NAD(P)H Oxidases in Rat Basilar Arterial Endothelial Cells

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Background and Purpose—Reactive oxygen species (ROS) may play a critical role in the regulation of vascular tone and development of vascular diseases, such as stroke. NAD(P)H oxidase is a major source of ROS in vascular cells, including endothelial cells. It has been considered that Nox2 and Nox4 are exclusively expressed among Nox homologues in the endothelial cells of noncerebral blood vessels. However, the precise molecular identity of the NAD(P)H oxidase in the endothelial cells of the cerebral arteries is not fully understood. We examined the expression of Nox homologues and their activation mechanism in the endothelial cells of the cerebral arteries.

Methods—We isolated and cultured basilar artery endothelial cells (BAECs) of Sprague-Dawley rats. Expression of NAD(P)H oxidase was examined by reverse-transcription-polymerase chain reaction (RT-PCR) and immunohistological staining.

Results—RT-PCR disclosed abundant expression of Nox4 with marginal Nox2 in BAEC. In addition, Nox1 was expressed highly both at mRNA and protein levels in BAECs. Immunohistological staining also showed the prominent expression of Nox1 in the endothelial cells of the basilar artery. With respect to the cytosolic components of NAD(P)H oxidases, BAECs expressed p67phox and, to a lesser extent, p47phox, Noxo1, and Noxa1. Both NADH and NADPH induced superoxide production of the BAEC membranes. The phagocyte-type cytosolic components, p47phox and p67phox, significantly enhanced the NADH-induced superoxide production of the BAEC membranes, whereas the components failed to increase the NADPH-induced superoxide production.

Conclusions—Nox1 is highly expressed in the endothelial cells of the cerebral arteries along with Nox2 and Nox4, and the endothelial NAD(P)H oxidase of the cerebral arteries may have a unique activation mechanism by the phagocyte-type cytosolic components. (Stroke. 2005;36:1040-1046.)

Key Words: cerebrovascular disorders ■ endothelium ■ free radicals ■ NAD(P)H oxidase

Vascular endothelial cells play a critical role in the regulation of vascular tone and structure. Reactive oxygen species, including superoxide and hydrogen peroxide, may modulate such endothelial functions as mediators of intracellular signaling.1,2 NAD(P)H oxidase, a superoxide-producing enzyme, is recognized as the major origin of reactive oxygen species in vascular cells, including the endothelial cells.3-5 The phagocyte NADPH oxidase, the prototype of vascular NAD(P)H oxidases, consists of 2 membrane components, gp91phox (also known as Nox2) and p22phox, and 4 cytosolic components, the small GTP-binding protein Rac, p47phox, p67phox, and p40phox.6 The superoxide production after phagocytosis absolutely requires the translocation of at least 3 cytosolic components, Rac, p47phox, and p67phox, to the phagosomal membranes to form a complex with the membrane components.6-9

In contrast to Nox2, activation mechanism of other Nox proteins, ie, Nox1, Nox3, Nox4, and Nox5, remains poorly understood.6 Among these homologues, Nox2 and Nox4 are expressed and may be functionally active in the endothelial cells,4,5 and both Nox1 and Nox4 may be major Nox proteins in the smooth muscle cells10 of the systemic arteries. However, Touyz et al11 have demonstrated recently that Nox2 and Nox4, but not Nox1, are expressed and functionally active in the smooth muscle cells of human peripheral arteries. Thus, it may be possible that endothelial cells from different vascular beds express different sets of Nox proteins.

In the cerebral blood vessels, NAD(P)H oxidase appears to modulate vascular activities.12 Didion et al13 have demonstrated that in the basilar artery and the cerebral arterioles, both NADH and NADPH affect vascular tone via NAD(P)H oxidase-mediated superoxide production. Paravicini et al14 have suggested that Nox4 is upregulated in the rat whole
basilar artery during chronic hypertension and may be associated with its enhanced vasodilator responses. In addition, a genetic research has reported that the C242T polymorphism of the p22\textsuperscript{phox} gene may be a risk factor for ischemic stroke.\textsuperscript{15} Thus, NAD(P)H oxidases may play an important role in both physiological and pathological functions of cerebral blood vessels. However, the detailed molecular identity and activation mechanism of the NAD(P)H oxidase in the endothelial cells of the cerebral arteries are not fully understood.

