Phosphorylated Endothelial NOS Ser1177 via the PI3K/Akt Pathway Is Depressed in the Brain of Stroke-Prone Spontaneously Hypertensive Rat

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Stroke-prone spontaneously hypertensive rats (SHRSP) demonstrate impaired endothelium-dependent relaxation and often develop brain injuries. We investigated whether the regulatory mechanism for endothelial NOS (eNOS) phosphorylation and activation is altered in the cerebral cortex of SHRSP at a younger age. Western blot analysis revealed a low ratio of phosphor-eNOS (Ser1177) to total eNOS in SHRSP at 10 weeks of age. In addition, urinary nitric oxide metabolites (ie, nitrate and nitrite) were decreased compared with normal control WKY rats. Likewise, Akt phosphorylation (especially Ser473) was significantly reduced, with no changes in total Akt. Furthermore, the amount of the phosphatidylinositol 3-kinase (PI3K) was upstream of Akt was diminished, although attenuation of the PI3K/Akt pathway was not an effect of mTOR, another downstream target of Akt. Our findings indicate that abnormalities of the PI3K/Akt pathway in the cerebral cortex are involved in the impaired eNOS phosphorylation and activation in SHRSP. Key Words: Hypertension—insulin signaling—nitric oxide—SHRSP.

Hypertension commonly contributes to cardiovascular and cerebrovascular diseases, such as myocardial infarction and stroke. In particular, stroke occurs in the majority of patients with various degrees of hypertension. Therefore, hypertension is considered an important risk factor for cerebrovascular disease.

The stroke-prone spontaneously hypertensive rat (SHRSP), a substrain of SHR established by selective breeding, provides a model for basic research on hypertension-associated cerebrovascular injury.1 Because of the marked elevation of blood pressure, almost all of these animals develop stroke. SHRSP also is used as a model for insulin resistance in the adipocytes and skeletal muscles.2,3

The PI3K-Akt pathway is known to be related to blood pressure regulation. One of the insulin signaling cascades,4 PI3K-Akt is stimulated by other factors, including insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF).5 Akt activation is a process involving phosphorylation at Ser473 and Thr308 by phosphoinositide-dependent protein kinases.6 Phosphorylated Akt, acting through its downstream effectors, has been implicated in the regulation of mechanisms important for glucose homeostasis and the control of cell functions, including growth, protein synthesis, survival, and endothelial nitric oxide (NO) production. Recent studies reported impaired PI3K-Akt signaling in the heart and aorta of SHRSP at a younger age.7,8 This process has not been investigated in the cerebral cortex of SHRSP, however.

Endothelial NOS (eNOS) lies downstream of Akt, and its activity is controlled by phosphorylation at the domain of Ser1177. NO, recognized as an important endothelial-derived vasodilator generated by eNOS from L-arginine,
plays an important role in the modulation of vascular tone and structure via a cyclic guanosine monophosphate mechanism.\textsuperscript{9-11} In contrast to eNOS, the mammalian target of rapamycin (mTOR), which also lies downstream of Akt, is an important molecule in the regulation of protein synthesis and cell growth. mTOR activity also requires phosphorylation at Ser2448 by Akt. Previous studies have demonstrated that focal ischemic brain injury significantly decreased the levels of phosphorylated Akt and mTOR and suppressed protein synthesis.\textsuperscript{12} Whether activation of both eNOS and mTOR in the cerebral cortex of SHRSP becomes impaired at a younger age is unclear.

Moreover, NO synthase not only includes eNOS, but also is inducible from macrophage NOS (iNOS) and neuronal NOS (nNOS). In previous studies, these NOSs demonstrated varying expression in the cerebral cortex of SHRSP at a younger age and depending on age or feeding procedure.\textsuperscript{13-17}

In the present study, we investigated the expressions of various types of NOS as well as differences in the PI3K-Akt-eNOS-NO pathway through comparison between SHRSP and WKY rats at age 10 weeks.

