NADPH oxidase activity is higher in cerebral versus systemic arteries of four animal species: role of Nox2

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Miller AA, Drummond GR, De Silva TM, Mast AE, Hickey H, Williams JP, Broughton BR, Sobey CG. NADPH oxidase activity is higher in cerebral versus systemic arteries of four animal species: role of Nox2. Am J Physiol Heart Circ Physiol 296: H220–H225, 2009. First published November 21, 2008; doi:10.1152/ajpheart.00987.2008.—We previously reported that NADPH oxidase activity is greater in intracranial cerebral versus systemic arteries of the rat. Here, we first tested whether NADPH oxidase activity is also greater in intracranial cerebral than systemic arteries of three other animal species, i.e., mouse, rabbit, and pig. Second, using Nox2-deficient mice, we evaluated the involvement of Nox2-containing NADPH oxidases in any such regional differences. NADPH-stimulated superoxide (O2-) production by basilar, middle cerebral arteries (MCA), and common carotid arteries (CA) and thoracic aorta (AO) from rat, mouse, rabbit, and pig was measured using lucigenin-enhanced chemiluminescence. Basal production of O2- and hydrogen peroxide (H2O2) by cerebral arteries, AO, and CA from wild-type (WT) and Nox2-/- mice was measured using L-012-enhanced chemiluminescence and Amplex Red fluorescence, respectively. Western blotting was used to measure Nox2 and SOD1–3 protein expression, and immunofluorescence was used to localize Nox2, in mouse arteries. In rats, WT mice, rabbits, and pigs, NADPH-stimulated O2- production by cerebral arteries was up to 40-fold greater than that in AO and CA. In WT mice, basal O2- and H2O2 production by cerebral arteries was ninefold and ~2.5-fold higher, respectively, than that in AO and CA and was associated with ~40% greater expression of Nox2 protein. Nox2 immunofluorescence was localized to the endothelium, and to a lesser extent the adventitia, in all mouse arteries and appeared to be more intense in endothelium of MCA than AO or CA. In Nox2-/- mice, NADPH-stimulated O2- production by cerebral arteries was ~35% lower than that in WT mice, whereas Nox2 deletion had no significant effect on O2- production by AO or CA. Thus NADPH oxidase activity is greater in intracranial cerebral versus systemic arteries of several animal species and is associated with higher cerebrovascular expression and activity of Nox2.

REACTIVE OXYGEN SPECIES (ROS) such as superoxide (O2-) serve as important cell signaling molecules for the regulation of normal vascular function (5, 15, 23, 25). Moreover, a wealth of evidence suggests that the excessive generation of vascular ROS plays a key role in the pathogenesis of several vascular diseases such as stroke and hypertension (6, 8, 10, 11, 16, 17, 22).

NADPH oxidases are now believed to be a major physiological source of ROS within cerebral and systemic vasculatures (7, 15). These enzymes are membrane bound and generate O2- by transferring electrons from NAD(P)H to molecular oxygen via a flavin-containing Nox catalytic subunit (3). To date, five Nox proteins have been discovered, namely Nox1 (21), Nox2 (also known as gp91phox) (12), Nox3 (4), Nox4 (20), and Nox5 (2). Studies have shown that Nox1, Nox2, and Nox4 are expressed in cerebral arteries (1, 14, 15, 17), suggesting that multiple isoforms of NADPH oxidase may be important for vascular ROS production in the brain. Recently it has become evident that marked differences exist between cerebral and noncerebral arteries with respect to the functioning of vascular NADPH oxidases under normal physiological conditions. Specifically, we have reported that the activity of NADPH oxidases is even up to 100-fold greater in intracranial arteries versus a range of systemic arteries of the rat (15). It is not known whether such regional differences in NADPH oxidase activity also exist in other animal species. Therefore, the first aim of this study was to determine whether the activity of NADPH oxidase is normally greater in cerebral versus systemic arteries from three other animal species (mice, rabbits, and pigs). Our second aim was to utilize Nox2-deficient mice to assess the importance of Nox2-containing NADPH oxidase activity in any such regional differences.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Ethics Committee. In total, six male Sprague-Dawley rats, 68 male C57Bl/6 wild-type (WT) mice, 17 Nox2-/- mice, six male New Zealand White rabbits, and six Sus scrofa pigs (3 male and 3 female; 31–87 kg; 9–24 mo old) were studied. Nox2-/- mice were backcrossed to the C57Bl6/J strain for at least 10 generations. O2- production by cerebral and systemic arteries. Basilar, middle cerebral arteries (MCA), and common carotid arteries and thoracic aorta were excised from rats, mice, rabbits, or pigs and cut into 5-mm ring segments. NADPH (100 μmol/l)-stimulated O2- production was measured by 5 μmol/l lucigenin-enhanced chemiluminescence in the presence of the Cu2+-chelating agent diethyldithiocarbamate (DETA; 3 mmol/l) as previously described (18). In some experiments, arteries were treated with the NADPH-oxidase inhibitor diphenyleneiodonium (DPI; 5 μmol/l). Basal O2- production was measured in mouse cerebral arteries (pooled basilar arteries and MCA), thoracic aortae, and common carotid arteries by 100 μmol/l L-012-enhanced chemiluminescence. In all experiments, background counts were subtracted and O2- production was normalized for dry tissue weight.

