NADPH Oxidase Activity and Function Are Profoundly Greater in Cerebral Versus Systemic Arteries

Alyson A. Miller, Grant R. Drummond, Harald H.H.W. Schmidt, Christopher G. Sobey

Abstract—Recent studies suggest that the superoxide generating enzyme NADPH oxidase may play a functional role in regulating cerebral vascular tone. We tested whether the activity, function, and expression of NADPH oxidase differs between rat cerebral and systemic arteries. Superoxide production by basilar (BA), middle cerebral (MCA), carotid (CA), renal (RA), and mesenteric (MA) arteries and aorta (AO) was measured using lucigenin-enhanced chemiluminescence. Superoxide production from NADPH oxidase was localized and semiquantified using dihydroethidium. Vascular functional responses were assessed in a myograph or organ bath. Vascular Nox4 protein expression was measured using Western blotting. Superoxide production (basal or in response to NADPH or angiotensin II) in the intracranial arteries, BA, and MCA was 10- to 100-fold greater than in AO, CA, RA, or MA. Similar results were found using either intact vessels or arterial homogenates, and were associated with 10-fold greater expression of Nox4 in the BA versus AO, CA, and MA. Superoxide production was attenuated by the NADPH oxidase inhibitors, diphenyleneiodonium, apocynin, and gp91ds-tat. NADPH and H_2O_2 were strong relaxing stimuli in the BA, where the H_2O_2 scavenger catalase, as well as apocynin, attenuated these relaxations and also augmented contractions to angiotensin II. NADPH oxidase activity is markedly higher in intracranial versus systemic arteries, in association with higher Nox4 expression. In cerebral arteries, endogenous H_2O_2 derived from NADPH oxidase activation appears to cause relaxation and is able to offset angiotensin II-induced constriction. These data are consistent with the concept that NADPH oxidase-derived reactive oxygen species modulate cerebral vascular tone under physiological conditions. **(*Circ Res*. 2005;97:1055-1062.)**

Key Words: cerebral ■ NADPH oxidase ■ reactive oxygen species ■ angiotensin II ■ hydrogen peroxide

Emerging evidence indicates that reactive oxygen species (ROS) are important molecules in the control of vascular reactivity.[1] Indeed, the superoxide anion (O_2^-) increases vascular tone via the inactivation of endothelium-derived nitric oxide (NO). Recent studies, however, have revealed that the by-product of O_2^- metabolism, hydrogen peroxide (H_2O_2), is a powerful endogenous vasodilator within the cerebral circulation.[2–5] Thus, ROS may represent important signaling molecules for regulating local cerebral blood flow.

Numerous investigators have reported that NADPH oxidases are the primary generators of ROS within the vasculature.[6–9] These enzymes are membrane-associated and generate O_2^- by transferring electrons to molecular oxygen via a flavin-containing "Nox" catalytic subunit.[10] In the systemic circulation, NADPH oxidase has been studied extensively, and it is now believed that excessive O_2^- generation by NADPH oxidase contributes to endothelial dysfunction associated with numerous cardiovascular diseases.[11] Although it has been recently established that NADPH oxidase is expressed and active in the cerebral circulation,[12–14] its function in this vascular bed is less clear. Application of the substrates for NADPH oxidase, NADPH or NADH, increases O_2^- production by cerebral arteries.[12–14] Interestingly, activation of NADPH oxidase using NADPH elicits profound cerebral vasodilatation in vitro[12] and in vivo,[13,14] raising the possibility that in contrast with the systemic circulation, NADPH oxidase-derived ROS may normally play a functional role in vasodilator responses in the cerebral circulation. No study, however, has directly compared the activity and function of NADPH oxidase between systemic and cerebral circulations. Therefore, the aim of the present study was to test whether NADPH oxidase activity and function is normally greater in rat cerebral versus systemic arteries.

Materials and Methods

All procedures were approved by the institutional animal ethics committee. In total, 145 male, department-bred Sprague-Dawley rats (300±5 g) were studied. Rats were euthanized by inhalation of 80% CO_2/20% O_2.

Original received March 10, 2005; resubmission received August 8, 2005; revised resubmission received September 14, 2005; accepted September 28, 2005.

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C.G.S. and G.R.D. are consultants for and have significant ownership interests in Radical Biotechnology Pty Ltd of Australia.

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O_2^- Production by NADPH Oxidase in Intact Cerebral and Systemic Arteries

Basilar, middle cerebral, common carotid, mesenteric (2nd order branch of the superior mesenteric artery), and renal arteries and thoracic aorta were excised and cut into 5 mm-long ring segments. O_2^- production was measured by 5 μmol/L lucigenin-enhanced chemiluminescence in the absence and presence of NADPH (100 μmol/L) as previously described.[15] In some experiments, arteries were treated with the Cu^{2+}-chelating agent diethyldithiocarbamate (DETCA; 3 mmol/L) for 30 minutes before start of assay (to inhibit endogenous Cu^{2+}/Zn^{2+} superoxide dismutase [SOD] activity), in the presence and absence of angiotensin II (0.1 μmol/L) and/or the NADPH oxidase inhibitors diphenyleneiodonium (DPI; 5 μmol/L), apocynin (300 μmol/L), and gp91ds-tat (RKKRRQRRRCSTRIRRQL-CONH2; 1 μmol/L). In experiments using apocynin and gp91ds-tat, arteries were incubated with drugs for 1 hour before addition of NADPH and DETCA. Additionally, experiments in NADPH-treated basilar arteries were performed in the presence of the NO synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L). Background counts were subtracted and O_2^- production normalized for dry tissue weight.

O_2^- Production by NADPH Oxidase in Cerebral and Systemic Artery Homogenates

Basilar, common carotid, and mesenteric (2nd order branch of the superior mesenteric artery) arteries and thoracic aorta were excised and cut into 5 mm-ring segments. Arteries were homogenized in either 70 μL (basilar and mesenteric) or 90 μL (carotid and aorta) of lysis buffer (composition in mmol/L: sucrose 250, HEPES 10) with protease inhibitors (Roche Complete Mini, #1836153) using 0.2 mL glass homogenizers. O_2^- production was measured by 5 μmol/L lucigenin-enhanced chemiluminescence in the presence of NADPH (100 μmol/L) and DETCA (3 mmol/L). Background counts were subtracted and values were normalized to protein content (as measured using the Bradford protein assay; BioRad).

