Vascular NAD(P)H Oxidase Triggers Delayed Cerebral Vasospasm After Subarachnoid Hemorrhage in Rats

Dong Eun Kim, MS; Young Suk Suh, MD; Mi-Sook Lee, MS; Ki Young Kim, PhD; Jeong Hyun Lee, MS; Hyun Seung Lee, MS; Ki Whan Hong, MD, PhD; Chi Dae Kim, MD, PhD

Background and Purpose—To clarify the role of vascular NAD(P)H oxidase in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage (SAH), both the activity and/or activation mechanisms of NAD(P)H oxidase in the cerebral vasculature and the effect of oxidase inhibition on SAH-induced cerebral vasospasm were assessed.

Methods—The changes in the luminal perimeter of the middle cerebral artery were measured histologically after SAH was induced according to a 2-hemorrhage model in rats. The NAD(P)H oxidase activity in the cerebral vasculature was measured with a lucigenin assay at different time intervals from 12 hours to 14 days after injection of autologous blood into cisterna magna. The membrane translocation of p47phox and the protein expression of membrane subunits (gp91phox and p22phox) of NAD(P)H oxidase were analyzed using Western blot analysis.

Results—The luminal perimeter of the middle cerebral artery started to decrease on day 1 and peaked on day 5 after a second injection of blood, and these changes were significantly ameliorated by treatment with an NAD(P)H oxidase inhibitor, diphenyleneiodonium. At 24 hours after the second injection of blood, both vascular production of superoxide anion and NAD(P)H oxidase activity were markedly increased with enhanced membrane translocation of p47phox, but by 48 hours the enzyme activity had regained normal values. However, no significant changes in the expression of gp91phox and p22phox were observed throughout the experiments.

Conclusions—These findings suggest that the activation of NAD(P)H oxidase through enhanced assembly of the oxidase components in the early stages of SAH might contribute to the delayed cerebral vasospasm in SAH rats. (Stroke. 2002;33:2687-2691.)

Key Words: NADPH oxidase ■ reactive oxygen species ■ subarachnoid hemorrhage ■ vasospasm ■ rats

Cerebral vasospasm after subarachnoid hemorrhage (SAH) is characterized by a delayed but prolonged contraction of the major cerebral arteries and seriously affects the prognosis of a patient, but the pathophysiological mechanisms of cerebral vasospasm remain unclear. A variety of mechanisms have been proposed to explain these phenomena, and in particular, oxidant stress has been implicated in the pathogenesis of cerebral vasospasm.1–3 Numerous reports have supported the significance of reactive oxygen species (ROS) in causing vasospasm through activation of the protein kinase C system and/or inactivation of NO.4,5

Convincing evidence has been presented to show that production of ROS occurs in nonphagocytic cells, including endothelial cells,6 vascular smooth muscle cells,7 and aortic adventitial fibroblasts,8 and superoxide anion has a variety of biological functions that accompany induction of gene expression,9 cellular proliferation,10,11 apoptosis,11,12 and hypertrophy.13 However, both the enzymatic source and the role of vascular production of ROS in the cerebral vasospasm in SAH remain unknown.

Among various sources for ROS such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, NO synthase, and mitochondrial electron transport, NAD(P)H oxidase has been considered as a major source of ROS in the vasculature.14 Vascular NAD(P)H oxidase is similar in structure to the neutrophil NAD(P)H oxidase, which consists of 4 major subunits of a membrane-associated cytochrome b558, comprising gp91phox and p22phox and 2 cytosolic components, p47phox and p67phox. On activation, the cytosolic constituents are translocated to the plasma membrane, where they bind to cytochrome (membrane assembly) and initiate superoxide synthesis.15–17 In the present study, to clarify the role of vascular NAD(P)H oxidase in the delayed cerebral vasospasm in SAH, both the activity and/or activation mechanisms of NAD(P)H oxidase in the cerebral vasculature and the effect of oxidase inhibition on SAH-induced cerebral vasospasm were assessed using a rat model of SAH.

