Exogenous NADPH Increases Cerebral Blood Flow Through NADPH Oxidase–Dependent and –Independent Mechanisms

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Objective—NADPH, a substrate for the superoxide-producing enzyme NADPH oxidase, produces vasodilation in the cerebral circulation. However, the mechanisms of the effect have not been fully elucidated. We used a peptide inhibitor of NADPH oxidase (gp91ds-tat) and null mice lacking the gp91phox subunit of NADPH oxidase to examine the mechanisms of the cerebrovascular effects of exogenous NADPH.

Methods and Results—Cerebral blood flow (CBF) was assessed by laser-Doppler flowmetry in anesthetized mice equipped with a cranial window. Superfusion with NADPH increased CBF (27% at 100 μmol/L) without affecting the EEG. The CBF increase was attenuated by the free-radical scavenger MnTBAP (∼54%, P<0.05) but not by the H2O2 scavenger catalase. The response was also attenuated by gp91ds-tat (∼64%, P<0.05) and by the nitric oxide synthase inhibitor Nω-nitro-L-arginine (∼44%, P<0.05). The increase in CBF produced by NADPH was attenuated in gp91-null mice (∼41%, P<0.05). NADPH increased production of reactive oxygen species, assessed by hydroethidine microfluorography, an effect blocked by MnTBAP or gp91ds-tat and not observed in gp91-null mice.

Conclusions—These data suggest that the mechanisms of the CBF increases produced by exogenous NADPH are multifactorial and include NADPH oxidase–dependent and –independent factors. (Arterioscler Thromb Vasc Biol. 2004;24:1860-1865.)

Key Words: hydroethidine ■ gp91phox ■ cerebral blood flow ■ laser-Doppler flowmetry ■ reactive oxygen species

Reactive oxygen species (ROS) have emerged as powerful signaling molecules involved in both normal and abnormal cellular functions.1 There is increasing evidence that ROS are critical regulators of vascular reactivity in the systemic and cerebral vasculatures.2 Thus, ROS participate in the increase in cerebral blood flow (CBF) produced by endothelium-dependent vasodilators, such as bradykinin or the calcium ionophore A23187,3–5 as well as in the disturbance in cerebrovascular regulation produced by hypertension, diabetes, aging, hyperhomocystinemia, and Alzheimer’s disease.6,7

NADPH oxidase, a superoxide-producing enzyme first discovered in phagocytes, has recently been identified as a major source of ROS in the vasculature.8,9 The typical NADPH oxidase is composed of membrane-bound (gp91phox, p22phox) and cytoplasmic (p40phox, p47phox, and p67phox) subunits.8,9 Assembly of the cytoplasmic and membrane-associated units, a process that requires phosphorylation of p47phox and the small GTPase Rac, leads to activation of the enzyme and production of superoxide.8,10 Although NADPH oxidase has been studied extensively in the systemic circulation, very little is known about its function in the cerebral circulation. However, recent studies suggest that NADPH oxidase is active in cerebral blood vessels as well.11,12 Administration of the NADPH oxidase substrates NADH or NADPH increases superoxide production in rabbit and rat cerebral blood vessels and produces vasodilation both in vitro and in vivo.11,12 These effects are attenuated by the NADPH oxidase inhibitor diphenylene iodonium (DPI),11,12 suggesting that they are mediated by NADPH oxidase. However, DPI inhibits all flavin-containing enzymes and lacks specificity for NADPH oxidase.13,14 Therefore, the findings of those studies, although suggestive, do not provide definitive evidence for an involvement of NADPH oxidase.

Null mice lacking the gp91phox subunit of NADPH oxidase have recently been introduced15 and have provided important insights into the role of NAPDH oxidase in the systemic circulation.16–18 Furthermore, a peptide inhibitor of NADPH oxidase (gp91ds-tat) has been developed.19 This peptide prevents the assembly of NADPH oxidase subunits, thereby inhibiting the activity of the enzyme.17,19 These new tools provide the opportunity to better understand the potential role of NADPH oxidase in the cerebral circulation. Therefore, in the present study, we used the peptide inhibitor gp91ds-tat and gp91-null mice to examine the mechanisms of the increase in CBF produced by NADPH. We found that neocortical superfusion of NADPH increases local ROS production and CBF and that these increases are markedly
attenuated by gp91ds-tat and are smaller in gp91-null mice than in wild-type controls. Furthermore, the increase in CBF is attenuated by the nitric oxide synthase (NOS) inhibitor N\(^-\)nitro-L-arginine (L-NNA). The results suggest that the CBF increases produced by NADPH are multifactorial and are mediated by NADPH oxidase–dependent and –independent mechanisms.

