Protein S Confers Neuronal Protection During Ischemic/Hypoxic Injury in Mice
Dong Liu, PhD; Huang Guo, PhD; John H. Griffin, PhD; Jose A. Fernández, MD, PhD; Berislav V. Zlokovic, MD, PhD

Background—Protein S is an antithrombotic factor that also exhibits mitogenic activity. Thus, we hypothesized that protein S may control cerebrovascular thrombosis in stroke and protect brain tissue from ischemic injury.

Methods and Results—We studied protein S in a murine in vivo model of stroke and an in vitro model of neuronal hypoxia/reoxygenation injury. Animals received purified human plasma–derived protein S or vehicle intravenously 10 minutes after initiation of middle cerebral artery occlusion followed by reperfusion. Protein S at 0.2 to 2 mg/kg significantly improved the motor neurological deficit by 3.8- to 3.2-fold and reduced infarction and edema volumes by 45% to 54% and 45% to 62%, respectively. Protein S at 2 mg/kg improved postischemic cerebral blood flow by 21% to 26% and reduced brain fibrin deposition and infiltration with neutrophils by 40% and 53%, respectively. Intracerebral bleeding was not observed with protein S. Protein S protected ischemic neurons in vivo and cultured neurons from hypoxia/reoxygenation-induced apoptosis in a time- and dose-dependent manner. Recombinant human protein S exerted protective effects from hypoxia-induced damage similar to the plasma-derived protein S both in vivo and in vitro.

Conclusions—Protein S is a significant neuroprotectant during ischemic brain injury with direct effects on neurons and antithrombotic effects. Thus, protein S could be a prototype of a new class of agents for clinical stroke with combined direct neuronal protective effects and systemic antithrombotic and antiinflammatory activities. (Circulation. 2003;107:1791-1796.)

Key Words: protein S ■ ischemia ■ stroke ■ thrombosis ■ neurons

Protein S is a physiological antithrombotic agent that inhibits prothrombinase complex activity on endothelial cells and platelets by inhibiting coagulation factors Va and Xa and is a cofactor for activated protein C, a serine protease that inactivates coagulation factors Va and VIIIa.1-7 The critical physiological antithrombotic role of protein S is revealed by the massive thrombotic complications suffered by infants homozygous for protein S deficiency.8,9 In adults, mild heterozygous deficiencies in protein S are associated with risk for venous and arterial thrombosis,10-13 ischemic stroke,14,15 and cerebral thrombophlebitis.16,17 Protein S binds to vascular cells and is a potent mitogen.18-20 Its structural homologue, growth arrest–specific gene-6 (gas6), is a survival factor.21-24 It has been suggested that protein S and gas6 are ligands for the Tyro3/Axl family of receptor tyrosine kinases.25 However, the extent to which protein S functions in vivo as a Tyro3 ligand is unclear.26 Ischemic strokes in humans are caused by thrombotic or thromboembolic vascular occlusions27 resulting in neurodegeneration. Protein S had significant antithrombotic activity in a rabbit model of peripheral arterial thrombosis.28 In contrast to tissue plasminogen activator (tPA), which is neurotoxic and may predispose to central nervous system bleeding,31 elevated levels of bovine protein S did not cause bleeding in experimental studies.28 Because of its potential as a survival factor and demonstrated antithrombotic activity,32 we hypothesized that protein S may control ischemic brain damage by promoting anticoagulation and directly protecting neurons.

Methods

Animals
Procedures were approved by the Institutional Animal Care and Use Committee. Male C57BL/6 mice (23 to 26 g; Taconic, Germantown, NY) were anesthetized with ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP). Animals were allowed to breathe spontaneously. Rectal temperature was maintained at 37±1°C. The right femoral artery was cannulated for monitoring of blood pressure and blood analysis.

