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Research Report

Increased ICP promotes CaMKII-mediated phosphorylation of neuronal NOS at Ser^{847} in the hippocampus immediately after subarachnoid hemorrhage

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ABSTRACT

Early brain injury has recently been identified as an indicator of poor prognosis after subarachnoid hemorrhage (SAH). Calmodulin-dependent protein kinase IIα (CaMKIIα) has been shown to phosphorylate neuronal NOS (nNOS) at Ser^{847}, resulting in a reduction in nNOS activity. In this study, we revealed chronological changes in the phosphorylation of nNOS at Ser^{847} in the hippocampus and cortex immediately after SAH. In a rat single-hemorrhage model of SAH, the hippocampus and adjacent cortex were collected up to 24 h after SAH. Samples from rats that were not injected with blood were used as controls. NOS was partially purified from the crude samples using ADP-agarose affinity chromatography. Western blot analysis revealed that nNOS phosphorylated (p-nNOS) at Ser^{847} was significantly increased in the hippocampus, but not in the cortex, at 1 h after SAH compared with that resulting from the control treatment. Immunoreactivity of p-nNOS at Ser^{847} was observed in interneurons of the hippocampus at 1 h after SAH. Injection of saline instead of blood also significantly induced p-nNOS at Ser^{847} levels in the hippocampus at 1 h after injection. The colocalization of CaMKIIα and nNOS was transiently increased in the hippocampus at 0.5 h after SAH. Our data suggest that immediately after SAH, an increase in intracranial pressure might induce transient cerebral ischemia, potentially promoting the phosphorylation of nNOS at Ser^{847} by CaMKIIα in the hippocampus. The activation of p-nNOS at Ser^{847} in the hippocampus may alleviate ischemic insults immediately after SAH to exert a neuroprotective effect against early brain injury.

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1. Introduction

Subarachnoid hemorrhage (SAH) occurs in approximately 6–8 out of every 100,000 people per year (Linn et al., 1996). Cerebral vasospasm, defined as delayed arterial narrowing, is a major cause of morbidity and mortality after SAH; this condition typically develops between 4 and 14 days and is most severe between 7 and 9 days after SAH. Previous investigations have focused on vasospasm and its sequelae. Early brain injury is a recent concept that considers overall brain injury and is thought to be the primary cause of mortality after SAH (Cahill et al., 2006). Apoptotic, inflammatory and ischemic pathways, all of which induce cell death, have been implicated in early brain injury (Cahill and Zhang, 2009).

Patients who experience severe SAH frequently exhibit deficits in learning and memory. A significant decrease in synapses of neurons in the CA1 area has been reported in rat SAH models (Han et al., 2014; Tariq et al., 2010), and this decrease may be responsible for the loss of long-terminal potentiation after SAH. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) plays important roles in hippocampal learning and *N*-methyl-D-aspartate receptor-dependent synaptic plasticity, which regulates long-term potentiation (Fukunaga and Miyamoto, 1999). CaMKII is a broadly specific Ser/Thr protein kinase that catalyzes the phosphorylation of over 100 protein and peptide substrates *in vitro*. Neuronal nitric oxide synthase (nNOS) has been demonstrated to be a potential physiological substrate of CaMKII (Hayashi et al., 1999; Komeima et al., 2000). However, chronological changes in the activation of nNOS after SAH have remained unclear to date. Therefore, this study was performed to determine whether nNOS is activated in a rat SAH model using Western blot and immunohistochemistry techniques.

2. Results

2.1. *Effects of SAH on the phosphorylation of nNOS at Ser^{847}*

We first examined the levels of phosphorylated nNOS (p-nNOS) at Ser^{847} in both the hippocampus and cortex after SAH. SAH induced a threefold enhancement in the phosphorylation of nNOS at Ser^{847} at 1 h after SAH in the hippocampus relative to the control treatment (Fig. 1A). At that time point, no significant increase in nNOS phosphorylation was detected in the cortex (Fig. 1B). Equal levels of nNOS were purified from crude fractions even after SAH, indicating that the SAH episode modulated nNOS primarily via its phosphorylation at Ser^{847} in the hippocampus.