### Methods

#### Mice

C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Me). Mice lacking the gp91phox subunit of NADPH oxidase\(^{1,2}\) and wild-type littermates were obtained from an in-house colony derived from breeding pairs purchased from Jackson Laboratories. Null mice were backcrossed to the C57BL/6J strain at least 6 times and genotyped as described.\(^{2,3}\) All mice were used at age 2 to 3 months.

#### General Surgical Procedures

All experimental procedures were approved by the Institutional Animal Use and Care Committee. As described in detail elsewhere,\(^{2,3}\) mice were anesthetized with isoflurane (induction 5%, maintenance 1% to 2%). One of the femoral arteries was cannulated for recording of arterial pressure and collection of blood samples. Mice were intubated and artificially ventilated with an oxygen-nitrogen mixture adjusted to provide a PaO\(_2\) of 120 to 140 mm Hg (Table I, available online at http://atvb.ahajournals.org). Rectal temperature was maintained at 37\(^\circ\)C with a thermostatically controlled rectal probe connected to a heating pad. After surgery, isoflurane was gradually discontinued and anesthesia was maintained with urethane (750 mg/kg IP) and \(\alpha\)-chloralose (50 mg/kg IP).

Throughout the experiment, the level of anesthesia was monitored by testing corneal reflexes and motor responses to tail pinch.

#### Monitoring of CBF

A small craniotomy (2×2 mm) was performed to expose the somatosensory cortex, the dura was removed, and the site was superfused with a modified Ringer’s solution (37\(^\circ\)C, pH 7.3 to 7.4; see Iadecola\(^{22}\) for composition). CBF was continuously monitored at the site of superfusion with a laser-Doppler probe (Vasamedic) positioned stereotaxically on the cortical surface and connected to a computerized data acquisition system. CBF values were expressed as percent increase relative to the resting level. Zero values for CBF were obtained after the heart was stopped by an overdose of isoflurane at the end of the experiment.

#### Electroencephalogram

The electroencephalogram (EEG) was recorded monopolarly from a stainless steel screw positioned over the dura overlaying the left somatosensory cortex (3 mm lateral and 1.5 mm caudal to bregma). The reference electrode was a clip attached to neck muscles. The EEG was recorded for 5 minutes, which corresponded to the time during which CBF was measured, and stored in a computer by using a data acquisition system (PowerLab, ADInstruments Ltd). Power analysis of the EEG was performed with a software module provided by PowerLab.

#### Detection of ROS by Hydroethidine

Hydroethidine (HE; 2 \(\mu\)mol/L dihydroethidium; Molecular Probes) in Ringer’s solution was topically superfused on the somatosensory cortex for 60 minutes by using an approach described by Suzuki et al.\(^{23}\) At the end of the superfusion, the brain was removed and frozen in Freon (-30\(^\circ\)C). Brain sections (thickness, 20 \(\mu\)m) were serially cut in a cryostat starting from the beginning to the end of the corpus callosum and collected at 100-\(\mu\)m intervals. This area includes the cortex underlying the cranial window. ROS production was assessed by methods published from our laboratory.\(^{21,24}\) In brief, brain sections were examined under an Eclipse E800 fluorescence microscope (Nikon) equipped with an ethidium filter set (Chroma Technology, No. 31014). Images were acquired with a computer-controlled digital monochrome camera (Coollens, Roper Scientific). Image acquisition was performed with the same fluorescence settings in all cases. Ethidium fluorescence was assessed in the brain area superfused with HE. The analysis of ROS production in the different conditions studied (see Experimental Protocol) was performed in a blinded fashion with the IPLab software (Scanalytics). After background subtraction of the camera dark current, pixel intensities of ethidium signals were quantified. Fluorescent intensities of all sections (20 to 30 per animal) were added, divided by the total number of pixels analyzed, and expressed as relative fluorescence units (RFU).

#### Experimental Protocol

CBF recordings were started after arterial pressure and blood gases were in a steady state (Table I). All pharmacological agents studied were dissolved in Ringer’s solution, unless otherwise indicated.

#### Topical Application of NADPH

NADPH (20, 100, or 1000 \(\mu\)mol/L; Sigma) was topically superfused on the somatosensory cortex until the evoked change in CBF reached a steady state (usually 3 to 5 minutes).