The goal of the present study was to elucidate which Nox proteins were expressed in the endothelial cells of the cerebral arteries and whether the cytosolic components of the NAD(P)H oxidase had the potential to enhance superoxide production of the endothelial membranes. Here we demonstrate that Nox1, as well as Nox2 and Nox4, is expressed both at mRNA and protein levels in cultured basilar arterial endothelial cells (BAECs). Immunohistological staining confirms in situ expression of Nox1 in the BAECs. Both NADH and NADPH induce superoxide production of the BAEC membranes in vitro. The phagocyte-type cytosolic components, p47\textsuperscript{phox} and p67\textsuperscript{phox}, enhance the NADH-induced, but not NADPH-induced, superoxide production of the membranes.

Materials and Methods

Cell Culture
Basilar arteries were isolated from male Sprague-Dawley (SD) rats at age 5 weeks and cultured as described previously.\textsuperscript{5,16} Briefly, a few days after incubating the arterial segments in DMEM including 10% fetal calf serum, colonies of endothelial cells with cobblestone pattern, which are typical for endothelial cells, proliferated from the basilar arterial segments. After the arterial segments were removed, only the primary BAEC colonies were gently scraped off and subcultured in other dishes. BAECs were identified by the expression of von Willebrand factor (vWF) and uptake of 3,3\textprime;dioctadecylindocarbocyanine iodide (DiI)–low-density lipoprotein.\textsuperscript{16} Cells at passages 2 to 7 were harvested with trypsinization and used for the following experiments.

Reverse-Transcriptase Polymerase Chain Reaction and Quantitative Real-Time Polymerase Chain Reaction
Total RNAs of BAECs, basilar arterial smooth muscle cells (BASMCs), rat colon, and human neutrophils\textsuperscript{5} were prepared with TRIzol (Invitrogen). One μg of total RNA was reverse-transcribed by Avian Myeloblastosis Virus (AMV)-transcriptase (Roche Diagnostics) as previously described.\textsuperscript{5} Using the complementary DNA produced by the AMV-transcriptase (Roche Diagnostics) as previously described,\textsuperscript{5} the reverse transcription mixture (20 μL) containing 2 μL of LightCycler (Roche Diagnostics, Basel, Switzerland) in the reaction mixture (20 μL) containing 2 μL of LightCycler-FastStart DNA Master SYBR Green 1 (Roche), 0.5 μmol/L each primer, and 3 mmol/L MgCl\textsubscript{2}. The copy numbers of mRNA were standardized by those of glyceraldehydephosphate dehydrogenase.

Cell Fractionation
Harvested BAECs and BASMCs were lysed by sonication in the presence of proteinase inhibitors, ie, 1 mmol/L of PMSF, 1 μg/mL of leupeptin, and 1 μg/mL of pepstatin A. The sonicate was centrifuged for 10 minutes at 10 000g. The resultant supernatant was further centrifuged for 60 minutes at 100 000g. The pellet was harvested as membrane fraction.

Western Blot
Equal amounts of membrane proteins (10 μg) were subjected to 10% sodium dodecyl sulfate/PAGE. After proteins were transferred to a PVDF membrane, immunoblots were probed with an anti-Nox1 antibody (1:200 dilution), which was made by immunizing rabbits with synthetic peptides corresponding to the 544 to 556 amino acid residues of human Nox1.\textsuperscript{17} Nox1-immunized serum was purified by affinity chromatography using the antigen peptide-conjugated agarose.\textsuperscript{17,18} The blots were developed using BCIP/NBT Color Development Substrate (Promega) to visualize the antibody.

