Brain-Specific Protein C Activation During Carotid Artery Occlusion in Humans

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Background and Purpose—Activation of plasma protein C (PC) zymogen by thrombin-thrombomodulin at the endothelial surface is an important endogenous antithrombotic mechanism. It is unknown whether activated protein C (APC) is generated in vivo in the cerebrovasculature, because there is only limited thrombomodulin expression in human brain vascular endothelium. Therefore, we tested the hypothesis that carotid occlusion produces brain-specific PC activation.

Methods—Blood samples were simultaneously collected from the ipsilateral internal jugular vein and radial artery before and during carotid cross-clamping and on “de-occlusion” in 8 awake patients undergoing routine carotid endarterectomy. Plasma PC zymogen and circulating APC levels were measured using enzyme immunocapture assay and expressed as percent of pooled plasma controls.

Results—Internal jugular vein APC levels increased 28% exclusively during carotid occlusion and then decreased 32% with de-occlusion (F=8.1, P<0.005). PC zymogen increased only 5.9% with occlusion (F=6.3, P<0.02), consistent with hemoconcentration. There were no changes in radial artery PC or APC levels.

Conclusions—These findings demonstrate brain-specific protein C activation in humans during carotid occlusion and suggest a protective role for endogenous APC generation during cerebrovascular occlusion. (Stroke. 1999;30:542-545.)

Key Words: cerebral ischemia ■ protein C ■ stroke ■ thrombomodulin

Impairments of the protein C (PC) antithrombotic mechanism are implicated in the pathogenesis of acute ischemic stroke.1,2 The antithrombotic enzyme, activated protein C (APC), functions by inhibiting activated clotting factors Va and VIIIa.3 Circulating APC is generated at the endothelial surface when thrombomodulin (TM) binds thrombin and terminates its prothrombotic actions, forming the TM-thrombin complex that activates PC from its zymogen precursor form. Although TM is abundant in most systemic vasculature, immunohistochemical studies4–7 have revealed only limited, if any, TM expression in the human cerebrovasculature. A restricted functional role for PC activation in the central nervous system is further suggested by findings8 that astrocyte coculture produces a 20-fold downregulation of endothelial cell TM mRNA production in vitro. Hence, the importance of the PC antithrombotic mechanism in brain has not yet been established, and it is unknown whether functional TM is present in sufficient quantity to generate APC during cerebrovascular insufficiency.

Circulating APC is a normal plasma component that provides systemic antithrombotic surveillance and can be directly measured by amidolytic assay after enzyme immunocapture.9 To test the hypothesis that cerebral vascular occlusion produces brain-specific PC activation in humans, we measured PC zymogen and circulating APC levels in blood sampled from the ipsilateral internal jugular vein (IJ) before and during carotid artery cross-clamping and immediately after “de-occlusion” in awake, stable patients undergoing routine carotid endarterectomy. Simultaneous PC zymogen and APC levels from radial artery (RA) blood served as controls to distinguish potential systemic from cerebral PC activation.

Subjects and Methods

Patients scheduled for elective carotid endarterectomy under regional anesthesia were considered for study entry. After surgical exposure, an 18-gauge butterfly catheter was placed directly in the IJ and anchored with a purse suture. An RA catheter was placed for routine intraoperative blood pressure monitoring. Intravenous heparin (5000-IU bolus) was administered 5 minutes before carotid cross-clamping, before blood sampling.

Blood samples were obtained from IJ and RA immediately before and within the first minute during manual carotid cross-clamping and within 3 minutes after de-occlusion. The initial 5 mL of blood was discarded, and then blood was collected directly into precooled syringes containing the anticoagulant EDTA (0.2 M; 1:9, vol/vol) and benzamidine chloride (0.3 M), a reversible APC inhibitor. Platelet-poor plasma was prepared within 20 minutes and stored at −70°C, with only 1 freeze-thaw cycle permitted.

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Circulating APC enzyme activity was measured by amidolytic assay following specific immunocapture of APC and PC from plasma, and total PC was measured after Protac (American Diagnostica) activation of the PC zymogen that was immunocaptured on a microplate. Levels of circulating APC and PC (mean±SEM) are expressed as percentages of pooled plasma from healthy adult control subjects. Repeated-measures ANOVA (Statistical Analysis Software, version 6.12; SAS Inc) was used to determine the significance of changes in APC activity and total PC levels in IJ and RA plasma samples.

