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.)

Key Words: cerebrovascular disorders ■ hypertension ■ pharmacology ■ potassium channels ■ vasodilation
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 animals 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.  

**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, PCO$_2$=37±1 mm Hg, P0$_2$=162±12 mm Hg) throughout each experiment. As described previously, 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, PCO$_2$=31±1 mm Hg, P0$_2$=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 (Diamtrak, 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.

**Drug Treatment**

H$_2$O$_2$ was obtained from Merck, and all other drugs were from Sigma. DPI was prepared at 10 mmol/L in dimethyl sulfoxide and diluted in saline (in vivo experiments) or Krebs-HEPES (in vitro experiments) such that the final concentration of dimethyl sulfoxide was ≤0.05%. All other drugs were dissolved and diluted in saline or Krebs-HEPES as appropriate.

**Data Analysis**

All results are expressed as mean±SEM. Statistical comparisons were made with the use of Student’s paired or unpaired t tests or ANOVA as appropriate. A value of P<0.05 was considered significant.

**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 mmol/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) and Cu$^{2+}$/Zn$^{2+}$–superoxide dismutase (SOD), relative to a “reference” sample, in basilar arteries from WKY and SHR. 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.

**Drugs**

H$_2$O$_2$ was obtained from Merck, and all other drugs were from Sigma. DPI was prepared at 10 mmol/L in dimethyl sulfoxide and diluted in saline (in vivo experiments) or Krebs-HEPES (in vitro experiments) such that the final concentration of dimethyl sulfoxide was ≤0.05%. All other drugs were dissolved and diluted in saline or Krebs-HEPES as appropriate.
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 O$_2^-$ production in isolated basilar arteries from WKY and SHR in the absence (a) and presence (b) of DETCA (3 mmol/L) to inactivate endogenous Cu$^{2+}$/Zn$^{2+}$-SOD. Vascular O$_2^-$ was assayed with the use of lucigenin-enhanced chemiluminescence; values are expressed as 10$^3$ 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 Cu$^{2+}$/Zn$^{2+}$-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 ($\Delta\Delta$Ct) and then expressed relative to a reference sample ($\Delta\Delta$Ct). *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±2 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 Mediated by NADPH-Oxidase, H$_2$O$_2$, and BK$_{Ca}$ 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 non-specific 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 H$_2$O$_2$. First, we established that exogenous H$_2$O$_2$ elicits concentration-dependent dilatation of the basilar artery, which was reproducible within the same animal (time control responses to 100 and 300 μmol/L H$_2$O$_2$ 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 H$_2$O$_2$ (Figure 5b) without altering responses to SNP (Figure 5c), suggesting that NADPH-induced vasodilatation is mediated at least in part by NADPH-oxidase-derived H$_2$O$_2$. Catalase also inhibited NADPH-induced responses by approximately 50% in SHR (not shown). Finally, inactivation of Cu$^{2+}$/Zn$^{2+}$-SOD with DETCA (3 mmol/L) also inhibited NADPH-induced vasodilatation (Figure 6a), whereas vasodilator responses to exogenous H$_2$O$_2$ were unaltered (Figure 6b).

Inhibition of BK$_{Ca}$ 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 H$_2$O$_2$ (eg, response to 300 μmol/L H$_2$O$_2$: control = 38 ± 6%; TEA-treated = 19 ± 7%; n = 6; P < 0.05) without affecting responses to the ATP-sensitive K$^+$ (K$_{ATP}$) 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 O$_2^-$, which is converted to H$_2$O$_2$ by Cu$^{2+}$/Zn$^{2+}$-SOD. H$_2$O$_2$ then elicits vasodilatation via opening of BK$_{Ca}$ channels in vascular smooth muscle cells. Importantly, Nox4 mRNA expression was 4.1-fold higher in basilar...
arteries from SHR versus WKY, and this was associated with greater NADPH-oxidase–dependent O$_2^-$ production and vasodilatation in SHR. To our knowledge this is the first study to demonstrate a functional consequence of increased vascular expression and activity of a Nox4-containing NADPH-oxidase in chronic hypertension.

**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 basilar artery. Thus, together with recent findings by others, there is now strong evidence that NADPH-oxidase is expressed and functional in cerebral blood vessels. Moreover, our present findings strongly suggest that activity of NADPH-oxidase is augmented in cerebral arteries during chronic hypertension. Inactivation of endogenous Cu$^{2+}$/Zn$^{2+}$-SOD with DETCA not only unmasked a small basal O$_2^-$ signal in basilar arteries from some SHR but also revealed that NADPH-stimulated O$_2^-$ production was 2.3-fold higher in these tissues than in those from age-matched WKY.

