Antithrombotic Effects of Combining Activated Protein C and Urokinase in Nonhuman Primates

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Background. We have determined in vivo the relative antithrombotic efficacy and hemostatic safety of combining low-dose activated protein C (APC) and urokinase (urinary plasminogen activator, u-PA), two natural proteins that regulate thrombogenesis.

Methods and Results. To model acute thrombotic responses of native blood under conditions of arterial flow, thrombogenic segments of Dacron vascular graft (VG) were incorporated into chronic exteriorized femoral arteriovenous (AV) access shunts in baboons. Thrombus formation on VG was determined by measuring 1) the deposition of autologous \(^{111}\)In platelets using real-time scintillation camera imaging, 2) the accumulation of \(^{125}\)I fibrin, 3) segment patency by Doppler flow analysis, and 4) blood tests for thrombosis, including plasma concentrations of platelet factor 4, \(\beta\)-thromboglobulin, fibrinopeptide A (FPA), and d-dimer. Treatments consisting of low-dose and intermediate-dose APC (0.07 or 0.25 mg/kg·hr), u-PA (25,000 or 50,000 IU/kg·hr), or the combination were administered for 1 hour by continuous intravenous infusion. In untreated controls, platelets and fibrin accumulated rapidly, reaching plateau values at 1 hour of 15.1±3.8x10\(^5\) platelets and 7.8±2.2 mg fibrin. Although the low-dose APC or u-PA alone did not decrease either platelet or fibrin deposition significantly, this combination moderately reduced both platelet and fibrin accumulation (7.3±2.6x10\(^5\) platelets, \(p<0.05\); 3.9±0.6 mg fibrin, \(p<0.05\)). Furthermore, intermediate-dose APC or u-PA reduced thrombus formation by half when administered alone (\(p<0.001\) for both platelet and fibrin deposition), and the combination markedly interrupted the accumulation of platelets (3.0±1.0x10\(^5\) platelets, \(p<0.001\)) and fibrin (1.3±0.6 mg fibrin, \(p<0.001\)). During active treatments, all VG segments remained patent. Hemostatic plug forming capability, as measured by template bleeding times, remained normal during all experiments (\(p>0.05\)). The \(T_{50}\) clearance time for APC activity was not affected by the concurrent administration of u-PA. u-PA alone increased the plasma levels of d-dimer, FPA, and, interestingly, APC, implying that during pharmacological activation of the fibrinolytic system, thrombin activity was released, and the protein C pathway was activated.

Conclusions. A combination of intermediate-dose APC and u-PA produce substantial and efficient antithrombotic effects without impairing hemostatic function. (Circulation 1991; 84:2454–2462)

Two physiological serine proteases—activated protein C (APC) and plasmin—exhibit potent antithrombotic effects, as evidenced by the capacity of APC to interrupt platelet-dependent thrombus formation in nonhuman primate models of arterial thrombosis\(^1\)-\(^3\) and the clinical usefulness of plasminogen activators (PAs).\(^4\)-\(^5\) APC, the activation cleavage product of plasma protein C (PC) and the endothelial thrombin-thrombomodulin complex,\(^6\) catalyzes the proteolytic inactivation of blood coagulation...
catalysts Va and VIIIa. Both clinical9–12 and experimental13 data indicate that the PC pathway is physiologically important for the regulation of thrombosis. Plasmin, derived from plasminogen cleavage by various PAs,14 is also physiologically important in the regulation of thrombus propagation. The clinical benefits of thrombolytic therapy are well documented, although there are a number of important associated complications, including increased risk of abnormal bleeding and failure to achieve reperfusion or early reocclusion.15 Because combining PAs with other antithrombotic agents16–18 may enhance thrombolytic efficacy without adversely affecting hemostatic safety, we have tested the hypothesis that combining low doses of APC and urinary plasminogen activator (u-PA) effectively and safely blocks the formation of platelet-rich arterial thrombus.