**Results**

We studied 8 white male patients of mean age 68±2 years (range, 59 to 77 years). Stroke risk factors included history of hypertension in all patients, diabetes mellitus in 63% (n=5), hypercholesterolemia in half, and current cigarette smoking in half. Medical histories included coronary artery disease (n=6), peripheral arterial occlusive disease (n=2), and prior nondisabling ischemic stroke (n=2). Two of the surgical candidates had angiographically verified carotid occlusion contralateral to the hemodynamically significant carotid stenosis. Four left-sided and 4 right-sided endarterectomies were performed. Clinical evaluation of the adequacy of cerebral blood flow during carotid cross-clamping was performed by awake-patient monitoring with assessment for changes in neurological status. Seven patients experienced no such changes and therefore did not require carotid bypass shunt placement. One patient with known contralateral carotid occlusion who became unresponsive during carotid cross-clamping rapidly recovered on de-occlusion, and endarterectomy with bypass shunt was subsequently completed without complication. IJ samples were obtained before carotid cross-clamping, during occlusion, and 3 minutes after de-occlusion from all patients. RA samples were available from 6 of the 8 patients because RA blood from 1 patient was inadequately processed and postocclusion RA sampling was not obtained from the patient with transient neurological worsening.

Circulating APC and PC zymogen mean levels for each group of samples are illustrated in the Figure. Repeated-measures ANOVA showed significant changes in IJ APC during endarterectomy (n=8, F=8.1, P<0.005), consisting of a 28% increase in APC during carotid cross-clamping followed by a 32% decrease on de-occlusion. After exclusion of the patient with neurological worsening, who had no increase in IJ APC level during carotid cross-clamping, 5 of 7 patients (71%) had increased IJ APC levels during the carotid occlusion. For the 6 patients with simultaneous IJ and RA APC levels at all time points, profile contrast analysis revealed a significant (42%) increase in IJ APC levels exclusively during carotid occlusion (F=7.75, P=0.04), followed by a 35% decrease to baseline IJ APC levels on de-occlusion (F=12, P=0.02). The RA circulating APC levels showed no significant change throughout the procedure (P=0.76).

There were modest changes in IJ PC levels during carotid endarterectomy (F=6.3, P<0.02), consisting of a 5.9% increase during carotid occlusion (F=6.5, P=0.015) followed by an 8% decrease on de-occlusion (F=7.9, P=0.04). The small but significant increase in IJ PC zymogen levels during carotid cross-clamping and subsequent decline on de-occlusion are attributed to transient hemoconcentration, based on prior studies showing a similar 5% increase in hematocrit from IJ samples during the cross-clamping phase of carotid endarterectomy.

The RA PC zymogen levels did not change (P=0.4).

**Discussion**

TM-dependent PC activation is an important native antithrombotic mechanism whose function in human brain has not been established. The present study demonstrates brain-specific PC activation during carotid artery occlusion in a substantial proportion of awake patients undergoing routine carotid endarterectomy. That enhancement of PC activation occurred only in the brain is based on the findings that IJ APC levels increased exclusively during carotid cross-clamping, while the lack of change in RA APC levels documents absence of any systemic PC activation. The increase in IJ APC was rapid (<60 seconds), and the levels quickly returned to baseline after de-occlusion (ie, at least within 3 minutes), further suggesting that the PC-activating mechanism is dynamically affected by factors related to cerebral perfusion.

Notably, the mean values for baseline APC levels in both the cerebral (IJ) and systemic (RA) circulation were approximately 25% lower than reference values from pooled healthy controls. These circulating APC levels in carotid endarterectomy patients are remarkably similar to the low APC levels we have previously reported using the same assay in a case-control study of patients with acute ischemic stroke, suggesting that APC deficiency may constitute a procoagu-
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Lant element in atherosclerotic disease. Interpretation of these findings is limited, because we did not directly compare the APC levels in our patients with those of a group of matched controls in this study. Further studies are needed to better understand the association between circulating APC deficiency and atherothrombotic risk.

In animal thrombosis models and in humans, APC can be an important inhibitor of arterial thrombus formation. Circulating APC levels in healthy humans and patients with severe hereditary PC deficiency are inversely related to markers of thrombin generation.\textsuperscript{1,11,12} Therapies that enhance PC activation reduce these markers of a prothrombotic state, and in a primate arterial and venous thrombosis model, significantly inhibit platelet and fibrin deposition, indicating that endogenous APC inhibits thrombin procoagulant activity in vivo.\textsuperscript{12–14} Recent studies\textsuperscript{15} demonstrate that neonatal death in homozygous PC-deficient mice occurs because of severe thrombosis in the brain microvasculature. In a porcine model,\textsuperscript{16} endogenous APC is rapidly generated in the microcirculation during coronary artery occlusion and provides a direct cardioprotective benefit. Furthermore, APC therapy is more effective than heparin in preventing reocclusion after thrombolytic therapy with recombinant tissue-type plasminogen activator in a canine coronary artery thrombosis model.\textsuperscript{17} Increasing APC also reduces the hypercoagulable state, organ failure, and death in disseminated intravascular coagulation,\textsuperscript{18–20} perhaps in part due to a direct anti-inflammatory action.\textsuperscript{21,22} Thus, prior studies suggest that PC activation has important protective effects in both arterial thrombosis models and inflammatory-prothrombotic conditions,\textsuperscript{23} processes synergistic in the pathogenesis of focal brain ischemia.\textsuperscript{2,24}