Localization and Semiquantification of O_2^- by Dihydroethidium

Dihydroethidium (DHE; 2 μmol/L) was used to localize and measure O_2^- production in frozen sections (16-μm) of basilar and common carotid arteries and thoracic aorta as previously described.[15,16] In the presence of O_2^-, DHE is oxidized to ethidium and oxyethidium, which intercalate between DNA strands, producing a nuclear staining pattern. Arterial sections were treated with NADPH (100 μmol/L) for 30 minutes before treatment with DHE. Some sections were treated with polyethylene glycol-SOD (PEG-SOD, 250 U/mL) or native Cu^{2+}/Zn^{2+}-SOD (250 U/mL) for 30 minutes before incubation with DHE. Fluorescent images were acquired as 8-bit (256 intensity levels) and were analyzed with Image-Pro Plus software (version 5.0.1.11, Media Cypernetics Inc). For each arterial section, pixels in the upper 50% intensity level were counted, background fluorescence was subtracted, and data were presented as total fluorescent units per mm^2 of cross sectional artery wall area.

Expression of Nox4

Expression of Nox4 in basilar, common carotid, and mesenteric arteries (2nd order branch of the superior mesenteric artery) and thoracic aorta was measured by Western blotting. Anti-Nox4 rabbit polyclonal antibodies were raised against the Nox4 peptide (aa 84 to 101; RGSQKVPSRRTRRLLDKS). We have previously demonstrated that preincubation of Nox4 antibody sera with Nox4 peptide (10 μg/mL) results in undetectable immuoreactive bands, implying specificity of the Nox4 antibody.[17] Arteries were homogenized in ice-cold lysis buffer (composition in mmol/L: sucrose 250; HEPES 10) with protease inhibitors (Roche Complete Mini, #1836153) using 0.2 mL glass homogenizers. Homogenates were cleared by centrifugation (10 000 relative centrifugal force; 5 minutes) and protein concentration was determined using the Bradford protein assay (BioRad). For blotting, gel sample buffer was added to homogenates and boiled for 5 minutes. Equal amounts of protein were loaded onto a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% skim milk for 1 hour at room temperature and then incubated overnight (4°C) with the Nox4 primary antibody (1:1000). Membranes were then incubated with a horseradish peroxidase anti-rabbit immunoglobulin for 1 hour at room temperature (1:1000; Chemicon Australia Pty). Equal protein loading was verified by α-actin immunostaining (monoclonal mouse α-actin antibody). Immunoreactive bands were detected by enhanced chemiluminescence (Progen Biosciences) and quantitated densitometrically with the use of a Kodak Image Station 440CF (Perkin-Elmer Life Sciences).

In Vitro Preparation

Basilar, common, carotid and mesenteric (2nd order branch of the superior mesenteric artery) arteries and thoracic aorta were excised and placed in cold, carbogen-bubbled (95% O_2, 5% CO_2) Krebs-bicarbonate solution (composition, in mmol/L; NaCl 118, KCl 4.5, $MgSO_4$ 0.45, KH_2PO_4 1.03, $NaHCO_3$ 25, glucose 11.1, $CaCl_2$ 2.5). Arteries were cut into 5-mm rings. Ring segments of basilar, carotid, and mesenteric arteries were then threaded onto 2 wires and mounted in a Mulvany-Halpern myograph (Danish Myo Technology A/S), and resting tension was increased to either 5 mN (basilar and mesenteric) or 15 mN (carotid). Segments of aorta were mounted in organ baths with a resting tension of 0.5 g. All rings were continuously bathed in warm (37°C) carbogen-bubbled Krebs-bicarbonate solution. After an equilibration period of 60 minutes, rings were exposed to a high potassium physiological salt solution (KPSS) containing 122.7 mmol/L KCl (equimolar replacement of NaCl with KCl) to induce vascular contraction defined as 100% of KPSS. In all rings, endothelial integrity was assessed by measuring acetylcholine-induced (ACh, 10 μmol/L) relaxations of serotonin (0.1 to 1 μmol/L; for basilar, aorta, mesenteric) or U46619-induced (0.03 μmol/L; for carotid) tone (50% to 60% of contraction to KPSS).

In Vitro Protocol

Rings were again contracted submaximally (50% to 60% of contraction to KPSS) with either serotonin (basilar, aorta, and mesenteric) or U46619 (carotid). Once contractions were stable, the effects of the following agonists on vascular tone were investigated: NADPH (10 and 100 μmol/L), sodium nitroprusside (SNP; 0.01 to 1 μmol/L), and H_2O_2 (10 to 1000 μmol/L). Responses were examined in the presence and absence of apocynin (300 μmol/L) or the H_2O_2 scavenger catalase (1000 U/mL). In some experiments, arteries were contracted submaximally (20% of contraction to KPSS) with KCl (20 mmol/L). Once contractions were stable, the effect of angiotensin II (100 nmol/L) on vascular tone in the presence and absence of catalase (1000 U/mL) or apocynin (300 μmol/L) was investigated. Arteries were treated with apocynin for 90 minutes before experiments commenced.

Drugs

H_2O_2 was purchased from Merck, DHE from Molecular Probes, angiotensin II from Auspep, apocynin from Fluka, and gp91ds-tat from SynPep Corporation. All other drugs were purchased from Sigma. DPI, DHE, and apocynin were prepared at 10 mmol/L in dimethyl sulfoxide (DMSO) and diluted in Krebs-HEPES solution such that the final concentration of DMSO was <0.05% to 0.001%. All other drugs were dissolved and diluted in either Krebs-HEPES (lucigenin/DHE experiments) or Krebs-bicarbonate (myograph/organ bath experiments) solution.

