased responsiveness to endogenous agonists as well as decreased adhesion and aggregation capacity (6). Thus, the ability of platelets to contribute to the generation and maintenance of stable clots is compromised post-CPB. A second common explanation of post-CPB bleeding is hyperfibrinolysis. Activation of the fibrinolytic pathway generates plasmin, a serine protease responsible for the breakdown of fibrin clot. However, plasmin has direct and indirect actions on other components of the hemostatic system, including platelets. The ability of plasmin to affect multiple components of the hemostatic system suggests that excessive plasmin activity could severely compromise the ability of the hemostatic system to form stable clots. Because aprotinin is a serine protease inhibitor, the most logical mechanism by which aprotinin achieves its hemostatic efficacy is via inhibition of plasmin. However, other hemostatic agents also inhibit plasmin, but do not have the same clinical efficacy as aprotinin, suggesting that the mechanism of aprotinin’s hemostatic effect is more than just inhibition of plasmin. A first step to understanding the hemostatic efficacy of aprotinin is to understand the pharmacological properties of aprotinin.

### APROTININ: PHARMACOLOGICAL PROPERTIES

Aprotinin is a serine protease inhibitor isolated from bovine lung tissue. The aprotinin molecule is a highly basic, hydrophilic polypeptide with a molecular weight of 6512 Daltons and an isoelectric point of 10.5 (7,8). The inhibitory activity of aprotinin is expressed in kallikrein inhibitor units (KIU), where one KIU is equivalent to the amount of aprotinin that decreases the activity of two biological kallikrein units by 50%. Aprotinin dosage is commonly expressed in KIU or milligrams (mg), where 1 mg aprotinin is equivalent to 7143 KIU (7,8).

Aprotinin competitively inhibits a wide variety of serine proteases via the formation of an enzyme-inhibitor complex. The dissociation constants (Kᵢ) of aprotinin are listed in Table 3. Three important points about the action of aprotinin are evident from the data in Table 3. First, the number of serine proteases listed demonstrates the broad specificity of aprotinin. Second, the wide range of Kᵢ values demonstrates the dose-dependent effects of aprotinin. Higher concentrations of aprotinin will have the po-

### Table 1. Common serine proteases.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Serine Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulation</td>
<td>FXIIa, FXIa, FIIa, FXa, FIXa, tissue factor/FVIIa, activated protein C</td>
</tr>
<tr>
<td>Fibrinolysis</td>
<td>Plasmin, kallikrein, tissue plasminogen activator, urokinase</td>
</tr>
<tr>
<td>Complement</td>
<td>Kallikrein, enzymes of the complement cascade</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>Elastase, cathepsin G, bradykinin</td>
</tr>
</tbody>
</table>

### Table 2. Endogenous serine protease inhibitors (serpins).

<table>
<thead>
<tr>
<th>Serine Protease Inhibitor</th>
<th>Serine Protease Inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III</td>
<td>FIIa, FXa, FIXa, FIIa, kallikrein, plasmin</td>
</tr>
<tr>
<td>Heparin co-factor II</td>
<td>FIIa, FIIa, FIXa, FXIIa complex</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor (TFPI)</td>
<td>FIIa, FIXa, kallikrein</td>
</tr>
<tr>
<td>α₁-antitrypsin</td>
<td>FXa, activated protein C, elastase</td>
</tr>
<tr>
<td>α₂-macroglobulin</td>
<td>FIIa, FIXa, FIIa, kallikrein</td>
</tr>
<tr>
<td>α₂-antiplasmin</td>
<td>FXa, FIXa, plasmin, kallikrein</td>
</tr>
<tr>
<td>Protease nexin-1</td>
<td>FIIa, urokinase, plasmin</td>
</tr>
</tbody>
</table>

### Table 3. Dissociation constants (Kᵢ) for serine proteases.

<table>
<thead>
<tr>
<th>Serine Protease</th>
<th>In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KIU/mL</td>
</tr>
<tr>
<td>Plasmin</td>
<td>1 nmol/L</td>
</tr>
<tr>
<td>Plasma Kallikrein</td>
<td>30–36 nmol/L</td>
</tr>
<tr>
<td>Elastase</td>
<td>3.5 μmol/L</td>
</tr>
<tr>
<td>Activated protein C</td>
<td>1.1–2.6 μmol/L</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>1.1 μmol/L</td>
</tr>
<tr>
<td>Urokinase</td>
<td>2.5–8.0 μmol/L</td>
</tr>
<tr>
<td>Tissue factor/FVIIa complex activator</td>
<td>9.8 μmol/L</td>
</tr>
</tbody>
</table>

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tential to inhibit a greater number of serine proteases, and thus, will influence hemostasis to a greater extent. Third is the disparity between the in vitro and in vivo (i.e., clinically effective concentration) $K_i$ values. This disparity is attributable to the inhibitory activity of aprotinin under such different conditions as in the presence or absence of endogenous serine protease inhibitors, fibrin, or unfractionated heparin (9–11). These observations suggest that the effective aprotinin concentration may change under different conditions of hemostatic activation and that calculation of the $K_i$ for a such given target protease as plasmin will require determination under conditions approximating the in vivo environment, such as during CPB (7).

