Anti-Inflammatory, Antithrombotic, and Neuroprotective Effects of Activated Protein C in a Murine Model of Focal Ischemic Stroke

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Background—Activated protein C (APC) contributes to systemic anticoagulant and anti-inflammatory activities. APC may reduce organ damage by inhibiting thrombin generation and leukocyte activation. Neutrophils and cerebrovascular thrombosis contribute to ischemic neuronal injury, suggesting that APC may be a potential protective agent for stroke.

Methods and Results—We examined the effects of APC in a murine model of focal ischemia. After middle cerebral artery occlusion/reperfusion, the average survival time in controls was 13.6 hours. Animals that received purified human plasma–derived APC 2 mg/kg IV either 15 minutes before or 10 minutes after stroke induction survived 24 hours and were killed for neuropathological analysis. APC 2 mg/kg given before or after onset of ischemia restored cerebral blood flow, reduced brain infarct volume (59% to 69%; \( P < 0.003 \)) and brain edema (50% to 61%; \( P < 0.05 \)), eliminated brain infiltration with neutrophils, and reduced the number of fibrin-positive cerebral vessels by 57% (\( P < 0.05 \)) and 25% (nonsignificant), respectively. The neuroprotective effect of APC was dose-dependent and associated with significant inhibition of ICAM-1 expression on ischemic cerebral blood vessels (eg, 61% inhibition with 2 mg/kg APC). Intracerebral bleeding was not observed with APC.

Conclusions—APC exerts anti-inflammatory, antithrombotic, and neuroprotective effects in stroke. Central effects of APC are likely to be related to improved maintenance of the blood-brain barrier to neutrophils and to reduced microvascular obstructions and fibrin deposition. (Circulation. 2001;103:1799-1805.)

Key Words: proteins ■ ischemia ■ blood cells ■ cerebrovascular disorders ■ thrombosis ■ nervous system

Endogenous activated protein C (APC) is part of a systemic anticoagulant and anti-inflammatory surveillance system.1–3 On both systemic and brain endothelial cells,1–6 thrombomodulin-bound thrombin converts protein C to APC, a serine protease that, with nonenzymatic plasma cofactors, protein S,1–3 and HDL particles,7 inactivates coagulation factors Va and VIIIa by highly specific proteolysis. APC may reduce organ damage by mechanisms independent of its anticoagulant activity.8,9 APC reduces ischemia/reperfusion-induced renal injury and endotoxin-induced pulmonary vascular injury by inhibiting leukocyte activation.10,11 In vitro studies confirmed that APC inhibits inflammatory responses of activated monocytes.12

Plasma protein C is an inverse risk factor for ischemic stroke,13 and reduced circulating APC14 and resistance to the anticoagulant effects of APC are potential risk factors for stroke.15,16 Cerebral ischemia17 and cardiopulmonary bypass surgery18 cause APC generation, consistent with the hypothesis that APC is protective.

Ischemic strokes in humans are due to thrombotic or thromboembolic vascular occlusions.19,20 Brain infiltration with neutrophils contributes to ischemic neuronal injury.21–24 It is not known whether APC may control leukocyte response in cerebral ischemia as in other injury models.8–11 APC has significant antithrombotic activity in vitro25 and in vivo,26–29 but its effects in stroke have not been studied. In contrast to other antithrombotic agents (eg, heparin or tissue plasminogen activator, tPA) that may predispose to central nervous system bleeding,30–33 elevated levels of APC do not cause bleeding in experimental studies.26–29 Here, we show that APC has significant protective effects in a murine model of focal ischemia.34

Methods

Animals

Procedures were approved by the Institutional Animal Care and Use Committee. Male C57BL/6 mice (23 to 26 g) were initially anesthetized with metofane and maintained with intraperitoneal ketamine.
(60 mg/kg) and xylazine (5 mg/kg). Atropine methyl nitrate (0.18 mg/kg IP) was given to prevent airway obstruction. Animals were allowed to breathe spontaneously. Rectal temperature was maintained at 37 ± 1°C. The right femoral artery was cannulated for continuous monitoring of blood pressure and blood analysis.

Stroke Model
A modified intravascular middle cerebral artery (MCA) occlusion technique was used to induce stroke. A nonsiliconized uncoated 6-0, 8-mm-long prolene suture with a rounded tip (diameter 0.20 mm) was advanced into the internal carotid artery to occlude the MCA for 1 hour, followed by 24 hours of reperfusion.

