Clinical Inhibition of the Seven-Transmembrane Thrombin Receptor (PAR1) by Intravenous Aprotinin During Cardiothoracic Surgery

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Background—Protease-activated receptor-1 (PAR1) is the principal thrombin receptor in the vasculature, and antagonists against this receptor are in preclinical trials. Aprotinin, already approved for clinical use to reduce transfusion requirements in cardiopulmonary bypass (CPB) surgery, has been shown to inhibit PAR1 activation in vitro. Here, we exploit CPB as a model for thrombin generation in humans to examine whether aprotinin can inhibit platelet PAR1 activation clinically.

Methods and Results—PAR1 expression and function on platelets was examined in coronary artery bypass grafting (CABG) patients randomized into 2 groups: (1) those receiving saline infusion during CPB (n=17) and (2) those receiving aprotinin (2x10^6 kallikrein inhibitor units [KIU] in pump prime, 2x10^6 KIU loading dose, followed by 0.5x10^6 KIU/h [n=13]). Platelets in the saline group showed loss of PAR1-specific function at 2 hours after CPB, but this was preserved in the aprotinin group (P<0.001). These effects were most likely targeted at PAR1 receptor cleavage, because (1) the level of thrombin generated during CPB did not vary significantly between groups, (2) expression of SPAN12, which detects only uncleaved PAR1 receptors, was preserved in the aprotinin but not the placebo group (P<0.05), and (3) supporting evidence in vitro showed reduced thrombin-induced PAR1 cleavage (P<0.001) and platelet aggregation (P<0.001) in the presence of aprotinin.

Conclusions—This study demonstrates that platelet PAR1 activation by thrombin can be inhibited by aprotinin. Our results extend the clinical mechanism of action of aprotinin and provide the first proof of principle that PAR1 can be inhibited clinically. This has implications beyond cardiac surgery for the development of therapeutic PAR1 blockade. (Circulation. 2004;110:2597-2600.)

Key Words: thrombin ■ platelets ■ cardiopulmonary bypass

The protease-activated receptor (PAR) family comprises 4 members, PAR1 through PAR4, which are widely expressed on platelets and cells of the vasculature.1 PAR1 is the principal thrombin receptor in humans, mediating thrombin-induced aggregation of platelets and adverse events, such as proinflammatory responses, angiogenesis, cell invasion, and neurodegeneration in other cell types; thus, antagonism of PAR-1 may have broad therapeutic significance.

A number of PAR1 antagonists have been developed on the basis of the unique mechanism of PAR1 activation. PAR1 is cleaved by the serine protease activity of thrombin, creating a new N-terminal sequence (SFLLRN) that can dock in the ligand-binding pocket of the transmembrane domain and transmit G protein–coupled signals into the cell. Synthetic peptide and nonpeptide mimetics based on either the ligand or transmembrane signaling structures have been developed.2,3 A hallmark of these antagonists is that cleavage of PAR1 still occurs on activation by thrombin, even though intracellular signaling is abrogated. Ligand mimetic antagonists have shown clear therapeutic promise in preclinical trials of thrombosis and restenosis,4,5 but clinical trial data are still awaited.

Aprotinin is a serine protease inhibitor that has been used in cardiothoracic surgery to prevent blood loss and preserve platelet function during cardiopulmonary bypass (CPB).6,7 The platelet dysfunction in bypass is thought to be due to selective activation of PAR1 and internalization of receptor after exposure to thrombin generated in the bypass circuit.8–10 Because aprotinin has been shown to selectively inhibit PAR1-dependent platelet activation in vitro,11 we tested its ability to block PAR1 activation in vivo in a randomized, placebo-controlled trial of CABG patients on bypass.

