Inhibition of Platelet-Dependent Thrombus Formation by Human Activated Protein C in a Primate Model

By András Gruber, John H. Griffin, Laurence A. Harker, and Stephen R. Hanson

The in vivo antithrombotic properties of human plasma activated protein C (APC), a natural anticoagulant enzyme, were investigated in a baboon model of thrombus formation on prosthetic vascular grafts. Infusion of 0.25 to 1.1 mg/kg/h purified, human, APC inhibited blood clotting, as measured by the activated partial thromboplastin time (APTT), and reduced vascular graft platelet deposition by 40% to 70%, as determined by the real-time scintillation camera imaging of 111In-labeled platelet deposition. APC infusion also preserved graft patency. Hemostatic plug formation remained normal, as measured by the template bleeding times. These results suggest that APC administration may produce immediate antithrombotic effects under arterial flow conditions.

© 1989 by Grune & Stratton, Inc.

A PHYSIOLOGIC anticoagulant enzyme, APC, the product of thrombin's action on protein C (PC) in the presence of endothelial cell thrombomodulin, inhibits blood clotting by proteolytic inactivation of blood coagulation cofactors Va and VIIIa. Heterozygous PC or protein S deficiency in some patients is associated with venous thromboembolism, while homozygous PC deficiency or acquired inhibitors of PC have been associated with severe and generalized thrombotic disease.

Although some studies document the role of PC as an antithrombotic protein, the importance of the PC pathway for platelet-dependent reactions in vivo remains undefined. Thus the relative antithrombotic and antihemostatic effects of infused human APC were determined using a nonhuman primate model of thrombus formation on arterial vascular grafts.

MATERIALS AND METHODS

Thrombosis model. Purified APC was administered to awake, healthy, male baboons weighing 10 to 11 kg and bearing chronic shunts between the femoral artery and vein (AV shunts). Thrombogenic Dacron vascular graft segments (5 cm in length, 4 mm internal diameter [ID]) were inserted as extension pieces between silicone rubber tubing segments comprising the high-flow shunt. The baboon was chosen as the experimental animal because of its primate vascular anatomy and close similarity to man with respect to the molecular and cellular composition and function of the hemostatic apparatus. Thrombus formation was measured in real time by scintillation camera imaging of autologous 111In-platelet deposition onto the segments of vascular graft. The time of graft occlusion and the mean blood-flow rates in the shunt were determined using a Doppler flow meter. The capacity of platelets to form hemostatic plugs was assessed by measuring template bleeding times, as described previously. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with federal guidelines.

Activated protein C. PC was purified from plasma factor IX concentrate using immunoaffinity chromatography as follows. Anti-human PC light-chain monoclonal antibodies (MoAbs) designated C3 were coupled to CNBr-activated Sepharose 4B (Pharmacia; 3 mg protein/mL gel) in coupling buffer (0.5 mol/L Tris, pH 7.4) was activated using thrombin-Sepharose beads. The resultant APC contained no detectable thrombin, as measured by clotting assay, and was >90% activated according to SDS-PAGE. The APC prolonged the activated partial thromboplastin time (APTT) of both human and baboon plasma in a concentration-dependent manner. The specific activity of our reference APC was 250 U/mg as determined in a clotting assay using an Electra 700 apparatus (Medical Laboratory Automation, Inc, Mount Vernon, NY) as follows. Initially, 0.5-3 µL of normal human plasma (NHP) or of APC were mixed with 50 µL of Protac solution, prepared according to the manufacturer's instructions (1 U/mL, American Diagnostica, Inc, New York) and then incubated in the Electra 700 cuvette for 460 sec at 37°C. Then 100 µL of APTT reagent (General Diagnostics, Morris Plains, NJ) and 100 µL of PC deficient plasma (George King Biomedical, Inc, Overland Park, KS) were added simultaneously, and the mixture was incubated for 200 sec prior to recalcification using 100 µL 25 mM CaCl2. There was no difference between the corresponding APTTs of NHP and APC.

The concentration of APC in all preparations was determined prior to each experiment in an amidolytic assay using the reference APC preparation.