Methods

Thrombosis Model

The effects of combining APC and u-PA have been studied in 16 juvenile male baboons (Papio anubis) weighing 8–12 kg and previously observed to be disease-free for 3 months. We used a standardized and reproducible arterial thrombosis model.3 To model acute platelet thrombosis under conditions of well-controlled flow and geometry, we incorporated 5-cm-long segments of thrombogenic knitted Dacron vascular grafts (VG) (0.4 cm i.d., 630 µl volume, U.S. Catheter, Inc.) into chronic nonthrombogenic silicone rubber arteriovenous exteriorized access shunts in baboons.1–3 The grafts were exposed to arterial blood flow for 1 hour beginning at time 0, with graft removal after 1 hour as described.1–3 The porous VGs quickly became actively thrombogenic toward circulating blood elements, primarily platelets. Platelet deposition was measured continuously and noninvasively using gamma scintillation camera imaging after labeling autologous platelets with 111In-oxine.3 Graft platelet radioactivity in controls increased 40–50-fold, reaching a plateau of 20–25 million platelets/µl in the VG by 1 hour. Concurrently, the fibrin content of the thrombus in the graft was determined at the study end point after labeling with homologous 125I fibrinogen (FGN).3 VG FGN radioactivity in controls increased fivefold in 1 hour compared with the fibrinogen radioactivity contained within the graft luminal blood volume. Because control grafts generally occlude within 1–2 hours after insertion,1–3 each perfusion experiment was terminated at 60 minutes. The blood flow in the permanent shunt was restored after removal of the VG. Primary hemostasis was assessed by measurements of standardized bleeding time.19 Each animal was subjected to a minimum of one and a maximum of four experiments within a 2-week period. In general, control experiments were performed initially and then followed on separate days by infusions of antithrombotic agents. Fifty thrombosis model experiments were performed, including controls, during an 18-month period.

The animals were carefully observed during and after each experiment for possible adverse reactions, including abnormal bleeding, cardiorespiratory vital signs, allergic reactions, and fever. All experiments were approved by the institutional animal care and use committee in accordance with federal guidelines.

Antithrombotic Agents

PC was initially purified from lyophilized human factor IX complex concentrate (FIXc) as described.1 The immunoglobulin (Ig) G fraction of serum from sheep immunized with human PC was passed over a PC-Sepharose column (containing a total of 30 mg human PC coupled to 10 ml gel) in the presence of 5 mM CaCl₂ (both FIXc and sheep IgG were kindly provided by Dr. H.P. Schwarz). Calcium-dependent anti-PC polyclonal antibodies were eluted from the column using 20 mM EDTA and dialyzed against 0.5 M NaCl and 0.05 M borate (pH 8.5); 20 mg of IgG was coupled to CNBr-activated Sepharose 4B (Pharmacia). PC from FIXc was absorbed onto this gel in the presence of 5 mM CaCl₂. The gel was thoroughly washed with 1 M NaCl, 5 mM CaCl₂, 0.05 Tris (pH 7.35), 0.02% Na-azide, and 0.02% Tween-20, and the PC was eluted with 1 M NaCl, 20 mM EDTA, and 0.05 M Tris (pH 7.35). The cycle was repeated several times, yielding approximately 3.6 mg PC each cycle without significant loss of capacity. The final PC products were more than 95% pure on sodium decyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and all protein bands cross-reacted with anti-human protein C light-chain murine monoclonal antibodies in Western blots. After extensive dialysis against 0.14 M NaCl and 0.01 M Tris (pH 7.35) (TBS), pooled lots of the PC preparations were activated using thrombin-Sepharose beads, as described.20 The final APC products were more than 95% pure on SDS-PAGE. Three different APC preparations were used. The specific anticoagulant activity of each APC preparation was measured in a coagulation assay, as described.1 With this assay, the anticoagulant activity of each APC preparation was identical to the anticoagulant activity of plasma protein C activated by the snake venom activator Protac (American Diagnostica, N.Y.). All three APC preparations used for infusions had a specific in vitro anticoagulant activity of 250 IU/mg. The relations between the anticoagulant activities of these various human plasma–derived APC preparations and their antithrombotic activities in nonhuman primate platelet-dependent thrombosis was not determined. The APC was passed through a bacterial filter, was frozen in liquid nitrogen, and was stored at −70°C, where it remained stable for more than 1 year. The APC was diluted with sterile saline containing 5% dextrose before use. The enzyme was found to remain stable in solution for at least 3 hours at room temperature.
APC was infused for 1 hour at two doses: a lower dose of 0.07 mg/kg·hr and an intermediate dose of 0.25 mg/kg·hr. One third of the total administered dose of agent was injected as a bolus in the access shunt at 0 minutes, and the remainder was administered by infusion throughout the 1-hour period of study. The intermediate-dose APC was chosen on the basis of previous experiments in baboons, indicating that this dose produced intermediate inhibitory effects on thrombus formation. The low dose of APC was chosen by extrapolating previous data to a dose where no significant effect on platelet deposition was expected.