**Materials and Methods**

**Animals**

Six-week-old male SPF (specific pathogen-free) stroke-prone spontaneously hypertensive rats (SHRSP/Izm) \( n = 10 \) and genetically normotensive Wistar-Kyoto rats (WKY/Izm) \( n = 7 \) were supplied from the Disease Model Cooperative Research Association. All rats were housed in a climate-controlled (temperature, 22–24°C; humidity, 40%–60%), light-regulated room with 12-hour light/dark cycles. All groups consumed an SP diet (Funa-hashi, Tokyo, Japan) and had free access to rat chow and water throughout the experiment. Body weight and blood pressure were measured every 2 weeks. At age 10 weeks, 24-hour urine samples were collected from the rats housed in metabolic cages, in which food was withdrawn but water was provided. Then the animals were anesthetized with intraperitoneal injections of pentobarbital sodium (Nembutal, 100 mg/kg). Brains were immediately extirpated, cleaned, and promptly frozen in liquid nitrogen (Nembutal, 100 mg/kg). Brains were immediately extirpated, cleaned, and promptly frozen in liquid nitrogen (Nembutal, 100 mg/kg). Brains were immediately extirpated, cleaned, and promptly frozen in liquid nitrogen (Nembutal, 100 mg/kg). Brains were immediately extirpated, cleaned, and promptly frozen in liquid nitrogen (Nembutal, 100 mg/kg). Brains were immediately extirpated, cleaned, and promptly frozen in liquid nitrogen (Nembutal, 100 mg/kg).

The cerebral cortex was homogenized with ice-cold homogenized buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% Nonidet-P40, 0.25% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 50 mM NaF, 2 mM Na3VO4, 30 mM Na pyrophosphate, 2 mM phenylmethanesulfonylfluoride, 1 mM benzamidine, 0.02 g/mL of trypsin inhibitor, 0.02 g/mL of leupeptin, and 0.02 g/mL of aprotinin. After incubation for 2 hours, lysates were centrifuged at 15,000 rpm for 20 min, and supernatants were isolated. Proteins were extracted by boiling the tissues in 0.5 mmol/L Tris/HCl (pH 6.8), glycerol, 10% SDS, 0.1% bromophenol blue, and 2-mercaptoethanol. The proteins (25 μg) were electrophoresed using 7.5%–12.5% SDS-polyacrylamide gel electrophoresis at 100 V for 2 hours. After fractionation, the proteins were transferred onto a polyvinylidene fluoride membrane (Amersham, Buckinghamshire, UK) at 100 mA for 2 hours. The membrane was blocked in Blocking One (Nacalai Tesque, Kyoto, Japan) for 20 minutes. After appropriate blocking, blots were incubated with anti-iNOS (1:5000), anti-nNOS, anti-eNOS, anti-phospho-eNOS (Ser1177), anti-Akt, anti-phospho-Akt (Ser473 and Thr308), anti-mTOR, anti-phospho-mTOR (Ser2448), and anti-PI3K (1:1000) in Antibody Solution 1 (Toyobo, Osaka, Japan) overnight. It was then washed with Tris-Tween buffered saline and finally incubated for 1 hour with a 1:5000 dilution of anti-rabbit, anti-mouse, and anti-goat IgG horse-radish peroxidase. Detection was achieved using an Amersham electrochemical luminescence kit, with β-actin serving as the SBP reading. The average of 3 measurements was taken as the SBP reading.

**Urinary Nitrate/Nitrite Assay**

Nitrate and nitrite (NO\textsubscript{2}/NO\textsubscript{3}) concentrations in the urine were measured by the Griess method using an NO\textsuperscript{2}/NO\textsuperscript{3} Assay Kit-C (Colorimetric; Dojindo, Kumaoto, Japan). The standard curve ranged from 0 to 100 μmol/l. Urine samples were read at 560 nm using a 96-well Spectra Microplate Autoreader (Sunrise Rainbow, Tecan, Austria).

**Primary Antibodies**

Immunoblotting was performed with commercially available antibodies. Anti-rabbit eNOS was purchased from Affinity BioReagents (Golden, CO). Anti-rabbit phospho-eNOS (Ser1177), anti-rabbit Akt, anti-rabbit phosphor-Akt (Ser473, Thr308), and anti-rabbit nNOS were purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse β-actin and anti-rabbit iNOS were obtained from Sigma-Aldrich (St. Louis, MO). Anti-goat PI3K was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Western Blot Analysis**

Blood Pressure Measurements

Systolic blood pressure (SBP) was measured using a sphygmomanometer (UR-1000; Ueda, Chiba, Japan) using the tail-cuff method. The average of 3 measurements was taken as the SBP reading.
as an internal control. The density of the bands was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses of the data were done using Student’s t test to determine the significance of differences. A P value < .05 was considered significant.