2.2. *Neurons in the hippocampus contain p-nNOS at Ser^{847} after SAH*

To further investigate the localization of nNOS phosphorylation to Ser^{847} in the hippocampus, we measured p-nNOS expression at Ser^{847} at 1 h after SAH via immunohistochemistry. Immunoreactivity of nNOS was primarily observed in the cytoplasm and processes of CA1 nonpyramidal neurons, and it was not detectably altered at 1 h after SAH compared with that resulting from the control treatment (Fig. 2A and B). Immunoreactivity of p-nNOS at Ser^{847} was observed in the cytoplasm and processes of CA1 nonpyramidal neurons at 1 h after SAH (Fig. 2D and E *arrowheads and arrows*) but not in the control (Fig. 2C).

2.3. *Effects of saline on the phosphorylation of nNOS at Ser^{847}*

To further investigate the mechanism underlying the induction of the phosphorylation of *nNOS* at Ser^{847} after SAH, we injected the same volume of saline or autologus blood into the cisterna magna and removed the hippocampus after 1 h. Immunoblot analyses revealed a significant increase in the phosphorylation of *nNOS* at Ser^{847} by approximately 4-fold following saline injection compared with that resulting from the control treatment (Fig. 3). This increase was nearly equivalent to that observed following autologous blood injection.

2.4. *Colocalization of nNOS and CAMKIIα in the hippocampus*

The Ser^{847} residue of nNOS has recently been identified as a potential site of phosphorylation by CaMKII *in vitro* and *in situ* (Hayashi et al., 1999; Komeima et al., 2000). Therefore, we examined the spatial relationship between CaMKIIα and nNOS by co-sedimentation assay using ADP-agarose affinity chromatography. The CaMKIIα level was not changed in the crude fraction of either the hippocampus or the cortex after SAH (Fig. 4). CaMKIIα co-sedimented with nNOS, as shown by ADP-agarose affinity chromatography of control samples from both the hippocampus and cortex. We next examined whether CaMKIIα was present in the nNOS fractions after SAH and found that it was significantly increased at 0.5 h after SAH in the hippocampus but not in the cortex (Fig. 4).

3. Discussion

In this study, we for the first time reveal the immediate activation of nNOS in the hippocampus after SAH. Intracisternal administration of autologous blood or saline significantly induced the phosphorylation of nNOS at Ser^{847} in the hippocampus. These findings suggest that an immediate increase in intracranial pressure (ICP) caused by SAH induces transient cerebral ischemia, which results in the activation of CaMKIIα and the phosphorylation of nNOS at Ser^{847} in the hippocampus.

Endovascular perforation and blood injection into the cisterna magna are the most common models used to study the mechanism underlying cerebral vasospasm after SAH in rats (Bederson et al., 1995; Jackowski et al., 1990; Solomon et al., 1985). Some studies have compared different experimental rat SAH models (Lee et al., 2009). The endovascular perforation model appears to more closely mimic the human situation. However, the high mortality rate (up to ~50%) and large variations in bleeding severity are major drawbacks to this model (Bederson et al., 1995). In contrast, the injection of autologous blood into the cisterna magna is easy to perform and results in a low mortality rate (Jackowski et al., 1990; Solomon et al., 1985). In a single-injection model, an immediate increase in ICP to approximately 50 mmHg after intracisternal injection of 0.3 mL of autologous