The profibrinolytic agent u-PA was a gift from Abbott Laboratories (Abbokinase, urokinase for injection, North Chicago, Ill.) and was reconstituted aseptically in sterile saline-dextrose before infusion. Because baboons are less sensitive to human urokinase than humans or chimpanzees, relatively large doses of u-PA were used in the experiments. The lower dose of u-PA was calculated to produce theoretically marginally efficient plasma levels of 200 IU/ml or less. Preliminary studies in the baboon indicated that infusion of twice the intermediate-dose u-PA (100,000 IU/kg·hr) in large part degraded circulating fibrinogen and in part interrupted VG thrombus formation, whereas the low-dose u-PA (25,000 IU/kg·hr) failed to affect either platelet deposition or hemostasis (data not shown). Thus, u-PA was used at a low dose of 25,000 IU/kg·hr and an intermediate dose of 50,000 IU/kg·hr.

APC and u-PA were combined at both doses (i.e., the low-dose APC with low-dose u-PA, and the intermediate-dose APC with intermediate-dose u-PA). Because most of the animals underwent a series of experiments, including controls without APC or u-PA, the low or intermediate doses alone, and the combination of either low or intermediate doses, the design was efficient for statistical analyses.

Laboratory Studies

Arterial blood was collected directly from the silicon rubber tubing of the chronic arteriovenous shunts into appropriate anticoagulants before, during, and after each study. Circulating free APC activity, APC–protein C inhibitor complexes (APC:PCI), fibrinopeptide A (FPA), fibrinogen (FGN), D-dimer, β-thromboglobulin (BTG), platelet factor 4 (PF4), hemoglobin levels, hematocrit, and activated partial thromboplastin times (APTT) were determined as described elsewhere. The immunoassays developed for respective human antigens were reliably used for measurements of baboon antigens because of good cross-reactivity.

Statistical Analysis

Results are given as mean±SD, unless otherwise stated. Comparative statistical analyses for each sample were performed using a nonparametric rank test (Wilcoxon). Pearson’s correlation coefficient and linear regressions were performed where indicated (TL

Gustafson, EPISTAT statistical program packages, 1984, Round Rock, Tex.). A probability value of less than 0.05 for the difference between samples and for correlations between variables was considered statistically significant.