The standard dosage regimens for aprotinin administration are listed in Table 4 (8). Several variations of these standard regimens have been implemented in clinical studies with varying success in reducing blood loss. The plasma concentration of aprotinin achieved by a given dosage regimen is what determines the pharmacological efficacy of aprotinin. Although the hemostatic efficacy of aprotinin seems to follow a tight dose–response relationship, such a dose–response relationship is not always apparent in patients during CPB (12–15). Large variations in the plasma aprotinin concentrations have been demonstrated with fixed dosage protocols in patients undergoing CPB (14). To ensure maximum efficacy, several dose administration protocols have been developed based on body weight or target effective plasma concentration of aprotinin (14,16).

The plasma aprotinin levels required to inhibit plasmin and kallikrein during CPB have been calculated to be between 50–125 KIU/mL and 200–250 KIU/mL, respectively (8). Clinical efficacy (i.e., decreased bleeding) has been demonstrated at aprotinin concentrations between 127–191 KIU/mL at the end of CPB (17). The mean plasma concentrations of aprotinin, using a high or low dose protocol, have been reported to be and 236 and 160 KIU/mL after 60 min of CPB, respectively. Although aprotinin concentrations of <200 KIU/mL were observed in 70% of the patients in the low-dose group during CPB, no differences in blood loss or transfusion requirements have been observed between high- and low-dose groups (14). Thus, reductions in blood loss seem be achieved at aprotinin concentrations required to inhibit plasmin rather than kallikrein (18,19).

In trying to discern the mechanism of action of aprotinin, it is important to distinguish between the biochemical efficacy and clinical efficacy of the drug. The biochemical efficacy will be influenced by the factors that affect the interaction of aprotinin with the target protease, such as pH, protein concentration, presence or absence of endogenous serine protease inhibitors, presence or absence of fibrin, and the type of anticoagulant (9–11). The clinical efficacy will be influenced by factors that influence the degree of blood activation during CPB, such as patient population, pre-operative medications, hemodilution, prime composition, use of ultrafiltration, temperature, the use of coronary suction, and the duration of CPB. The greatest clinical efficacy of aprotinin seems to occur with high-risk patients and in conditions of greater blood activation (20,21).

The comparison of aprotinin with such other antifibrinolytic agents as tranexamic acid or epsilon aminocaproic acid has demonstrated a greater hemostatic efficacy of aprotinin, despite similar effects on the biochemical indices of hemostasis (3,22,23). These observations suggest that aprotinin has more than one site of action or possibly more than one mechanism of action. In physiological terms, aprotinin is not considered a true antifibrinolytic agent, because it does not directly inhibit plasmin formation. Rather aprotinin inhibits excessive plasmin activity (12,24). Because excessive plasmin can have multiple effects on the hemostatic system, the distinction between direct plasmin inhibition versus plasmin formation may help to explain the greater efficacy of aprotinin as compared to true antifibrinolytic agents.

Another property that may explain the clinical efficacy of aprotinin is aprotinin’s resistance to attack by endogenous proteinases, which is not true of the endogenous serine protease inhibitors (i.e., serpins) (25). Thus, in conditions associated with increased proteolytic activity, such as CPB, serpins such as α2-antiplasmin or antithrombin III may be consumed by proteolytic breakdown. α2-antiplasmin is the primary endogenous inhibitor of plasmin, and an acquired α2-antiplasmin deficiency has been associated with bleeding attributable to the inability to regulate plasmin activity (26,27). Thus, aprotinin by its ability to inhibit serine proteinases may attenuate the proteolytic consumption of serpins, resulting in the preservation of the antiproteinase capacity of human plasma (25).

By this mechanism, aprotinin may preserve the balance between the endogenous procoagulant and anticoagulant enzyme cascades, allowing rapid re-establishment of hemostasis at the termination of CPB.

Finally, the highly basic nature of the aprotinin molecule allows aprotinin to bind to acidic glycoproteins and mucopolysaccharides, proteins commonly found in the

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**Table 4.** Common dosage regimen for aprotinin.