APC plasma level. The purified human APC was dilute with TBS containing 5% dextrose prior to infusion and was administered continuously for 24 h using an infusion pump. Arterial blood was drawn into 0.3 mol/L benzamidine-0.13 mol/L citrate (nine parts blood, one part anticoagulant) to block all serine protease activity as well as complex formation between APC and its inhibitors. The plasma was stored at −70°C. C3 antibodies were immunoaffinity purified on CNBr-activated Sepharose beads coupled to human PC. Wells on microtitre plates were coated with the purified C3 antibody (100 to 200 µL/well, 50 to 120 µg/mL in 0.1 mol/L NaCl, 0.01 mol/L carbonate buffer, pH 9.6, at least two days, 4°C), blocked with 3% bovine serum albumin (BSA) or 1% casein (0.15 mol/L NaCl, 0.01 mol/L benzamidine, 2 mmol/L EDTA, 0.02% Na-azide, 0.01 mol/L Tris, pH 8.5), and then 2.5 µL plasma

From the Department of Immunology and the Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, CA.

Submitted June 21, 1988; accepted November 5, 1988.

Supported by research grants from the NIH (HL 24891, HL 31469, and HL 31930).

Address reprint requests to John H. Griffin, PhD, Department of Immunology 1MM7, Research Institute of Scripps Clinic, 10666 North Torrey Pines Rd, La Jolla, CA 92037.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.

0006-4971/89/7303-0030$3.00/0

Blood, Vol 73, No 3 (February 15), 1989: pp 639-642

639
samples in 100 μL of this buffer per well were incubated (one hour, 37°C) for adsorption of PC antigen to the immobilized antibody. Subsequently, unbound constituents (eg, PC inhibitors) and the reversible inhibitor of APC (ie, benzamidine) were washed away and thus did not affect the C3-bound enzyme. The control wells were incubated with immunoaffinity-purified goat-polyclonal antihuman PC antibodies (1 mg/mL) for ten minutes to block the amidolytic activity of the C3-bound APC. Finally 100 μL of 0.7 mmol/L chromogenic substrate (S-2401 or S-2366, Kabi, Stockholm) for the APC was added to each well. Amidolytic activity was measured from the change of absorbance at 405 nm. The APC levels of the samples were calculated from the amidolytic activity of standards using dilutions of the reference human APC (0.005 to 5.0 μg/mL) in baboon plasma containing benzamidine and applied to the wells as described above. Standards prepared from dilutions of APC in blocking buffer gave similar values to plasma standards, suggesting that APC is protected from its plasma inhibitors under these conditions.

Other laboratory studies. APTT was determined within ten minutes from arterial blood sampling. Arterial blood was also collected into 1 mol/L EDTA, 1 mol/L benzamidine anticoagulant (100 parts blood, one part anticoagulant) before and 45 minutes after starting the infusion of APC into baboons. D-dimer antigen levels were determined using an ELISA kit (American Diagnostica, New York), while t-PA and plasminogen antigen levels were measured using dilutions of the reference human APC (0.005 to 0.34 mg/mL). In these animals the circulating plasma APC levels averaged 0.25 ± 0.45 μg/mL (± SEM; range 0.2 to 0.34 mg/mL; n = 20) during five to 45 minutes of the infusion. Four other animals received 1.1 mg/kg APC, and the plasma levels averaged 1.58 ± 0.01 μg/mL (range 0.85 to 2.90 μg/mL; n = 20) during five to 45 minutes of the infusion. The measurements of APTT and APC amidolytic activity during the infusion period were comparable (Figs 1 A and B), suggesting a direct relationship between the clotting time and the APC level in vivo. APTT and APC levels were unchanged in control animals infused with buffer or PC (data not shown).

In contrast to the enhanced in vitro lysis of exogenous thrombin-induced blood clots of dogs infused with bovine APC16 or cats with human APC,17 but in agreement with findings in the squirrel monkey model,20 human APC did not appear to induce measurable fibrinolysis in baboons in vivo, since the plasma levels of D-dimer, plasminogen, and t-PA antigen did not change appreciably following APC infusion (Table 1, Student's t-test, baseline v 45 minutes, P > .05, n = 4 for all measurements).

**Table 1. Fibrinolytic Assessment During APC Infusion**

<table>
<thead>
<tr>
<th></th>
<th>Baseline Value</th>
<th>45-Minute Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC Dose</td>
<td>0.25 mg/kg/h</td>
<td>1.10 mg/kg/h</td>
</tr>
<tr>
<td>D-dimer</td>
<td>0.45 ± 0.08</td>
<td>1.69 ± 0.19</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>210 ± 13</td>
<td>217 ± 25</td>
</tr>
<tr>
<td>t-PA</td>
<td>2.9 ± 0.1</td>
<td>3.8 ± 1.3</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