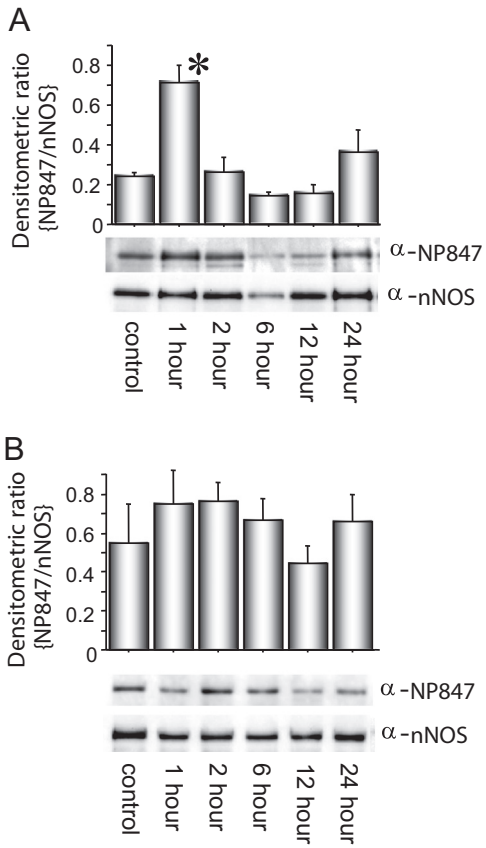

Fig. 1 – Phosphorylation of nNOS at Ser^{847} after SAH. At 1, 2, 6, 12 and 24 h after intracisternal injection of autologous blood (300 μL), as indicated below the panel, nNOS was affinity-purified via ADP-agarose affinity chromatography from the hippocampus (A) and cortex (B), and these samples were subjected to Western blot using anti-nNOS (α-nNOS) and anti-p-nNOS at Ser^{847} (α-NP847) antibodies. The histogram shows the amount of α-NP847 relative to that of α-nNOS in the membrane. The mean values ± SEM for five animals are shown. Control; control basilar artery not subjected to SAH. $^{*}P<0.05$ denotes a significant difference between the control and SAH groups based on ANOVA followed by Fisher's PLSD.

blood or saline has been observed (Jackowski et al., 1990). An even higher increase in ICP (to ~100 mmHg) has been reported after intracisternal injection of 0.3 mL of autologous blood at a higher injection velocity over 15 s. Using the same procedure, injection of 0.3 mL saline has been shown to increase ICP to up to 70 mmHg. Intracisternal single injection of autologous blood (0.2 mL) over 60 s also has been shown to increased ICP to up to 33 mmHg (Lee et al., 2009). The degree of increase in ICP might depend on the dose and injection rate of autologous blood or saline. These previous reports confirm that cerebral perfusion pressure (CPP) decreases after intracisternal injection of autologous blood. It has been suggested that elevated ICP and reduced CPP induce cerebral ischemia (Grote and Hassler, 1988). Clinically, an MRI study has revealed that 40 out of 61 patients suffer from cerebral ischemia at 1–3 days after the onset of SAH (Frontera et al., 2014). In the case of poor-grade SAH, acute-stage diffusion-weighted imaging has revealed ischemic findings in 81% of patients within 24 h after SAH (Sato et al., 2010). Based on our data, the administration of the same volume of autologous blood or saline induced an equivalent level of phosphorylation of nNOS at Ser^{847}. Both treatments significantly increased the p-nNOS level at Ser^{847} compared with that resulting from the control treatment. These data suggest that early cerebral ischemia due to increased ICP, rather than extravasated blood, might be a cause of early brain injury after SAH.

Nitric oxide (NO), a well known endothelium-derived relaxing factor, is produced by endothelial nitric oxide synthase (eNOS) in the intima and by nNOS in the adventitia of the cerebral arteries, and NO synthase dysfunction appears to be responsible for delayed cerebral vasospasm. The loss of nNOS immunoreactivity in the adventitia of vasospastic arteries deprives these arteries of NO, leading to the induction of delayed vasospasm (Pluta et al., 1996). eNOS dysfunction plays a key role in the development and/or persistence of vasospasm after SAH (Iuliano et al., 2004). NO-targeted therapies for cerebral vasospasm have been intensively studied. Simvastatin has been shown to increase eNOS activity via the phosphatidylinositol 3-kinase/Akt pathway and to attenuate cerebral vasospasm (McGirt et al., 2002; Sugawara et al., 2008). Moreover, decreased NO availability, which plays an important role in acute cerebral vasoconstriction and ischemic brain damage, has been observed during the acute stage of SAH (Schwartz et al., 2000; Sehba et al., 1999, 2000). NO is scavenged by hemoglobin immediately after SAH (Goretski and Hollocher, 1988), and its level recovers as hemoglobin is metabolized into methemoglobin. NOS is a critical regulatory enzyme in the central nervous system that catalyzes the production of NO from arginine. Previous studies have revealed time-dependent changes in the protein expression of NOS with various cerebral diseases. Cerebral ischemia or traumatic brain injury increases nNOS expression (Gahm et al., 2000; Zhang et al., 1994), and the number of nNOS-positive cells has been shown to markedly increase at 1 to 3 h after SAH, which may have occurred in response to reduced CBF (Sehba et al., 2004). However, in the hippocampus, the number of nNOS-positive cells remains unchanged during the first 6 h after SAH (Sehba et al., 2004). These data suggest that different regional nNOS-related responses might occur immediately after the onset of SAH, especially in the hippocampus.