<table>
<thead>
<tr>
<th>Dosage Regimen</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High dose</strong></td>
<td>280 mg (2 × 10⁶ KIU) intravenously after induction of anesthesia, but before sternotomy followed by 70 mg/h (5 × 10⁶ KIU/h) by intravenous infusion for the duration of surgery plus 280 mg added to the priming fluid of the CPB circuit</td>
</tr>
<tr>
<td><strong>Low dose</strong></td>
<td>One-half the high-dose regimen (140 mg loading dose, 35 mg/h infusion dose, 140 mg prime dose)</td>
</tr>
<tr>
<td><strong>Pump prime only</strong></td>
<td>280 mg in priming fluid of CPB circuit</td>
</tr>
</tbody>
</table>

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plasma membranes of cells. This property of aprotinin provides the potential for the accumulation of high concentrations at a membrane surface as well as intracellular sites of action (28). Aprotinin has been shown to bind to the plasma membranes of leukocytes, platelets, and monocytes and to influence their response to various stimuli as well as the activities of membrane associated enzymes (7, 29, 30). The ability of aprotinin to interact at the cellular level potentially broadens the range of serine proteases inhibited at the aprotinin concentrations achieved during CPB.

APROTININ: CONTACT PATHWAY

Cardiopulmonary bypass is considered to be a massive procoagulant stimulus with activation of both the intrinsic and extrinsic coagulation cascades as well as the components of the inflammatory response. Initially, it was thought that the procoagulant response to CPB was primarily mediated by activation of the intrinsic, or contact, cascade via activation of factor XII. However, recent data suggest that activation of the extrinsic coagulation cascade is primarily responsible for the initiation of coagulation during CPB (31–35). This observation does not mean that the contact pathway is not activated during CPB, rather it means that contact pathway is not responsible for the initiation of coagulation. Current models of coagulation suggest that the intrinsic pathway, at the level of factor XI, is responsible for the amplification and maintenance of coagulation rather than the initiation of coagulation (36, 37). On the other hand, the contact system, at the level of factor XII, functions as the biological modulator of the vascular system via activation of the kallikrein-kininogen-kinin, fibrinolytic, and complement pathways (38). Two products of contact activation (i.e., factor XII activation) are the serine proteases, kallikrein, and plasmin. The importance of kallikrein and plasmin generation to the function of the hemostatic system explains one mechanism by which aprotinin may decrease post-operative bleeding.

KALLIKREIN

Kallikrein is generated by the activation of at least two pathways, one dependent and one independent of activated factor XII (FXIIa) (Figures 1 and 2). The FXII-dependent pathway is initiated by the binding of factor XII (FXII) to negatively charged surfaces, leading to the generation of trace amounts of FXIIa via an autoactivation mechanism (Figure 1). Prekallikrein circulates in the plasma bound to high molecular weight kininogen (HMWK), which also binds to negatively charge surfaces. The interaction of trace FXIIa, prekallikrein, and HMWK at a foreign surface result in the formation of kallikrein. High molecular weight kininogen also serves as a cellular receptor for prekallikrein on platelets, vascular endothelial cells, and granulocytes via the kininogen binding receptor, cytokeritan-I. Prekallikrein-HMWK binding to these cells causes the activation of a membrane bound cysteine protease, which then converts prekallikrein to kallikrein (39, 40). This interaction provides the second pathway for kallikrein formation, a pathway that is independent of FXIIa, or contact activation.

Kallikrein is a multifunctional enzyme with actions on contact activation, fibrinolysis, inflammation, the vascular endothelium, and platelets (Figure 1). Kallikrein plays a fundamental role in the activation and amplification of the contact pathway via a feedback activation of FXII. Kallikrein also plays a major role in the activation of the fibrinolytic system by stimulating the conversion of plasminogen to plasmin by three mechanisms. These mechanisms include direct activation of plasminogen, direct activation of urokinase plasminogen activator (uPA) formation, and indirect activation of tissue plasminogen activator (tPA) release from vascular endothelial cells via the formation of bradykinin (BK). The inflammatory actions of kallikrein include activation of the complement pathway and direct activation of neutrophils (38, 41). Bradykinin, a byproduct of HMWK degradation, mediates the endothelial and platelet effects of kallikrein. Bradykinin is a potent vasodilator, enhances vascular permeability and inhibits thrombin-stimulated endothelial activation (39). Bradykinin also induces the release prostacyclin and nitric oxide from vascular endothelial cells, both of which are potent platelet inhibitors. Finally, bradykinin inhibits thrombin-induced activation of platelets (39, 40). Thus, kallikrein is an important mediator of the contact pathway, because it can directly and indirectly attenuate hemostasis and activate inflammation at multiple levels.

The influence of kallikrein on fibrinolysis, platelet function, and inflammation implies that inhibition of kallikrein by aprotinin should have significant effects on hemostasis (Figure 1). The in vivo Ki value of 200–250 KIU/mL for kallikrein suggests that high-dose regimen would be required to significantly inhibit the effects of kallikrein during CPB. Studies have demonstrated that high-dose aprotinin does inhibit kallikrein during CPB, and thus may be another mechanism by which aprotinin reduces post-operative blood loss (42–44).

