Modification of Endothelial NO Synthase Through Protein Phosphorylation After Forebrain Cerebral Ischemia/Reperfusion

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Background and Purpose—Production of NO by endothelial NO synthase (eNOS) is thought to play a neuroprotective role after cerebral ischemia. The vascular endothelial growth factor (VEGF) contributes to activation of eNOS by Ca²⁺/calmodulin and also stimulates the protein kinase Akt, which directly phosphorylates eNOS on Ser¹¹⁷⁷ and increases enzyme activity. Although the expression of VEGF has been studied in ischemic stroke models, the activation of eNOS after cerebral ischemia has not been investigated. The purpose of the present study was to clarify molecular mechanisms underlying the regulation of eNOS activity through protein phosphorylation in postischemic processes.

Methods—Sprague-Dawley rats were subjected to forebrain cerebral ischemia for 15 minutes with hypotension and reperfusion for up to 24 hours. Western blot analysis and ELISAs were used to study the temporal profiles of Akt, phospho-Akt at Ser⁴³⁷, eNOS, phospho-eNOS at Ser¹¹⁷⁷, and VEGF expression, respectively. Immunohistochemical studies were performed to examine the spatial expression patterns of phospho-Akt at Ser⁴³⁷ and phospho-eNOS at Ser¹¹⁷⁷.

Results—Increase in phospho-Akt at Ser⁴³⁷ was observed transiently 0.5 to 2 hours after reperfusion, whereas elevation of phospho-eNOS at Ser¹¹⁷⁷ and VEGF expression was observed from 6 hours after reperfusion. Endothelial cells in the microvessels were the major source of eNOS phosphorylated at Ser¹¹⁷⁷ at the 12-hour time point.

Conclusions—Increase in Ser¹¹⁷⁷ phospho-eNOS occurs in endothelial cells of microvessels after ischemic episodes with temporal expression of VEGF, pointing to a contribution to the autoregulation of postischemic brain damage. (Stroke. 2004;35:000-000.)

Key Words: cerebral blood flow ■ nitric oxide ■ stroke

Nitric oxide is a putative neurotransmitter in the brain and peripheral nervous system and an important mediator of vascular homeostasis and blood flow.¹ It is generated by 3 different types of NO synthase (NOS), the constitutive calcium/calmodulin–dependent neuronal and endothelial isoforms and the inducible calcium-independent isoform.² NO produced by endothelial NOS (eNOS) reduces apoptosis and confers protection against stroke, whereas pathological concentrations of NO from inducible NOS and neuronal NOS (nNOS) induce apoptosis and are neurotoxic.³,⁴ Inhibition of eNOS activity decreases cerebral blood flow and promotes tissue damage after focal ischemia.⁵

Increase in eNOS protein levels after transient or permanent focal ischemia and global cerebral ischemia has been reported in infarcted areas,⁶–⁸ but mechanisms of postischemic modification of eNOS in terms of the upregulation remain to be clarified. Several recent studies have demonstrated that insulin, estrogen, and vascular endothelial growth factor (VEGF) cause eNOS phosphorylation and result in endothelial NO release through the phosphatidylinositol 3'-kinase (PI3-kinase)-Akt-dependent pathway. However, to our knowledge, eNOS phosphorylation after cerebral ischemia has not been identified in vivo.

Therefore, the aim of the present study was to evaluate phosphorylated eNOS levels in the cortex after forebrain ischemia in vivo using Western blot analysis and immunohistochemical methods. We also examined by ELISA the expression of VEGF as a regulator of eNOS.⁹

Materials and Methods

Materials
β-Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Oriental Yeast, and 2'-5'-ADP-agarose and other chemicals, unless otherwise specified, were from Sigma.
Forebrain Ischemic Model

All experiments were performed in accordance with guidelines for the care and use of laboratory animals in the physiological sciences as approved by the Physiological Society of Japan.

We adopted a forebrain ischemia model by Smith et al.10 that consistently gives flow rates of <5% of control in cortex.11 Anesthesia was induced in male Sprague-Dawley rats (350 to 400 g, Chubu Kagaku Shizai Ltd, Nagoya, Japan) using methohexitol sodium (50 mg/kg IP). Animals were then intubated and ventilated with 1.0% halothane in an oxygen/nitrous oxide (30%70%) gas mixture. Temperature was monitored with a rectal probe and maintained between 36.5 and 37.5°C with a heating pad and lamp. The right femoral artery and vein were catheterized with polyethylene tubing (PE-50) to allow blood sampling and the monitoring of arterial blood pressure during ischemia. Arterial blood gases were examined 5 minutes before ischemia induction and 15 minutes after blood reperfusion. Heparin (150 IU/kg) was administered intravenously before induction of ischemia. Blood was withdrawn from the femoral vein catheter to cause hypotension with a mean arterial blood pressure of 50 mm Hg, and both carotid arteries were clamped. At the end of the 15-minute ischemia period, clamps were removed, blood was reinfused through the femoral vein catheter, and all wounds were sutured.