The procedure of carotid endarterectomy routinely includes heparin pretreatment and surgical carotid cross-clamping, precautionary measures to inhibit thrombin generation and reduce risk of cerebral thromboembolism. Intraoperative neurological monitoring confirms that this procedure was well tolerated in those patients, which demonstrated increased IJ APC levels during carotid occlusion. Reductions in cerebral blood flow of patients experiencing acute ischemic stroke are greater and usually involve thrombin generation and thrombus formation, potent stimuli for APC generation.\textsuperscript{3,25} Hence, this study likely underestimates the magnitude of PC activation that occurs during natural thrombo-occlusive stroke.

Increased thrombin generation during carotid occlusion may account for enhanced TM-dependent PC activation in the brain. However, we did not directly measure the extent of thrombin generation in cerebral circulation and cannot rule out other potential mechanisms for the APC-generating response. Plasmin may directly activate PC, and it modulates APC antithrombotic function in a complex and concentration-dependent manner.\textsuperscript{26} It is possible that carotid artery occlusion may alter cerebrovascular endogenous fibrinolysis profiles by increasing tissue-type plasminogen activator activity, as has long been recognized to occur after venous occlusion.\textsuperscript{27} Subsequent generation of plasmin could then produce direct activation of PC from its zymogen precursor form. Carotid occlusion could also potentially lead to factor Xa generation that can activate PC in the absence of vascular endothelial TM via a process augmented by the presence of heparin.\textsuperscript{28–30} Further studies are needed to determine the precise mechanism(s) of PC activation and its relation to exogenous heparin administration and endogenous fibrinolysis profiles in the cerebral circulation.

Mechanisms that account for the heterogeneity in PC activation response are unclear. Numerous factors, such as the adequacy of collateral flow, patterns of venous outflow, and the extent of thrombin generation, could alter the cerebral PC activation response. The magnitude of cerebral hypoperfusion, known to vary considerably during carotid cross-clamping,\textsuperscript{31} was not measured in this study. We also did not standardize the side of endarterectomy or directly measure hemato logic parameters in the venous pool specific to the hemisphere hemodynamically challenged, as the right IJ normally drains bilateral superficial cortical veins via the superior sagittal sinus while the left IJ drains bilateral deeper subcortical regions.\textsuperscript{32} Beyond dilutional effects, these differing patterns of venous outflow could influence PC activation, because human brain demonstrates reduced constitutive TM expression in deep subcortical versus superficial cerebral vessels.\textsuperscript{3} The internal jugular vein may also drain a small proportion of blood from intracranial vessels that are not part of the brain parenchyma itself, such as the choroidal vein, and may provide some retrograde drainage of noncerebral blood via valveless veins such as those exiting the pterygoid plexus.\textsuperscript{33} Whether these potential minor sources of noncerebral IJ venous blood have TM-bearing blood vessels contribute to the APC-generating response during carotid occlusion cannot be determined from this study. The importance of other key PC regulatory factors including the endothelial cell PC receptor, the plasma PC inhibitor and its acceleration by heparin, as modulators of the APC-generating response in cerebral circulation is unknown.\textsuperscript{3,34}

In summary, this study supports the hypothesis that the PC activation mechanism is functional in human brain and rapidly upregulated by the hemodynamic challenge of cerebral artery occlusion. We previously reported that a deficiency in circulating APC is a distinct characteristic of the inflammatory-prothrombotic state following acute ischemic stroke.\textsuperscript{3} Plasma from patients with ischemic stroke also exhibits reduced anticoagulant response to exogenously added APC that is not attributable to the factor V Leiden mutation.\textsuperscript{3,36} These findings, along with recent controversy regarding thrombolytic therapy, highlight a need for further studies investigating potential cerebral protective effects of endogenous or therapeutically administered APC in acute ischemic stroke.\textsuperscript{3,36} Moreover, research is needed to determine whether native antithrombotic mechanisms in the cerebral microcirculation are related to clinical outcomes or may be pharmacologically augmented by vitamin therapy to lower homocysteine, a modifiable hematologic risk factor linked to TM-PC system integrity.\textsuperscript{38–41}

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