Data Analysis

All results are expressed as mean±SEM. Statistical comparisons were performed using either 1-way ANOVA with Bonferroni multiple comparisons post hoc-test or using Student paired or unpaired *t* test, as appropriate. $P<0.05$ was considered statistically significant.

Results

O_2^- Production by NADPH Oxidase in Cerebral and Systemic Arteries

Basal O_2^- production (counts measured in the absence of NADPH) by intact basilar and middle cerebral arteries was

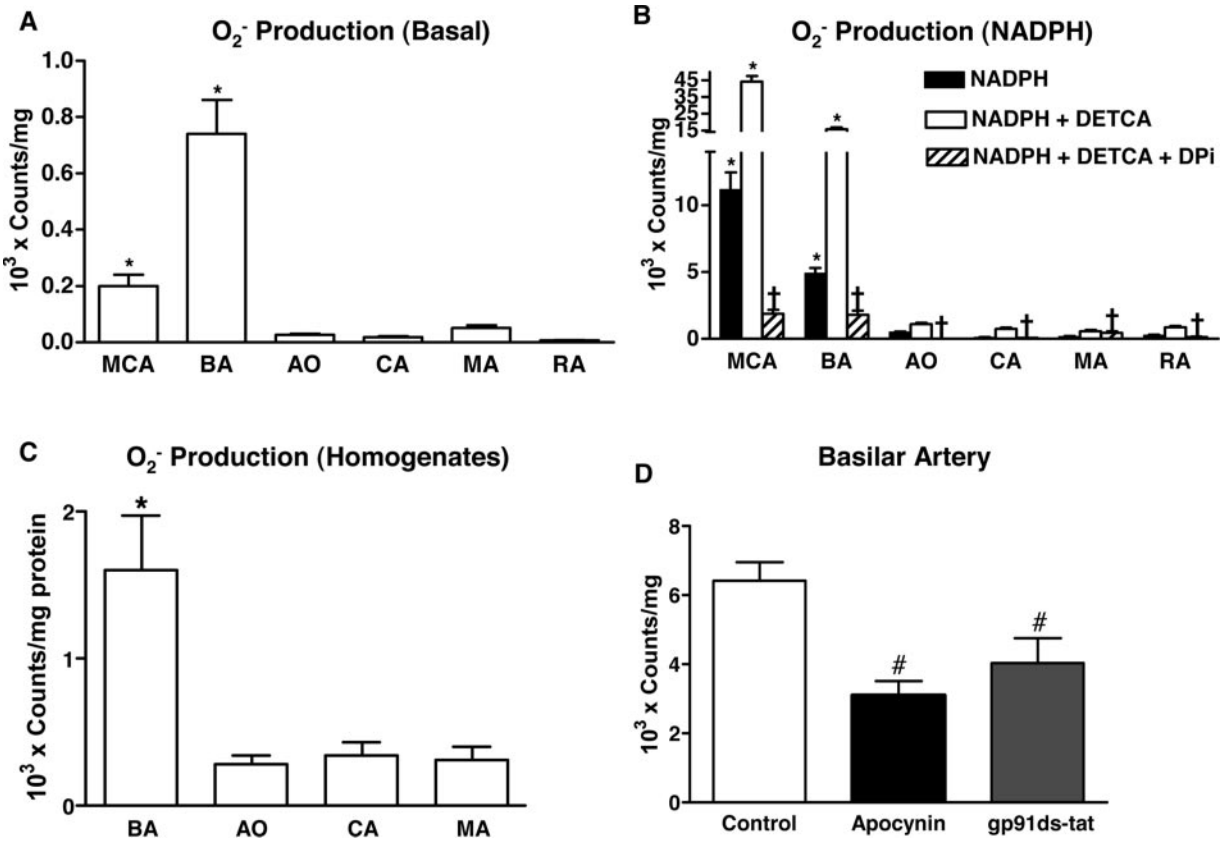

Figure 1. Vascular O_2^- production by cerebral and systemic arteries as measured by lucigenin-enhanced chemiluminescence. A, Basal O_2^- production (in the absence of NADPH) in intact middle cerebral (MCA), basilar (BA), common carotid (CA), mesenteric (MA), and renal arteries (RA) and thoracic aorta (AO). B, The effect of NADPH (100 μmol/L) on O_2^- production by intact arteries in the absence and presence of the Cu^{2+}/Zn^{2+}-SOD inhibitor, DETCA (3 mmol/L), and the NADPH oxidase inhibitor DPI (5 μmol/L). C, The effect of NADPH (100 μmol/L) on O_2^- production by arterial homogenates in the presence of DETCA (3 mmol/L). D, The effect of apocynin (300 μmol/L) and gp91ds-tat (1μmol/L) on NADPH-stimulated O_2^- production (control; in the presence of DETCA) by intact basilar arteries. Values are given as mean±SEM (10^3 counts per mg of dry tissue; n=5 to 6). *$P<0.05$ vs O_2^- production in similarly-treated AO, CA, MA, and RA; †$P<0.05$ vs NADPH/DETCA-treated arteries; # $P<0.05$ vs control (1-way ANOVA).