H2O2 production by cerebral and systemic arteries. The Amplex Red fluorescence assay (Molecular Probes) was used to measure basal H2O2 production by cerebral arteries (pooled basilar arteries and

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MCA), thoracic aortae, and common carotid arteries from mice. H₂O₂ standards (0–0.125 μM) and arteries were transferred to a 96-well plate. Fluorescence was determined in 100-μl solutions containing 15 μM Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazinone) and 0.1 U/ml horseradish peroxidase. Fluorescence was measured in a fluorimeter (Flexstation; Molecular Devices) using an excitation filter of 530 nm and an emission filter of 590 nm. Background fluorescence was subtracted and H₂O₂ (picomoles) accumulation was minute normalized for dry tissue weight.

**Protein expression of Nox2 and SOD isoforms.** Protein expression of the NADPH oxidase catalytic subunit Nox2 and SOD isoforms SOD1, SOD2, and SOD3 was measured in mouse cerebral (pooled basilar arteries and MCA) arteries, thoracic aortae, and common carotid arteries using Western blotting. Anti-Nox2 mouse monoclonal antibodies were obtained from Upstate (Chemicon). Arteries were homogenized in Laemmli buffer containing 25% glycerin, 12.5% Laemml, 7.5% sodium dodecyl sulfate, 25% 1M Tris-HCl (pH 8.0), and 0.25 mg/ml bromophenol blue over liquid nitrogen. Homogenates were then sonicated, heated (37°C for 10 min), and cleared by centrifugation (13,000 rpm for 10 min at 4°C). Protein concentration was then determined using the reducing agent-compatible and detergent-compatible (RC DC) protein assay (Bio-Rad). Equal amounts of protein were loaded onto a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% skim milk for 1 h at room temperature and then incubated overnight (4°C) with the appropriate primary antibody (1:300 for Nox2 and SOD1 and 1:1,000 for all other antibodies in 5% skim milk). Membranes were then incubated with a horseradish peroxidase-conjugated anti-mouse (Nox2) or anti-rabbit (for all other antibodies) immunoglobulin for 1 h at room temperature and then incubated overnight (4°C) at 4°C. The following day, the tissues were washed in 0.01 M PBS (3 × 10 min) and incubated in a FITC-conjugated goat anti-mouse IgG (1:200 for aorta and carotid and 1:500 for MCA; Zymed) for 3 to 4 h at 4°C. The tissues were then washed in 0.01 M PBS (3 × 10 min) and mounted in buffered glycerol (0.5 M Na₂CO₃ added dropwise to 0.5 M NaHCO₃ to pH 8.6, combined 1:1 with glycerol). Tissue-mounted slides were viewed and photographed on a Leica confocal scanning laser system.

**Drugs.** L-012 was purchased from Wako (Japan), Amplex Red fluorescence assay kit was from Molecular Probes, and all other drugs were from Sigma. DPI was prepared at 10 mmol/l in DMSO and diluted in Krebs-HEPES such that the final concentration of DMSO was <0.05%. All other drugs were dissolved and diluted in Krebs-HEPES.

**Data analysis.** All results are expressed as means ± SE. Statistical comparisons were performed using either one-way ANOVA with Bonferroni’s multiple comparisons post hoc test or Student’s t-test. P < 0.05 was considered statistically significant.