**Results**

**Blood Pressure and Body Weight**

Mean SBP was already significantly higher in the SHRSP group than in the WKY group at 6 weeks of age. The elevation became more apparent in SHRSP with aging, even though SHRSP had lower body weight than WKY at 8 and 10 weeks (Table 1).

**Change in NO Production**

Twenty-four-hour urinary NO metabolite excretion was significantly lower in SHRSP compared with WKY (Fig 1).

**Various Types of NOS Protein Expression**

To investigate whether the expression of NO synthases (which are vasodilators) differed between WKY and SHRSP, we used antibodies to measure iNOS, nNOS, and eNOS. The expression of iNOS, nNOS, and eNOS in the cerebral cortex did not differ between the 2 groups (Fig 2).

**Effects of Phosphorylation eNOS via the PI3K/Akt Pathway**

The phosphorylation of eNOS at Ser1177 in the cerebral cortex of SHRSP was significantly lower than that in WKY, as determined by Western blot analysis with the phosphospecific antibody. The ratio of phosphor-eNOS to total eNOS was decreased by about 50% (Fig 3A). Activation of the serine/threonine kinase Akt leads to phosphorylation and activation of eNOS, which in turn induces NO production and vasorelaxation. Therefore, we also investigated the phosphorylation of Akt. The results of Western blot analysis demonstrate significantly reduced cerebral cortex expression of Akt phosphorylated at Ser473 in SHRSP compared with WKY. Immunoblot analysis with antibodies against total Akt found equal protein levels in both groups, thus demonstrating that the decrease in phosphor-Akt (Ser473) was not due to decreased expression of Akt (Fig 3B). Indeed, a marked reduction in the ratio of phosphor-Akt (Ser473) to total Akt was found in SHRSP compared with WKY (Fig 3C). In addition, there was no difference in Thr308 between SHRSP and WKY (Fig 3D). Akt is located downstream of PI3K. Given the importance of the PI3K in the regulation of downstream signaling molecules, we assessed PI3K protein levels by Western blot analysis using its specific antibodies. The total amount of PI3K was significantly lower in SHRSP compared with WKY (Fig 3E).

**Effects of Phosphorylation mTOR**

Although mTOR is located downstream of PI3K-Akt pathway, the ratio of phosphor-mTOR (Ser2448) to total mTOR did not differ between SHRSP and WKY (Fig 4).

**Discussion**

Our findings demonstrate that at the developmental stage of hypertension, PI3K/Akt/eNOS signaling was disturbed in the cerebral cortex of 10-week-old SHRSP compared with age-matched WKY, but there was no difference in mTOR, another downstream target of Akt, between the 2 strains. SHRSP developed from SHR is considered a model for essential hypertension in humans, in which stroke is a common complication. Previous studies have demonstrated abnormalities of the renin-angiotensin system, catecholamines, vasopressin, and vasoactive intestinal peptide in SHRSP. 18 Our data demonstrate that SBP was already significantly higher in SHRSP compared with WKY at 6 weeks of age. Several studies have reported that impaired NO release from the endothelial cells is related to the hypertensive state of SHRSP. 19 We found reduced NOx excretion in the urine of SHRSP, providing additional evidence of these effects. NO release is known to play crucial roles in the regulation of a broad range of functions and that NO is generated by NOS, which has 3 distinct isoforms. eNOS is

<table>
<thead>
<tr>
<th>SBP, mm Hg</th>
<th>Body weight, g</th>
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<tr>
<td><strong>Age, weeks</strong></td>
<td><strong>WKY (n = 7)</strong></td>
</tr>
<tr>
<td>6</td>
<td>119.3 ± 2.6</td>
</tr>
<tr>
<td>8</td>
<td>128.6 ± 3.5</td>
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<td>10</td>
<td>146.3 ± 3.6</td>
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All values are mean ± SEM.