PLASMIN

Plasmin is the second important mediator generated by the activation of the contact system. Plasmin is generated from plasminogen via a conversion reaction stimulated by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The release or activation of both plasminogen activators is dependent upon the for-
tion kallikrein, as indicated above. Kallikrein, FXIIa, and FXIa can also directly stimulate plasminogen conversion. Thus, at least five pathways exist for the generation of plasmin.

During physiological fibrinolysis, plasminogen binds to fibrin and is then converted to plasmin by tPA or uPA. In this way, fibrin degradation is tightly regulated at the site of fibrin formation. However, when plasmin is generated by mechanisms not associated with the plasminogen–fibrin interaction, free plasmin is generated. Excess free plasmin can overwhelm the capacity of the endogenous plasmin inhibitors, resulting in plasmin degradation of clotting factors and interactions with several of the cellular components of hemostasis.

As with kallikrein, plasmin has multiple effects on the hemostatic system (Figure 1). Free plasmin can degrade clotting factors, affect platelet function, and influence vascular endothelial cell function. In addition to fibrin breakdown compromising clot stability, excessive fibrin degradation products (FDP) can interfere with fibrin polymerization and compete with fibrinogen for platelet glycoprotein IIb/IIIa (GP IIb/IIIa) receptors, thus affecting platelet-platelet interaction and clot stability. Free plasmin also degrades fibrinogen, the substrate necessary for thrombin and platelet-platelet interactions. Both low- and high-dose aprotinin protocols have demonstrated inhibition of plasmin during CPB as determined by decreases in D-dimer, FDP, and plasmin–antiplasmin complex levels (13,21,45,46).

Plasmin has also been shown to both activate and inhibit platelet function. This duality of plasmin action is dependent on concentration and duration of plasmin interactions (47,48). Low concentration of plasmin induces platelet dysfunction because of subtle cleavage of GP IIb/IIIa receptors, which reduces platelet–platelet interactions and decreases platelet aggregation capability. On the other hand, at high concentrations, plasmin activates platelets (47,48). Platelet activation by high concentrations of plasmin could, over time, decrease the number of functional platelets, and thus influence post-operative bleeding.
Initial studies with washed platelets suggested that plasmin cleaved platelet glycoprotein receptors, thus causing platelet dysfunction (49). However, more recent studies using platelet-rich plasma have demonstrated that plasmin causes redistribution of GPIb receptors into the surface connected canalicular system rather than actual receptor degradation (50). The redistribution of the GPIb receptors versus degradation may explain the transient nature of the platelet dysfunction, the rapid recovery of platelet adhesive function following plasmin neutralization by aprotinin, and the reported efficacy of aprotinin administration after CPB (19,51,52).

The functional integrity, versus number, of the GPIIb/IIIa receptors may also be affected by plasmin as demonstrated by a decreased fibrinogen binding capacity of the GPIIb/IIIa receptor in the presence of plasmin (53). Recent studies have also demonstrated that plasmin inhibits thrombin-induced platelet activation via the cleavage of the protease activated receptor (PAR) (47,54,55). Thus, the interaction of plasmin at the platelet level affects the both ability of the platelet to respond to stimuli as well as over-all platelet function.

In vitro studies have demonstrated platelet protective effects of aprotinin. Pretreatment of platelets with aprotinin (250 KIU/mL) inhibited the plasmin-induced decrease of the ristocetin-induce agglutination reaction and attenuated the down-regulation of GPIb receptors on platelets (56,57). In addition, aprotinin decreased the agonist-induced expression of GPIIb/IIIa receptors, suggesting a reduced level of activation in the presence of aprotinin (58). Aprotinin also has been shown to reduce heparin binding to platelets and thus may decrease heparin-induced platelet dysfunction (58,59).

Aprotinin has also demonstrated a platelet protective effect in vivo as demonstrated by an attenuation of platelet activation and the preservation of GPIb receptors in

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**Figure 2.** Extrinsic coagulation pathway — the role of the monocyte. The question mark (?) indicates possible, yet unproved site of action of aprotinin. Abbreviations: APC = activated protein C, APR = aprotinin, OFR = oxygen free radical, PC = protein C, TM = thrombomodulin, TF = tissue factor.
patients undergoing CPB (23,43,60–62). These results suggest that aprotinin maintains a viable platelet population during CPB that is better able to contribute to hemostasis post-CPB (55,61). Most CPB studies have shown, however, that aprotinin does not prevent the decrease in platelet number at the initiation of CPB. The lack of effect of aprotinin on platelet number supports the concept that platelet function is more important to the re-establishment of hemostasis post-CPB than is platelet number (6,63).