The occurrence of apoptosis in the dentate gyrus and CA1 regions of the hippocampus, and to a lesser extent in the cerebral cortex, has been demonstrated at 24 h after SAH (Park et al., 2004). Platelet aggregation in the cerebral microvasculature of the hippocampus as early as 10 min after SAH may contribute to decreased cerebral blood flow and ischemic injury (Sehba et al., 2005). A caspase inhibitor has been shown to suppress the activation of apoptosis via the neurovascular protection of cerebral endothelial cells (Park et al., 2004). TUNEL-positive cells are scattered in the brain parenchyma within 10 min after SAH, and substantially more TUNEL-positive cells appear at 24 h (Friedrich et al., 2012). Autopsies of SAH patients have revealed that apoptotic death occurs in the dentate gyrus beyond 24 h after SAH onset *in vivo* (Nau et al., 2002). Microdialysis analyses have revealed that extracellular glutamate concentrations increase within minutes after SAH in both the hippocampus and cortex (Bederson et al., 1998), suggesting the occurrence of cerebral ischemia. This decrease in acute cerebral blood flow after SAH is related to the degree of delayed cell death (Prunell et al., 2005). Taken together, ischemic injury due to increased ICP

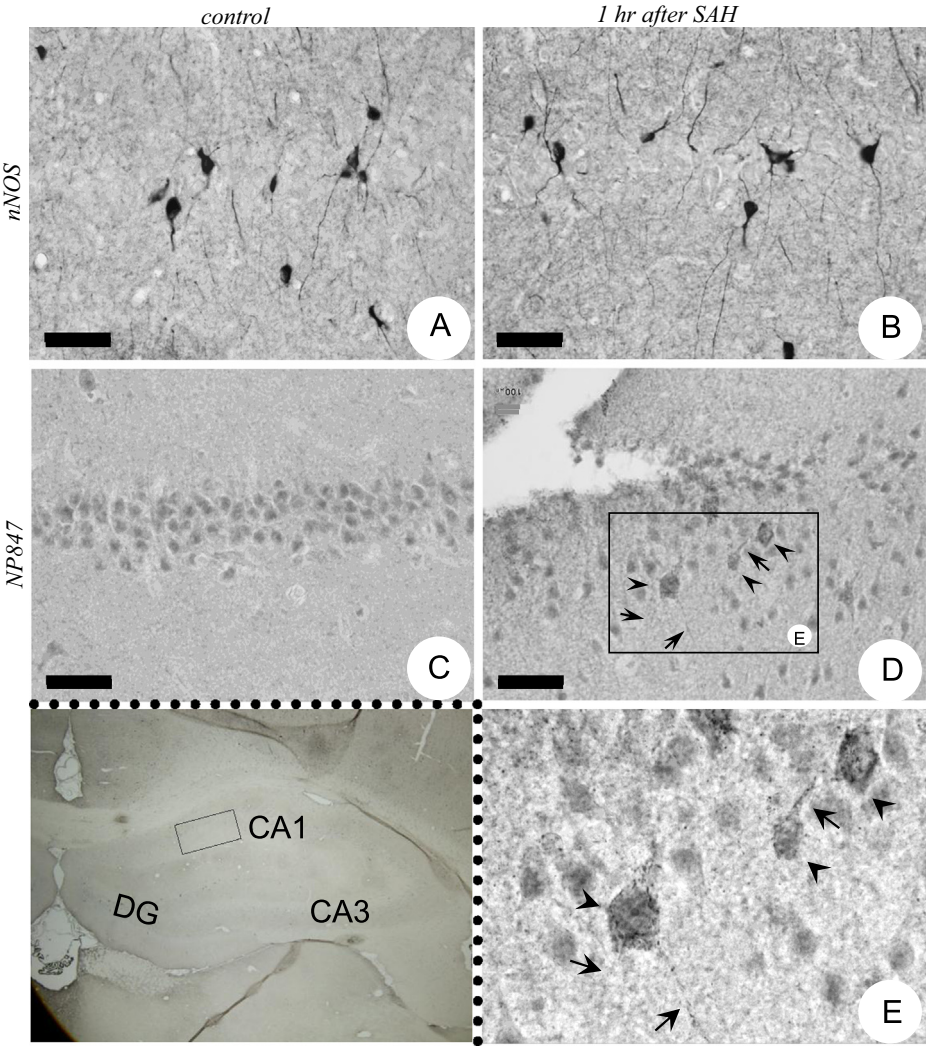

Fig. 2 – Immunohistochemical staining for nNOS and p-nNOS at Ser^{847} (α-NP847) expression in the hippocampus after SAH. Rats were perfused with 4% paraformaldehyde after sham operation (A and C) or at 1 h (B, D and E) after injection of autologous blood. Ten-micrometer coronal slices were immunostained with antibodies recognizing either nNOS (A and B) or p-nNOS at Ser^{847} (C, D and E), using the ABC method. The left hippocampus is shown (left lowest panel), and the CA1 area, especially that in the rectangle, was carefully examined. The area within the rectangle, as labeled in D, is shown at higher magnification in E. Immunoreactivity for nNOS was preserved at 1 h after SAH (B) compared with that of the sham (A). Note that positive staining using NP847 was observed in the cytoplasm (D and E, *arrow heads*) and processes (D and E, *arrows*) of CA1 nonpyramidal neurons at 1 h after SAH compared with that of the sham (C). Scale bars=50 μm, DG: dentate gyrus.

immediately after SAH might play an important role in the induction of apoptosis.