**RESULTS**

**NADPH oxidase activity is greater in cerebral versus systemic arteries.** In rat, WT mouse, rabbit, and pig, NADPH-stimulated O₂⁻ production by intact middle cerebral arteries (MCA), basilar, thoracic aorta, and common carotid arteries from rat (A), wild-type mouse (B), rabbit (C), and pig (D) in the absence and presence of the NADPH oxidase inhibitor diethyldithiocarbamate (DETCA; 3 mmol/l). Values are given as means ± SE (10³ counts/mg of dry tissue; n = 3–12). *P < 0.05 vs. similarly treated aorta and carotid arteries; ***P < 0.05 vs. NADPH-treated arteries (1-way ANOVA).
Greater cerebrovascular NADPH oxidase activity is associated with higher Nox2 protein expression. Protein expression of Nox2 was ~40% higher in cerebral arteries (pooled MCA and basilar) than in aorta and carotid arteries of WT mice (Fig. 2A). By contrast, SOD1, SOD2, and SOD3 protein expression did not differ between artery types (Fig. 2B–D).

Localization of Nox2. Nox2 immunoreactivity was predominantly observed in the endothelium and to a lesser extent in the adventitia of MCA, aorta, and carotid arteries (Fig. 3). No specific Nox2 immunoreactivity was located in the vascular smooth muscle. Consistent with our Western blot data (Fig. 2), Nox2 immunofluorescence appeared to be more intense in endothelial cells of MCA than of aorta and carotid.

Role of Nox2 in basal and NADPH-stimulated ROS production. Basal \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) production by mouse cerebral (pooled MCA and basilar) arteries was higher than levels generated by aorta and carotid arteries \( (P < 0.05; \text{Fig. 4}) \). Basal \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) production was similar between WT and Nox2\(^{-/-} \) mice in all artery types (Fig. 4). By contrast, NADPH-stimulated \( \text{O}_2^- \) production was ~35% lower in cerebral (pooled MCA and basilar) arteries of Nox2\(^{-/-} \) versus WT mice (Fig. 5). \( \text{O}_2^- \) production in aorta and carotid from Nox2\(^{-/-} \) mice was similar to levels in WT mice, suggesting a key role for Nox2-containing NADPH oxidases specifically in cerebrovascular NADPH oxidase activity.

DISCUSSION

We have previously reported that the activity of the ROS-generating enzyme NADPH oxidase is profoundly greater in rat intracranial arteries versus a range of systemic arteries. In this study, we extend this finding by reporting that this phenomenon exists in other animal species. Indeed, \( \text{O}_2^- \) production by NADPH oxidase is up to 40-fold greater in intracranial cerebral than systemic arteries from mouse, rabbit, and pig. The greater level of NADPH oxidase-derived \( \text{O}_2^- \) production in mouse cerebral arteries was associated with higher \( \text{H}_2\text{O}_2 \) production and also greater expression of Nox2 protein. Furthermore, NADPH-stimulated \( \text{O}_2^- \) production by cerebral, but not systemic, arteries was decreased in Nox2\(^{-/-} \) mice, suggesting that the activity of Nox2-containing NADPH oxidases contributes to the regional differences in arterial NADPH oxidase activity.

NADPH oxidases are now believed to be a major source of vascular ROS within the cerebral circulation (15). Indeed, numerous investigators have shown that the application of the