higher (up to 120-fold) than levels generated by the aorta and carotid, mesenteric, and renal arteries ($P<0.05$, Figure 1A). Similarly, in the presence of NADPH, O_2^- production by basilar and middle cerebral arteries was markedly higher (10- to 90-fold) than levels generated by aorta and carotid, mesenteric, and renal arteries ($P<0.001$, Figure 1B). When arteries were treated with the Cu^{2+}/Zn^{2+}-SOD inhibitor DETCA (3 mmol/L), O_2^- levels in all vessels were augmented; however, O_2^- levels in basilar and middle cerebral arteries remained profoundly greater than levels in systemic arteries ($P<0.01$, Figure 1B). In arterial homogenates, NADPH-stimulated O_2^- production (in the presence of DETCA) was also substantially higher in basilar arteries than in the aorta and carotid and mesenteric arteries ($P<0.01$, Figure 1C). The NADPH oxidase inhibitor DPI virtually abolished NADPH-stimulated O_2^- production in all intact arteries ($P<0.01$, Figure 1B). Moreover, in basilar arteries, the selective NADPH oxidase inhibitors apocynin and gp91ds-tat inhibited NADPH-stimulated O_2^- production ($P<0.05$, Figure 1D). The NOS inhibitor L-NAME (100 μmol/L) had no significant effect on NADPH-stimulated O_2^- production by basilar arteries (control, 5.9±0.6; L-NAME 6.6±0.6×10^3 counts/mg of dry tissue; n=6, $P>0.05$, 1-way ANOVA), suggesting that NADPH oxidase (and not NOS) was the primary source of vascular O_2^-. In the presence of NADPH (100 μmol/L) and DETCA (3 mmol/L), angiotensin II (30 minutes, 0.1 μmol/L) increased O_2^- production in all arteries. Angiotensin II-induced increases in O_2^- production were 30- to 120-fold greater in basilar arteries than in similarly-treated aorta and carotid and mesenteric arteries ($P<0.01$, Figure 2).

Localization of O_2^- Production by NADPH Oxidase

O_2^- production by NADPH oxidase was localized and measured in arterial sections of basilar and carotid arteries and aorta using DHE. In the presence of NADPH, ethidium/oxyethidium fluorescence was observed throughout the vascular wall of all arteries, but was substantially higher in basilar arteries than in carotid arteries or aorta ($P<0.001$, Figure 3A through 3C). In basilar arteries, fluorescence intensity was uniform throughout all cell types, whereas in carotid arteries and aorta, fluorescence was more intense in endothelial and adventitial cells than in vascular smooth muscle cells (Figure 3A through 3C). Treatment of sections with PEG-SOD inhibited fluorescence in all arteries ($P<0.01$, Figure 3D through 3F), confirming that O_2^- was the major ROS detected by DHE. By contrast, treatment with native Cu^{2+}/Zn^{2+}-SOD did not attenuate the signal (data not

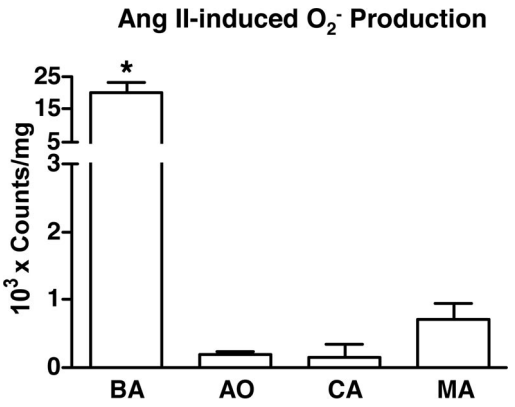

Figure 2. Angiotensin II (Ang II, 100 nmol/L)-induced increase in O_2^- production by intact basilar (BA), carotid (CA), and mesenteric (MA) arteries and thoracic aorta (AO). Experiments were performed in the presence of NADPH (100 μmol/L) and the Cu^{2+}/Zn^{2+}-SOD inhibitor DETCA (3 mmol/L). The data represent counts in angiotensin II-treated rings minus counts in vehicle-treated rings from the same animal. Vascular O_2^- production was assayed using lucigenin-enhanced chemiluminescence. Values are given as mean±SEM (10^3 counts per mg of dry tissue; n=6). *$P<0.05$ vs AO, CA, and MA (1-way ANOVA).

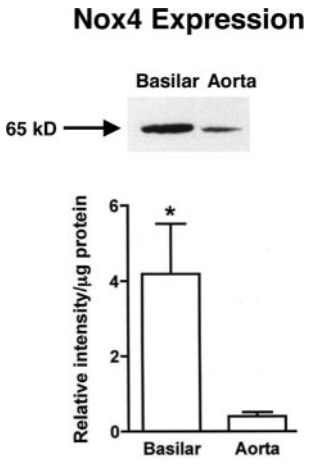

Figure 4. Representative Western blot showing expression of the NADPH oxidase catalytic subunit Nox4 in basilar and thoracic aorta (top). Also shown is a summary of densitometric data (bottom). The values are expressed as relative intensity/μg of protein and are given as mean±SEM (n=5). *$P<0.05$ vs aorta (paired t test).

shown), indicating that the DHE fluorescence reflected intracellular O_2^-.

Nox4 Expression

Expression of Nox4 protein was 10-fold greater in basilar arteries than in the aorta (Figure 4; $P<0.05$). Expression of Nox4 protein in common carotid and mesenteric arteries was similar to that found in aorta (data not shown).

Effect of NADPH on Vascular Tone

ACh (10 μmol/L) elicited similar relaxation responses in all arteries (basilar, 82±4%; aorta, 82±3%; carotid, 92±2%; mesenteric, 87±3%), indicating a functional endothelium. NADPH (10 to 100 μmol/L) elicited concentration-dependent relaxations of all arteries (Figure 5A). NADPH-induced relaxations of basilar arteries, however, were significantly greater than responses of carotid arteries and aorta ($P<0.01$, 1-way ANOVA). This enhanced relaxation was selective for NADPH, as relaxations to SNP were similar in all 4 arteries (Figure 5B). Furthermore, apocynin (300 μmol/L) virtually abolished NADPH-induced relaxation in the basilar artery but had no effect on ACh-induced (0.1 μmol/L) relaxation, suggesting that NADPH oxidase activity was responsible for mediating these effects ($P<0.05$, paired t test; Figure 5C and 5D; data shown only for 100 μmol/L NADPH).