Previously, we demonstrated that transient forebrain ischemia induces the phosphorylation of nNOS at Ser^{847} by CaMKIIα in the hippocampus (Osuka et al., 2002). This is a direct phosphorylation that leads to a reduction in its enzymatic activity *in vitro* and *in situ* (Hayashi et al., 1999; Komeima et al., 2000). After transient forebrain ischemia, activated CaMKII, which is colocalized with nNOS, attenuates nNOS activity in hippocampal neurons (Osuka et al., 2002) to prevent the excessive production of harmful NO. nNOS phosphorylation at Ser^{847} may exert protective effects against neuronal apoptosis via downstream heme oxygenase-1 expression (Kasamatsu et al., 2014). In this study, based on Western blot analysis of co-sedimentation assay followed by ADP-agarose affinity chromatography, CaMKII was found to be associated with nNOS at 30 min after SAH in the nNOS fraction of the hippocampus but not with that of the cortex. In the case of traumatic brain injury, the immediate activation of CaMKII within 30 min after injury in the hippocampus has been reported, suggesting that CaMKII signaling pathways might be involved in cell injury and impaired plasticity after traumatic brain injury (Atkins et al., 2006; Folkerts et al., 2007). Based on these data, an increase in ICP immediately after SAH induces transient forebrain ischemia, which activates CaMKIIα, resulting in the phosphorylation of nNOS at Ser^{847} in the hippocampus. This signal transduction pathway may contribute to the attenuation of early brain injury in the hippocampus after SAH.

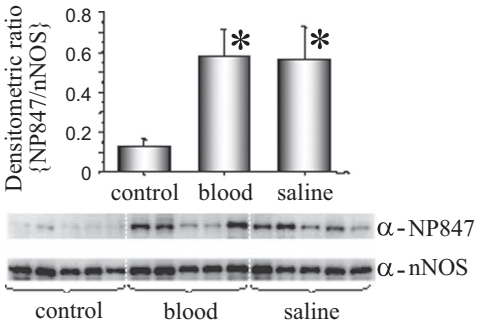

Fig. 3 – The phosphorylation of nNOS at Ser^{847} after intracisternal injection of autologous blood (300 μL) or saline (300 μL). At 1 h after intracisternal injection, as indicated below the panel, nNOS was affinity-purified from the hippocampus via ADP-agarose affinity chromatography and then subjected to Western blot using anti-nNOS (α-nNOS) and anti-p-nNOS at Ser^{847} (α-NP847) antibodies. The histogram shows the amount of α–NP847 relative to that of α-nNOS in the membrane. The mean values ± SEM for five animals are shown. Control; control basilar artery not subjected to injection. *P < 0.05 denotes a significant difference compared with the control based on ANOVA followed by Fisher's PLSD.