APC 2 mg/kg or vehicle was administered 15 minutes before or 10 minutes after the MCA occlusion via the femoral vein (n = 6 to 7 per group). APC dose-response studies used 0.1, 0.5, and 2 mg/kg of APC or vehicle administered intravenously 10 minutes after the MCA occlusion (n = 3 to 5 per group). APC was purified as previously described.

Regarding APC administration after the onset of ischemia, we felt that adding APC at 10 minutes, when blood flow was at a minimum, would give a reasonable test of the bioactivity of APC during ischemia, because the time course of pathophysiological changes in the present murine model is different from that of human strokes, and the occlusion is experimentally removed after 1 hour. Administration of APC after 2 to 3 hours in this murine model would actually be during the reperfusion phase, which may not be relevant to the human clinical situation, because complete reopening of major occluded blood vessels in humans who experience ischemic stroke might not typically happen spontaneously 1 hour after the onset of ischemic stroke.

Cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (Transonic Systems). Laser Doppler flowmetry probes (0.8 mm in diameter) were positioned on the cortical surface 2 mm posterior to the bregma, both 3 and 6 mm to each side of midline. The procedure was considered successful if a ≥85% drop in CBF was observed immediately after placement of the suture. Head temperature was monitored with a 36-gauge thermocouple probe in the temporalis muscle (model 9000, Omega).

Survival was monitored for 24 hours. Neurological examinations were performed at 24 hours and scored as follows: no neurological deficit (0), failure to extend left forepaw fully (1), turning to left (2), circling to left (3), unable to walk spontaneously (4), and stroke-related death (5).

Arterial blood gases (pH, PaO₂, PaCO₂) were measured before and during MCA occlusion with an ABL 30 Acid-Base Analyzer (Radiometer). Unfixed 1-mm coronal brain slices were incubated in 2% triphenyltetrazolium chloride in phosphate buffer (pH 7.4). Serial coronal sections were displayed on a digitizing video screen (Jandel Scientific). Brain infarct and edema volume were calculated with Swanson correction.

Histopathology and Fibrin Detection
Leukocytes were stained with anti-CD11b antibody (DAKO Corp) (1:250 dilution) directed against leukocyte Mac-1. Neutrophils were detected by dichloroacetate esterase staining (Sigma). Fibrin was localized with anti-fibrin II antibody (NYB-T2G1, Accurate Chemical & Scientific).

Survival and Motor Scores at 24 Hours After MCA Occlusion/Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival Time, h (Mean ±SD)</th>
<th>No. of Mice</th>
<th>Scores at 24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.6 ± 3.24</td>
<td>0 1 2 3 4 5</td>
<td>Score (Mean ± SD)</td>
</tr>
<tr>
<td>APC 15 min before MCAO, 2 mg/kg</td>
<td>23.7 ± 0.33*</td>
<td>0 2 2 1 0 1</td>
<td>4.50 ± 0.79</td>
</tr>
<tr>
<td>APC 10 min after MCAO, mg/kg</td>
<td></td>
<td></td>
<td>2.33 ± 1.51†</td>
</tr>
<tr>
<td>2</td>
<td>24.0 ± 0.00*</td>
<td>1 3 1 0 0 0</td>
<td>1.25 ± 0.96*</td>
</tr>
<tr>
<td>0.5</td>
<td>24.0 ± 0.00*</td>
<td>0 1 1 1 0 0</td>
<td>2.00 ± 1.00*</td>
</tr>
<tr>
<td>0.1</td>
<td>14.3 ± 6.43‡</td>
<td>0 0 0 0 1 2</td>
<td>4.33 ± 1.15‡</td>
</tr>
</tbody>
</table>

 APC or vehicle was administered 15 minutes before or 10 minutes after stroke induction. The difference between control animals treated with vehicle before or after MCA occlusion (MCAO) was not significant, and data for controls were pooled. *P < 0.05 for survival by Kruskal-Wallis test for APC-treated vs control group; †P < 0.01 for scores by χ² test with Fisher's transformation test for APC-treated vs control group; ‡P = NS.