#### Effects of DPI, ROS Scavengers, and NOS Inhibitors

The CBF response to NADPH (100 \(\mu\)mol/L) was first obtained during Ringer’s superfusion. The superfusion solution was then switched to Ringer’s containing 1 of the following agents: the flavoprotein inhibitor DPI (3 \(\mu\)mol/L; Sigma),\(^{25}\) the superoxide and \(\text{H}_2\text{O}_2\) scavenger Mn(III)tetrakis(4-benzoic acid) porpyrin (MnTBAP; 100 \(\mu\)mol/L; Calbiochem),\(^{21,26}\) the \(\text{H}_2\text{O}_2\) scavenger catalase (400 U/mL; Sigma),\(^{27}\) or the nonselective NOS inhibitor L-NNA (1 \(\mu\)mol/L; Sigma).\(^{22}\) DPI and MnTBAP were dissolved in dimethyl sulfoxide and Tris buffer (pH 8.0), respectively, and then further diluted in Ringer’s to a final concentration of both vehicles to be <0.5\%.\(^{2,3}\) The CBF response to NADPH was tested again 30 to 40 minutes after superfusion of inhibitors, a time interval selected on the basis of preliminary experiments. In some studies, the selectively neuronal NOS (nNOS) inhibitor 7-nitroindazole (7-NI; 50 mg/kg; Sigma)\(^{28}\) was administered IP 30 to 40 minutes before obtaining the CBF responses to NADPH. The nonspecific NADPH oxidase inhibitor DPI was used for comparison with previous studies in which the cerebrovascular effects of NADPH were investigated.\(^{11,12}\)

#### Effect of gp91ds-tat

In some experiments, the NADPH oxidase peptide inhibitor gp91ds-tat (YGRRKKRRQRQRCCSTRRQR-NH\(_2\)) was used.\(^{19}\) The scrambled sequence (sgp91ds-tat) (YGRRKKRRQRQCSTRRQRT-NH\(_2\)) served as a control. The peptide interferes with NADPH oxidase activity by targeting a sequence that is essential for the binding of gp91phox with p47phox.\(^{19,19}\) To facilitate entry into cells, the gp91phox sequence is linked to the HIV-tat peptide, an amino acid sequence that is internalized by all cells.\(^{27}\) The peptides were synthesized (BioSynthesis) with or without a fluorescent tag (carboxytetramethylrhodamine, TAMRA). Effects on CBF were assessed 40 minutes after gp91ds-tat (1 \(\mu\)mol/L) or sgp91ds-tat (1 \(\mu\)mol/L) superfusion. After the experiment, the brain was removed and frozen, and the area under the window was sectioned in a cryostat (thickness, 20 \(\mu\)m). The penetration of the peptide in the cortex underlying the cranial window was verified in all cases by using a fluorescence microscope. In experiments with HE, peptides without a fluorescent tag were used.

#### Assessment of Cerebrovascular Reactivity After gp91ds-tat or in gp91-Null Mice

The endothelium-dependent vasodilator acetylcholine (10 \(\mu\)mol/L) or the endothelium-independent agent adenosine (400 \(\mu\)mol/L), was topically applied to the cortex and the CBF response was monitored. Functional hyperemia was evaluated by assessing the increase in
CBF evoked by mechanical stimulation of the facial whiskers (60 seconds, 3 to 5 Hz). CBF reactivity to hypercapnia was tested by introducing CO₂ into the circuit of the ventilator and elevating arterial PCO₂ to 50 to 60 mm Hg. Peptides (gp91-ds-tat; sgp91-ds-tat) were topically superfused for 40 minutes before assessment of cerebrovascular reactivity.

Assessment of ROS Production by HE

The protocol for these experiments was identical to those in which the effect of NADPH on CBF was studied. Ringer’s solution containing HE alone or HE plus MnTBAP (100 μmol/L), gp91ds-tat (1 μmol/L), or sgp91ds-tat (1 μmol/L) was superfused for 45 minutes, followed by cocapsulation of NADPH (100 μmol/L) for 15 minutes. At the end of superfusion, the brain was removed and processed for ROS assessment as described earlier.

Data Analysis

Data in text and figure are expressed as mean ± SEM. Two-group comparisons were analyzed by the 2-tailed t test for dependent or independent samples, as appropriate. Multiple comparisons were evaluated by ANOVA and Tukey’s test. Statistical significance was considered for probability values <0.05.