Stroke Model
A modified intravascular middle cerebral artery (MCA) occlusion technique33,34 was used to induce stroke. A nonsiliconized uncoated
6-0 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 23 hours of reperfusion. Protein S, human plasma–derived (0.2, 0.5, and 2 mg/kg; n = 5 to 6 per group) or human recombinant (protein SREC, 2 mg/kg; n = 4), or vehicle (n = 6) was administered intravenously 10 minutes after the MCA occlusion. Protein S was purified as previously described.4

Cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (LDF; Transonic Systems).33,34 LDF 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 the midline. The procedure was considered successful if ≥80% 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).

Neurological examinations were performed at 24 hours and scored as follows:33: 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 after MCA occlusion with an ABL 30 Acid-Base Analyzer (Radiometer). Dissociated cell suspensions were plated at 5 × 105 cells per well on 12-well Corning tissue culture dishes coated with poly-d-lysine in serum-free Neurobasal medium plus B27 supplement (Gibco BRL). Absence of astrocytes was confirmed by negative staining for glial fibrillary acidic protein. Cultures were maintained in a humidified 5% CO2 incubator at 37°C for 5 days before treatment. To induce hypoxia/reoxygenation injury, 5-day-old cultures were treated for 12 hours with 95% N2/5% CO2 in serum-free DMEM without glucose, followed by 12 hours of exposure to normoxic conditions with 5 mmol/L glucose.33 Protein S, human plasma–derived (1 to 1000 ng/mL) or recombinant, was added throughout the entire 24-hour study. Cultures were fixed for 10 minutes with 4% formaldehyde in PBS at 4°C and double-stained with Hoechst 33258 (1 μmol/L) or recombinant, was added throughout the entire 24-hour study. Cultures were fixed for 10 minutes with 4% formaldehyde in PBS at 4°C and double-stained with Hoechst 33258 (1 μmol/L) and TUNEL to determine nuclear morphological changes and number of apoptotic cells. Cells were counted <30 times in 10 to 12 fields. The number of cells per field varied from 10 to 20. The percentage of TUNEL-positive neurons was expressed per total number of cells in the ischemic and nonischemic hemisphere in 6 to 10 cultures used for both the time-course and dose-response studies.

**Histopathology and Fibrin Detection**

Leukocytes were stained with anti-CD11b antibody (Dako Corp) (1:250 dilution) directed against leukocyte Mac-1.33 The number of CD11b-positive cells in tissue was given per square millimeter. Counting was performed in 10 random fields in the ischemic hemisphere by 2 independent observers blinded to the specimen source or timing. The amount of fibrin was measured in 1-mm-thick brain sections by Western blotting using anti-fibrin II antibody (NYB-TZG1, Accurate Chemical & Scientific Corp) (1:500 dilution).33,34 Double-labeling for in situ DNA fragmentation in neurons was performed on paraffin sections stained with terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) (NYB-T2G1, Accurate Chemical & Scientific Corp) (1:500 dilution) and tau.33 Tau was detected with a rabbit polyclonal anti-tau antibody (Chemicon, 1:100) and a secondary rhodamine conjugated goat anti-rabbit IgG (Molecular Probes, 1:150).

**Protein S ELISA Assay**

Human protein S in plasma of mice at 1 hour after they received protein S injections (0.2, 0.5, 2.0, and 6.0 mg/kg) was quantified by ELISA. Nunc Maxisorp microplates were coated with 20 μg/mL of polyclonal rabbit purified IgG anti-protein S (DAKO Corp) in 0.1 mol/L Na carbonate, pH 9.0 (150 μL/well) overnight at 10°C and then blocked with buffer (200 μL/well) containing 50 mmol/L Tris, 100 mmol/L NaCl, pH 7.4, 2% BSA for 2 hours. Aliquots (150 μL) of plasma diluted 1/400 and 1/1600 in 50 mmol/L Tris, 100 mmol/L NaCl, 0.02% Tween-20, 0.5% BSA were added to wells and incubated for 2 hours. After washing with TBS, 0.02% Tween-20, polyclonal horseradish peroxidase–labeled rabbit antibody from Dako Corp (5 μg/mL) was used with OPD substrate from Sigma to detect bound protein S. Standard curves, valid for 5 to 125 ng/mL protein S, were made with dilutions (1/200 to 1/6400) of pooled normal human plasma (assumed to contain 25 μg/mL protein S) from George King Inc. A plasma pool from 10 normal male mice gave no signal in this assay, whereas the same plasma containing purified human protein S (final concentration of 25 μg/mL) gave a standard curve indistinguishable from pooled human plasma.

