Neuroprotective Effects of Activated Protein C Through Induction of Insulin-Like Growth Factor-1 (IGF-1), IGF-1 Receptor, and Its Downstream Signal Phosphorylated Serine-Threonine Kinase After Spinal Cord Ischemia in Rabbits

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**Background and Purpose**—Activated protein C (APC) has beneficial effects on ischemia reperfusion injury in neuron. However, the possible mechanism of such beneficial effects is not fully understood. The aim of this study was to investigate the effects and possible mechanisms of APC on ischemic spinal cord damage.

**Methods**—After induction of spinal cord ischemia, APC (group A) or vehicle (group I) was injected intravenously. Severity of ischemic damage was analyzed by counting the number of motor neurons. To investigate the mechanisms by which APC prevents ischemic spinal cord damage, we performed immunoreactivity and Western blotting of insulin-like growth factor 1 (IGF-1), IGF-1 receptor, and phosphorylated serine-threonine kinase (p-Akt).

**Results**—APC eased the functional deficits and increased the number of motor neurons after ischemia. Immunoreactivity of IGF-1 in group A was stronger than in group I at 8 hours after reperfusion but was at the same level at 1 day. Induction of IGF-1 receptor and the downstream factor p-Akt was stronger and more prolonged in group A.

**Conclusions**—These results indicate that induction of IGF-1, IGF-1 receptor, and p-Akt might partially explain the neuroprotective effects of APC after transient spinal cord ischemia in rabbit. (*Stroke*. 2006;37:000-000.)

Key Words: spinal cord ■ ischemia ■ paraplegia ■ insulin-like growth factor 1 ■ protein C

Paraplegia after operation on the thoracic aorta is an unpredictable and disastrous complication. The reported incidence of paraplegia ranges from 2.3% to 23% for operations on the thoracic aorta.1 The mechanism of spinal cord injury during operations on the thoracic aorta is believed to be related primarily to direct spinal cord ischemia. Ischemia can occur when there is permanent exclusion of the essential intercostal arterial blood supply to the spinal cord or temporary interruption of the spinal cord blood flow.2 To date, no method has been developed that totally prevents paraplegia.

Various agents have been reported as promising therapy for stroke or spinal cord ischemia during thoracic aortic surgery. Among them, there is an increasing number of reports that investigate the efficacy of activated protein C (APC).3-5 APC is an important physiological anticoagulant that is produced from protein C by the action of the thrombin–thrombomodulin complex on endothelial cells. APC plays a significant role in the regulation of anti-inflammatory processes by inhibiting cytokine production by monocytes and has been reported to attenuate tissue or organ injury in various pathological conditions.3,6,7 It has been well demonstrated that APC can reduce hypoxia-induced apoptosis in neuron and cerebral endothelial cells.4 In spinal cord ischemia, APC has been reported to have beneficial effects in ischemia-reperfusion injury with its inhibitory effects on the production of tumor necrosis factor-α (TNF-α).8 However, the possible mechanisms involving APC as an effective factor in protecting motor neurons of the spinal cord from ischemic injury has not been fully examined, including intracellular signal transduction. Recently, a novel concept has been proposed that TNF receptor play a role in neurodegeneration via cellular death signal through “silencing of survival signals,” such as phosphatidylinositol 3-kinase (PI3K), that are activated by the insulin-like growth factor 1 (IGF-1) receptor.24 A variety of pathological insults, including ischemia, have been reported to upregulate several growth factors, and these can play important roles in modulating the survival of brain and spinal cord neurons. Among various growth factors,
IGF-1 has been reported to be upregulated in focal brain ischemia and to promote cell survival in ischemic brain injury associated with increased Akt activity. It has also been demonstrated that growth factor–induced neuronal survival was greatly associated with both PI3K and its downstream effector phosphorylated serine-threonine kinase (p-Akt). Therefore, it is possible that APC exerts its beneficial effects via affecting the induction of IGF-1 and its downstream molecule in addition to the inhibition of TNF-α production. In the present study, we examined the protective effects of APC and immunoreactivities of IGF-1, IGF-1 receptor, and p-Akt for possible neuroprotective mechanism after transient spinal cord ischemia.

Materials and Methods

Animal Models

Japanese domesticated white rabbits, weighing 2.5 to 3 kg, were divided into 3 groups as follows: sham control group (sham), transient ischemia with vehicle group (group I), and transient ischemia with human activated protein C (Anact C; kindly provided by Teijin Pharma, Osaka, Japan) group (300 U/kg; group A). Experimental and animal care protocols were permitted by the animal care committee of the Osaka University School of Medicine. Anesthesia was induced by intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane (in oxygen) inhalation.

