Increased NADPH-Oxidase Activity and Nox4 Expression During Chronic Hypertension Is Associated With Enhanced Cerebral Vasodilatation to NADPH In Vivo

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Background and Purpose—We examined the importance of NADPH-oxidase in reactive oxygen species production in cerebral arteries and its effect on vascular tone in vivo. Furthermore, we investigated whether chronic hypertension affects function or expression of this enzyme in cerebral vessels.

Methods—Superoxide generation was detected in isolated rat basilar arteries with the use of lucigenin-enhanced chemiluminescence. mRNA expression of NADPH-oxidase subunits was assessed by real-time polymerase chain reaction. Basilar artery diameter was measured with the use of a cranial window preparation in anesthetized rats.

Results—NADPH-stimulated superoxide production was 2.3-fold higher in arteries from spontaneously hypertensive rats (SHR) versus normotensive Wistar-Kyoto rats (WKY) and could be blocked by the NADPH-oxidase inhibitor diphenyleneiodonium. Higher NADPH-oxidase activity was also reflected at the molecular level as mRNA expression of the NADPH-oxidase subunit Nox4 was 4.1-fold higher in basilar arteries from SHR versus WKY. In contrast, expression of Nox1, gp91phox, p22phox, and p47phox did not differ between strains. Application of NADPH to basilar arteries caused larger vasodilatation in SHR than WKY. Vasodilatation to NADPH could be attenuated by diphenyleneiodonium, as well as diethyldithiocarbamate (Cu2+/Zn2+—superoxide dismutase inhibitor), catalase (H2O2 scavenger), or tetraethylammonium (BK Ca channel inhibitor).

Conclusions—Activation of NADPH-oxidase in cerebral arteries generates superoxide, which is dismutated by Cu2+/Zn2+—superoxide dismutase to H2O2. H2O2 then elicits vasodilatation via activation of BK Ca channels. Upregulation of Nox4 during chronic hypertension is associated with elevated cerebral artery NADPH-oxidase activity. (Stroke. 2004; 35:584-589.)

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thermore, we examined whether chronic hypertension affects the activity, function, and molecular expression of NADPH-oxidase in cerebral arteries.

**Materials and Methods**

All procedures were approved by the institutional animal ethics committee. In total, 80 male Wistar-Kyoto rats (WKY) (weight, 332±12 g; mean arterial pressure=92±2 mm Hg) and 54 male spontaneously hypertensive rats (SHR) (weight, 460±22 g; mean arterial pressure=165±6 mm Hg) up to 10 months old were studied. In specific experimental subgroups in which comparisons were made between WKY and SHR, rats from the 2 strains were age matched.

**Measurement of O$_2^-$ Production by Isolated Cerebral Arteries**

WKY (n=9) and SHR (n=11) were anesthetized by inhalation of 80% CO$_2$/20% O$_2$ and killed by decapitation. Basilar arteries were excised and cut into two 5-mm ring segments, and O$_2^-$ production was then measured by 5 μmol/L lucigenin-enhanced chemiluminescence as described previously.$^{10}$

**Measurement of Cerebral Artery Diameter In Vivo**

WKY (n=47) and SHR (n=19) were anesthetized with pentobarbital sodium (50 mg/kg IP). Supplemental pentobarbital was administered via a femoral vein cannula (10 to 20 mg/kg per hour). Blood gases were monitored and adjusted (pH 7.39±0.01, P$_{CO_2}$=37±1 mm Hg, P$_O_2$=162±12 mm Hg) throughout each experiment. As described previously,$^{11}$ a craniotomy was performed over the ventral brain stem, the basilar artery was exposed, and the cranial window was superfused with artificial cerebrospinal fluid at 2 mL/min. Cerebrospinal fluid sampled from the cranial window had the following pH and gases: pH 7.44±0.01, P$_{CO_2}$=31±1 mm Hg, P$_O_2$=136±1 mm Hg.

Basilar artery diameter was monitored with a microscope coupled to a video monitor and measured with a computer-based tracking program (Diamatrak, Montech).

**In Vivo Protocol**

After surgical preparation, a 20-minute stabilization period was allowed before topical application of drugs. Artery diameter was measured under baseline conditions and again when responses to drugs were stable. A 15- to 20-minute period was allowed between applications of vasodilators. Vascular responses were expressed as percent change in artery diameter compared with baseline.