**Cell Culture**

Primary neuronal cortical cultures were established from fetal C57BL/6 mice at 16 days of gestation as described previously.36 Dissociated cell suspensions were plated at 5 × 105 cells per well on 12-well Corning tissue culture dishes coated with poly-d-lysine in

**Figure 1.** CBF during MCA occlusion/reperfusion in controls (A) and protein S–treated mice (B). Vehicle or protein S (2 mg/kg) was given 10 minutes after initiation of MCA occlusion. CBF values (mean ± SD) in ischemic and nonischemic hemisphere in 6 controls and 6 protein S–treated mice; *P < 0.05 between 2 groups.

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**TABLE 1.** CBF During MCA Occlusion (60 min) Followed by Reperfusion

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| Vehicle   | 17.5 ± 2.5     | 17.7 ± 1.8       |
|           | 58.2 ± 3.8     | 52.1 ± 2.9       |
| Protein S, mg/kg | 17.9 ± 1.5     | 16.8 ± 1.3       |
|           | 70.6 ± 2.5*    | 65.8 ± 3.4*      |
| 2.0       | 16.0 ± 2.1     | 16.9 ± 3.2       |
|           | 65.7 ± 1.6*    | 58.5 ± 3.3       |
| 0.5       | 15.3 ± 3.5     | 16.0 ± 2.3       |
|           | 60.1 ± 4.7*    | 59.7 ± 4.9       |

CBF during MCA occlusion/reperfusion in control and protein S–treated mice. Vehicle or protein S was given 10 minutes after initiation of MCA occlusion. CBF values (mean ± SD) were averaged over studied period at time of occlusion or reperfusion and expressed as a percentage of baseline. *P < 0.05.
Statistics

Physiological variables, injury, infarction, and edema volumes were compared by Welch’s 2-sample t test (for samples with unequal variances) or Student’s t test (for samples with equal variances). Nonparametric data (neurological motor scores) was subjected to Kruskal-Wallis testing.

Results

Animals treated with protein S had no significant differences in mean arterial blood pressure, PaO2, PaCO2, pH, hematocrit, head temperature, or blood glucose compared with control animals (not shown). Protein S administration did not influence CBF under basal conditions. During MCA occlusion, CBF in the control group dropped to 17% to 18% of baseline ($P<0.001$); treatment with protein S (0.2 to 2 mg/kg) did not improve CBF during the occlusion phase (Figure 1; Table 1). During postischemic reperfusion, CBF returned to 58% to 52% of baseline in the control group (Figure 1A; Table 1). Protein S at 2 mg/kg significantly improved CBF during postischemic reperfusion by 21% to 26% ($P<0.05$; Table 1). Effects of the lower doses of protein S were either marginal or lacking (Table 1).

Control mice developed significant motor neurological deficit (Table 2). Even at the lowest dose (0.2 mg/kg), protein S significantly improved motor neurological score by 3.8-fold; at higher doses, 0.5 mg/kg and 2 mg/kg, protein S significantly reduced motor deficit by 3.2-fold (Table 1). The effects on scores of protein SREC at 2 mg/kg were comparable to plasma-derived protein S (not shown).