Spinal cord ischemia was conducted as in our previous report. Briefly, a 5F pediatric balloon-tipped catheter (model 405; Braun) was inserted through the right femoral artery and placed just 1.0 cm caudal to the left renal artery. In groups I and A, 15 minutes of spinal cord ischemia was achieved by inflating the balloon to obstruct blood flow to the spinal cord. Our previous experiments confirmed that 15 minutes of transient spinal cord ischemia was sufficient for selective and delayed motor neuron death. Saline or APC (300 U/kg in saline) was administered intravenously at the onset of ischemia. The halothane inhalation was discontinued at 30 minutes after reperfusion. During the experiment, arterial blood pressure and heart rate are monitored, and arterial gas sample was measured at the onset of ischemia, reperfusion, and the end of halothane inhalation. Immediately after death, the spinal cord was quickly removed with the plunger of a 1-mL syringe. All rabbits survived until they were euthanized. All sham Bioscience). The images of Western blot studies were quantified statistically significant. Parametric data are present as mean ± SD.

Statistical Analysis

The Mann–Whitney U test was used to compare the neurological scores and cell numbers. Quantitative analyses of the optical density of Western blots were analyzed by ANOVA. P value < 0.05 was considered statistically significant. Parametric data are present as mean ± SD.

Histological Study

Sections taken 7 days after reperfusion in all groups were stained with hematoxylin and eosin (H/E) and examined by light microscopy. With H/E, cells were considered dead if the cytoplasm was diffusely eosinophilic and viable if cells showed basophilic stripping (ie, contained Nissl substance).

IGF-1, IGF-1 Receptor, and p-Akt Immunocytochemistry

We also performed immunohistochemical studies to investigate changes of expression of IGF-1, IGF-1 receptor, and p-Akt 8 hours, 1 day, or 2 days after reperfusion in groups I and A 2 days after reperfusion in the sham group (n = 3 each). To determine the direct effect of APC on spinal cord, we examined that rabbits received drug injection at the same dose without induction of ischemia. Those animals were euthanized after 8 hours of drug injection. Spinal cord sections were rinsed in 0.1 mol/L PBS and blocked in 2% normal horse serum for 2 hours at room temperature. Then they were incubated with primary antibodies in 10% normal horse serum and 0.3% Triton X-100 for 20 hours at 4°C. The primary antibodies used were as follows: goat polyclonal anti–IGF-1 antibody (SC-7144; Santa Cruz Biotechnology, Inc), mouse monoclonal anti–IGF-1 receptor (22215; Upstate), and mouse polyclonal p-Akt antibody (05669; Upstate); and dilutions were at 1:200 in each antibody. Slides were washed in PBS and incubated for 3 hours with biotinylated anti-mouse IgGs (PK-6102; Vector Laboratories) and biotinylated anti-goat IgGs (PK-6105; Vector Laboratories) at 1:200 dilution in PBS containing 0.018% normal horse serum. Subsequently, they were incubated with avidin–biotin–horseradish peroxidase complex (PK-6102; Vector Laboratories). Sections were colored with diaminobenzidine/H2O2 solution, and the cytoplasm was counterstained with H/E. A set of sections was stained in a similar way without the primary antibodies to ascertain specific binding of the antibodies to the proteins.

Western Blot Analysis

To investigate changes of IGF-1, IGF-1 receptor, and p-Akt expression, we performed Western blot analyses. Tissue samples were homogenized in lysis buffer, and then the homogenates were centrifuged at 10,000g for 10 minutes at 4°C. The supernatants were used as protein samples. SDS-PAGE was performed in 10% polyacrylamide gel under nonreducing conditions. The electrophoresis running buffer contained 25 mmol/L Tris base, 250 mmol/L glycine, and 0.1% sodium dodecyl sulfate. Proteins on the gel were then transferred to a polyvinylidene fluoride membrane (LC2002; Invitrogen) with a transfer buffer (NuPAGE Transfer buffer; NP0006; Invitrogen) and 10% methanol.