Immunohistological Staining
Animal procedures were approved by the Animal Care and Use Review Committee at the Kyushu University. SD rats at 5 weeks old were anesthetized with amobarbital (100 mg/kg intraperitoneal) and perfused transcardially with 2% paraformaldehyde in phosphate-buffered saline (PBS) (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na\textsubscript{2}HPO\textsubscript{4}, and 1.4 mmol/L KH\textsubscript{2}PO\textsubscript{4}; pH 7.4). The brain and aorta were removed and fixed with 4% formaldehyde. Paraffin sections were cut at 3 μm, deparaffinized, and treated with 10 mmol/L citrate, pH 6.0, in 500-watt microwave oven for 15 minutes for antigen retrieval before immunostaining. After inactivation of endogenous peroxidase with 0.3% H\textsubscript{2}O\textsubscript{2} in methanol for 30 minutes at room temperature, the sections were preincubated for 1 hour with 10% dry milk and 5% bovine serum albumin in PBS. The samples were subsequently incubated with the anti-Nox1 antibody (1:200 dilution) for 120 minutes at 37°C, washed in PBS with 0.1% Triton X-100, and probed with biotinylated anti-rabbit IgG antibody (Nichirei Corp, Tokyo, Japan) for 30 minutes at room temperature. After washing in PBS with 0.1% Triton X-100, the sections were reacted with peroxidase-conjugated streptavidin (Nichirei Corp) for 30 minutes at room temperature, and the sites of peroxidase were visualized with diaminobenzidine (Nichirei Corp) and H\textsubscript{2}O\textsubscript{2}, and counterstained with hematoxylin in 2 minutes.

Preparation of Recombinant Proteins
Plasmid constructions (pGEX-2T–p47\textsuperscript{phox}, pGEX-2T–p67\textsuperscript{phox}, N (amino acids 1 to 242), and pGEX-2T–Rac2 (Q61L)) were performed as described previously.\textsuperscript{5} Proteins fused to GST were expressed in Escherichia coli strain BL21 and purified by glutathione-Sepharose-4B (Amersham Biosciences).

A Cell-Free Activation System of the NADPH Oxidase Using Chemiluminescence
Superoxide levels were measured by chemiluminescence detected with 5 μmol/L lucigenin (Sigma) or an enhancer containing luminol-based detection system DIOGENES (National Diagnostics), as described previously with minor modifications.\textsuperscript{5,7} Briefly, the membranes (10 μg of protein) were suspended in PBS at pH 7.0, containing 10 μmol/L FAD, 1 mmol/L EGTA, 1 mmol/L Na\textsubscript{2}HPO\textsubscript{4}, and a final concentration of 5 μmol/L lucigenin or 50 μL DIOGENES. After incubation of the membranes for 1 minute with or without the recombinant proteins, GST–p47\textsuperscript{phox}, GST–p67\textsuperscript{phox}, and GST–Rac2 (Q61L), in the presence of 100 μmol/L sodium dodecyl sulfate, 0.5 mmol/L NADH or NADPH was added to the reaction mixture to start the reaction. The chemiluminescence was measured using a luminometer (MiniLumat LB9506; EG&G Berthold, Germany). The reaction was stopped by addition of superoxide dismutase (200 U/mL).

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Statistical Analysis

Values are expressed as means±SEM. One-way factorial ANOVA followed by Scheffe multiple comparison test was used to compare the NAD(P)H-induced superoxide production of the BAEC membranes. Values of \( P<0.05 \) were considered significant.

Results

Identification of Rat BAECs

We isolated the endothelial colonies proliferated from the basilar arterial segment and subcultured the cells. The cells showed a characteristic cobblestone-like appearance in contrast to the BASMCs (Figure 1A). We confirmed that the BAECs abundantly expressed vWF, a specific protein for endothelial cells, but not smooth muscle cell-specific \( \alpha \)-actin (Figure 1B). These findings suggest that the contamination of the smooth muscle cells in the BAEC may be negligible.

Multiple Noxs Are Expressed in BAECs

To test the possibility that the endothelial cells of the cerebral arteries express Nox proteins differently from those of noncerebral arteries, which are considered to express exclusively Nox2 and Nox4,\(^4,5\) we examined expression of Nox homologues in cultured BAECs. RT-PCR showed that BAECs expressed Nox1 and Nox4 and, to a lesser extent, Nox2 (Figure 2A). Real-time
PCR showed that the order of mRNA abundance in BAECs was as follows: Nox4 > Nox1 > Nox2 (Table). The Nox1 mRNA levels were relatively high to those of BASMC, which are established as Nox1-expressing cells (Figure 2A). In addition, we confirmed, by Western blot, that Nox1 existed as a unique protein in the membrane fraction at molecular weight 63 kDa in BAECs (Figure 2B). The protein levels appeared to be comparable or slightly higher than those of the BASMC (Figure 2B). However, other Nox homologues, Nox3 and Nox5, were not detected by RT-PCR in BAECs (data not shown).