APC was administered into the AV shunt proximal to the vascular graft as a bolus comprising approximately 25% to 30% of the total dose given immediately prior to insertion of the vascular graft, followed by continuous infusion throughout the subsequent 50 to 60 minutes. Four baboons received a total dose of 0.25 mg/kg APC (range 0.2 to 0.34 mg/kg). In these animals the circulating plasma APC levels averaged 0.48 ± 0.02 μg/mL (± SEM; range 0.27 to 0.75 μg/mL; n = 20) during five to 45 minutes of the infusion. Four other animals received 1.1 mg/kg APC, and the plasma levels averaged 1.58 ± 0.11 μg/mL (range 0.85 to 2.90 μg/mL; n = 20) during five to 45 minutes of the infusion. The measurements of APTT and APC amidolytic activity during the infusion period were comparable (Figs 1 A and B), suggesting a direct relationship between the clotting time and the APC level in vivo. APTT and APC levels were unchanged in control animals infused with buffer or PC (data not shown).

In contrast to the enhanced in vitro lysis of exogenous thrombin-induced blood clots of dogs infused with bovine APC16 or cats with human APC,17 but in agreement with findings in the squirrel monkey model,20 human APC did not appear to induce measurable fibrinolysis in baboons in vivo, since the plasma levels of D-dimer, plasminogen, and t-PA antigen did not change appreciably following APC infusion (Table 1, Student's t-test, baseline v 45 minutes, P > .05, n = 4 for all measurements).
In the control experiments, platelet deposition onto the vascular grafts averaged $14 \times 10^9$ platelets in the four of six grafts that were patent at 60 minutes, a value similar to results reported previously. In previous control studies we have also observed elevated plasma levels of fibrinopeptide A and the platelet-specific $\alpha$-granule proteins, platelet factor 4 and $\beta$-thromboglobulin. Platelet thrombus formation associated with these highly thrombogenic vascular grafts was also unaffected by conventional antithrombotic therapies with heparin, aspirin, or the combination of these agents. In contrast, platelet accumulation in the vascular graft was decreased by infused APC in a dose-dependent manner in all experiments, and this reduction persisted for at least one hour after discontinuing the APC infusion (Fig 2). The inhibition of platelet deposition between five and 45 minutes in response to APC was dose dependent ($P < .01$ by analysis of variance, $n = 20$). The difference for inhibition between the two doses of APC was significant as early as 30 minutes after initiating the infusion (Student's $t$ test, $P < .02, n = 4$). At 30 minutes platelet deposition was inhibited by 43% and 76% at the lower and higher APC doses, respectively, while at 45 minutes the respective values for inhibition of platelet deposition were 42% and 77%.

Whereas all of the grafts in untreated animals occluded between 30 and 80 minutes, none of the grafts in APC-treated animals occluded within two hours following graft insertion. No decrease in blood flow (range 100 to 200 mL/min; 13 to 26 cm/sec) was detected (data not shown). The template bleeding times measured during the infusions of the lower and the higher APC doses were not significantly prolonged vs control preinfusion values: $8.4 \pm 1.7$ min ($n = 4$) vs $7.1 \pm 1.1$ min ($n = 7$) and $6.4 \pm 0.8$ min ($n = 4$) vs $4.9 \pm 0.8$ min ($n = 9$), respectively, (mean $\pm$ SEM, $P > .05$ for all measurements by Student's $t$ test). No adverse cardiovascular or hemorrhagic effects were observed during or after the infusion of APC.

Thus a potent antithrombotic effect was produced by human APC. APC has previously been shown to inhibit disseminated intravascular coagulation in rabbits, to reduce venous thrombosis in dogs, and to block the lethal effects of Escherichia coli infusions into baboons. Since APC is a physiologic antithrombotic protein that prevents thrombus formation under arterial flow conditions, it may also be therapeutically useful for immediate and transient inhibition of platelet-dependent arterial thrombosis in man (eg, for prevention of arterial reocclusion in patients who have undergone angioplasty, carotid endarterectomy, or successful coronary reperfusion by thrombolytic therapy).
addition, since APC did not compromise primary hemostasis, assessed here by bleeding time assays, it may contribute to antithrombotic protection for thromboembolic complications associated with other surgical procedures.

ACKNOWLEDGMENT
We are grateful to James Koziol for help with the statistical analyses and to John Dietrich, Jill Janik, Ulla Marzec, Paul McFadden, and Betty Richter for technical assistance.

REFERENCES
Inhibition of platelet-dependent thrombus formation by human activated protein C in a primate model

A Gruber, JH Griffin, LA Harker and SR Hanson