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Fig. 2. Representative Western blots showing protein expression of the NADPH oxidase catalytic subunit Nox2 (A) and \( \text{O}_2^- \) dismutase (SOD) isoforms SOD1 (B), SOD2 (C), and SOD3 (D) in cerebral arteries (pooled basilar and middle cerebral), thoracic aorta, and common carotid arteries from wild-type mice. A summary of densitometric data is also shown. Values are expressed as relative density per microgram protein normalized to GAPDH expression and are given as means ± SE \( (n = 4–8) \). *\( P < 0.05 \) vs. aorta and carotid.
substrate for NADPH oxidase, NAD(P)H, elicits a large increase in $O_2^\cdot$ production by intact cerebral arteries and homogenates of cerebral vessels from rats, mice, and rabbits (5, 13, 15, 17, 19). Moreover, this effect can be attenuated by the NADPH oxidase inhibitors DPI, apocynin, and gp91ds-tat (5, 13, 17, 19), suggesting that NADPH activates cerebrovascular NADPH oxidase. Over the past few years, NADPH oxidase has attracted considerable attention as a key culprit in vascular dysfunction associated with several vascular diseases, including those affecting the cerebral circulation (6, 8, 9, 22, 24). In contrast, the role of this enzyme for the normal functioning of cerebral blood vessels is less clear. Application of NAD(P)H to cerebral arteries from rats, mice, and rabbits elicits robust vasodilatation in vitro and in vivo (5, 13–15, 17, 19), mediated by either $O_2^\cdot$ or the downstream metabolite $H_2O_2$. Recently it has become evident that stark differences exist between cerebral and noncerebral arteries with respect to the activity of NADPH oxidases under normal physiological conditions (15). Specifically, using two separate approaches, we found that NADPH- and angiotensin II-stimulated $O_2^\cdot$ production by NADPH oxidase is one to two orders of magnitude higher in rat intact cerebral arteries than in a wide range of systemic arteries (15). In the present study, we confirm this previous finding and report that this phenomenon is not unique to the rat cerebral circulation. Indeed, using the chemiluminescence probe lucigenin, we found that in mice, rabbits, and pigs NADPH-stimulated $O_2^\cdot$ production is up to 40-fold higher in intact intracranial cerebral (i.e., MCA and basilar) compared with systemic arteries (i.e., aorta and carotid). Importantly, these findings were made in the presence of the SOD1/SOD3 inhibitor DETCA; thus it seems unlikely that differing rates of $O_2^\cdot$ inactivation by SODs account for these regional differences in $O_2^\cdot$ generated by NADPH oxidases. Furthermore, it is very likely that the observed differences in NADPH oxidase activity between cerebral and systemic arteries are not simply attributable to differences in vessel size. For example, we previously found that second order branches of the rat superior mesenteric artery, which have a comparable diameter with that of rat basilar and MCA (~250 μm), have similar (i.e., relatively low) levels of NADPH oxidase activity to other larger noncerebral arteries (e.g., aorta, common carotid, renal) (15). In accordance with previous studies (5, 13, 14, 17), the NADPH oxidase inhibitor DPI virtually abolished NADPH-stimulated $O_2^\cdot$ production by arteries from all animal species, consistent with NADPH oxidases being a primary source of $O_2^\cdot$ detected by this assay. We have previously reported that basal $O_2^\cdot$ production by rat intracranial arteries is greater than levels generated by systemic arteries (15). In the present study, we found that basal $O_2^\cdot$ production is also higher in mouse cerebral versus systemic arteries and that this is associated with higher production of the downstream metabolite $H_2O_2$. Levels of $O_2^\cdot$ and $H_2O_2$ in cerebral and systemic arteries were not altered in Nox2−/− versus WT mice, suggesting that Nox2-containing NADPH oxidase is unlikely to be the source of
basal ROS in the cerebral circulation. It has been previously reported that beside Nox2, mRNA and protein for Nox1 and Nox4 are expressed in cerebral arteries (1, 14, 15, 17). Therefore, it is possible that Nox1- and/or Nox4-containing NADPH oxidases may represent a major source of basal ROS in the cerebral circulation and may even partly compensate for Nox2 activity in Nox2−/− mice.

There is now some evidence that cerebral arteries may have a different composition of Nox isoforms than noncerebral arteries. We have previously reported that the expression of Nox4 protein is greater than 10-fold higher in rat basilar arteries versus aorta, carotid, and mesenteric arteries (15). Ago et al. (1) reported that mRNA for Nox subunits is greater in basilar than in aortic endothelial cells. Consistent with these findings, we found here that the protein expression of Nox2 is ~40% higher in mouse cerebral arteries than in aorta and carotid. Furthermore, protein expression of all three SOD isoforms did not differ between vascular beds, consistent with our interpretation of the O2•− assay findings made with DETCA that the regional differences are not attributable to different rates of metabolism O2•− by SOD and that such differences are likely to be physiologically relevant. Using immunochemistry, we found that Nox2 immunoreactivity was predominantly localized to endothelial and to a lesser extent adventitial cells of MCA, aorta, and carotid. Moreover, consistent with our protein expression data, Nox2 immunoreactivity appeared to be more intense in endothelial cells of the MCA than in aorta and carotid.

To test for a role of Nox2-containing NADPH oxidases in the regional differences in vascular NADPH oxidase activity, we next measured O2•− production by cerebral and systemic arteries from Nox2−/− mice and WT controls. In Nox2−/− mice, NADPH-stimulated O2•− production by cerebral arteries was selectively lower than levels generated by arteries from WT mice. This finding suggests that Nox2-containing NADPH oxidase is an important source of O2•− specifically in cerebral arteries and that higher activity of this isoform plays a key role in the higher cerebrovascular NADPH oxidase activity. Since Nox1 and Nox4 are also expressed in cerebral arteries (1, 14, 15, 17), it is conceivable that Nox1- and/or Nox4-containing NADPH oxidases may account for the remainder of the NADPH-stimulated O2•− production by mouse cerebral arteries.

In summary, the findings of this study reveal that in healthy animals from four mammalian species, NADPH oxidase activity is greater in cerebral than in systemic arteries from several animal species. Furthermore, we provide evidence that higher cerebrovascular Nox2 activity may, in part, account for these regional differences.

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