Results

Effect of NADPH on Resting CBF and Neural Activity

In C57BL/6J mice, neocortical superfusion with NADPH (20, 100, or 1000 μmol/L) increased resting CBF dose-dependently (P<0.05, n=6, ANOVA and Tukey’s test) (Figure 1). The increase in CBF was independent of changes in arterial pressure and blood gases (Table I). Because NADPH oxidase is also present in neurons, we then examined whether the increase in CBF was secondary to increases in local neural activity. NADPH oxidase superfusion (100 μmol/L, n=5) did not affect the pattern of EEG frequency distribution, as shown by power analysis (Figure 2).

Effect of ROS Scavengers and NOS Inhibitors on the Increase in CBF Produced by NADPH

Topical superfusion with MnTBAP (100 μmol/L, n=6) did not affect resting CBF (Ringer’s, 22±2 vs MnTBAP, 23±2 perfusion units [PU]; P>0.05), but it attenuated the increase in CBF evoked by NADPH by 54% (P<0.05) (Figure 3A). In contrast, catalase (400 U/ml, n=6) did not alter the CBF increase produced by NADPH (P>0.05) (Figure 3A). Because NADPH modulates NOS activity, we also investigated the role of NOS in the increase of CBF. The nonselective NOS inhibitor l-NNa (1 mmol/L, n=6) reduced resting CBF by 19% (Ringer’s, 26±2 vs l-NNa, 21±1 PU; P<0.05) and the CBF increase produced by whisker stimulation by 62% (Ringer’s, 21±2% vs l-NNa, 8±2%; P<0.05). Furthermore, L-NNa attenuated the increase in CBF produced by NADPH by 44% (P<0.05) (Figure 3B). In contrast, the nNOS inhibitor 7-NI (50 mg/kg, n=6) did not alter the CBF increase by NADPH by 18% (Ringer’s, 22±1 vs 7-NI, 18±1 PU; P<0.05) and the CBF response evoked by whisker stimulation by 63% (Ringer’s, 19±3% vs 7-NI, 7±2%; P<0.05), but did not attenuate the CBF increase evoked by NADPH (P<0.05) (Figure 3B).

Effect of DPI and gp91ds-tat on the Increase in CBF Produced by NADPH

The flavoprotein inhibitor DPI (3 μmol/L, n=6) did not affect resting CBF (Ringer’s, 20±1 vs DPI, 22±2 PU; P>0.05), but it attenuated the elevation in CBF evoked by NADPH by 42% (P<0.05) (Figure 3A). To implicate more specifically NADPH oxidase in the response, we also used the peptide inhibitor gp91ds-tat. Topical superfusion with gp91ds-tat (1 μmol/L, n=6) did not affect resting CBF (Ringer’s, 21±1 vs gp91ds-tat, 21±2 PU; P>0.05), but it attenuated the CBF increase evoked by NADPH by 64% (P<0.05) (Figure 4A). In contrast, CBF...
responses to whisker stimulation, acetylcholine, or adenosine were not affected (Figure 4B). Neocortical application of the control peptide sgp91ds-tat (1 μm/L, n=6) had no effect on resting CBF or on the increase in CBF produced by NADPH (P>0.05) (Figure 4A).

Effect of NADPH on CBF in gp91-Null Mice
To provide further evidence for an involvement of NADPH oxidase, gp91-null mice were studied. The increase in CBF produced by whisker stimulation, acetylcholine, and hypercapnia did not differ between wild-type and gp91-null mice (P>0.05, n=6 per group) (Figure 5A). However, the increase in CBF produced by NADPH was markedly attenuated (Figure 5B). The attenuation was 41% at a concentration of 100 μm/L (P<0.05, n=6 per group) (Figure 5B). Interestingly, the residual CBF response to NADPH in gp91-nulls was attenuated further by DPI (−35%; P<0.05, n=6) and by the NOS inhibitor L-NNA (−47%; P<0.05, n=6), but not by 7-NI (P>0.05, n=6) (Figure 5C).