Responses of the basilar artery to the following agonists were investigated: NADPH (10 and 100 μmol/L), H$_2$O$_2$ (100 and 300 μmol/L), sodium nitroprusside (SNP) (10 and 100 μmol/L), papaverine (10 and 100 μmol/L), and aprakalim (10 and 30 μmol/L). Responses were examined in the absence and presence of diphenyl-nitrosoiodonium (DPI) (5 μmol/L), catalase (1000 U/mL), diethyldithiocarbamate (DETCA) (3 μmol/L), or tetraethylammonium (TEA) (1 mmol/L).

In all experiments, control responses to each agonist were obtained before topical application of inhibitor drugs. After 20-minute treatment with 1 of these inhibitors, responses to vasoactive agents were reexamined in the continued presence of the inhibitor. Only 1 inhibitor was investigated per animal.

**Reverse Transcription Reaction**

For each mRNA sample, basilar arteries were pooled from 3 WKY or 3 SHR, and total RNA was extracted with the use of a commercially available kit (SV Total RNA Isolation System, Promega) and quantified spectrophotometrically (absorbance at 260 nm). Total RNA was subsequently used for reverse transcription (RT) in a final reaction volume of 100 μL as described previously.$^{10}$

**Real-Time Polymerase Chain Reaction**

Real-time polymerase chain reaction (PCR) and the ΔΔCt method were used as previously described to examine mRNA expression of the NADPH-oxidase subunits (gp91phox, Nox1, Nox4, p22phox, p47phox) and Cu$^{2+}$/Zn$^{2+}$–superoxide dismutase (SOD), relative to a “reference” sample, in basilar arteries from WKY and SHR.$^{12}$ Primers and probes for target genes were designed with the use of Primer Express software (PE Biosystems) and the published mRNA sequences (Table, available online at http://stroke.ahajournals.org).

18S RNA was used as an internal standard and was measured with the use of commercially available primers and probe (PE Biosystems; Table, available online at http://stroke.ahajournals.org). Probes were labeled at the 5’ end with the fluorescent dyes FAM (for target genes) and VIC (for 18S) and at the 3’ end with the quencher molecule TAMRA.$^{12}$

**Results**

**Basilar Artery O$_2^-$ Production by NADPH-Oxidase**

O$_2^-$ was not detected under basal conditions in basilar arteries from either WKY (aged 26±4 weeks; n=9) or SHR (aged 26±3 weeks; n=11) (Figure 1a). Treatment with NADPH increased O$_2^-$ production to a similar extent in each strain (Figure 1a). To determine whether any differences in cerebral artery O$_2^-$ production between the 2 strains were being masked by endogenous Cu$^{2+}$/Zn$^{2+}$-SOD activity, arteries were treated with the Cu$^{2+}$-chelating agent DETCA (3 μmol/L). Although we were still unable to detect a chemiluminescent signal in arteries from WKY, DETCA treatment unmasked a small O$_2^-$ signal in rings from SHR (Figure 1b). Furthermore, in DETCA-treated SHR arteries, NADPH-stimulated O$_2^-$ production was 2.3-fold higher than in similarly treated WKY rings (Figure 1b; n=8 to 9; P<0.05). The NADPH-oxidase inhibitor DPI abolished NADPH/DETCA-stimulated O$_2^-$ generation by basilar arteries from both WKY and SHR (Figure 1b), suggesting that NADPH-oxidase was the primary source of cerebral vessel O$_2^-$ in both strains.

**mRNA Expression of NADPH-Oxidase Subunits and Cu$^{2+}$/Zn$^{2+}$-SOD**

To determine whether increased NADPH-oxidase activity was reflected at the molecular level, real-time PCR was used to compare mRNA expression of the NADPH-oxidase subunits gp91phox, Nox1, Nox4, p22phox, and p47phox in basilar arteries from WKY (aged 30±1 weeks) and SHR (aged 30±1 weeks). Relative expression of Nox4 was 4.1-fold higher in SHR (ΔCt=19.41±0.25) compared with WKY (ΔCt=21.45±0.26; n=4; Figure 2a). In contrast, there were no differences in relative expression of other NADPH-oxidase subunits (each n=4 from 12 rats; Figure 2b to 2e), nor was there any change in expression of Cu$^{2+}$/Zn$^{2+}$-SOD (Figure 2f).
Cerebral Vasodilatation In Vivo