Brain samples were collected at the end of the 15-minute ischemia, as well as after 0.5, 2, 6, 12, and 24 hours of reperfusion. Rats with all surgical procedures, but without carotid occlusion and hypotension, were used as controls. Because there are many more microvessels in cortex than in hippocampus, we used cortex rather than hippocampus for tissue analysis in our forebrain ischemia model. The bilateral cortices adjacent to the hippocampus were immediately isolated on ice, frozen in liquid nitrogen, and kept at −80°C until analysis.

Sample Preparation for Western Blot Analysis

Samples were prepared from 4 different animals in each group. Brain tissues were homogenized using a homogenizer in 10 volumes of homogenization buffer containing 50 mmol/L Tris base/HCl, pH 7.5, 0.1 mmol/L dithiothreitol, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonfyl fluoride (PMSE), 1.25 g/mL pepstatin A, 0.2 μg/mL aprotinin, 0.2 μg/mL leupeptin, 5 mmol/L tetrahydrobiopterin, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 2 mmol/L sodium pyrophosphate, and 1% Nonidet P-40 (NP-40). Homogenates were then centrifuged at 15,000 rpm at 4°C for 10 minutes. Protein concentrations of the supernatants were determined by the Bradford method using BSA as the standard. Supernatant fractions were applied as crude fractions.

For preparation of the NOS fraction, the enzyme was partially purified with 2′,5′-ADP-agarose, as described previously.13 Briefly, 15 μL of 2′,5′-ADP-agarose with the same concentration of crude fraction (1250 μg/200 μL) was incubated gently for 1 hour at 4°C. The 2′,5′-ADP-agarose was washed with 200 μL of the homogenization buffer without NP-40, and NOS was eluted with 50 μL of 10 mmol/L NADPH.

Western Blotting Analysis

Extract samples and 25 μg of crude samples were subjected to 7.5% SDS-PAGE, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with primary polyclonal antibodies against Ser 1177-phosphorylated eNOS (Cell Signaling Technology, Beverly, Mass) at a dilution of 1:200, Ser 473-phosphorylated Akt (Cell Signaling Technology, Beverly, Mass) at a dilution of 1:500, or actin (Sigma, St Louis, Mo) at a dilution of 1:200 for 45 minutes at room temperature. Membranes were then incubated with secondary antibodies, which were visualized using an enhanced chemiluminescence (ECL) or ECL plus Western blotting detection system (Amersham). Phospho-eNOS and Akt immunoblots were stripped from PVDF membranes and rebotted with primary monoclonal eNOS (Transduction Laboratories, Lexington, Ky) at a dilution of 1:2000 and polyclonal Akt (Cell Signaling Technology, Beverly, Mass) at a dilution of 1:500 for 45 minutes at room temperature. Finally, membranes were developed with the ECL system. eNOS immunoblots were stripped again and rebotted with primary monoclonal nNOS (Transduction Laboratories, Lexington, Ky) at a dilution of 1:3000 and developed with the ECL system. Band intensities were quantitated by densitometric scanning using the NIH IMAGE program.

VEGF Immunoassay

Cortical tissue lysates were prepared at different times after cerebral ischemia. The homogenizing lysis buffer contained 10 mmol/L Tris base/HCl, pH 7.5, 1 mmol/L EDTA, 0.1 mol/L sodium chloride, 100 μg/mL PMSF, 1 μg/mL aprotinin, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS; homogenates were centrifuged in a microcentrifuge for 10 minutes, and supernatants were collected. Concentrations of VEGF in the lysates were quantitated using a commercially available VEGF ELISA kit (Quantikine M; R&D Systems) and the protein concentration by the Bradford method.

Immunohistochemistry

Rats subjected to a 15-minute ischemia followed by a 12-hour reperfusion were perfused with ice-cold 200 mL 4% paraformaldehyde in 0.1 mol/L sodium phosphate, pH 7.4. Serial coronal cryostat sections (10 μm) were stained according to the avidin-biotinylated peroxidase complex (ABC) technique at room temperature. The staining sequence was as follows: 2% goat serum for 30 minutes; primary polyclonal antibodies (Cell Signaling Technology, Beverly, Mass) against phospho-Ser1177 eNOS at a dilution of 1:75; phospho-Akt at a dilution of 1:60, Akt at a dilution of 1:180, and monoclonal antibodies (Transduction Laboratories, Lexington, Ky) against ENOS at a dilution of 1:500; biotinylated anti-rabbit or mouse IgG for 1 hour; and ABC for 1 hour. Sera for the blocking step, biotinylated antibodies, and ABC were purchased from Vector Laboratories. Reaction products were developed by incubation in 0.05% 3,3′-diaminobenzidine tetrachloride and 0.01% H2O2 in 50 mmol/L Tris-HCl, pH 7.5, for 10 minutes. Rats with all surgical procedures, but without carotid occlusion and hypotension, were used as controls.