Vascular Responsiveness to Endogenous and Exogenous H_2O_2

In basilar, carotid, and mesenteric arteries, relaxations to NADPH (10 to 100 μmol/L) were either abolished or inhibited by catalase (1000 U/mL; $P<0.01$; Figure 6A; data shown only for 100 μmol/L NADPH). In contrast, NADPH-

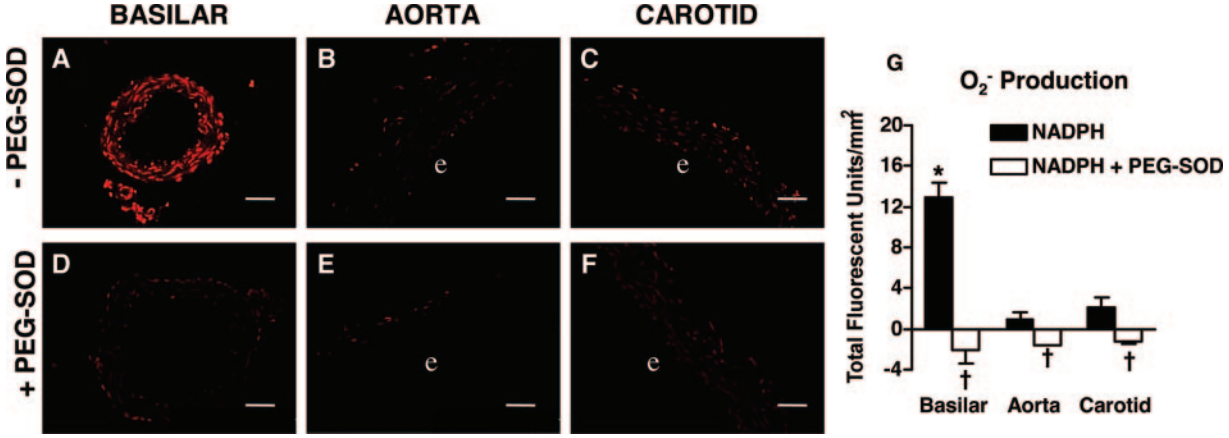

Figure 3. In situ detection of NADPH-stimulated (100 μmol/L) O_2^- production by ethidium/oxyethidium fluorescence in sections of basilar arteries (A), aorta (B), and carotid (C) arteries. All experiments were performed in the presence of NADPH (100 μmol/L). PEG-SOD inhibited fluorescence in all arteries (D through F). The intensity of red fluorescence represents the amount of O_2^- generated. e indicates the orientation of the endothelium. Magnification, ×400; scale bar, 50 μm. G, Summary of quantitative data for ethidium/oxyethidium fluorescence, presented as fluorescent units per mm² of artery wall. Values are given as mean±SEM (n=3 to 6). *$P<0.05$ vs fluorescence intensity in aorta and carotid arteries; †$P<0.05$ vs NADPH-treated arteries.

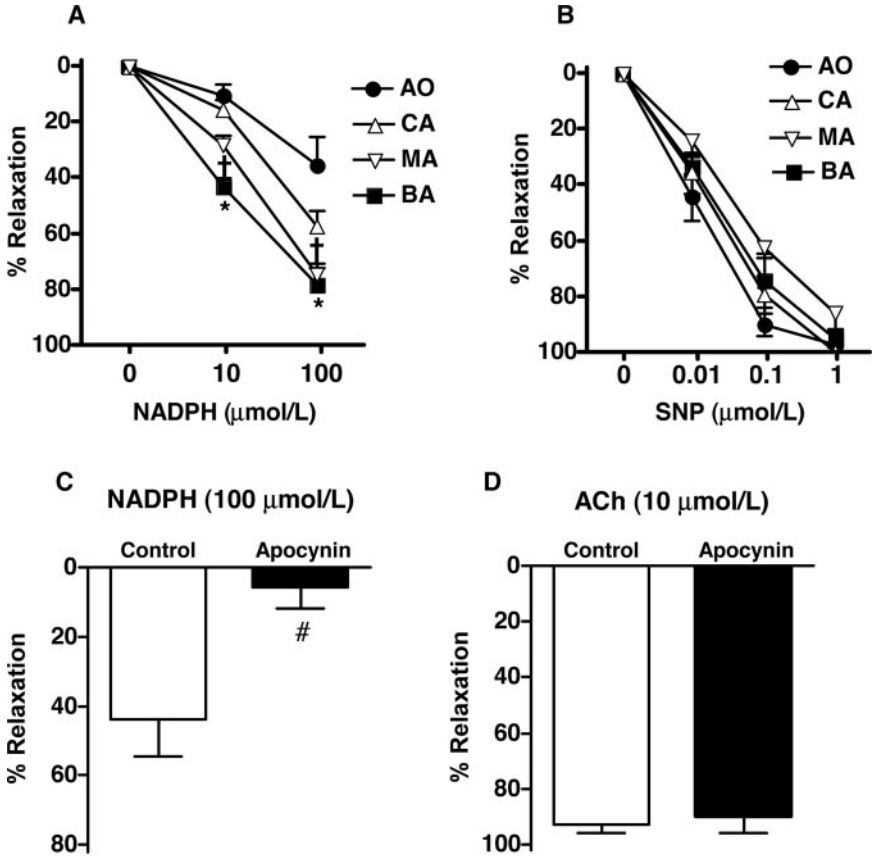

Figure 5. Effect of NADPH (A; 10 to 100 μmol/L) and SNP (B; 0.01 to 1 μmol/L) on vascular tone of thoracic aorta (AO) and common carotid (CA), mesenteric (MA), and basilar (BA) arteries. AO, MA, and BA were pre-contracted (≈50% of contraction to KPSS) with serotonin and CA with U46619 before addition of NADPH or SNP. Also shown is the effect of apocynin (300 μmol/L) on responses of pre-contracted BA to NADPH (C; 100 μmol/L) and ACh (D; 10 μmol/L). Values are expressed as percent relaxation of serotonin- or U46619-induced tone and are given as mean±SEM (n=5 to 8). *$P<0.05$ vs relaxations in AO and CA; †$P<0.0.05$ vs relaxations in AO (1-way ANOVA); #$P<0.0.05$ vs control (paired *t* test).

induced relaxations of the aorta were potentiated by catalase ($P<0.01$). Interestingly, in the presence of catalase, NADPH elicited small, transient contractions of basilar and carotid arteries (≈20% of pre-contracted tone, Figure 6A). Catalase had no effect on relaxations to SNP (1 μmol/L, Figure 6B), however. These experiments suggest that relaxations to NADPH are mediated by endogenous H_2O_2, presumably derived from NADPH oxidase, in all arteries except the aorta.