Materials and Methods

Animal Preparation

The experimental protocols used in this study were approved by the University of Pusan National University Animal Research Committee. Male Sprague-Dawley rats (300 to 400 g) were anesthetized with...
ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP), and the animals were allowed to breathe spontaneously. Aided by a surgical microscope, the atlantooccipital membrane was tapped carefully with a 27-gauge needle. After 0.3 mL of cerebrospinal fluid was aspirated, an equal amount of autologous blood from the femoral artery was injected during a period exceeding 3 minutes. The rats were then placed in a head-down prone position at a 30-degree angle for 30 minutes to hold the blood in the basal cisterns. For the second injection, the same procedure was repeated after 48 hours (day 2). Sham-operated rats were injected with 0.9% sterile NaCl solution instead of blood. To determine the role of NAD(P)H oxidase on SAH-induced vasospasm, diphenyleneiodonium (DPI) was injected into the cisterna magna at a dose of 5 μg/kg diluted in 25 μL of cerebrospinal fluid 20 minutes before injection of blood.

**Morphological Findings With Light Microscope**

After planned death by an overdose of anesthetic agents, rat hearts were cannulated and perfused with a phosphate buffer solution containing 2% glutaraldehyde. The perfusion rate was maintained constant at 7.8 mL/min by using a peristaltic pump (Cole-Parmer). The brain was fixed with 2% glutaraldehyde and embedded in paraffin, sliced, and stained with hematoxylin and eosin for light microscopy. The luminal perimeter of the middle cerebral artery (MCA) taken from each portion was measured using Image Plus Imaging software (Media Cybernetics).

**Measurement of Superoxide Production**

Frozen, enzymatically intact, 30-μm-thick sections of MCA were incubated with 5 μmol/L dihydroethidium (Molecular Probes) in a humidified chamber (37°C) protected from light. Thirty minutes after the incubation, the images were obtained with a laser scanning confocal microscope (LSM 510, Carl Zeiss Inc). For ethidium bromide detection, a 543-nm He-Ne laser combined with a 585-nm long-pass filter was used. The average fluorescent intensity at the maximal response time period (usually 25 minutes) was presented as units per square millimeter of artery.

**Measurement of NAD(P)H Oxidase Activity**

NAD(P)H oxidase activity was measured with the lucigenin assay, which is specific for superoxide anion. Briefly, cerebral vasculatures including basilar artery, cerebral arteries (anterior, middle, and posterior cerebral arteries), and pial arteries were isolated and homogenized with a motor-driven tissue homogenizer for 2 minutes in 50 mmol/L phosphate buffer containing 0.01 mmol/L EDTA and protease inhibitors. The homogenate was centrifuged at 1000 × g for 10 minutes to remove unbroken cells and debris. Protein content was determined by using the BCA protein assay kit (Sigma). The assay was performed in 50 mmol/L phosphate buffer, pH 7.0, containing 1 mmol/L EGTA, 150 mmol/L sucrose, 5 μmol/L lucigenin as the detector, and NADPH as the substrate (final volume, 150 μL). NADH and NADPH were used at a final concentration of 100 μmol/L, and the reactor was started by the addition of 25 μg of protein. The photon emission was measured every 15 seconds for 10 minutes in a microtiter plate luminometer (Miroclumat LB96P, EG&G).

**Western Blot Analysis**

Cerebral vasculature was initially homogenized in Tris-HCl buffer containing the protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL each of antipain, aprotinin, bestatin, leupeptin, soybean trypsin inhibitors, pepstatin A, and 0.1% 2-mercaptoethanol). The supernatant from the low-speed centrifugation was subjected to 30 000 g centrifugation for 45 minutes at 4°C to separate membrane and cytosolic fractions. The membrane and cytosolic fractions containing 30 μg of protein were used for immunoblotting. Antibodies used were polyclonal goat anti-human p47phox, gp91phox, p22phox, and horseradish peroxidase–conjugated anti-goat IgG (all from Santa Cruz Biotechnology). For the positive control, β-actin (mouse monoclonal IgG, Santa Cruz Bio-technology) and goat anti-mouse IgG (Upstate Biotech) were used. Blots were developed using ECL reagent (Amersham), and band intensity was compared by using a densitometer (GS-710 calibrated imaging densitometer, Bio-Rad).