Results

Platelet Thrombus Formation

The time courses of platelet deposition onto segments of thrombogenic VG are shown in Figure 1. The thrombosis model was highly reproducible (i.e., when control platelet deposition values [n=15] were analyzed over the period during which these experiments were performed, linear regression analysis and calculation of the correlation coefficient for these data showed that there was no increasing or decreasing trend for the 60-minute platelet deposition values in controls during the 18-month experimental period; r=0.007, y=15.03+0.005x, p=0.98). In control experiments, 15.1±3.8×10⁶ platelets were deposited in VG (n=15) that were patent at 1 hour. Control experiments in which with VG occluded before 1 hour were not included in the calculations. Values of platelet deposition after a 60-minute infusion of low-dose APC and u-PA were compared with the control values. The mean±SD of these values, in order of increasing effectiveness of therapy for the indicated agent regimen, were 1) low-dose APC (0.07 mg/kg·hr; 12.9±4.7×10⁶, n=5, p>0.2), 2) low-dose u-PA (25,000 IU/kg·hr; 11.3±5.0×10⁶, n=4, p>0.05), and 3) low-dose combination (7.3±2.6×10⁶, n=5, p<0.001). Thus, platelet deposition was not significantly inhibited by low-dose APC or low-dose u-PA alone but rather was significantly reduced by the low-dose combination (Figure 1A). Similarly (Figure 2A), low-dose APC and u-PA had marginal effect on fibrin accumulation on the graft segments when infused alone and further impaired fibrin deposition when used in combination (7.8±2.2 mg fibrin deposited in 1 hour in 12 controls compared with 5.2±0.8, n=5, p<0.01 for low-dose APC; 4.1±0.4, n=2, p<0.05 for low-dose u-PA; and 3.9±0.6, n=6, p<0.001 for low-dose combination).

When intermediate-dose APC and u-PA were administered alone, platelet deposition was detectably decreased (Figure 1B). The order of increasing effectiveness was 1) intermediate-dose u-PA (50,000 IU/kg·hr; 7.5±2.0×10⁶, n=5, p<0.001), 2) intermediate-dose APC (0.25 mg/kg·hr; 5.9±1.5×10⁶, n=5, p<0.001), and 3) intermediate-dose combination (3.0±1.0×10⁶; n=5, p<0.001). The low-dose combination, intermediate-dose u-PA, and intermediate-dose APC regimens were not statistically different from each other in the inhibition of platelet deposition (p>0.05 for each). However, the intermediate-dose combination (Figure 1B) significantly reduced platelet deposition to a lower value than any of the other regimens (p<0.05 in each case). Individual doses of APC (0.25 mg/kg·hr) or u-PA (50,000 IU/kg·hr) markedly decreased
fibrin deposition, and the combination of intermediate doses abolished fibrin accumulation (Figure 2B) (intermediate-dose APC: 3.8±0.5 mg, n=5, p<0.001; intermediate-dose u-PA: 2.4±1.0 mg, n=6, p<0.001; and the intermediate-dose combination: 1.3±0.6 mg, n=6, p<0.001). Fibrin deposition was greater for the intermediate dose of APC than with the intermediate dose of u-PA (p<0.05), and fibrin deposition was significantly less in the intermediate-dose combination experiments (p<0.02 versus other groups). Thus, APC and u-PA individually and in combination inhibited thrombus formation in a dose-dependent manner, and the combinations were more effective than either agent alone. Combination of APC with u-PA exhibited antithrombotic activities that were approximately the additive sum of each agent alone. Furthermore, low-dose APC inhibited fibrin deposition even when the inhibition of platelet deposition was not statistically significant.

Measurement of Blood Flow

Initial mean blood flow rates through the thrombogenic graft segments averaged 193±35 ml/min (n=11), as measured by Doppler flowmeter. There was a progressive decrease in blood flow in untreated control experiments, and three of 18 control grafts occluded within 60 minutes. Previously, we reported that all untreated control grafts occlude within 120 minutes. None of the grafts occluded within 60 minutes when APC, u-PA, or the combination was infused into the animal, and blood flow rates remained constant throughout each 60-minute treatment period.