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2597
Methods

Patients
Patients referred for elective CABG were enrolled with regional ethics committee approval and after giving informed consent. Patients were randomized to receive saline infusion during bypass (group 1; n=17) or “high-dose” aprotinin (2×10⁶ kallikrein inhibitor units [KIU] in the pump prime and 2×10⁶ KIU loading dose, followed by 0.5×10⁶ KIU/h on CPB; group 2, n=13). Surgeons were blinded as to treatment group, and all patients underwent a routine surgical procedure and recovery using standardized techniques. Each patient received 300 IU/kg porcine heparin (Monoparin, CP Pharmaceuticals) to achieve an activated coagulation time (ACT) >480 seconds (Hemochron Jr with kaolin). ACT was measured at baseline, after heparinization, and every 30 minutes during CPB. Heparin was reversed with 200 mg protamine sulfate (Celltech Pharmaceuticals) and assessed by normalization of the ACT.

Thrombin Generation During Bypass
Thrombin generated during bypass was measured by thrombin-antithrombin complex formation in plasma, collected preoperatively, 5 minutes into bypass, at end of bypass, and 2 hours, 24 hours, and 5 days after bypass. Samples were stored at −70°C before analysis by ELISA (Affinity Biologicals).

Platelet PAR1 Expression and Function Ex Vivo
Expression of uncleaved PAR1 was detected with the antibody SPAN12 (Beckman Coulter). Blood was drawn preoperatively and at 2 hours and anticoagulated with sodium citrate, and SPAN12 expression was determined by flow cytometry of platelet-rich plasma as described previously. All steps were performed at 4°C to prevent internalization of cleaved receptors. PAR1 function was analyzed by tricolor flow cytometry in whole blood that measured the formation of thrombin receptor agonist peptide (TRAP)-6–dependent platelet:leukocyte conjugates in whole blood. This test reflected PAR1 function by flow cytometry of platelet-rich plasma that measured the formation of TRAP-6–inducible platelet:leukocyte conjugates. Whole blood was diluted (3:7) in modified Tyrode’s buffer, and 100-μL aliquots were incubated at room temperature with TRAP-6 (SFLLRN hexapeptide; Bachem Ltd; 20 μmol/L) or buffer. After 10 minutes’ incubation, samples were fixed with 1% paraformaldehyde and stained with the following monoclonal antibodies: pan-leukocyte marker CD45-FITC, the monocyte marker CD14-ECD, and the platelet marker CD42b-PE (Beckman Coulter). Leukocyte populations were identified with CD45-FITC and CD14-ECD fluorescence, and then CD42b-PE was used to calculate the percentage of platelets adherent to each leukocyte population.

In Vitro PAR1 Expression and Platelet Aggregation
Washed platelets were obtained from platelet-rich plasma, and aggregation was performed in response to 3 nmol/L thrombin (Sigma Aldrich) as described previously. The PAR1 peptide antagonist FLLRN (Bachem) was included at 500 μmol/L and aprotinin at 50, 100, and 200 KIU/mL. To detect PAR1 cleavage, platelets were activated with thrombin for 10 minutes, stained with SPAN12 at 2 hours and anticoagulated with sodium citrate, and SPAN12 expression was determined by flow cytometry of platelet-rich plasma (as assessed by thrombin-antithrombin complex formation in plasma) measured by loss of SPAN12 expression. Results are expressed as mean relative fluorescent intensity (RFI) of staining, calculated by dividing mean fluorescent staining intensity with SPAN12 antibody at any given time point by staining intensity of class-matched control antibody ± SEM. 2h Post indicates 2 hours after CPB.

Statistical Analysis
Comparisons between multiple groups were analyzed by ANOVA followed by Newman-Keuls post hoc test, except for platelet aggregation (Figure 2A), which used a Dunnett post hoc test (“thrombin” as reference group). A Student’s t test was used to compare thrombin-antithrombin levels between aprotinin and saline groups (Figure 1A). Significance was assumed at P<0.05.