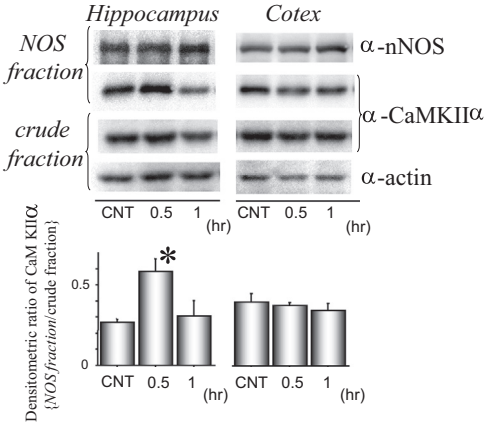

Fig. 4 – Immunoblot analysis of CaMKIIα in the brain after SAH. At 0.5 and 1 h after intracisternal injection of autologous blood (300 μL), as indicated below the panel, the hippocampus and adjacent cortex were removed. The 1% NP-40-soluble fraction (crude fraction) and the nNOS-containing fraction (NOS fraction), as prepared by ADP-agarose affinity chromatography, from the hippocampus or the cortex were subjected to Western blot using anti-nNOS (α-nNOS), anti-CaMKIIα (α-CaMKIIα) and anti-actin (α-actin) antibodies. CNT; control hippocampus and cortex not subjected to SAH. The histogram shows the amount of CaMKIIα in the NOS fraction relative to that in the crude fraction. The mean values ± SEM for four animals are shown. *P < 0.05 denotes a significant difference compared with the control based on ANOVA followed by Fisher's PLSD.

In conclusion, our results show that the phosphorylation of nNOS at Ser^{847} is differentially regulated by CaMKIIα between the hippocampus and cortex immediately after SAH. The phosphorylation of nNOS in the hippocampus decreases its enzymatic activity and inhibits the production of excess NO after SAH, which might play a role in protection against early brain injury. Further studies using NOS gene knockout mice may explain the precise roles of CaMKIIα and nNOS in early brain injury in the hippocampus after SAH.

4. Experimental procedures

4.1. Materials

β-NADPH was purchased from Oriental Yeast (Osaka, Japan). All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO).

4.2. Experimental model of SAH and saline injection

The experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and with the approval of the Animal Care and Use Committee of Aichi Medical University. All efforts were made to minimize the number of animals used and their suffering.

Anesthesia was induced in male Sprague-Dawley rats (300 to 350 g, Chubu Kagaku Shizai Ltd., Nagoya, Japan) using chloral hydrate (400 mg/kg, IP). The animals were then intubated and ventilated with 1.0% halothane in an oxygen/nitrous oxide (30%/70%) gas mixture. The temperature was monitored using a rectal probe and was maintained at between 36.5 and 37.5 °C, using a heating pad and lamp. The right femoral arteries were exposed and catheterized using polyethylene (PE-50) tubing to enable blood sampling. A midline skin incision was created from the middle of the calvarium to the cervical spine during stereotactic surgery. The atlanto-occipital membrane was exposed under a microscope, and a 27-gauge needle was inserted into the cisterna magna. SAH was induced via the infusion of 300 μL of autologous arterial blood over a 1-min period. Instead of blood, the same dose of saline (300 μL) was also injected using the same procedure. The rats were maintained in a head-down position for 5 min to ensure that the blood or saline contacted the basilar artery, after which the needle was withdrawn, and all wounds were sutured. Animals that did not receive an injection of blood or saline were used as controls.