The primary antibodies used were the same as those used for immunocytochemistry mentioned above. After washing in PBS, membranes were incubated with horseradish peroxidase–conjugated anti-goat IgG (SC-2020; Santa Cruz Biotechnology Inc) and horseradish peroxidase–conjugated anti-mouse IgG (RPN2124; ECL Plus Western blotting Reagent Pack; Amersham Bioscience) at 1:1000 dilution in PBS for 90 minutes, respectively. The blots were developed using an ECL Plus detection method (RPN2132; Amersham Bioscience). The images of Western blot studies were quantified by plotting a 2D densitogram using the image analysis program NIH Image, version 1.63 (Research Services Branch; NIH; National Institutes of Health).

Results

Neurological Assessment

There was no significant difference in blood pressure, heart rate, and blood gas data in all groups during the experiment. All rabbits survived until they were euthanized. All sham-operated controls (n = 5) demonstrated normal neurological function. 5 (0.2 ± 0.0). Almost all rabbits in group I demonstrated severe paraparesis by 7 days (2.6 ± 0.89). In group A, the decline of neurological function was eased significantly compared with that of group I (4.2 ± 0.83; P = 0.019)

Histological Study

In sham-operated control animals, the spinal cord was intact with many large motor neurons in the anterior horn...
However, 7 days after blood flow restoration in group I, 60% of the motor neurons in the spinal cord were damaged (Figure 1B). Seven days after reperfusion in group A (Figure 1C), there were fewer damaged motor neurons. The number of intact motor neurons in group A was significantly larger than in group I ($P = 0.0066$; Table).

**Immunohistochemical Study**

Spinal cords of sham-operated animals showed slight immunoreactivity of IGF-1 receptor (see Figure 3A) but did not show IGF-1 and p-Akt immunoreactivities (Figures 2A; see Figure 4A). After 15 minutes of transient ischemia, IGF-1 (Figure 2B), IGF-1 receptor (Figure 3B), and p-Akt (Figure 4B) were induced in motor neurons 8 hours after reperfusion, and the immunoreactivities were almost lost in the motor neurons at 1 day (Figures 2C, 3C, and 4C, respectively) in group I. In group A, motor neurons showed stronger immunoreactivity for IGF-1 (Figure 2E) at 8 hours and prolonged and stronger immunoreactivity for IGF-1 receptor (Figure 3E and 3F) and p-Akt (Figure 4E and 4F) until 1 day after reperfusion compared with group I. No different profile of the expression of IGF-1, IGF-1 receptor, and p-Akt did show the animals received injection of APC without ischemia compared with that of sham control (data not shown).

**Western Blot Analysis**

Representative results of Western blot analysis are shown in Figure 5. With antibodies against IGF-1, a slight band was
detected in sham control samples, but that was slightly enhanced at 8 hours after blood flow restoration; however, it became scarcely detectable 1 day after reperfusion in group I. In group A, the band was more remarkable at 8 hours after reperfusion, but it became to the same level of sham group at 1 day (Figure 5A). With antibodies against IGF-1 receptor, a weak band was detectable in sham control samples, but those at 8 hours after blood flow restoration revealed a single band with a molecular weight of 95 kDa, which became scarcely detectable 1 day after reperfusion in group I. In group A, a single detectable band remained until 1 day after reperfusion, but it was almost lost at 2 days (Figure 5B). With antibodies against p-Akt, no band was detected in sham control samples, but those at 8 hours after blood flow restoration revealed a single band with a molecular weight of 60 kDa, which became scarcely detectable 1 day after reperfusion in group I. In group A, a single detectable band remained until 1 day after reperfusion, but it was almost lost at 2 days (Figure 5C). The membrane without the primary antibodies revealed no band (data not shown). With quantitative analysis, we found that APC affected expression profiles of IGF-1, IGF-1 receptor, and p-Akt (*P<0.01; **P<0.0001; Figure 5D through 5F, respectively).

Discussion

We demonstrated that APC preserved more motor neurons and attenuated delayed lower limb dysfunction after transient spinal cord ischemia. The immunohistochemical study revealed that APC induced IGF-1 more strongly at early stage of reperfusion and prolonged the expression of IGF-1 receptor and p-Akt, which is considered downstream and survival signal transduction of IGF-1, in motor neuron in spinal cord. Several mechanisms of anti-inflammatory effect of APC have been documented. One of its main anti-inflammatory mechanisms is to inhibit the production of TNF-α by inhibiting neutrophil accumulation after spinal cord transient ischemia. APC prevented neutrophil accumulation by inhibiting the production of TNF-α.8 In rats administered with lipopolysaccharide (LPS), APC prevented pulmonary vascular injury by inhibiting neutrophil activation through inhibition of TNF-α production.7 TNF-α is a multifactorial cytokine that promotes inflammation and intracellular death signaling cascades. Within the central nervous system, TNF-α has been well documented for its ability to initiate neurological apoptotic cascades, resulting in neurological damage.17,18 However, the exact mechanisms of inhibition of TNF-α production from activated monocytes are not fully understood. In monocytes stimulated by LPS, APC inhibited coupling of LPS and CD14, which initiated the production of cytokines.19