Blood Measurements of Thrombosis, APC, and Plasmin Activity

In vivo activation of platelets and coagulation was evaluated by measuring plasma levels of the platelet-specific storage proteins, PF4 and BTG, and thrombin's cleavage product of fibrinogen, FPA.
The circulating levels of D-dimer reflected the plasmin cleavage of cross-linked fibrin, and determinations of FGN concentrations served to assess the combined effect of the consumption of FGN by thrombin cleavage and the degradation of FGN by plasmin. The results are summarized in Table 1. The highest increase in FPA levels was observed after infusion of intermediate-dose u-PA. However, the administration of both low- and intermediate-dose APC prevented significant changes in FPA levels and prevented or attenuated the increase caused by low- or intermediate-dose u-PA infusions, respectively (Table 1). FGN levels at 60 minutes decreased moderately in the intermediate-dose u-PA experiments (p<0.05) and were not different than control values at 60 minutes in the other treated animals (p>0.05 for each; Table 1). The FGN level during control experiments did not decrease significantly (2% decrease, p>0.2) but was moderately reduced during infusion of low-dose u-PA, intermediate-dose u-PA, and the combination; the average reductions were 13%, 18%, and 13%, respectively (p<0.05 in each case).

An increase in D-dimer levels was observed after infusion of intermediate-dose APC, u-PA, and the combination; these increases were 54%, 194%, and 166%, respectively (Table 1), indicating that these agents stimulated fibrinolysis. The markers of platelet activation, BTG and PF4, were increased at 60 minutes in all experiments (more than 170% of baseline values, p<0.05 for each) (Table 1). However, infusions of low-dose APC and the low-dose combination as well as intermediate-dose APC, u-PA, or their combination were associated with smaller increases in BTG. Furthermore, intermediate-dose APC alone or its combination with u-PA was associated with smaller increases in PF4. This suggested that these regimens reduced the platelet activation (p<0.05 in each case).

Circulating levels of APC activity at 60 minutes are shown in Figure 3. The APC level achieved during the infusion of intermediate-dose APC alone (0.25 mg/kg · hr) was 0.58±0.07 μg/ml (n=5); it was 0.53±0.15 μg/ml (n=5) for the intermediate-dose combination. These levels were equivalent (p>0.05). Similarly, the APC levels with low-dose APC alone and with the low-dose combination were indistinguishable (p>0.05) (0.11±0.02 μg/ml and 0.13±0.03 μg/ml, respectively). Interestingly, endogenous APC activity increased from background (less than 2 ng/ml) to 3.0±2.1 ng/ml at 60 minutes in control studies with Dacron grafts, and APC was 27±12 and 23±7.4 ng/ml (range, 11–38 ng/ml) after the low- and intermediate-dose u-PA infusions, respectively (p<0.001 for each group). After terminating the infusions of APC at 60 minutes, APC activity diminished with an initial T50 of 13.2±1.5 and 13.7±2.6 minutes in the intermediate-dose APC and intermediate-dose combination experiments, respectively (p>0.05). Thus, the circu-
TABLE 1. Plasma Markers of Thrombin and Plasmin Activity in Baboons Receiving APC and/or u-PA Infusion

<table>
<thead>
<tr>
<th></th>
<th>BTG (µg/l)</th>
<th>PF4 (µg/l)</th>
<th>FGN (mg%)</th>
<th>FPA (nM)</th>
<th>D-dimer (µg/l)</th>
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<td><strong>Control</strong></td>
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<tr>
<td>0 Min</td>
<td>3.65±2.63</td>
<td>5.69±2.38</td>
<td>309±98</td>
<td>4.19±2.3</td>
<td>415±183</td>
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<tr>
<td>n</td>
<td>16</td>
<td>16</td>
<td>15</td>
<td>14</td>
<td>13</td>
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<tr>
<td>60 Min</td>
<td>37.14±14.68</td>
<td>24.35±13.79</td>
<td>303±100</td>
<td>13.05±5.99</td>
<td>443±179</td>
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<td>16</td>
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<td>P1</td>
<td>&lt;0.001</td>
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<td>%</td>
<td>1,018</td>
<td>428</td>
<td>98</td>
<td>311</td>
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**Intermediate-dose**