4.3. Sample preparation and Western blot analysis

Brain samples were obtained via decapitation under deep anesthesia at 1, 2, 6, 12 or 24 h after SAH and at 1 h after saline injection. Hippocampus and cortex samples obtained at the same level were immediately isolated on ice, frozen in liquid nitrogen and stored at −80 °C until use. Brain samples from rats that were not subjected to SAH or saline injection of saline were used as controls.

The samples were homogenized using a homogenizer in 15 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 EGTA, 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1.25 μg/mL pepstatin A, 0.2 μg/mL aprotinin, 5 nmol/L tetrahydrobiopterin (BH_4), 1 mmol/L sodium orthovanadate,

50 mmol/L sodium fluoride, 2 mmol/L sodium pyrophosphate, and 1% Nonidet P-40 (NP-40). The homogenates were then centrifuged at 18,000*g* at 4 °C for 10 min. The protein concentrations of the supernatants were determined via the Bradford method using bovine serum albumin as the standard. The supernatant fractions were used as crude fractions.

To prepare the nNOS fractions, NOS was partially purified using 2′,5′-ADP-agarose as described previously (Richards and Marletta, 1994). Briefly, 20 μL of 2′,5′-ADP-agarose and the same concentration of crude fraction (1000 μg/200 μL) were gently incubated for 1 h at 4 °C. The agarose was washed with 200 μL of homogenization buffer lacking NP-40 and nNOS and was eluted from the 2′-5′-ADP-agarose using 50 μL of 10 mmol/L β-NADPH.

The extracts (10 μL) were subjected to 7.5% SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with a primary polyclonal antibody specific for p-nNOS at Ser^{847} (NP847) at a dilution of 1:200 overnight at 4 °C. The membranes were then incubated for 30 min at room temperature with a horseradish peroxidase-conjugated secondary antibody, and antibody binding was visualized via enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK). The p-nNOS immunoblots were stripped from the PVDF membranes and reprobed using a primary monoclonal antibody against nNOS (Transduction Lab., Lexington, KY) at a dilution of 1:3000 for 45 min at room temperature. Finally, the membranes were developed via ECL, and the band intensities were quantified via densitometry using ImageQuant software.

4.4. *Immunohistochemistry*

The rats were perfused with 200 mL of ice-cold 4% paraformaldehyde in 0.1 mol/L sodium phosphate (pH 7.4) at 1 h after SAH. The brains were removed, placed in fixation solution for 3 h, and rinsed with 0.1 mol/L lysine hydrochloride in 0.1 mol/L phosphate-buffered saline for 3 additional hours. 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 or horse serum for 30 min; a primary polyclonal antibody against p-nNOS at Ser^{847} at a dilution of 1:100 and a primary monoclonal antibody against nNOS at a dilution of 1:2000 overnight; biotinylated anti-rabbit or mouse IgG for 1 h; and ABC for 1 h. Sera for the blocking step, biotinylated antibodies, and ABC were purchased from Vector Laboratories (Burlingame, CA). The reaction products were developed via incubation in 0.05% 3,3′-diaminobendizine tetrachloride and 0.01% H_2O_2 in 50 mM Tris–HCl (pH 7.5) for 10 min. Rats that were not subjected to SAH were used as controls. The CA1 area of the hippocampus was meticulously examined.

4.5. *Colocalization of nNOS and CaMKIIα*

SAH was induced via the infusion of 300 μL of autologous arterial blood over a 1-min period as described above. Samples of the hippocampus and adjacent cortex were obtained following decapitation under deep anesthesia at 0.5 or 1 h after SAH ($n=4$ for each group). Brain samples from rats that were not subjected to SAH were used as controls ($n=4$). Crude fractions and NOS fractions were prepared from each sample. Western blot analyses were performed using antibodies against nNOS (Transduction Lab., Lexington, KY), CaMKIIα (Transduction Lab., Lexington, KY) and actin (Sigma, St. Louis, MO) incubated at a dilution of 1:3000 for 45 min at room temperature, as described above.

4.6. *Statistical analysis*

The data are expressed as the mean values ± SEM. Significant differences between the groups were assessed via one-way ANOVA followed by Fisher's PLSD for multiple comparisons. Significance was considered to be $P<0.05$.

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