**Statistical Analysis**

Data are mean±SEM or percentage of control. Statistical comparisons between groups were performed with the 2-tailed Student t test or Dunnett multiple-comparison test after ANOVA. A value of P<0.05 was considered statistically significant.

**Results**

The physiological parameters measured before and after SAH are listed in Table 1. There were no significant differences among the groups in terms of mean arterial blood pressure, Pco2, Po2, and pH.

**Time Course of SAH-Induced Changes in the MCA Perimeter**

The luminal perimeter of the MCA was quantitatively analyzed by image analysis of photomicrographs obtained with light microscopy. The baseline value of the MCA perimeter was 487.2±49.5 μm. In the 2-hemorrhage model, the perimeter of the MCA started to decrease on day 1, and the constriction peaked on day 5 (312.6±40.3 μm) after the second injection of autologous blood into cisterna magna. However, no significant change in the MCA perimeter was observed in the 1-hemorrhage group (Figure 1).

**Effect of DPI on the SAH-Induced Cerebral Vasospasm**

Light microscopic examination of the MCA in the SAH rats revealed substantial corrugation of the internal elastic lamina,
whereas arterial corrugation was less prominent in animals treated with DPI. The values obtained from the quantitative measurements of the cross-sectional perimeter of the MCA are shown in Figure 2. Five days after the second injection of blood into cisterna magna, the MCA perimeter was decreased by \( \approx 36\% \) in comparison with that in the control group. The MCA constriction in SAH rats was significantly attenuated by treatment with DPI in a dose of 5 \( \mu g/kg \) that did not alter the basal perimeter of MCA in the control rats (457.2 \( \pm \) 52.3 \( \mu m \) in DPI-treated rats versus 487.2 \( \pm \) 49.5 \( \mu m \) in control rats). In another series of experiments using rat pial artery, the decreases in diameter of the pial artery under suffusion of phenylephrine (an adrenergic \( \alpha \)-agonist) and U46619 (a thromboxane mimetic) were little altered in the presence of DPI (10 \( \mu mol/L \)).

**Vascular Production of Superoxide Anion**

As shown in Figure 3, the superoxide anion was detected in the MCA segments, as measured by the red fluorescence of the ethidium-DNA complex. A low-intensity fluorescence was identified in the MCA from the control rats, and the intensity was significantly increased in the samples from SAH rats.

**Time Course of Changes in the Vascular NAD(P)H Oxidase**

Figure 4 shows the time course of the changes in NAD(P)H oxidase in the cerebral vasculature after injection of autologous blood into the cisterna magna. At 12 and 24 hours after the second injection of blood, the activity of NAD(P)H oxidase in the cerebral vasculature was significantly increased (\( P<0.05 \) at 12 hours; \( P<0.01 \) at 24 hours), after which the activity recovered to the control values by 48 hours. These values were maintained for 12 days after the second injection of blood.

**Effect of Several Inhibitors on the NAD(P)H Oxidase Activity**

Enhanced NAD(P)H oxidase activity in the cerebral vasculature at 24 hours after the second injection of blood was assessed by measuring the superoxide level under treatment with several inhibitors, such as the following (all at 10 \( \mu mol/L \)): DPI, allopurinol (an inhibitor of xanthine oxidase), sulfaphenazole (a selective cytochrome P450 2C9 inhibitor), \( N^\epsilon \)-monomethyl-L-arginine (L-NMMA; a NO synthase inhibitor), and indomethacin (a cyclooxygenase inhibitor). As shown in Figure 5, NAD(P)H-evoked oxidase activity in SAH rats was significantly decreased by addition of DPI, but not by allopurinol, sulfaphenazole, L-NMMA, or indomethacin, respectively.