Figure 1. CBF during MCA occlusion/reperfusion in control (A) and APC-pretreated (B) mice. Vehicle or APC 2 mg/kg was given 15 minutes before MCA occlusion (I). CBF values (mean ± SD) are shown in ischemic (open symbols) and nonischemic (solid symbols) hemispheres in 7 controls and 6 APC-treated mice; *P < 0.02 to < 0.005 between 2 groups.
Fibrin in 1-mm-thick brain hemisections was quantified by Western blotting as described. For dual staining, fibrin was localized first with the NYB-T2G1 antibody and detected with 3,3'9-diaminobenzidine (DAB) substrate (Vector Laboratories), followed by detection of leukocytes with anti-CD11b antibody and the Vector SG peroxide substrate. For immunostaining of intracellular adhesion molecule-1 (ICAM-1) on endothelium, brains were placed in cryo-embedding medium, then frozen in liquid nitrogen, and 8- to 10-μm-thick sections were fixed in acetone and incubated with the monoclonal rat anti-mouse ICAM-1 antibody (B D Biosciences). Sections were treated with biotinylated goat anti-rat antibody, avidin-biotin-peroxidase complex, and the peroxidase substrate aminoethylcarbazol (Vector) and counterstained with hematoxylin. Blood vessels exhibiting red precipitation were considered positive.

Routine controls included deletion of primary antibody, deletion of secondary antibody, and/or the use of an irrelevant primary antibody. Fibrin-positive and ICAM-1-positive vessels were expressed as percentage per mm². The number of CD11b and dichloroacetate esterase–positive cells in tissue was given per mm². Counting was performed in 10 random fields in the ischemic hemisphere by 2 independent observers blinded to the specimen source or timing.

**Spectrophotometric Hemoglobin Assay**

After triphenyltetrazolium chloride staining, 1-mm-thick brain hemisections were homogenized and treated with Drabkin’s reagent (Sigma) to determine hemoglobin as described. Bovine erythrocyte hemoglobin or mouse blood added to brain homogenates was used for standard curves.

**Statistics**

Physiological variables and infarct and edema volumes were compared by Student’s t test and ANOVA. Nonparametric data (neurological outcome scores) were subjected to the χ² test with Fisher’s transformation. Survival was compared by the Kruskal-Wallis test.

**Results**

Animals treated with APC 2 mg/kg 15 minutes before or 10 minutes after the MCA occlusion had no significant differences in mean arterial blood pressure, PaO₂, PaCO₂, pH, hematocrit, head temperature, or blood glucose compared with control animals (data not shown). APC administration did not influence CBF under basal conditions in the absence of occlusion/reperfusion. During MCA occlusion, the CBF in the control group dropped to 9% to 13% of baseline (P<0.001), whereas pretreatment with APC 2 mg/kg resulted in 25% improvement (P=0.05; Figure 1). During postocclusion reperfusion, the CBF in the ischemic hemisphere returned to 30% to 40% of baseline in the control group (Figure 1A) but to 70% to 85% in APC-pretreated mice (2 mg/kg) (Figure 1B).

Stroke-related death within 24 hours (score 5) was observed in 8 of 12 control animals (Table). Mean survival time for the control group was 13.6 hours. Ten of 11 mice treated with 2 mg/kg APC either 15 minutes before occlusion or 10 minutes after occlusion survived 24 hours, and 1 APC-treated animal died at 23 hours. All 3 animals treated with 0.5 mg/kg APC at 10 minutes after stroke induction survived 24 hours.

The motor neurological scores in mice given 2 mg/kg APC 15 minutes before and 10 minutes after the MCA occlusion were improved by 2- to 2.7-fold compared with control group mice. Also, 0.5 mg/kg APC given 10 minutes after MCA occlusion improved neurological outcome significantly. The protective effect of APC was not apparent, however, at an APC dose of 0.1 mg/kg on the basis of survival time and neurological function (Table) and the volume of brain injury and effects on CBF (Figure 7). APC-treated animals were killed at 24 hours to determine the volume of brain injury.

**Figure 2.** Brain injury in control and APC-pretreated mice. A, Volumes of brain infarct and edema (mean±SEM) from 7 controls and 6 APC-treated mice; *P<0.01, **P<0.05. B, Infarct area in 5 coronal sections of brains in A (mean±SEM); *P<0.05, **P<0.01. Vehicle or APC 2 mg/kg was given 15 minutes before MCA occlusion.