Statistical Analysis

Data are expressed as mean±SE values. Statistical analyses were performed by 1-way ANOVA followed by Fisher post hoc test. Statistical significance was concluded at the P<0.05 level.

Results

Physiological Parameters

Major physiological parameters for the animals were unaltered. No significant changes in mean arterial blood pressure, temperature, or arterial blood gas data were detected among any of the experimental groups by repeated-measure ANOVA (Table).

Effects of Ischemia on Phosphorylation of Akt at Ser1177

We first examined the levels of phosphorylated Akt at Ser1177 in crude fractions after transient ischemia. A 6-fold enhancement was evident after a 30-minute reperfusion, relative to nonischemic control samples (Figure 1). Even after a 2-hour reperfusion, Akt phosphorylation was significant. Equal levels of actin and Akt were detected in crude fractions even after reperfusion, indicating that similar amounts of proteins were loaded and that an ischemic episode modulates Akt primarily through phosphorylation at Ser1177.
Induction of VEGF protein after forebrain cerebral ischemia. Brain lysates were prepared from control rats and various times after cerebral ischemia and VEGF concentrations were measured with a VEGF ELISA kit. Graphs illustrate mean±SE data from 4 independent experiments. *P<0.05 vs the control at each time point.

Phosphorylation of eNOS and Akt After Transient Ischemia

After a 12-hour reperfusion, immunoreactivity against eNOS was apparent in the endothelial cells of microvessels without any remarkable change from the control case (Figure 4A and 4B). No immunoreactivity of phospho-Ser1177 eNOS was detected in control rat cortex, whereas intense immunoreactivity was observed in the endothelial cells of microvessels after a 12-hour reperfusion (Figure 4C and 4D). Akt was detected mainly in the neurons in the cortex but weakly observed in the endothelial cells of microvessels (Figure 4E and 4F). Phospho-Ser1177 Akt was observed not only in the neurons but also in the endothelium after a 12-hour reperfusion (Figure 4F).

Discussion

The present study demonstrated, for the first time to our knowledge, significant increase in phosphorylated eNOS at Ser1177 from 6 hours after forebrain cerebral ischemia in vivo. Upregulation of nNOS after cerebral ischemia has been reported,13,14 but our data showed that levels of nNOS in NOS fractions after forebrain ischemia did not change significantly for...
Akt phosphorylation in endothelial cells after cerebral ischemia. Our results show that eNOS is phosphorylated at Ser\(^{1177}\) immediately after ischemia has occurred within 4 hours of reperfusion, in agreement with our previous finding of induction of VEGF and phosphorylation of eNOS at Ser\(^{1177}\) (Akt-dependent) signaling pathway. Akt phosphorylation induced by cerebral ischemia is detected not only in hippocampus but also cerebral cortex after cerebral ischemia. Recent reports have provided evidence that Ser\(^{1177}\) phosphorylation activates eNOS, whereas Thr\(^{495}\) phosphorylation inhibits activity. This regulation of eNOS involves phosphatases and multiple protein kinases, including Akt, and the AMP-activated protein kinase. The serine/threonine kinase Akt, also known as protein kinase B, enhances survival with cerebral ischemia through a PI3-kinase–dependent signaling pathway. Akt phosphorylation induced by cerebral ischemia is detected not only in hippocampus but also cerebral cortex after cerebral ischemia occurring within 4 hours of reperfusion, in agreement with our results. Phosphorylation of Akt immediately after ischemia has been found to be mainly localized in astrocytes or neurons in cerebral cortex, our findings suggest that this is not directly correlated with phosphorylation of eNOS.

In conclusion, our results show that eNOS is phosphorylated in endothelial cells of microvessels in the cortex within 6 hours of reperfusion, which may correlate with the phosphorylation of eNOS in our model.
after forebrain ischemia. This could increase enzyme activity and production of NO and thus contribute to regulation of cerebral blood flow after cerebral ischemia. Further studies are now needed to explore the molecular mechanisms that regulate induction of VEGF and modification of eNOS in the ischemic brain in vivo.

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References

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