|                |            |            |           |          |                |
| APC            | 60 Min     | 10.24±4.64 | 9.62±2.75 | 316±15   | 3.90±0.49      | 638±111         |
| n              | 5          | 5          | 5         | 4        | 5              |
| P2             | <0.001     | <0.001     | >0.5      | <0.001   | <0.05          |
| %              | 627        | 220        | 96        | 93       | 154            |
| u-PA           | 60 Min     | 19.17±6.85 | 16.41±5.45| 286±73   | 21.92±6.26     | 1,222±524       |
| n              | 6          | 6          | 6         | 5        | 6              |
| P2             | <0.001     | >0.05      | <0.05     | <0.01    | <0.001         |
| %              | 525        | 289        | 93        | 523      | 294            |
| APC plus u-PA  | 60 Min     | 6.50±5.55  | 9.98±4.04 | 288±82   | 7.65±4.95      | 1,103±572       |
| n              | 6          | 6          | 4         | 5        | 6              |
| P2             | <0.001     | <0.001     | >0.2      | >0.2     | <0.001         |
| %              | 178        | 175        | 93        | 183      | 266            |

**Low-dose**

|                |            |            |           |          |                |
| APC            | 60 Min     | 23.52±7.21 | 16.20±4.22| 338±75   | 5.18±1.30      | 496±216         |
| n              | 6          | 6          | 5         | 6        | 6              |
| P2             | <0.05      | >0.05      | >0.2      | <0.001   | >0.2           |
| %              | 644        | 285        | 109       | 124      | 120            |
| u-PA           | 60 Min     | 33.06±9.11 | 25.15±9.36| 295±90   | 13.95±7.09     | 744±354         |
| n              | 5          | 4          | 5         | 4        | 5              |
| P2             | >0.5       | >0.5       | >0.05     | >0.5     | >0.05          |
| %              | 906        | 442        | 95        | 333      | 179            |
| APC plus u-PA  | 60 Min     | 21.38±8.14 | 15.54±4.55| 285±66   | 6.32±1.46      | 566±183         |
| n              | 5          | 5          | 5         | 5        | 5              |
| P2             | <0.05      | >0.05      | >0.05     | <0.001   | >0.05          |
| %              | 585        | 268        | 92        | 151      | 136            |

APC, activated protein C; u-PA, urinary plasminogen activator; BTG, β-thromboglobulin; PF4, platelet factor 4; FGN, fibrinogen; FPA, fibrinopeptide A. All results are shown as mean±SD as determined from blood samples at 0 and 60 minutes in controls and after 60 minutes of APC and u-PA infusion experiments. Number of animals is indicated after each parameter and range from four to 16. P1 is the probability value in comparison of control samples at 0 and 60 minutes. P2 values indicate comparisons at 60 minutes in control and low- and intermediate-dose APC and u-PA experiments. All p values were determined using the Wilcoxon rank test. The 60-minute mean values are also expressed as a percentage of the control (0 min) mean values for the corresponding parameters.

...ating level of APC was proportional to the amount of APC infused, and its clearance rate was independent of u-PA coinfusion. Importantly, infusion of u-PA alone significantly increased circulating endogenous APC levels. The correlation between the observed increase in FPA and APC levels after infusion of u-PA was weak (r=0.45, p>0.05).

The plasma levels of APC:PCI complexes were measured in the intermediate-dose experiments. The value at 60 minutes was 51.2±20.3 ng/ml for infusion of u-PA alone, 2.18±0.64 µg/ml for the APC infusion, and 2.53±0.41 µg/ml for the combination infusion. The concentrations of APC:PCI complexes were below the detection limit of the assay (10 ng/ml) in the control experiments. Thus, both endogenously generated APC and infused exogenous APC were inhibited by plasma PCI in vivo.