Nox1 Is Highly Expressed in the Endothelial Cells of the Intact Basilar Artery

To confirm in situ expression of Nox1 in the endothelial cells of the basilar artery, we performed immunohistological staining of Nox1 on the basilar artery of SD rats at 5 weeks old. Interestingly, the staining of Nox1 was detected in all vascular cell types but was stronger in the endothelial cells and adventitial cells of the basilar artery than that in the smooth muscle cells (Figure 3A). This finding was in contrast to the staining pattern of the aorta, which showed diffuse staining of Nox1 mainly in the medial layer (Figure 3B). We further confirmed that the expression levels of Nox1 mRNA in the cultured BAECs were higher than those of the aorta by use of real-time PCR (Table).

Expression of Cytosolic Components of NAD(P)H Oxidases in BAECs

We next examined the expression of cytosolic components of NAD(P)H oxidase, p47\textsuperscript{phox} and p67\textsuperscript{phox}, and their recently identified homologues, Noxo1 and Noxa1,\textsuperscript{19–21} in cultured BAECs. RT-PCR showed that p67\textsuperscript{phox} was abundantly expressed, whereas its homologue Nox1 was at very low levels (Figure 4). However, p47\textsuperscript{phox} and its homologue Noxo1 were expressed at lower levels in the BAECs than each positive control (p47\textsuperscript{phox} in neutrophil and Noxo1 in colon) (Figure 4).

NAD(P)H-Dependent Superoxide Production of BAECs

To examine the superoxide-producing activity of BAECs, we prepared the membrane fraction of BAECs, which was supposed to contain Nox1, Nox2, and Nox4. Both NADH and NADPH promptly increased superoxide levels produced by the BAEC membranes in the chemiluminescence assay with use of 2 different superoxide-detecting chemiluminescence reagents, 5 \textmu mol/L lucigenin and luminol-mediated DIOGENES (Figure 5B and 5C). The superoxide levels increased by NADPH were larger than those by NADH with use of

### mRNA Abundance in BAECs and Aortic Endothelial Cells

<table>
<thead>
<tr>
<th>Copy Number</th>
<th>BAEC</th>
<th>AoECs</th>
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<tbody>
<tr>
<td>Nox1</td>
<td>343±81</td>
<td>40±21</td>
</tr>
<tr>
<td>Nox2</td>
<td>32±15</td>
<td>10±5</td>
</tr>
<tr>
<td>Nox4</td>
<td>26 254±7195</td>
<td>19 837±3082</td>
</tr>
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AoECs indicates aortic endothelial cells.

Copy numbers of Nox mRNA per 0.05 \mu g total RNA were obtained by real-time PCR.

Values are expressed as means±SEM (n=4).

![Figure 3. Expression of Nox1 in the basilar artery and the aorta of SD rats. Immunohistochemical localization of anti-Nox1 antibody in the basilar artery (A) and the aorta (B) of SD rats at 5 weeks old was examined. The sections were incubated with control rabbit IgG (left) or the Nox1 antibody (right), probed with biotinylated anti-rabbit IgG antibody, and reacted with peroxidase-conjugated streptavidin. The sites of peroxidase were visualized with diaminobenzidine and \textsubscript{2}H\textsubscript{2}O\textsubscript{2} (arrowheads) and counterstained with hematoxylin.](http://stroke.ahajournals.org/Downloaded from http://stroke.ahajournals.org/ by guest on August 8, 2017)
Figure 4. Expression of cytosolic components of NAD(P)H oxidase in BAECs. Expression of the phagocyte-type cytosolic components, p47\textsuperscript{phox} and p67\textsuperscript{phox}, and their homologues, Nox1 and Nox1 in BAECs (EC), neutrophil (N), and colon (C) was examined by RT-PCR. The PCR products were subjected to 1.2% agarose-gel electrophoresis, and stained with ethidium bromide.

Discussion

In the present study, we provide 2 major new findings. First, Nox1 is highly expressed at protein levels in the endothelial cells of the basilar artery both in the isolated cultured cell levels and the vessel wall in situ. Nox1 may be functionally active in the endothelial cells. This is the first report so far to confirm the expression of Nox1 in the endothelial cells of any vascular beds. Second, the phagocyte-type cytosolic components of the NAD(P)H oxidase, p47\textsuperscript{phox} and p67\textsuperscript{phox}, are expressed and have a potential to enhance the superoxide production of BAECs in an NADH-dependent manner in vitro.