Our finding that higher O$_2^-$ production in cerebral arteries from SHR versus WKY was only observed after DETCA treatment suggests that Cu$^{2+}$/Zn$^{2+}$-SOD adequately disposes of the excess O$_2^-$ produced during hypertension. However, this is not to say that levels of ROS downstream from O$_2^-$ remain unaltered. Dismutation of O$_2^-$ by SOD yields H$_2$O$_2$, a membrane-permeable ROS that elicits a range of cellular responses. Although H$_2$O$_2$ is normally rapidly metabolized by enzyme systems such as catalase and glutathione peroxidase, increased dismutation of O$_2^-$ in the absence of compensatory catalase and/or peroxidase activity will lead to higher levels of H$_2$O$_2$ in the cerebral vasculature during hypertension. Indeed, a recent clinical study provided direct evidence that activities of catalase and glutathione peroxidase in whole blood are reduced in hypertensive patients, which probably contributes to the elevated plasma H$_2$O$_2$ levels in hypertensive patients compared with normotensive controls. Because H$_2$O$_2$ is a powerful cerebral vasodilator, elevated NADPH-oxidase–dependent O$_2^-$ production in the presence of normal Cu$^{2+}$/Zn$^{2+}$-SOD expression and activity may therefore have important consequences for cerebral vascular tone.

**Functional Consequences of NADPH-Oxidase Activity in Cerebral Arteries**

In the systemic vasculature, ROS generally cause vasoconstriction, mainly via the O$_2^-$–mediated inactivation of endothelial NO. In contrast, a number of ROS have been reported to dilate cerebral arteries. In particular, both exogenous and endogenous H$_2$O$_2$ dilate rat cerebral arterioles in vivo via activation of BK$_{ca}$ channels. Having first confirmed that exogenous H$_2$O$_2$ similarly dilates the rat basilar artery, we tested whether activation of NADPH-oxidase elicits vasodilatation and, if so, whether this effect is ultimately mediated via the generation of H$_2$O$_2$ and activation of BK$_{ca}$ channels. In support of this proposal, NADPH elicited dilator responses of the basilar artery in vivo that were sensitive to treatment with the NADPH-oxidase inhibitor DPI at a concentration that did not affect responses to the NO synthase–dependent vasodilator acetylcholine. Vasodilator responses to NADPH were also inhibited by the H$_2$O$_2$ scavenger catalase and the inhibitor of BK$_{ca}$ channels TEA. Furthermore, NADPH-induced vasodilatation could also be inhibited by the Cu$^{2+}$/Zn$^{2+}$-SOD inhibitor DETCA, suggesting that SOD activity is required to generate H$_2$O$_2$ at a sufficient rate from NADPH-oxidase–derived O$_2^-$ to enable accumulation of H$_2$O$_2$ and decreased vascular tone. Interestingly, none of these inhibitor treatments had any significant effect on baseline diameter of the normotensive WKY basilar artery, suggesting that NADPH-oxidase activation may not play a major role in regulating basal cerebral artery tone under physiological conditions.

Chronic hypertension is a major risk factor for stroke. We found that vasodilatation in response to NADPH was significantly greater in chronically hypertensive rats than in age-matched normotensive controls. It is unclear what consequences (beneficial or detrimental) higher activity of NADPH-oxidase may have for the regulation of cerebral vascular tone during hypertension. However, ROS-mediated vasodilatation, which appears to represent a physiological mechanism in cerebral vessels during normotension, could be beneficial in hypertension by maintaining cerebral blood flow, particularly during cerebral ischemic episodes common in stroke. Evidence from the present study consistent with such a protective effect is that DPI and catalase each constricted the basilar artery of SHR but not WKY. In further support of a protective role for NADPH-oxidase in the 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.
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.

Acknowledgments

This work was supported by funds from a grant-in-aid (G00M0659) from the National Heart Foundation of Australia and a project grant (208969) from the National Health and Medical Research Council of Australia (NHMRC). Dr Sobey is supported by a NHMRC R.D. Wright career development award (209160). Dr Drummond is supported by a NHMRC Peter Doherty postdoctoral fellowship (007044). T. Paravicini and S. Chrisbolobis are supported by Australian postgraduate awards.

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Stroke. 2004;35:584-589; originally published online January 22, 2004; doi: 10.1161/01.STR.0000112974.37028.58

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