**Measurements of Hemostatic Function**

The standardized template bleeding time averaged 4.7±0.9 minutes (n=14; range, 3.6–7.2 minutes) before therapy infusion and in control animals. The bleeding times during infusion of APC, u-PA, or the combination were not significantly increased (p>0.05 for each regimen). The longest bleeding value for any group was 6.6±1.7 minutes (n=5; range, 4.5–8.8 minutes), observed with infusion of the intermediate-dose combination. Thus, hemo-
static plug formation, as assessed by the template bleeding time assay, was not compromised by any of the treatment regimens applied. There was no evidence in any of the animals studied of spontaneous external or internal bleeding or of oozing from venipuncture or bleeding time sites.

The APTTs at 60 minutes were prolonged by 11.3±7.3 seconds in the low-dose APC experiments and by 11.5±6.1 seconds in the low-dose combination experiments (p<0.05). APTT prolongation in the intermediate-dose APC infusion experiments was not determined. It has been previously reported that infusion of 0.25 mg/kg·hr of APC prolonged the APTT by 40 seconds in the baboon.12 Hemoglobin and hematocrit levels did not change significantly in controls or after infusion of the antithrombotic agents (data not shown).

Adverse effects (e.g., fever, cardiovascular abnormalities, or allergic reactions) were not observed in 32 experiments during or after infusion of human APC and/or human urokinase into baboons.

**Discussion**

The present study demonstrates that low-dose combinations of APC and u-PA produce efficient and safe dose-dependent antithrombotic effects for aspirin- and heparin-resistant experimental arterial thrombosis in baboons. When APC and u-PA are combined at very low doses that individually are ineffective in reducing platelet thrombus formation, substantial antithrombotic effects are achieved without impairing hemostatic function (Figure 1A). Furthermore, increasing the doses of APC and u-PA to levels that individually cause significant effects produces marked interruption of thrombus formation when the agents are given together (Figure 1B), while not affecting hemostasis significantly. Parallel decreases are produced in the deposition of both platelets and fibrin for APC and u-PA used alone or in combination (Figures 1 and 2). The additional finding that thrombotic occlusion of graft segments did not occur during infusion of APC or u-PA suggests that even minimal or incomplete therapies may modify important outcomes.

Plasma-derived and recombinant APC1,2 exhibits potent dose-dependent antithrombotic effects in nonhuman primate models of arterial thrombosis that are platelet dependent and resistant to therapy with aspirin and heparin.25 The efficacy of the thrombolytic enzyme u-PA in preventing platelet-dependent thrombus formation in this model has not been demonstrated previously; however, u-PA is a well-established thrombolytic agent.4,26 Although there are no relevant data available regarding the use of APC as an antithrombotic agent in humans, the first infusion of APC into normal subjects, and in one patient with disseminated intravascular coagulation, has recently been reported.27 Combined administration of prourokinase with heparin has been shown to improve the efficacy of coronary thrombolysis in humans.18 The potential clinical usefulness of combining APC with u-PA has not been previously evaluated. In designing the present preclinical study, doses of APC and u-PA have been selected that

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**Figure 3.** Bar graphs of circulating activated protein C (APC) activity after infusion of APC and urinary plasminogen activator (u-PA). Bars represent the concentration of APC (ng/ml) determined in arterial blood samples (mean values). APC activity was measured using an amidolytic enzyme capture assay.1 Panel A: Control findings are compared with levels observed during low- and intermediate-dose APC infusions, alone or combined with u-PA. Panel B: Control findings are compared with levels observed after infusion of u-PA alone.
produce no or marginal effects or moderately impair platelet and fibrin deposition in baboons.