**Figure 3.** Incidence and topography of infarct at level of optic chiasm in control (A) and APC-pretreated (B) mice. Key for incidence is given in A. Vehicle (n=7) or APC 2 mg/kg (n=6) was given 15 minutes before MCA occlusion.
Pretreatment with APC 2 mg/kg reduced brain infarct volume by 59% (P < 0.02) and edema volume by 50% (P < 0.05) (Figure 2A). Injury was reduced in each of the 5 coronal sections (Figure 2B). All control mice had significant injury in the ipsilateral cortex and lateral striatum (Figure 3); >50% of the mice exhibited changes in the medial striatum, whereas <50% had changes in the dorsomedial and ventromedial cortex. APC 2 mg/kg limited brain injury to a small, well-localized area in the lateral cortex and significantly reduced injury in other regions (Figure 3).

Intravascular fibrin deposition was frequently found in the ischemic hemisphere in control mice (Figure 4A, 4D, and 4E, top). Fibrin was also found in the perivascular space in ischemic tissue in control animals (Figure 4B), suggesting leakage of fibrinogen across the blood-brain barrier. Significant migration of leukocytes (neutrophils) into ischemic tissue was observed in all control animals (Figures 1C, 4E top, 4F top, and 4H top), and “white thrombi” were found in several vessels (Figures 4D and 4F top). Pretreatment with APC 2 mg/kg completely eliminated deposition of neutrophils from brain tissue and ischemic vessels (Figure 4C bottom, 4E bottom, and 4F bottom), reduced deposition of fibrin in microvessels (Figure 4E bottom), and eliminated perivascular staining for fibrin (not shown).

The number of CD11b-positive leukocytes (Figure 5A) and the number of dichloroacetate esterase–positive neutrophils (Figure 5B) were the same in control mouse tissue, suggesting that most (if not all) CD11b-positive cells were neutrophils. With APC pretreatment (2 mg/kg), the number of neutrophils in tissue was close to background levels (Figure 5A and 5B).

**Figure 4.** Effects of APC 2 mg/kg pretreatment on leukocyte infiltration and fibrin deposition in ischemic hemisphere in mice subjected to 1 hour of transient MCA occlusion. A, Brain capillaries with intraluminal fibrin deposits in controls (arrowheads). B, Perivascular fibrin deposits in controls. A and B, Fibrin was localized with NYB-T2G1 antibody and detected with DAB as brown reaction product. C, CD11b-positive leukocytes (brown DAB substrate) in capillaries (arrow) and parenchyma (arrowheads) in control mice (top) and their absence in APC-treated mice (bottom). D, Dual staining for vascular fibrin (brown DAB substrate) and CD11b-positive cells (dark blue-gray SG substrate) in large venule and capillary (arrow) in control mice. E, Fibrin-positive vessels (brown, arrows) and CD11b-positive leukocytes (dark blue-gray cells, arrowheads) in controls (top) and APC-treated mice (bottom). Dual staining as in D. F, Dichloroacetate esterase staining indicates numerous neutrophils in tissue (arrowheads) and in vessel (arrow) in controls (top) and their elimination by APC treatment (bottom).
The number of fibrin-positive ischemic microvessels was reduced by 57% by APC pretreatment (2 mg/kg) (Figure 5C). Weak intraluminal staining for fibrin, reduced numbers of positive vessels, and absence of perivascular fibrin deposits resulted in an 8.2-fold decrease in the amount of fibrin at the level of the optic chiasm in the ischemic hemisphere in mice pretreated with 2 mg/kg APC versus control mice based on quantitative Western blot analysis (Figure 6). Background levels of hemoglobin in the ischemic hemisphere (Figure 5D) confirmed the absence of detectable intracerebral bleeding in APC-treated animals.

The effects of administration of APC after occlusion were studied in a separate set of experiments. APC 2 mg/kg given 10 minutes after the MCA occlusion reduced infarct size (A) and edema volume (B) by 69% ($P < 0.03$) and 61% ($P < 0.05$), respectively (Figure 7A and 7B), restored the CBF toward control values (Figure 7C), and eliminated brain accumulation of neutrophils (Figure 7D). The decrease in the number of fibrin-positive vessels in the ischemic hemisphere was 25%, which was not significant in comparison to vehicle-treated controls (Figure 7E). APC reduced volumes of brain infarct and edema in a dose-dependent fashion (Figure 7A and 7B) and produced a dose-dependent restoration in CBF during reperfusion (Figure 7C). Immunostaining for ICAM-1 in the ischemic hemisphere indicated that APC administration after the onset of ischemia reduced the number and intensity of ICAM-1–positive blood vessels (Figure 8A and 8B). The number of ICAM-1–positive blood vessels was reduced by 61% (Figure 8C).