Results
Aprotinin Inhibits Platelet PAR1 Activation During CABG Surgery With Bypass
Thirty patients were enrolled in the study, all of whom had uncomplicated postoperative recoveries. Thrombin generation (as assessed by thrombin-antithrombin complex formation) was sharply increased perioperatively, consistent with previous reports, but did not differ significantly between groups up to 2 hours after bypass (Figure 1A). At 24 hours, however, thrombin generation was significantly reduced in the aprotinin group compared with the saline group (P=0.037) but was no longer reduced by 5 days. The functional status of PAR1 perioperatively was probed by examination of the formation of TRAP-6–inducible platelet:leukocyte conjugates in whole blood. This test re-
revealed a significant drop in PAR1 function in the saline treatment group, which was most pronounced at 2 hours after bypass (Figure 1B). Platelets from the aprotinin group, however, were significantly protected compared with saline ($P<0.001$). Spontaneous platelet:leukocyte conjugate formation and platelet responses to ADP or epinephrine remained unchanged during bypass and between groups (data not shown), which demonstrates that platelet desensitization was selective for PAR1. Expression of the SPAN12 epitope, which detects uncleaved PAR1, confirmed that significantly less receptor activation took place in the aprotinin than in the saline group ($P<0.05$; Figure 1C).

**Inhibition of PAR1 Receptor Cleavage In Vitro**

In vitro experiments used washed platelets, free of detectable clotting factors and other soluble plasma proteins, and thrombin at $3 \text{nmol/L}$, a concentration that selectively activates PAR1 (ie, not PAR4). In this model, aggregation was essentially abolished by the peptide-mimetic FLLRN (Figure 2A) and significantly inhibited by 50, 100, and 200 KIU/mL aprotinin (respectively, $37.5 \pm 6.5\%$ inhibition [mean±SEM, n=10; $P<0.001$], $41.3 \pm 11.02\%$ [$P<0.05$], and $43.63 \pm 3.41\%$ [$P<0.001$]). Loss of SPAN12 epitope was inhibited by aprotinin ($P<0.001$) but not by the PAR1 peptide antagonist FLLRN ($P>0.05$), which suggests a distinct mechanism of action for aprotinin aimed at preventing PAR1 cleavage.

**Discussion**

Our results in CABG patients on bypass constitute the first clinical data showing that PAR1 activation by thrombin can be inhibited clinically. The sparing of PAR1 receptor cleavage, both in vivo and in vitro, rules out the likelihood that effects seen clinically were due to indirect targeting of plasma proteases such as plasmin or kallikrein. It is also unlikely that effects seen clinically were due to differences in thrombin generation, because this did not vary significantly between groups up to 2 hours after bypass.

Interestingly, blockade of platelet PAR1 by aprotinin does not exacerbate bleeding, because platelets maintain their ability to be activated by other stimuli, such as collagen and ADP. Thus, aprotinin minimizes participation of thrombin-activated platelets in the coagulation cascade, thereby exerting a net antithrombotic effect, whereas the hemostatic capacity of platelets in surgical wounds is maintained.

The PAR1 sparing mechanism identified here is distinct from other peptidomimetics in that (1) aprotinin inhibits both signaling and receptor cleavage in response to thrombin, whereas peptidomimetics inhibit signaling, not receptor cleavage, and (2) aprotinin does not inhibit signaling caused by TRAP-6 (Figure 1B and Poullis et al), whereas peptidomimetics do. However, the obvious hypothesis, that aprotinin as a serine protease inhibitor directly inhibits the catalytic activity of thrombin in solution, is unlikely, because the systemic concentration of aprotinin achievable during bypass is $\sim 60$-fold below the inhibitory constant ($K_i$) for thrombin.

The PAR1-sparing mechanism described here places aprotinin into a distinct category of PAR1 antagonists, with implications beyond its primary use as a hemostatic agent during cardiac surgery. The present study demonstrates that PAR1 activation by thrombin can be inhibited therapeutically; however, we are not advocating extending the therapeutic role of aprotinin beyond its current license. Aprotinin is uniquely well suited to cardiac surgery with CPB, because it combines a well-documented antifibrinolytic hemostatic benefit with platelet preservation, protection from the systemic inflammatory response, and significant reduction in perioperative stroke. Such a broad pharmacotherapeutic profile may be less beneficial in other disease states in which fibrinolysis is not as important. This study may also explain the reduction in perioperative stroke, because stroke patients have been shown to manifest similar platelet exhaustion due to cleavage of PAR1.

In conclusion, we have demonstrated that aprotinin inhibits PAR1 activation by thrombin during CPB, with clinical implications for platelet function and perioperative stroke, and we have proven the principle that PAR1 can be inhibited clinically.

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**References**


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