Although many studies have demonstrated an attenuation of CPB-induced platelet dysfunction, this has not been a consistent finding. Several studies have demonstrated that aprotinin significantly reduces post-operative blood loss without an effect on platelet morphology or function (64–66). The reasons for these contradictory findings are not known, but could be attributable in part to differences in study design or study parameters. Further studies will be required to understand better the contributions of platelets and aprotinin to post-operative bleeding.

In addition to the adhesive and aggregation properties, platelets also contribute to hemostasis via the rapid and localized generation of thrombin (67). The procoagulant activity of platelets requires the expression of specific phospholipids [i.e., phosphatidylserine (PS)], which provide an appropriate surface for the formation of the tenase and prothrombinase complexes required for the subsequent generation of thrombin. In vitro studies have demonstrated that inhibition of the platelet GPIIb/IIIa receptors decreases the number of PS binding sites and the thrombin generating capacity of platelets (68–70). These observations suggest that an inability to express functional GPIIb/IIIa receptors may compromise the ability of platelets to generate thrombin in quantities sufficient to form stable clots. Because aprotinin may protect GPIIb/IIIa function during CPB, the possibility exists that the protective effect of aprotinin on platelet function may be associated with the protection of platelet thrombin generating capacity (58). Neither the contribution of platelet procoagulant activity to post-CPB hemostasis, nor the influences of aprotinin on platelet procoagulant activity have been examined.

Plasmin has also been shown to have effects on the vascular endothelium, another essential cellular component of hemostasis. Treatment of cultured vascular endothelial cells with plasmin resulted in a reduction in the thromboresistant properties of the vascular endothelial cells as demonstrated by decreased tPA synthesis and release, increased plasminogen activator inhibitor-1 synthesis, decreased thrombomodulin activity, and increased vascular permeability (71–73). Pretreatment of the endothelial cells with aprotinin (200 KIU/mL) attenuated these plasmin-induced effects (72). These results suggest that excessive plasmin generation can damage the vascular endothelium and that these damaging effects may be related to the pathophysiology of bleeding (72).

APROTININ: ACTIVATED PROTEIN C

Based on the actions of aprotinin discussed thus far, it seems that aprotinin promotes a procoagulant environment by inhibiting fibrinolysis, protecting platelet function, and preserving the regulatory mechanisms of hemostasis. Aprotinin may further contribute to the procoagulant environment by inhibition of activated protein C. The protein C pathway is an important antithrombotic mechanism located on the vascular endothelium (74). Thrombin activates the protein C pathway via an interaction with thrombomodulin, a high affinity thrombin receptor expressed on endothelial cells. Formation of the thrombomodulin–thrombin complex inactivates thrombin and activates protein C. Activated protein C (APC) is a serine protease that in turn inactivates FVa and FVIIa and effectively turns off local thrombin generation. In addition, APC inhibits plasminogen activator inhibitor-1 (PAI-1), an inhibitor of the plasminogen activator, uPA. Aprotinin has been shown to inhibit APC in a time- and concentration-dependent manner (75–77). Furthermore, the affinity of aprotinin for APC increases in the presence of heparin, with a maximal effect demonstrated at a heparin concentration of 4 U/mL (75). Based on the calculated in vivo Kᵢ value for APC, the inhibitory effect of aprotinin on APC should be evident at the therapeutic level reached during CPB, especially with the use of a high-dose protocol. The inhibitory effect of aprotinin on APC would, thus, result in thrombin generation and inhibition of plasmin generation; in other words, the generation of a more procoagulant environment.

In in vitro studies using plasma from healthy volunteers, aprotinin was shown to produce a factor V (FV) Leiden type defect; that is, APC resistance. This defect was accentuated (i.e., made more hypercoagulable) when the plasma from patients with FV Leiden defect was used (77). This observation suggested a high risk for an aprotinin-induced prothrombotic state for patients with a FV Leiden defect. Interestingly, aprotinin did not produce a hypercoagulable state during CPB in FV Leiden patients. Rather, a less hypercoagulable state was evident (78). The mechanism for the decrease in hypercoagulability by aprotinin was not described, but suggests that factors are present in vivo that modify the interaction of aprotinin with APC and prevent the development of a hypercoagulable state during CPB.