Protein S–treated animals were killed at 24 hours to determine brain injury. Within the range studied, from 0.2 to 2 mg/kg, protein S significantly reduced the volumes of total brain injury, infarction, and edema by 43% to 58%, 45% to 54%, and 45% to 62%, respectively (Figure 2, A–C). As shown in Figure 2D, the infarction area was significantly reduced in 4 of 7 coronal sections with 0.5 mg/kg of protein S. Similar neuroprotective effects were obtained with recombinant protein SREC when studied at 2 mg/kg (Figure 2, A–C). All control mice had significant injury in the lateral cortex and striatum on the side of the occlusion (Figure 3); $\leq50\%$ of mice exhibited changes in the medial striatum, whereas $<50\%$ had changes in the dorsomedial and ventromedial cortex (Figure 3A). Protein S 2 mg/kg limited brain injury to a small, well-localized
area in the striatum and spared most of the cortex (Figure 3B).

At 2 mg/kg, protein S reduced the amount of fibrin deposited in the ischemic hemisphere by 40% ($P<0.05$; Figure 4B), the number of CD11b-positive leukocytes by 53% ($P<0.01$; Figure 4C), and the number of TUNEL-positive neurons in the ischemic cortex by 67% (Figure 4, D–E). The ischemia-induced changes in tau accumulation in neuronal soma were prevented by protein S.

Mean levels of circulating human protein S at 1 hour after injection (n=4 mice) were 4.9, 11.0, 51.8, and 155 µg/mL for injections of 0.2, 0.5, 2.0, and 6.0 mg/kg, respectively.

The direct effects of protein S in vitro on cultured mouse cortical neurons were studied. Neurons cultured under normoxic conditions occasionally exhibited TUNEL-positive staining (Figure 5A, left). In contrast, during ischemic hypoxia/reoxygenation injury, most cultured neurons were TUNEL-positive and exhibited nuclear condensation and/or fragmentation (Figure 5A, middle). In the presence of protein S, there was ~70% reduction ($P<0.05$) in the number of apoptotic cells (Figure 5A, right, and Figure 5B). Recombinant protein SREC had a neuroprotective effect comparable to that of plasma-derived protein S (Figure 5B). Under the present experimental conditions, protection of neurons from apoptotic death by protein S was time-dependent (Figure 5C) and dose-dependent, with the half-maximal effect, EC$_{50}$, at 75 nmol/L (Figure 5D).

**Discussion**

The data presented demonstrate neuroprotective, antithrombotic, and antiinflammatory effects of protein S in a murine in vivo model of ischemic stroke and direct neuronal protective effects of protein S in vivo and in vitro in cultured cortical neurons challenged by hypoxia/aglycemia followed by reoxygenation. Protein S significantly reduced motor neurological deficit, infarct volume, and edema volume within the range studied, from 0.2 to 2 mg/kg. The effect of protein S on postischemic CBF was significant with 2 mg/kg but marginal with an intermediate dose (0.5 mg/kg) and absent with a low dose (0.2 mg/kg). It is noteworthy that the same low dose of protein S (0.2 mg/kg) significantly reduced the infarction and edema volumes by 45% and the motor neurological score by 3.8-fold despite the lack of an observable effect on CBF.

![Figure 3](image-url). Incidence and topography of infarction at level of optic chiasm in control mice (A) and mice receiving plasma-derived protein S (B). Key for incidence is given in A. Vehicle (n=6) or protein S (2 mg/kg, n=6) was given 10 minutes after initiation of MCA occlusion.

![Figure 4](image-url). Deposition of fibrin, CD11b-positive leukocytes, and TUNEL-positive neurons in ischemic brain in control mice and protein S 2 mg/kg–treated mice. A, Signal on Western blot for fibrin from standard curve was linear between 0.15 and 3 µg of fibrin β-chain/0.1 mL; 3 µg/0.1 mL was arbitrarily set as 1. B, Western blot analysis of 10 mg brain homogenate (mean±SEM) in controls (n=3) and protein S–treated mice (n=3) at level of optic chiasm. C, CD11b-positive leukocytes (mean±SEM) from 6 controls (open bars) and 6 protein S–treated mice (closed bars) in ischemic and nonischemic hemisphere. *$P<0.05$. D, TUNEL-positive neurons (green) double-stained with tau (red) in ischemic cortex of vehicle-treated (ischemia) and protein S–treated mice. E, TUNEL-positive neurons in control and protein S–treated mice were counted in 10 random fields (magnification ×40) in ischemic cortex (n=6). *$P<0.01$. 