**Membrane Translocation of p47phox**

The results of quantitative analysis of changes in the amount of p47phox in the cytosol and the membrane fractions are summarized in Figure 6. At 24 hours after the second injection of autologous blood into the cisterna magna, the value in the cytosol fraction was significantly lower than that in the sham-operated group (\( P<0.01 \)). In the membrane fractions, the value was significantly higher than that in the sham-operated group.

![Figure 2](image1.png)

**Figure 2.** Effect of DPI (5 \( \mu g/kg \)) on SAH-induced changes in luminal perimeter of middle cerebral artery. Values were obtained from data at 5 days after second injection of blood and are mean \( \pm \) SEM from 6 animals. **\( P<0.01 \) vs control; \#\( P<0.05 \) vs vehicle of SAH.**

![Figure 3](image2.png)

**Figure 3.** A, Typical fluorescence microscope images showing superoxide production in MCA segments from control and SAH rats. B, Summary of intensity of ethidium-DNA fluorescence detected in MCA segments expressed as mean \( \pm \) SEM of 5 experiments. MCA segments were prepared 24 hours after secondary cisternal injection of autologous blood. **\( P<0.01 \) compared with the value obtained from control.**

![Figure 4](image3.png)

**Figure 4.** Time course changes of NAD(P)H oxidase activity in cerebral vasculature from 1-hemorrhage (\( \square \)) and 2-hemorrhage (\( \bullet \)) rats. Oxidase activity in the cerebral vasculature was assessed by measuring NAD(P)H-induced superoxide production with lucigenin chemiluminescence. Arrows indicate time of intracisternal administration of autologous blood. Values are given in nmol/min per mg protein and are mean \( \pm \) SEM from 5 measurements. *\( P<0.05 \), **\( P<0.01 \) vs baseline values on day 0.
Furthermore, the time course of the changes in the amounts of membrane-associated p47phox was similar to those of the changes in the oxidase activity.

Expression of gp91phox and p22phox
Because gp91phox and p22phox, the membrane-associated components of the vascular NAD(P)H oxidase, have been shown to be present and functional in the vasculature, we elucidated the protein expression of gp91phox and p22phox in sham-operated and SAH rats by performing Western blot analysis. As shown in Figure 7, the levels of gp91phox and p22phox expression in SAH rats were not different from those of sham-operated rats. These data suggest that the protein expression of gp91phox and p22phox may not be responsible for the increased activity of NAD(P)H oxidase in the cerebral vasculature from SAH rats.

Discussion
Although cerebral vasospasm in SAH patients is a major cause of morbidity and mortality, the precise mechanisms for the delayed cerebral vasospasm remain unclear. Cerebral vasospasm after SAH was attenuated not only by local administration of superoxide dismutase but also by overexpression of Cu/Zn-superoxide dismutase in the transgenic mice, suggesting the pivotal role of oxygen radicals in the development of cerebral vasospasm. In the present experiment, we demonstrated that the time course of the development of vasospasm in the rat 2-hemorrhage model was similar to that of vasospasm after a SAH in the clinical situation. Minimal narrowing was present by 1 to 2 days and had progressively increased by 5 days after the second injection of autologous blood into cisterna magna. The delayed cerebral vasospasm in SAH rats was significantly attenuated by pretreatment with DPI, an NAD(P)H oxidase inhibitor. Considering other reports with our results, NAD(P)H-derived superoxide might be closely involved in the pathogenesis of cerebral vasospasm in SAH.