*P < .01 vs WKY.
known to regulate vascular tone, nNOS is involved in neural signaling, and iNOS modulates immune function. No NOS isoform showed any difference in expression between SHRSP and WKY at 10 weeks of age. Expression of each NOS isoform in the cerebral cortex of SHRSP at a younger age has been demonstrated by previous studies; for example, one study found higher protein expression of iNOS in SHRSP than WKY at 14 weeks of age, although another study found no difference between the 2 strains at 20 weeks of age. iNOS was reported to be already increased in SHRSP before stroke occurrence, presumably due to inflammatory stimulation in the development of cerebrovascular lesions. But we did not detect elevated iNOS expression in the present study, suggesting that inflammatory response to cerebrovascular lesions begins later than 10 weeks of age. On the other hand, eNOS expression was previously reported to be already decreased in the brain cortex of SHRSP at 6 weeks of age compared with WKY, but no difference was reported between SHRSP and WKY at 15 weeks. Because NO generation from eNOS would initially induce vasodilation to attenuate the development of stroke lesions, we consider eNOS activity to play a more important role than NOS expression at younger ages.

We have demonstrated for the first time decreased phosphorylation of eNOS at Ser1177 in the cerebral cortex of SHRSP compared with WKY. Because eNOS phosphorylation is downstream of Akt activation, our findings suggest that in SHRSP, decreased eNOS phosphorylation might be the result of a diminished PI3K/Akt pathway, considering that the decreased total PI3K expression and reduced Akt phosphorylation, especially at Ser473, occurred with no change in the total amount of Akt. This pathway has multiple stimuli, including VEGF, IGF-1, insulin, and estrogen. Previous studies have shown that insulin might modulate NO production via the PI3K/Akt pathway, and that Akt signaling was impaired in SHR. In addition, the defective Akt-eNOS signaling in SHR causes the prehypertensive SHR to develop hypertension. More recently, IGF-1/insulin can modify vascular responses via PI3K/NO pathways in the aorta of WKY rats, but the vascular effects of IGF-1/insulin in the hypertensive SHRSP may be attenuated due to abnormalities in pathway regulation related to NO bioavailability. Likewise, impaired IGF-1/Akt signaling, which decreases the total amount of PI3K in the heart, occurs at an early age and might contribute to the development of hypertension in SHRSP. The SHRSP strain is used not only as a model for human essential hypertension, but also as a model for insulin resistance in adipocytes and skeletal muscles. This is also related to the defect in the PI3K/Akt pathway. Consequently, we speculate that blunting of this pathway in the brain of SHRSP is one trigger for the development of hypertension and stroke.

Whereas inactivation of the PI3K/Akt/eNOS pathway has been associated with deleterious effects in the brain,

![Figure 1](https://example.com/figure1.png)  
**Figure 1.** Urinary nitrate/nitrite excretion at 10 weeks of age in WKY (n = 7) and SHRSP (n = 8). Data are mean ± SEM. *P* < .05 vs WKY.

![Figure 2](https://example.com/figure2.png)  
**Figure 2.** Western blot analysis of the protein expression of iNOS (A), nNOS (B), and eNOS (C) in the cerebral cortex at 10 weeks of age in WKY (n = 4) and SHRSP (n = 4). β-actin served as protein quantity control with each experiment. Data are mean ± SEM.
Akt also mediates signaling, such as mTOR phosphorylation, in response to a variety of physiological stimuli. Many neurodegenerative diseases are characterized by neuronal death via apoptosis, and modulation of mTOR activity might possibly offer some protection against these effects. In particular, diseases involving oxygen and nutrient deprivation, such as stroke, would be expected to inhibit the mTOR pathway. Moreover, previous reports have demonstrated that transient focal ischemic brain injury significantly decreases the levels of phosphorylated Akt and mTOR. In the present study, phosphorylation of mTOR was similar in SHRSP and WKY despite lower Akt activation in SHRSP. Thus, in the cerebral cortex of SHRSP at age 10 weeks, there is...
a disturbance of PI3K/Akt/eNOS signaling only with no effect on mTOR, suggesting that decreased mTOR signaling might not yet occur at 10 weeks.

In summary, our study demonstrated markedly decreased phosphorylation of eNOS in the cerebral cortex of SHRSP at 10 weeks of age, apparently associated with impairment of the PI3K/Akt pathway with no change in mTOR. Controlling the effects of cerebral PI3K/Akt/eNOS signaling may help improve hypertensive cerebral disorders, such as stroke, in earlier stages. These findings may contribute to the development of therapy for patients with hypertensive brain disease.

References