We next compared the sensitivity of cerebral and systemic arteries to exogenous H_2O_2. In all arteries, exogenous H_2O_2 (10 to 1000 μmol/L) induced a biphasic response: A small transient contraction followed by a large, sustained relaxation. There were no differences between arteries with respect to H_2O_2-induced contractions (data not shown); however, relaxations to H_2O_2 (100 to 1000 μmol/L) were greater in basilar and mesenteric arteries when compared with aorta and carotid arteries ($P<0.01$, Figure 7A). In all arteries, catalase abolished relaxations to H_2O_2 ($P<0.01$, Figure 7B).

Effect of Angiotensin II on Vascular Tone

Angiotensin II (100 nmol/L) elicited contractions of all arteries (Figure 8A), but those contractions were smaller in the basilar artery than in systemic arteries ($P<0.05$, 1-way ANOVA). Treatment with catalase resulted in a 3-fold increase in angiotensin II-induced contractions of the basilar artery, but not the systemic arteries ($P<0.01$, 1-way ANOVA; Figure 8A). Similarly, in the presence of apocynin, there was a 3-fold increase in angiotensin II-induced contractions of the basilar artery ($P<0.05$, paired *t* test; Figure 8B).

Discussion

The findings of this study reveal striking differences between intracranial cerebral arteries and a range of systemic arteries with respect to the activity, function, and expression of the ROS-generating enzyme NADPH oxidase. Specifically, using 2 separate techniques, we found that basal and stimulated

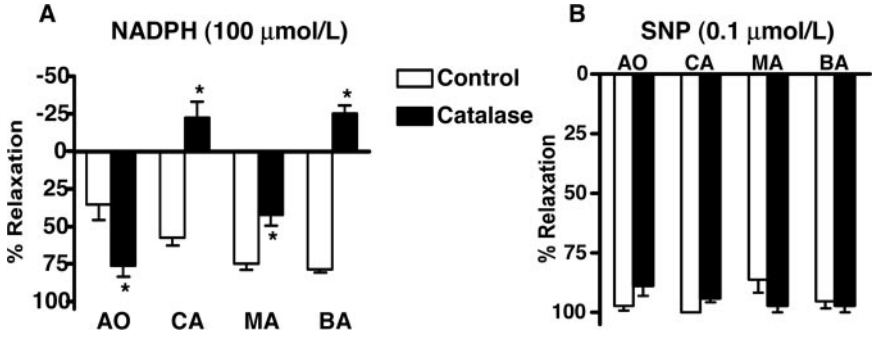

Figure 6. The effect of catalase (1000 U/mL) on relaxations of thoracic aorta (AO) and common carotid (CA), mesenteric (MA), and basilar (BA) arteries to (A) NADPH (100 μmol/L) and (B) SNP (0.1 μmol/L). Values are expressed as percent relaxation of serotonin- or U46619-induced tone and are given as mean± SEM (n=6 to 7). *$P<0.05$ vs control (1-way ANOVA).

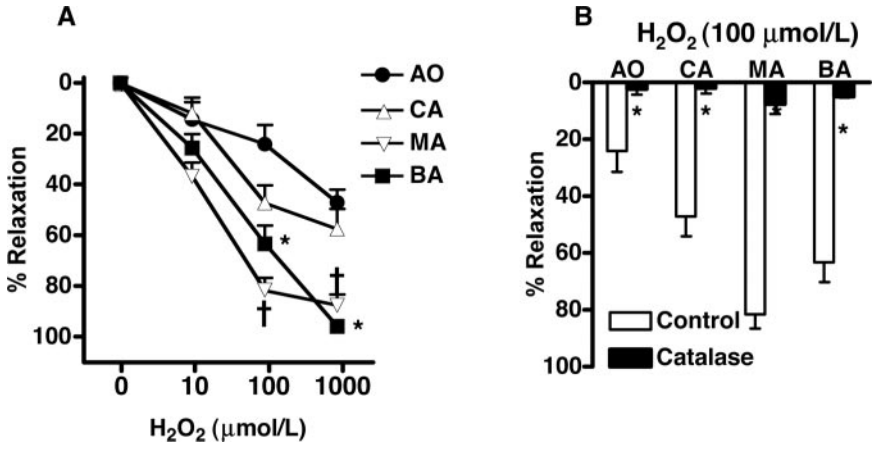

Figure 7. A, The effect of H_2O_2 (10 to 1000 μmol/L) on the tone of thoracic aorta (AO) and common carotid (CA), mesenteric (MA), and basilar (BA) arteries. *$P<0.05$ vs relaxations in AO and CA; †$P<0.0.05$ vs relaxations in AO (one-way ANOVA).B, The effect of catalase (100 U/mL) on relaxations to H_2O_2 (100 μmol/L). *$P<0.05$ vs control. Values are expressed either as percent relaxation of serotonin- or U46619-induced tone and are given as mean±SEM (n=6).

O_2^- generation from NADPH oxidase is 1 to 2 orders of magnitude higher in cerebral arteries than in systemic arteries. The higher level of NADPH oxidase-derived O_2^- in the basilar artery was associated with a 10-fold higher expression of the catalytic subunit protein Nox4. Activation of NADPH oxidase in vitro elicited relaxation of all arteries, but this response was greatest in cerebral arteries. Thus, we report for the first time that the activity, expression, and function of NADPH oxidase is profoundly greater in cerebral than systemic arteries. Like NADPH, angiotensin II was found to be a much stronger stimulus for NADPH oxidase activity in the basilar artery, where its contractile efficacy appears to be modulated by endogenous H_2O_2. These findings support the concept that NADPH oxidase-derived ROS may play a physiological role in regulating cerebral blood flow.