![Figure 5](image-url). 

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Thus, in the present model, the neuroprotective effects of protein S might be more important in preventing brain injury than its antithrombotic properties.

Significant obstructions in CBF in focal stroke might result from microvascular occlusions caused by fibrin deposition, vascular accumulation of neutrophils, and brain swelling. Previous studies reported significant anticoagulant activity of protein S in vitro and in vivo. The present study confirmed reduced fibrin deposition and reduced infiltration of brain tissue with leukocytes in the presence of protein S. At higher doses, protein S alleviated ischemic cerebral coagulopathy and reduced ischemic microvascular obstructions with blood cells, thereby limiting development of brain thrombosis and contributing to restoration of postischemic brain perfusion. However, cerebroprotective effects were observed with lower doses of protein S, which apparently did not improve the postischemic CBF significantly. Therefore, we considered as a possible mechanism the direct neuroprotective cellular effects of protein S.

Remarkably, our studies demonstrated that protein S protects ischemic cultured neurons exposed to hypoxia/reoxygenation injury. In the presence of protein S, the numbers of cultured neurons that were TUNEL-positive and exhibiting either nuclear shrinkage, chromatin condensation, and/or nuclear fragmentation were significantly reduced in a dose-dependent manner. These in vitro studies provide initial evidence that protein S has direct neuroprotective effects, but additional studies using different models are needed to better define these effects. Cell binding and mitogenic effects of protein S have been demonstrated in vascular smooth muscle cells, whereas the antiapoptotic effects of gas6, a structural homologue of protein S, are well established. Whether a protein S receptor is a transmembrane tyrosine kinase remains debatable. Direct antiapoptotic activity of protein S has not been described previously. Studies suggest that both gas6 and protein S are ligands for the Tyro3/Axl family of receptor tyrosine kinases. However, the role of Tyro3/Axl receptor is controversial, and the neuronal protein S receptor is unknown.

Bleeding and intracerebral hemorrhage are potential life-threatening complications with antithrombotic therapy for stroke (eg, thrombolytic treatment with tPA). IPA is directly toxic for brain cells. This and previous studies found that administration of protein S does not cause bleeding. However, a number of neuroprotective agents that appeared promising in animal stroke models have failed clinical trials. The time course of pathophysiological changes in the present murine model is different from that in human strokes, and the occlusion in this model is removed after 1 hour. In human clinical situations, spontaneous reopening of major occluded blood vessels in patients with ischemic stroke does not typically happen within 1 hour after the insult. Although protein S could serve as a prototype of a new class of agents for clinical stroke with combined direct cellular protective effects on neurons during cerebral ischemia and systemic antithrombotic and antiinflammatory activities, further evaluations are needed.

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References


Figure 5. Neuroprotective effects of protein S in an in vitro model of ischemic injury in cultured mouse cortical neurons subjected for 12 hours to hypoxia/aglycemia followed by 12 hours of reoxygenation. A, TUNEL-positive neurons (top) and neurons showing chromatin condensation and/or nuclear fragmentation by Hoechst staining (bottom) under normoxic conditions (left), hypoxia/reoxygenation (middle), and hypoxia/reoxygenation with protein S (500 nmol/L, right), B, TUNEL-positive neurons in absence and presence of plasma-derived protein S (A) or protein S (B) corrected for basal values of apoptosis. C, Time-dependent antiapoptotic effect of protein S. Dose-response for neuronal protective effect of protein S. Hypoxia/reoxygenation in absence (open squares) and presence (solid squares) of protein S. Mean±SEM from 3 to 5 cultures. **P<0.01. *P<0.05.


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