**Discussion**

Data presented here demonstrate significant anti-inflammatory, antithrombotic, and neuroprotective dose-dependent effects of APC in a murine model of transient focal cerebral ischemia. APC markedly diminished both leukocyte and fibrin deposition and promoted CBF restoration. APC reduced detectable ICAM-1 at the blood-brain barrier, thereby preventing adhesion of neutrophils to the ischemic vessel wall and their subsequent transport across the blood-brain barrier. Neutrophils can contribute to ischemic injury. Inhibition of neutrophils with anti–Mac-1 antibodies results in significant neuroprotection during cerebral ischemia, and mice deficient in Mac-1 are less susceptible to cerebral ischemia/reperfusion injury. The role of neutrophil adhesion in the pathogenesis of stroke was also demonstrated in homozygous null ICAM-1 mice. APC protects against ischemia/reperfusion renal damage and endotoxin-induced vascular pulmonary injury by inhibiting leukocyte activation. Thus, APC probably reduces cerebrovascular and brain damage by attenuating or eliminating leukocyte response in ischemic tissue.

Significant obstructions in CBF in focal stroke might result from massive microvascular occlusions due to vascular accumulation of neutrophils and fibrin deposition. Animals that lack a key fibrinolytic factor, for example tPA–/– mice, develop substantial ischemic brain thrombosis and injury even when the CBF is moderately reduced. Previous studies reported significant anticoagulant activity of APC. In the present study, the effects of APC are perhaps more striking on neutrophil accumulation than on fibrin cerebrovascular deposition, in particular when APC was administered after stroke induction. In effect, APC alleviates...
both ischemic microvascular obstructions with blood cells and ischemic cerebral coagulopathy, thereby contributing to restoration of postischemic blood flow. Furthermore, at this point the possibility that APC may also have direct protective effects on neurons cannot be excluded.

The present study indicated that APC does not adversely affect hemostatic function or produce intracerebral hemorrhage, consistent with previous studies demonstrating that administration of APC does not cause bleeding.\textsuperscript{26–29} In contrast, bleeding and intracerebral hemorrhage are potential life-threatening complications with antithrombotic therapy for stroke, including thrombolytic treatment with tPA\textsuperscript{32,33} or anticoagulant treatment with heparin.\textsuperscript{30,31}

The results presented here give further insight into previous clinical studies. For example, prospective epidemiological studies suggest that protein C may be protective against stroke in humans.\textsuperscript{13} Low plasma levels of protein C in stroke patients may be caused by protein C depletion due to excessive thrombin generation and rapid APC clearance, whereas low circulating APC may result from depletion of protein C, increased levels of APC inhibitors, or reduced APC-generating capacity.\textsuperscript{14–17} It has been suggested that generation of APC during cerebral ischemia and after cardiopulmonary bypass surgery is protective.\textsuperscript{17,18}

In conclusion, the dose-dependent neuroprotective effects of APC demonstrated here in a murine model of focal ischemic stroke suggest the potential relevance of APC as a neuroprotective agent with multiple actions that may be beneficial for clinical applications in stroke.

**Figure 7.** Brain infarct (A) and edema (B), CBF during reperfusion (C), neutrophils (D), and fibrin-positive vessels (E) in ischemic hemisphere in mice treated with vehicle or APC after stroke induction. Vehicle (open bars) or APC 2 mg/kg (solid bars), 0.5 mg/kg (light gray bars), and 0.1 mg/kg (dark gray bars) was given 10 minutes after MCA occlusion. Mean±SEM from 3 to 5 animals. *\textit{P}<0.05.

**Figure 8.** Effects of APC on ICAM-1–positive blood vessels (arrows) in ischemic hemisphere. Immunocytochemical analysis of ICAM-1 in controls (A) and after treatment with 2 mg/kg APC given 10 minutes after MCA occlusion (B). C, Percentage of ICAM-1–positive vessels (mean±SEM) in control (\textit{n}=4; open bars) and APC-treated (\textit{n}=4; solid bars) mice. *\textit{P}<0.05.
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References

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