NADPH Oxidase Activity and Cerebral Arteries

NADPH oxidase is normally expressed and functional in both the cerebral and systemic vasculature.[6–8,12–14] When increased to supranormal levels by cardiovascular risk factors, NADPH oxidase activity is thought to be linked to endothelial dysfunction associated with various cardiovascular diseases.[11] However, it is not known what role, if any, is played by NADPH oxidase-derived ROS in modulating vascular function in the systemic circulation under normal physiological conditions. In contrast, findings from recent studies have raised the possibility that NADPH oxidase-derived ROS may serve as an important physiological vasodilator mechanism in the cerebral circulation.[13,14] Using the chemiluminescent probe lucigenin, we found that either angiotensin II or the substrate for NADPH oxidase, NADPH, increased O_2^- production in intact cerebral and systemic arteries, and this effect was inhibited by the NADPH oxidase inhibitors DPI, apocynin, and gp91ds-tat. Moreover, inhibition of NOS had no effect on O_2^- production in basilar arteries, providing strong evidence that NADPH oxidase was the primary source of O_2^- detected by this assay. A striking observation in these experiments was that O_2^- production by NADPH oxidase was profoundly greater in intracranial cerebral arteries (ie, basilar and middle cerebral) versus systemic arteries (ie, aorta, carotid, renal, and mesenteric). Furthermore, a considerably higher NADPH oxidase activity was also found in arterial homogenates of cerebral versus non-cerebral vessels, confirming that this phenomenon is not dependent on the presence of whole arterial tissue or even intact cells, and is consistent with a regional difference in enzyme subunit expression levels (see below). Because detected levels in cerebral arteries remained up to 75-fold higher even when Cu^{2+}/Zn^{2+}-SOD was inhibited by DETCA, it seems likely that this regional difference is, in large part, due to differing levels of NADPH oxidase activity and not simply varying degrees of O_2^- inactivation. In addition to the quantitative chemiluminescence data, we localized and measured O_2^- production in NADPH-treated basilar arteries, aorta, and carotid arteries using the oxidative fluorescent dye dihydroethidium. Ethidium/oxyethidium fluorescence was visible in endothelial, vascular smooth muscle, and adventitial cells of basilar and carotid arteries and the aorta, indicating that

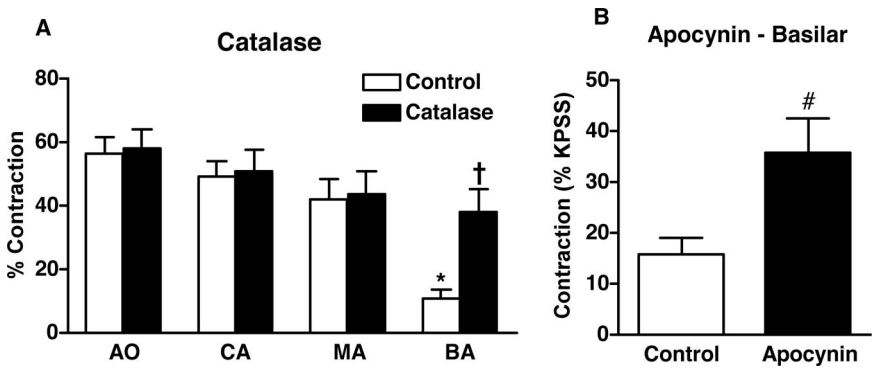

Figure 8. A, The effect of angiotensin II (100 nmol/L) on the vascular tone of thoracic aorta (AO) and common carotid (CA), mesenteric (MA), and basilar (BA) arteries in the absence and presence of catalase (1000 U/mL). *$P<0.05$ vs contractions to angiotensin II in AO, CA, and MA; †$P<0.0.05$ vs BA control (one-way ANOVA). B, Effect of apocynin (300 μmol/L) on responses of BA to angiotensin II (100 nmol/L). All arteries were pre-contracted (≈20% of contraction to KPSS) with KCl before addition of angiotensin II. Values are expressed as a percent of maximal contraction to KPSS and are given as mean±SEM (n=6 to 8). #$P<0.05$ vs control (paired *t* test).

NADPH oxidase is expressed and active throughout the vasculature of all these arteries. Although the range and resolution of O_2^- detection may differ between the 2 methodologies, making it difficult to strictly compare quantitative findings from each approach, using both assays we found markedly greater levels of O_2^- production in basilar arteries versus systemic and/or non-intracranial arteries. Thus, together these results provide strong evidence that NADPH oxidase activity is substantially greater in the cerebral circulation versus systemic vasculature under physiological conditions.

Nox4 Expression

We have previously reported that Nox4 mRNA expression is abundant in the rat basilar artery and is augmented during chronic hypertension.[13] We now report that Nox4 protein expression is markedly higher in the basilar artery than in systemic arteries. Although the associated higher expression of Nox4 protein in cerebral versus systemic arteries seems likely to contribute to the higher relative production of ROS by cerebrovascular NADPH oxidase, we cannot exclude the possibility that other Nox subunits, such as Nox2 and Nox1, might also contribute substantially to this regional difference. Of relevance here is our finding that the inhibitors of the association of cytosolic and membrane-associated NADPH oxidase components, apocynin and gp91ds-tat, both inhibited the NADPH-induced superoxide production by ≈50% in the basilar artery. Furthermore, a recent study has reported that when expressed in cultured cells, Nox4, unlike Nox2, does not appear to require cytosolic components to function.[18] Thus, it is conceivable that other Nox proteins, including Nox2 and Nox1, may also contribute to the molecular basis of higher NADPH oxidase activity in cerebral arteries.

NADPH Oxidase Function and Cerebral Arteries

We next tested for any regional differences in the regulation of vascular tone associated with these differing levels of NADPH oxidase activity. Recent studies in rats, rabbits, and mice have shown that NADPH causes cerebral vasodilatation in vivo and in vitro.[12–14] We confirmed here that application of NADPH to isolated basilar arteries elicits powerful cerebral vascular relaxation. Because in systemic arteries activation of NADPH oxidase and generation of O_2^- is generally thought to increase vascular tone via the inactivation of endothelium-derived NO,[19,20] it was somewhat surprising to find that NADPH consistently elicited relaxations of all 3 systemic arteries. Most notable, however, was the finding that cerebral (basilar) arteries were more responsive in terms of relaxation to NADPH than systemic arteries. This enhanced vasodilatation was relatively specific for NADPH, as relaxations to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator SNP were similar in all artery types. Of importance, apocynin virtually abolished NADPH-induced relaxation of the basilar artery, providing strong evidence that the relaxant effect of NADPH is NADPH oxidase-dependent. Interestingly, apocynin appears to inhibit NADPH-induced cerebral relaxation more effectively than it inhibits NADPH-induced O_2^- production. This may reflect differences in the accessiblity of apocynin to NADPH oxidase in the different experimental procedures, or may perhaps suggest that only apocynin-sensitive Nox homologues contribute to the vasodilatation.