APROTININ: ANTICOAGULANT EFFECT

Although the over-all consequence of aprotinin administration is the achievement of a more procoagulable en-

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vironment, an anticoagulant effect of aprotinin has also been described. In vitro, the anticoagulant effect was attributed to the inhibition of kallikrein and the subsequent lack of amplification of the contact activation pathway (25). Such an effect would be expected to be minimal in the in vivo setting unless there was overactivation of the kallikrein-kininogen-kinin system, which may be possible in the presence of endotoxin (79). Because the evidence suggests that the initiation of coagulation during CPB is via the extrinsic system, the contribution of the kallikrein-kininogen-kinin system to the anticoagulation effect of aprotinin would be expected to minimal (32–35). However, in vivo an anticoagulation effect of aprotinin has been demonstrated as a dose-dependent augmentation of the anticoagulant effect of heparin (13,80,81). In addition, several CPB studies have demonstrated a decrease in thrombin generation during CPB in the presence of aprotinin, again suggesting a possible anticoagulant effect (82–85). However, the actual contribution of aprotinin to overall anticoagulation during CPB has not been determined.

Intravascularly, the initiating step of the extrinsic pathway is the expression of tissue factor on the surface of activated endothelial cells and monocytes (Figure 2). Although tissue factor expression by endothelial cells plays an important role in hemostasis, this role has not been extensively studied with regards to coagulation alterations during CPB. However, the role of monocytes in the initiation of coagulation has been examined.

Activated monocytes express tissue factor by adherence to foreign surfaces, interaction with activated platelets via a P-selectin mediated mechanism, and via such inflammatory mediators as tumor necrosis factor and oxygen free radicals (86–91). Tissue factor expression requires de novo synthesis, mediated by a nuclear factor $\kappa$B (NF$\kappa$B)-dependent pathway (87). The requirement for de novo synthesis of tissue factor suggests a time course of tissue factor expression of hours, suggesting that the contribution of this coagulation pathway would not be important during CPB. However, rapid expression of tissue factor has been recently demonstrated (87). Tissue factor functions as a receptor for FVIIa and FVIIla. The formation of the tissue factor/FVIIla complex permits the activation of FX, and the subsequent formation of thrombin.

Monocytes can also support FX activation independent of both FxIla and tissue factor/FVIIla (92,93). The monocyte-mediated activation of FX involves the expression of the CD11b (Mac-1) receptor on the surface of monocytes and the subsequent binding of FX. Factor X binding to CD11b stimulates the release of cathepsin G, a serine protease, which then proteolytically activates FX (92). The expression of Fxa on the surface of the monocyte can support the formation of the prothrombinase complex and the subsequent formation of thrombin (90). The expression of CD11b on monocytes is increased during CPB, suggesting a potential FxIla- and tissue factor-independent pathway for coagulation activation during CPB (35).

As with the intrinsic pathway, aprotinin can also interact with the activation of the extrinsic pathway. In vitro studies have demonstrated that aprotinin (1493 KIU/mL) can directly inhibit tissue factor/FVIIa-initiated coagulation (94,95). However, the concentration of aprotinin required to inhibit the expression of tissue factor or the tissue factor/FVIIa complex is three to five times the plasma concentration of aprotinin achieved with the high-dose regimen during CPB.

In simulated models of CPB, aprotinin at 650–876 KIU/mL attenuated the expression of both tissue factor and CD18/CD11b on the surface of monocytes and decreased thrombin generation (96,97). Two CPB studies have demonstrated that aprotinin significantly reduced the expression of CD11b on neutrophils (98,99). However, the effect of the reduced neutrophil expression of CD11b on thrombin generation was not examined. Nonetheless, these results suggest that the monocyte surface may be a possible site of action for an anticoagulation effect of aprotinin in vivo.

Finally, the observation that aprotinin can bind to cell surfaces (29,30) suggests another possible site where aprotinin could inhibit cell associated serine proteases. Aprotinin binding to cell surfaces could allow aprotinin to accumulate at the cell surface to a concentration sufficient to inhibit membrane bound proteases or intracellular serine proteases (100). The accumulation of high concentrations of aprotinin at the membrane surface could affect the extrinsic pathway by inhibiting serine proteases associated with the membrane surface, such as tissue factor/FVIIa and cathepsin G. Inhibition of tissue factor/FVIIa would block initiation of the extrinsic pathway, and inhibition of cathepsin G would block the activation of FX bound to the CD11b receptor. By whatever mechanism, the anticoagulant effect of aprotinin would support the concept that enhanced anticoagulation better preserves the components of hemostasis during CPB, thus permitting a more rapid re-establishment of hemostasis post-CPB (81,83).

**APROTININ: VASCULAR ENDOTHELIUM**

Although the vascular endothelium plays a vital role in the maintenance of hemostasis and is activated and/or damaged during CPB, the consequences with respect to post-operative bleeding have not been thoroughly examined (84,101). Aprotinin did not affect the markers of endothelial damage during CPB, suggesting that aprotinin does not protect the endothelium per se (84). In vitro however, aprotinin (260 KIU/mL) modified the release of prostacyclin and thromboxane $A_2$ (TXA$_2$) from cultured endothelial cells, such that the prostacyclin/TXA$_2$ ratio

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was altered. The author speculated that such an alteration could result in increased platelet aggregation at the endothelium and improve vessel sealing (102). Other studies with different endothelial cell models were unable to demonstrate similar results with respect to prostacyclin and TXA₂ production (103,104). However, in a saphenous vein model, aprotinin did attenuate the vasoconstrictor response to TXA₂. The author speculated that the reduced vasoconstrictor response could promote platelet adhesion and platelet plug stability, and thus potentially reduce bleeding (104).