Recent in vitro and in vivo studies provided further and remarkable insights into the mechanisms of the neuroprotective activities of APC. In addition to inhibition of TNF-α...
production, which is supposed to have indirect and noxious effects on cells, APC acts directly on cells and alters gene expression profiles, inhibits apoptosis, and downregulates inflammation. In an in vitro model involving hypoxia-induced apoptosis of human brain endothelial cells, protease-activated receptor-1 and endothelial protein C receptor were required for APC to exert its antiapoptotic effects. In these cells, APC blunts hypoxia-induced increases in p53 messenger RNA and protein, reduces proapoptotic Bax, and increases antiapoptotic Bcl-2, thereby inhibiting mitochondrial-dependent apoptosis. Murine ischemic stroke model studies have provided in vivo evidence for the physiological roles of protease-activated receptor-1 and endothelial protein C receptor in neuroprotective activities of APC.

In the present study, we showed the stronger and prolonged expression of IGF-1, IGF-1 receptor, and the downstream signaling molecule p-Akt associated with attenuation of spinal cord ischemic damage after treatment with APC. A variety of pathological insults, such as ischemia and heat shock, has been reported to upregulate several growth factors, and they can play important roles in modulating survival of brain and spinal cord neurons. It has been demonstrated that there is a significant upregulation of IGF-1 and IGF-1 receptor in the CA1 and CA3 regions and the dentate gyrus after 15 minutes of 2-vessel occlusion ischemia and 1 hour to 4 days of reperfusion. IGF-1 is a local effector of growth hormone that is structurally similar to proinsulin and functional similarity to insulin. IGF-1 and its receptor provide cytoprotection against various noxious stimuli in a number of cell types, including neuronal cells and myocytes subjected to ischemia and reperfusion. Wang et al reported that IGF-1 had a significant effect on ameliorating brain injury after transient focal brain ischemia with affecting the expressions of cdk5 and its activator p35. Nakao et al reported that IGF-1 protected motor neuron cells from ischemic spinal cord injury associated with differential regulation of Bcl-xL and Bax proteins. It has been well established that stimulation of IGF-1 receptor coupled with tyrosine kinase activates PI3K and subsequently, p-Akt, which are representative survival signals. Therefore, enhanced expression of IGF-1 and prolonged representation of IGF-1 receptor and its important signal component p-Akt after treatment with APC might partly explain its beneficial effects. However, the exact mechanisms for stronger and prolonged expression of survival cell signals after treatment with APC are still unclear. One possible hypothesis is as follows. Recently, a new concept of neurodegeneration with TNF receptor inducing cellular death signal through silencing of survival signals, such as PI3K, that are activated by the IGF-1 receptor, has been proposed. This is achieved via TNF-α-mediated impairment in the ability of IGF-1 to phosphorylate tyrosine residues in the major insulin receptor substrate (IRS) docking proteins in murine granule neurons, IRS2. IGF-1 and its receptor induced by transient spinal cord ischemia might be silenced by simultaneously produced TNF-α, followed by inactivation of its downstream signal p-Akt. The mechanism of regulation of IGF-1 receptor expression in spinal cord is not fully understood. In human pancreatic cancer cells, activated p-Akt has been reported to upregulate IGF-1 receptor. Thus, it is possible that a decline of tissue TNF-α with treatment of APC after spinal cord transient ischemia failed to interrupt IGF-1 receptor signal transduction, leading to activation of downstream survival signal of p-Akt, which might upregulate IGF-1 receptor. This disruption of intracellular cross-talk effect of TNF-α leading to prolonged survival signal transduction of IGF-1 system might explain one of the anti-inflammatory mechanisms of APC in ischemia-reperfusion spinal cord injury.

Summary
This report showed that APC protected motor neurons of the spinal cord from ischemic injury. APC affected the expression of IGF-1, IGF-1 receptor, and p-Akt, which might be associated with the inhibition of cross-talk effect of TNF-α. APC is a strong candidate for use as a therapeutic agent in the treatment of ischemic spinal cord injury in the near future.
References


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Stroke. published online February 16, 2006;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

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