NADPH (10 and 100 μmol/L) elicited concentration-dependent dilatation of the basilar artery in WKY (Figure 3a and 3b). In most animals (approximately 70%), NADPH elicited a transient peak dilatation at either or both concentrations, followed by a sustained steady state response (Figure 3a). Peak responses to 10 and 100 μmol/L NADPH were 16±4% (n=10) and 29±3% (n=23), respectively. However, since this peak response did not occur in all animals, we have focused on the steady state responses that were reproducible within the same animal (time control steady state responses to 10 and 100 μmol/L NADPH in WKY: first 8±2% and 17±6%; second 8±1% and 14±1%; n=4). Overall, steady state responses to 10 and 100 μmol/L NADPH in WKY were 7±1% and 13±1% (n=30), respectively.

Figure 1. Effect of NADPH and DPI on O₂⁻· production in isolated basilar arteries from WKY and SHR in the absence (a) and presence (b) of DETCA (3 mmol/L) to inactivate endogenous Cu²⁺/Zn²⁺-SOD. Vascular O₂⁻· was assayed with the use of lucigenin-enhanced chemiluminescence; values are expressed as 10⁵ counts per second per milligram dry tissue weight (n=8 to 9). *P<0.05 vs similarly treated WKY rings, †P<0.05 vs NADPH/DETCA-treated rings.

Figure 2. Real-time PCR detection of NAPDH-oxidase subunit mRNA. Expression of Nox4 (a), Nox1 (b), gp91phox (c), p22phox (d), p47phox (e), and Cu²⁺/Zn²⁺-SOD (f) in basilar arteries from WKY and SHR (each n=4 from 12 rats) was normalized to the respective 18S rRNA content for each sample (∆Ct) and then expressed relative to a reference sample (∆ΔCt). *P<0.05 vs WKY.

Figure 3. Change in basilar artery diameter in response to cumulative application of either NADPH or SNP in WKY and SHR. A, Experimental recording shows typical vasodilator responses to NADPH in WKY. B, Group data show responses to NADPH in age-matched WKY (aged 24±1 weeks; baseline=262±7 μm; n=18) and SHR (aged 24±2 weeks; baseline=230±14 μm; n=9; P<0.05) rats. C, Group data show responses to SNP in WKY (n=18) and SHR (n=9). Values are expressed as percent change in artery diameter from baseline. *P<0.05.
NADPH-Induced Vasodilatation Is Enhanced in Hypertension

NADPH-induced vasodilatation was approximately 70% greater in age-matched SHR (24 ± 2 weeks; n = 9) than WKY (24 ± 1 weeks; n = 18; Figure 3b). This enhancement was selective for NADPH since responses to SNP were similar in WKY and SHR (Figure 3c).

NADPH-Induced Vasodilatation Is Mediated by NADPH-Oxidase, H₂O₂, and BKCa Channels

We performed further studies to elucidate features of the mechanism of NADPH-induced vasodilatation. Treatment of the cranial window with DPI or catalase had no significant effect on baseline artery diameter in WKY (−0.7 ± 2.5% [n = 7] and 2.6 ± 4.2% [n = 8], respectively). However, both DPI and catalase caused a substantial constriction in SHR (−8.4 ± 1.6% [n = 6] and −8.7 ± 2.3% [n = 5], respectively; both P < 0.05). DPI inhibited NADPH-induced vasodilator responses by approximately 50% in both WKY (Figure 4a) and SHR (not shown), without affecting responses to a nonspecific vasodilator, papaverine (Figure 4b), or an endothelial NO-dependent vasodilator, acetylcholine (Figure 4c). Combined treatment of the cranial window with N-nitro-L-arginine methyl ester (30 μmol/L) and indomethacin (10 μmol/L) had no effect on responses to NADPH but abolished vasodilatation to acetylcholine (n = 6; data not shown), confirming no contribution of NO synthase or cyclooxygenase to the response to NADPH.

We next tested whether vasodilator responses to NADPH may be mediated by endogenous H₂O₂. First, we established that exogenous H₂O₂ elicits concentration-dependent dilata-

tion of the basilar artery, which was reproducible within the same animal (time control responses to 100 and 300 μmol/L H₂O₂ in WKY: first = 9 ± 3% and 31 ± 12%; second = 9 ± 6% and 31 ± 7%; n = 3). Second, we found that catalase (1000 U/mL) inhibited vasodilatation to both NADPH (Figure 5a) and H₂O₂ (Figure 5b) without altering responses to SNP (Figure 5c), suggesting that NADPH-induced vasodilatation is mediated at least in part by NADPH-oxidase–derived H₂O₂. Catalase also inhibited NADPH-induced responses by approximately 50% in SHR (not shown). Finally, inactivation of Cu²⁺/Zn²⁺-SOD with DETCA (3 mmol/L) also inhibited NADPH-induced vasodilatation (Figure 6a), whereas vasodilator responses to exogenous H₂O₂ were unaltered (Figure 6b).