Among various sources for ROS such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, NO synthase, and mitochondrial electron transport, NAD(P)H oxidase has been considered as a major source of ROS in the vasculature and has been implicated in numerous cellular processes and vascular diseases. Recently, Wang et al have demonstrated that NAD(P)H oxidase localized in the adventitia served as a primary site for superoxide production. In agreement with these reports, our previous experiment clearly demonstrated that periartrial blood application to the aortic segments significantly augmented the NAD(P)H-

Figure 5. Activity of NAD(P)H oxidase in the cerebral vasculature was assessed by measuring NAD(P)H-induced superoxide production with lucigenin chemiluminescence. Values are expressed as nmol/min per mg protein. SAH-induced increase (Veh) in superoxide production was significantly attenuated by pretreatment with DPI (10 μmol/L) but not by sulfaphenazole (SFP, 10 μmol/L), allopurinol (Allo, 10 μmol/L), L-NMMA (NMA, 10 μmol/L), or indomethacin (IND, 10 μmol/L), respectively. Values are mean±SEM from 10 experiments. Cont indicates control. **P<0.01 vs control; #P<0.05 vs vehicle of SAH.

Figure 6. Changes in amounts of p47phox in the cytosol (C) and membrane (M) fractions of cerebral vasculature from sham-operated (Control) and SAH rats. Results are expressed as percentage of the total amount of p47phox. In SAH rats, cerebral vasculature was isolated 24 hours after secondary cisternal injection of autologous blood. Values are mean±SEM from 5 measurements. **P<0.01 vs control group.

Figure 7. Protein expression of gp91phox and p22phox in the cerebral vasculature from sham-operated (Control) and SAH rats. Bar graph summarizes data obtained from 5 experiments, expressed as percentage change over levels of β-actin (mean±SEM). In SAH rats, cerebral vasculature was isolated 24 hours after secondary cisternal injection of autologous blood.
dependent production of superoxide. Furthermore, the expression of intercellular adhesion molecule–1 by autologous blood was well correlated with generation of superoxide. Thus, it was suggested that NAD(P)H oxidase–derived superoxide was implicated in periartrial blood-induced vasospasm via increased expression of intercellular adhesion molecule–1 with subsequent mobilization of inflammatory cells.

The regulation of NAD(P)H oxidase activity in cardiovascular cells occurs on at least 2 levels. First, activation of the oxidase can be mediated by intracellular second messengers, including calcium.24 On activation, the cytosolic components translocate to the plasma membrane, and the functional NAD(P)H oxidase is assembled.25 Secondly, oxidase activity can also be modulated by upregulation of the membrane component mRNAs. In the present experiments, at 24 hours after the second injection of autologous blood into cisterna magna, both the oxidase activity and the membrane translocation of p47phox were markedly increased, but by 48 hours they had regained normal values that were maintained up to 14 days after SAH. However, there were no significant changes in the protein expression of gp91phox and p22phox throughout the experiments. These findings suggest that the enhanced activity of NAD(P)H oxidase in the early stages of SAH rats is mediated by the enhanced assembly of oxidase through increased membrane translocation of cytosolic components of oxidase (p47phox) instead of overexpression of membrane subunits of oxidase.

With regard to SAH, it is well known that ROS are involved in cerebral vasospasm not only by protein kinase C–dependent augmentation of contraction but also by suppression of vasodilation, which is mediated by endothelium-derived relaxing factor.4,26 Considering these previous reports and our experimental finding that DPI has a preventive effect on the SAH-induced cerebral vasospasm even though there was a time discrepancy between vascular NAD(P)H oxidase activation and cerebral vasospasm that was maximized 5 days after second injection of blood, it is suggested that vascular production of ROS in the early stage of SAH may participate in the development of delayed cerebral vasospasm through the activation of yet-uncharacterized signaling pathways as well as induction of redox-sensitive genes that coordinate delayed cerebral vasospasm. However, further studies are required to elucidate the precise afferent pathways involved in the activation of NAD(P)H oxidase and efferent pathways of oxidative stress, as well as the role of NAD(P)H oxidase in the delayed cerebral vasospasm in SAH rats.

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