Nox1 Is Highly Expressed in the Endothelial Cells of the Cerebral Arteries Along With Nox2 and Nox4

It has been considered that among Nox proteins, Nox2 and Nox4 are exclusively expressed in the endothelial cells.\(^4\) Consistent with our previous report,\(^5\) we show that BAECs express Nox4 abundantly and Nox2 marginally by real-time PCR. In addition to these Nox proteins, BAECs express Nox1 highly (Table). The significant expression of Nox1 may be specific for the BAECs, because the Nox1 expression level in the aortic endothelial cells was marginal. These findings are consistent with the hypothesis by Touyz et al\(^1\) that different vascular beds might have a different set of Nox proteins even in the same vascular cell type.

Substrate Specificity and Roles of the Cytosolic Components

It remains unsolved whether vascular NAD(P)H oxidases preferentially use NADH or NADPH as an electron donor. It may be because multiple Nox proteins are expressed simultaneously and their expression levels are different in each vascular cell. The phagocyte NADPH oxidase, which consists of only Nox2 among Nox homologues, exclusively uses NADPH as an electron donor.\(^6\) However, there remains a possibility that Nox1 and Nox4 use both NADH and NADPH.\(^3\)

In the present study, both NADH and NADPH induce superoxide production in BAECs. With use of lucigenin, the superoxide levels increased by NADPH were significantly larger than those by NADH in BAECs, which is similar with the finding by Didion et al.\(^13\) Moreover, we found, using both lucigenin and DIIOGENES, that the phagocyte-type cytosolic components enhance superoxide levels produced by the BAEC membranes in an NADH-dependent, but not NADPH-dependent, manner by 2-fold (Figure 4), which is in contrast to the phagocyte membranes: the cytosolic components drastically enhance superoxide levels produced by the phagocyte membranes exclusively in an NADPH-dependent manner\(^5,6\) (data not shown). These findings indicate that available Nox2 proteins are quite small and/or that the cytosolic components augment other Nox activity, such as Nox1 and Nox4, in BAECs. Geiszt et al\(^22\) have demonstrated that p47\textsuperscript{phox} and p67\textsuperscript{phox} augment Nox1 activity, albeit at much lower levels compared with Nox2 activity. In contrast to Nox1 and Nox2, Nox4 is considered constitutively active and might not require cytosolic components for further activation.\(^5,6,23\) Thus, our findings suggest that p47\textsuperscript{phox} and p67\textsuperscript{phox} augment Nox1 activity to increase superoxide levels produced by the BAEC membranes.

Roles of Nox1 in the Endothelial Cells of the Cerebral Arteries

Recent evidence has suggested the functional importance of vascular NAD(P)H oxidases in both physiology and pathophysiology, such as regulation of vascular tone,\(^13,14\) oxygen-sensing,\(^23\) and development of atherosclerosis.\(^1\) We show that 3 different Nox proteins exist simultaneously in BAECs. Each Nox protein may participate in each specific role in BAEC, probably by localizing in the distinct subcellular
region: Nox1 is localized in caveolae, Nox2 is in the perinuclear region, and Nox4 is in focal adhesions. It was originally demonstrated that Nox1 might mediate growth of smooth muscle cells in response to growth factors, such as angiotensin II and platelet-derived growth factor. These growth factors promptly enhance Nox1 expression and superoxide production in the cells. Thus, it is possible that Nox1 is also associated with the growth of endothelium. Future studies such as knockout mice will elucidate precise role of each Nox protein in the physiology and pathophysiology of the cerebral arteries.

In conclusion, the endothelial cells of the cerebral arteries have a unique set of Nox homologues. Nox1 is highly expressed along with Nox2 and Nox4 and may be functionally active in the endothelial cells. In addition, the endothelial NAD(P)H oxidase of the cerebral arteries is regulated by a unique mechanism: the phagocyte-type cytosolic components, p47phox, p67phox, and Rac2 (Q61L), in the presence of SDS for 60 seconds. 0.5 mmol/L NADH or NADPH was added to the reaction mixture. As indicated, SOD was preincubated in the mixture for 60 seconds. With use of 5 μmol/L lucigenin (B) or DIOGENES(C), chemiluminescence change was continuously monitored. Total chemiluminescence change in the membrane fraction alone was set to 100. Values are means±SEM (n=4, *P<0.05, n.s., not significant vs membrane fraction alone).

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