Inhibition of BKCa channels with 1 mmol/L TEA attenuated responses to NADPH (eg, response to 100 μmol/L NADPH: control = 12 ± 2%; TEA-treated = 5 ± 2%; n = 6; P < 0.05) and also H₂O₂ (eg, response to 300 μmol/L H₂O₂: control = 38 ± 6%; TEA-treated = 19 ± 7%; n = 6; P < 0.05) without affecting responses to the ATP-sensitive K⁺ (KATP) channel opener aprikalim (not shown). Neither catalase, DETCA, nor TEA altered the baseline diameter of the WKY basilar artery.

Discussion

ROS are established to elicit cerebral vasodilatation under both normal and disease conditions. This study demonstrates that activation of NADPH-oxidase in cerebral arteries in vivo causes generation of O₂⁻, which is converted to H₂O₂ by Cu²⁺/Zn²⁺-SOD. H₂O₂ then elicits vasodilatation via opening of BKCa channels in vascular smooth muscle cells. Importantly, Nox4 mRNA expression was 4.1-fold higher in basilar
NADPH-Oxidase Activity and Expression in Cerebral Vessels

It is well established that NADPH-oxidase is a major source of ROS in the systemic vasculature and that its activity is upregulated in pathophysiological states such as hypertension. Although we were unable to detect O$_2^-$ in isolated basilar arteries from WKY under basal conditions, ex vivo treatment with NADPH caused a marked increase in O$_2^-$ generation. Furthermore, NADPH-stimulated O$_2^-$ production was inhibited by DPI, indicating an involvement of NADPH-oxidase.

Messenger RNA for Nox4, gp91phox, Nox1, p22phox, and p47phox was found to be present in the WKY cerebral circulation is the recent finding that in a Japanese population, the C242T polymorphism of p22phox is a novel risk factor for ischemic cerebrovascular disease. In a sepa-
rate study, Guzik et al found that the C242T polymorphism is independently associated with decreased NADPH-oxidase activity. Taken together, our findings and these recent epidemiological data are compatible with an important protective role for NADPH-oxidase in the cerebral circulation.

Increased Cerebral Artery Nox4 Expression in Hypertension

To shed some light on which isoform of NADPH-oxidase is involved in augmented cerebrovascular O$_2^-$ production in hypertension, we measured mRNA expression of gp91phox and the putative vascular homologues of this catalytic subunit, Nox1 and Nox4. Interestingly, while levels of gp91phox and Nox1 were not different, expression of Nox4 was markedly higher in basilar arteries from SHR. This finding is somewhat at odds with previous studies examining the effects of pathophysiological stimuli on Nox4 expression. For example, in vitro treatment of rat aortic smooth muscle cells with angiotensin II downregulates Nox4 expression, while in aortas of renin-overexpressing or angiotensin II–treated hypertensive rats, Nox4 mRNA levels were only modestly higher (ie, $<70\%$) than in normotensive controls. Indeed, we too found that aortic expression of Nox4 was unaltered in SHR (not shown), which may suggest that this subunit is selectively upregulated in certain vascular beds under pathophysiological conditions to act as a protective mechanism against reduced blood flow.

In summary, this study demonstrates that activation of NADPH-oxidase in cerebral arteries in vivo causes vasodilatation via the generation of H$_2$O$_2$ and opening of BK$_{ca}$ channels. Importantly, during chronic hypertension NADPH-oxidase activity is elevated in cerebral vessels, and this is presumably the result of increased expression of the catalytic subunit Nox4. The functional consequence of this is enhanced vasodilator responses on activation of the enzyme in vivo. It is presently unclear whether the consequences of increased NADPH-oxidase activity in the cerebral circulation are likely to be mainly beneficial or detrimental. However, if it were beneficial, caution should be taken in future attempts to inhibit effects of ROS in systemic vascular diseases so as not to inadvertently compromise cerebral blood flow.

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References

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