APROTININ: INFLAMMATION

The inflammatory and hemostatic pathways are interrelated, merging at the kallikrein-kininogen-kinin, complement, extrinsic coagulation, monocyte, and platelet activation pathways. The inflammatory response is also mediated by serine proteases, and thus regulated by serine protease inhibitors. Aprotinin can inhibit several of the serine proteases associated with the inflammatory response, but at concentrations higher than those required to inhibit plasmin or kallikrein (Table 3). However, aprotinin has been shown to have some anti-inflammatory effects at the doses efficacious for hemostasis. Again, an understanding of the pathways involved may lead to some clues as to how aprotinin works to attenuate the inflammatory response during CPB.

Neutrophils

The anti-inflammatory actions of aprotinin have been demonstrated at several points within the CPB-induced inflammatory response. With respect to neutrophils, aprotinin significantly decreased neutrophil activation as measured by elastase release and/or CD11b expression (99,105,106). In addition, low-dose aprotinin decreased the neutrophil expression of CD11b to the same extent as one gram of solumedrol, a known anti-inflammatory agent (98). Aprotinin was also shown to decrease the expression of P-selectin on platelets and the number of platelet–leukocyte conjugates. Both of these effects would be expected to reduce the magnitude of the inflammatory response (107). Finally, during major vascular surgery, aprotinin was found to preserve the ability of leukocytes to function postoperatively. Thus, as with platelet function, reduced leukocyte activation during surgery would help to protect leukocyte function for the post-operative period (108).

Cytokines

The levels of cytokines, the chemical mediators of the inflammatory response, are also affected by aprotinin during CPB. Aprotinin attenuated the increases of interleukin (IL)-8, IL-6, and tumor necrosis factor-α normally observed during CPB (8,109). In addition, high-dose aprotinin increased the level of the anti-inflammatory cytokine, IL-10 (110).

The importance of IL-10 as a modulator of the inflammatory response has been demonstrated by several in vitro and in vivo studies. Addition of IL-10 to a simulated model of CPB significantly reduced leukocyte activation, and thus decreased the magnitude of the inflammatory response (111). Addition of IL-10 also inhibited the activation of the coagulation and fibrinolytic pathways in a human model of endotoxemia (112). And finally, IL-10 was shown to inhibit tissue factor expression in isolated monocytes (113). These findings suggest that IL-10 could mediate several of the anti-inflammatory and hemostatic effects of aprotinin. However, the mechanism of the aprotinin-induced increase in IL-10 levels is not known.

Nitric oxide

Nitric oxide is also considered an important mediator of the inflammatory response. Aprotinin has been shown to be a competitive inhibitor of nitric oxide synthase-I (NOS-I) and NOS-II (Kᵢ = 2380 KIU/mL and 3710 KIU/mL, respectively), the intracellular enzymes responsible for the production of nitric oxide (114). These in vitro results suggest that very high plasma concentrations of aprotinin would be required to inhibit nitric oxide production at the level of NOS. However, a high concentration of aprotinin could be achieved by the ability of aprotinin to accumulate on cellular surfaces, thus permitting inhibition of NOS. In vivo, aprotinin has been shown to dose-dependently decrease airway nitric oxide levels at aprotinin levels commonly used during CPB (28,115). These results suggest that one mechanism of action of aprotinin may be via the inhibition of NOS at the cellular level. Whether or not aprotinin decreases systemic nitric oxide production during CPB has not been examined.

Oxygen free radicals

Aprotinin also has anti-oxidant properties. In vitro studies revealed that aprotinin inhibited oxygen free radical production and lipid peroxidation in isolated human neutrophils stimulated with the chemotactic peptide f-Met-Leu-Phe (fMLP). The required aprotinin concentration for free radical inhibition was 571 KIU/ml (23,116). An anti-oxidant effect of aprotinin has also been demonstrated during CPB using high- and low-dose protocols (117,118). Cardiopulmonary bypass is normally associated with a change in the redox potential, expressed as a shift toward a more oxidative state. Aprotinin minimized this change in redox potential, resulting in a less oxidative state (118). The mechanism by which aprotinin attenuated the shift in redox potential was not examined, but the results do suggest an anti-oxidant effect by aprotinin.