An interesting aspect of the present study is the finding that FPA levels are significantly elevated (versus control results) by the infusion of u-PA (Table 1). These data are concordant with previous reports that systemic thrombolytic therapy increases thrombin activity in blood.28,29 Possible sources of increased thrombin activity include release of fibrin-bound thrombin by fibrinolysis,30 activation of prothrombin in blood in the presence of PAs,31,32 or other unknown mechanisms. Presumably, increased thrombin activity, as evidenced by an increase in FPA levels, is responsible for the activation of endogenous protein C observed after the infusion of u-PA (i.e., 27 versus 3 ng/ml APC; Figure 3), although some direct activation of the PC pathway by the fibrinolytic system in vitro has also been observed (Gruber et al, personal observations). It can also be hypothesized that endogenous APC might be captured in thrombi and subsequently released into the circulation upon fibrinolysis. Thus, the efficiency of thrombolytic therapy may involve enhancement of the APC antithrombotic mechanism. Measurements of thrombin-antithrombin complexes, plasmin-antiplasmin complexes, and APC activity in samples from patients undergoing thrombolysis could provide further information about relations among plasminogen activation, thrombin release, and APC generation.

The increase in D-dimer level after the administration of the intermediate dose of u-PA (to 1,222±524 μg/l) suggests that the antithrombotic effect of u-PA was, at least in part, the result of thrombolysis. The present study also showed a significant increase in D-dimer levels after intermediate-dose APC infusions compared with controls (p < 0.05), although the data were insufficient to correlate the D-dimer levels with the dose of APC infused. Thus, there is no conclusive evidence in this study that APC enhances fibrinolysis. However, because APC presumably reduces the quantity of fibrin to be lysed, these data do not exclude the possibility that APC enhances fibrinolysis in vivo, as suggested by the results of in vitro studies.33

Interestingly, infusions of APC alone or u-PA alone reduce circulating markers of platelet activation (BTG and PF4) in a dose-dependent manner (Table 1). Because neither APC nor u-PA inhibits platelets directly, this result may reflect an inhibition of secondary mechanisms supporting the participation of platelets in thrombus formation. For example, thrombin is a potent platelet agonist, and APC inhibits thrombin production by destroying the cofactor activities of FV and FVIIIa. Urokinase less obviously exhibits direct antithrombin activity; it generates thrombin in vivo and in vitro. The pathophysiological role of thrombin generation during fibrinolytic therapy is not known. However, u-PA infusion also increases endogenous APC levels, and the antiplatelet effects of u-PA could be related to this effect, as discussed above. An inhibitory role of FGN deg

radation products in platelet-dependent arterial thrombus formation also cannot be excluded. Finally, direct binding of infused u-PA to specific receptors on platelets34 could result in locally enhanced fibrinolytic and fibrinogenolytic activity. These observations, in concert with previous results,23,35,36 suggest that formation of platelet-rich arterial thrombi depends on the incorporation of fibrin or FGN molecules as well as on local prothrombin activation.

APC is a natural serine protease, and it is inhibited by several serine protease inhibitors.37,38 Because u-PA, another serine protease, competes for protein C inhibitor,39 it can be hypothesized that combined infusion of the two agents would alter the circulating half-lives of both APC and u-PA, resulting in higher levels of the enzymes and therapeutic synergism. However, infusion of intermediate-dose u-PA (50,000 IU/kg·hr) with intermediate-dose APC (0.25 mg/kg·hr) failed to prolong the T½ of APC or produce significantly higher circulating APC plasma levels (Figure 3). Thus, at these therapeutically effective APC and u-PA doses, there was no pharmacologically significant competition for protease inhibitors in nonhuman primates, presumably due to the excess blood concentration of several inhibitors for each protease.

In summary, the combined infusion of APC with u-PA enhances antithrombotic efficacy without compromising safety during short-term therapy for arterial-type thrombosis in baboons. These findings suggest that the combination of low doses of APC and u-PA may be useful in humans during thrombolytic therapy for myocardial infarction; after percutaneous transluminal coronary angioplasty; or in coronary, cerebral, or retinal artery thrombosis.

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