The identity of the NADPH oxidase-derived ROS responsible for mediating cerebral vasodilatation to NADPH is not well defined. We previously reported that NADPH-induced dilatation of rat basilar arteries in vivo were inhibited by catalase and DETCA, suggesting that H_2O_2 (or a downstream ROS) generated from the dismutation of O_2^- by Cu^{2+}/Zn^{2+}-SOD was responsible for the vasodilatation.[13] In contrast, a recent study in mice found that O_2^- and not H_2O_2 was partially responsible for cerebral vasodilatation to NADPH.[14] In the present study, we found that catalase abolished NADPH-induced relaxation of cerebral arteries without affecting responses to SNP, indicating that the effect of NADPH was mediated by endogenous H_2O_2 (or a downstream ROS thereof) and that the effects of catalase were independent of contractile agents used in arterial preparations. We confirmed here previous reports that H_2O_2 is a powerful endogenous cerebral vasodilator.[3,4] Thus, cerebral arteries appear to be not only very sensitive to the relaxant effect of H_2O_2, but also likely to generate much higher amounts of H_2O_2 in response to stimuli of NADPH oxidase by virtue of a much higher activity of this enzyme relative to systemic arteries. The greater cerebral vasodilator response to NADPH is therefore likely to be a consequence of both higher ROS generation by NADPH oxidase and greater sensitivity to H_2O_2 of cerebral arteries.

The present findings using extracellular NADPH as an activator of NADPH oxidase do, however, raise an important question: How does extracellular NADPH increase NADPH oxidase activity when the substrate binding domain is believed to be intracellular?[21] One potential explanation is that application of NADPH to the extracellular milieu results in elevated intracellular levels of the pyridine nucleotide. NADPH is a relatively large, highly charged molecule, however, and is therefore unlikely to cross the plasma membrane by simple diffusion. Hence, to influence the intracellular concentration of NADPH, extracellular NADPH would either have to be actively transported across the plasma membrane or pass through a channel. Alternatively, NADPH could conceivably act as a reducing equivalent for regeneration of intracellular NADPH from $NADP^+$ by donating electrons either to a plasma membrane spanning electron transport protein or to a membrane permeable electron acceptor (ie, analogous to the mitochondrial citrate/pyruvate shunt). To date, there is no information on the existence of such mechanisms in vascular cells, or indeed in any other mammalian cell types, nor is there any published evidence on whether extracellular NADPH influences the intracellular concentration of the pyridine nucleotide. Nevertheless, the observations that NADPH-induced ROS production and cerebral vasodilatation in vitro and in vivo can be attenuated by specific NADPH oxidase inhibitors and gene knockdown approaches (present study and others[9,12–14,22]) suggest that NADPH, either directly or indirectly, triggers NADPH oxidase activation.

Potential Functional Consequences of Higher Cerebral NADPH Oxidase Activity

It is well established that the activity of NADPH oxidase is increased by vasoactive hormones such as angiotensin II.[8]

Indeed, we found here that angiotensin II potentiated vascular O_2^- production, and this effect was markedly greater (100-fold) in basilar arteries compared with aorta. Interestingly, this is analogous to our previous finding in spontaneously hypertensive rats, where O_2^- production from NADPH oxidase was selectively enhanced in basilar arteries.[13] Thus, vasodilator ROS production stimulated by angiotensin II in cerebral arteries would be expected to offset and hence protect against angiotensin II-induced cerebral vasospasm. Consistent with this hypothesis is our present finding that catalase and apocynin can substantially and selectively augment cerebral vasoconstriction by angiotensin II. Mechanical forces, including cyclic stretch and laminar and oscillatory shear stress, have also been shown to increase NADPH oxidase activity in endothelial cells,[23,24] and we have recently reported that NADPH oxidase-derived ROS partly mediate flow-dependent responses of the basilar artery in vivo.[25] Thus, it is possible that NADPH oxidase-derived ROS serve as important secondary messengers and/or paracrine signaling factors in endothelial cells of the cerebral circulation, increasing blood flow in response to hormonal and physical stimuli. It is therefore noteworthy that the present results support the concept that the phenomenon of high NADPH oxidase activity in cerebral arteries is physiologically relevant and is not limited to studies using exogenous NADPH as a stimulus.

In summary, this study has demonstrated that under normal conditions, NADPH oxidase activity is profoundly greater in cerebral versus systemic arteries. Activation of NADPH oxidase in cerebral arteries can cause powerful vasodilatation and offset angiotensin II-induced vasoconstriction via H_2O_2 derived from the dismutation of NADPH oxidase-derived O_2^-. These findings are associated with markedly higher expression of the Nox4 catalytic subunit of NADPH oxidase in cerebral than in systemic arteries. Our findings raise the prospect that NADPH oxidase-derived ROS may play an important role in the control of cerebral vascular tone. If so, caution may need to be taken in the future use of therapies to inhibit the effect of ROS in systemic vascular disease so as not to inadvertently compromise cerebral blood flow.

Acknowledgments

These studies were supported by a Project Grant from the National Health and Medical Research Council of Australia (NHMRC) (ID 350477). C.G.S. is a Senior Research Fellow of the NHMRC. G.R.D. is the recipient of a Monash Fellowship from Monash University.

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Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. 2005;97:1055-1062; originally published online October 6, 2005;
doi: 10.1161/01.RES.0000189301.10217.87
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571