The anti-oxidant/anti-inflammatory effect of aprotinin
has also been demonstrated by the preservation of the hemorrhheologic properties of blood cells (119). The importance of blood cell hemorrhheology has been demonstrated by the relationship between the decrease in red blood cell deformability and a bleeding tendency after CPB (120).

The anti-inflammatory effects of aprotinin have not been demonstrated in all CPB studies, especially in studies using low-dose regimens (43,121–123). Although the lack of the anti-inflammatory effect of aprotinin may be dose-dependent, perfusion techniques may also contribute to the lack of observed efficacy. As demonstrated in heparin-coated circuit studies, biomaterial-independent stimulation may overwhelm the anti-inflammatory properties of aprotinin and thus mask any effect.

Although the evidence suggests that aprotinin has anti-inflammatory actions, how aprotinin achieves these actions, or at what level within the inflammatory cascade these actions take place, is not known. The observations of anti-inflammatory actions at hemostatically efficacious aprotinin dosages suggest that one of aprotinin’s anti-inflammatory actions may be mediated at the level of the kallikrein-kininogen-kinin pathway. As indicated in Figure 1, kallikrein mediates the formation of complement and bradykinin, both of which contribute to the development of an inflammatory response. In addition, kallikrein directly activates leukocytes, the cellular mediators of the inflammatory response (41).

A second mechanism by which aprotinin could conceivably attenuate the inflammatory response is via interaction at the cellular level. As already discussed, the ability of aprotinin to bind to cellular membranes could result in the accumulation of aprotinin in sufficient quantities to inhibit membrane-bound serine proteases or the permeation of aprotinin into the cell. Inhibition of the cytokine-induced activation of NFκB by a serine protease inhibitor in cultured endothelial cells suggests another potential mechanism of aprotinin (100).

Nuclear factor-κB is found in the cytoplasm associated with inhibitor-κB (IκB). Cellular activation by various proinflammatory mediators results in phosphorylation of IκB, which liberates NFκB, and allows NFκB to translocate to the nucleus where it mediates the regulation of several genes associated with both hemostasis and inflammation (Table 5). This mechanism of activation permits a rapid cellular response to the extracellular environment (124). The phosphorylation of IκB is mediated by at least two mechanisms, which include a serine protease activated by proinflammatory mediators, or a tyrosine phosphorylase activated under conditions of oxidative stress (125). The anti-inflammatory actions of aprotinin could be achieved by either mechanism, by direct inhibition of the serine protease or indirectly by anti-oxidant actions.

The speculation that aprotinin influences NFκB activity was examined in a series of in vitro studies using a lung epithelial cell line. In these studies, it was found that aprotinin (0.13 KIU/mL), but not tranexamic acid or aminocaproic acid, significantly inhibited cytokine-induced nitric oxide production via reduction in iNOS expression, a product regulated by NFκB (28,126,127). These researchers speculated that aprotinin inhibits the serine protease responsible for the degradation of IκB, and thus the NFκB-mediated gene expression for iNOS.

Finally, the anti-inflammatory cytokine IL-10 has been shown to inhibit NFκB translocation selectively in a dose-dependent manner (128). Because aprotinin has been shown to increase IL-10 levels during CPB (110), it is possible that aprotinin mediates its anti-inflammatory actions via IL-10 at the cellular level. Based on the serine protease inhibitor and antioxidant properties of aprotinin, it is conceivable that aprotinin could mediate both the hemostatic and anti-inflammatory actions at the cellular level.

**SUMMARY**

The ability of aprotinin, a serine protease inhibitor, to improve post-operative hemostasis has been well documented. The inhibition of several key serine proteases within the hemostatic and inflammatory systems explains the superior efficacy of aprotinin compared to other serine protease inhibitors or antifibrinolytic agents. The primary mechanism by which aprotinin mediates its hemostatic efficacy is most likely by inhibition of plasmin, and at higher concentrations, kallikrein. Both of these serine proteases interact at multiple levels within the hemostatic system, including fibrinolysis, platelet function, vascular endothelial function, and inflammation. However, inhibition of plasmin and kallikrein do not explain all the observed biochemical and clinical effects of aprotinin. Both in vivo and in vitro studies have suggested additional sites of action of aprotinin. The ability of aprotinin to bind to cell surfaces expands the number of serine proteases that could be inhibited by aprotinin at plasma concentrations achieved with high- or low-dose regimens.

Based on all the current information, the efficacy of aprotinin is probably not attributable to inhibition of just
one serine protease. Rather, aprotinin attributable to its broad specificity, probably inhibits serine proteases in several cascades, each individually insignificant, but together significantly improves the function of the entire hemostatic system after CPB. Further studies are required to elucidate the mechanism(s) by which aprotinin achieves its hemostatic efficacy. Such a determination would also facilitate the understanding of the mechanisms responsible for post-CPB bleeding.

REFERENCES