NADPH-dependent ROS Production
To determine whether NADPH increased local ROS generation, the HE method was used. Superfusion with HE did not alter resting CBF (Ringer’s, 19±1 vs HE, 19±1 PU; P>0.05) or the CBF increase evoked by NADPH (Ringer’s, 26±3 vs HE, 26±2%; P>0.05; n=5 per group). NADPH (n=5) significantly (P<0.05) increased the ethidium signal reflecting ROS production (Figure 6A and 6B; P<0.05). The increase was observed in the pial surface and pial vessels in the area of superfusion, as well as in the underlying brain parenchyma (Figure 6A and 6B). The ethidium signal evoked by NADPH was blocked by MnTBAP (n=5) or by gp91ds-tat (n=5), but not by the scrambled peptide sgp91ds-tat (n=5) (Figure 6C). Furthermore, NADPH failed to increase the ethidium signal in gp91-null mice (n=5) (Figure 6C).

Discussion
We found that topical application of NADPH increases resting CBF in the mouse somatosensory cortex, an effect that is not related to changes in neural activity. The CBF increase was attenuated by the free-radical scavenger MnTBAP, but not by catalase, suggesting that it was partly mediated by superoxide generated in response to NADPH. We then sought to provide evidence of whether NADPH oxidase was the source of the ROS. We found that the increase in CBF produced by NADPH was attenuated by DPI, an agent commonly used as an inhibitor of NADPH oxidase, and by the more specific peptide inhibitor gp91ds-tat. Furthermore, we found that the response to NADPH was attenuated in mice lacking the gp91phox subunit of NAPDH oxidase. In parallel experiments, we demonstrated that NADPH increases ROS production, assessed by the HE method, and that such an increase is attenuated by MnTBAP or gp91ds-tat, but not by the scrambled peptide sgp91ds-tat. Furthermore, the NADPH-induced ROS increase was not observed in gp91-null mice. We also found that the NOS
inhibitor l-NNA attenuated the CBF increase, an effect observed also in gp91-null mice. These data provide evidence that although a gp91phox-containing NADPH oxidase contributes to the increase in CBF produced by exogenous NADPH, NO is also involved in the response.

Recent studies in rats and rabbits have provided evidence that NADPH produces cerebrovasodilation. However, previous findings were entirely based on DPI, a nonspecific inhibitor of NADPH oxidase. The present results, using gp91-null mice and the NADPH oxidase assembly inhibitor peptide gp91ds-tat, provide the strongest evidence to date that NADPH increases ROS production and CBF in the brain and that a component of the CBF increase is dependent on NADPH oxidase. Furthermore, the findings unveil a previously unrecognized role of NO in the vasodilation evoked by NADPH.

The findings of the present study cannot be the results of differences in arterial blood pressure or blood gases because these parameters were carefully controlled and did not differ among the groups of mice studied. For the following reasons, the effects of the gp91ds-tat peptide on the CBF response to NADPH cannot be attributed to nonspecific vascular actions. First, the peptide did not alter resting CBF or its reactivity to the endothelium-dependent vasodilator acetylcholine, to the endothelium-independent vasodilator adenosine, or to functional hyperemia produced by whisker stimulation. Second, the scrambled peptide gp91ds-tat did not alter the CBF response to NADPH. Third, the effects of the peptide were identical to those observed in gp91-null mice. Similarly, the reduced CBF responsiveness to NADPH in gp91-null mice cannot be attributed to reduced vascular reactivity because the increases in CBF produced by whisker stimulation, acetylcholine, or hypercapnia were not altered in these mice. Therefore, the findings of the present study cannot result from instability of the experimental preparation or to nonspecific effects of the experimental approaches used.

The results of the present study suggest that exogenous NADPH leads to activation of NADPH oxidase and production of ROS, which either directly or indirectly mediates the vasodilation. One question concerns the cell type in which NADPH oxidase is located. In the cerebral cortex, NADPH oxidase is likely to be present in neurons, glia, and cerebral blood vessels. We have no direct evidence to attribute the increase in CBF produced by NADPH to a specific cell type. Although NADPH did not affect neural activity, the possibility that the source of ROS is neuronal or glial cannot be ruled out. Indeed, we found that cortical superfusion with NADPH increased ROS production, not only in cerebral blood vessels but also in other brain cells. However, because ROS are short lived, their source must be within or near their sites of action. In vessels, gp91phox is present in endothelial cells and adventitial fibroblasts. However, the observation that DPI attenuates further the increase in CBF produced by NADPH in gp91-null mice would suggest that non-gp91phox-containing NADPH oxidases also play a role. However, the possibility that the DPI-induced attenuation is mediated by effects on other flavoproteins, such as NOS, cannot be ruled out.

In vascular cells, NADPH oxidase has been reported to be intracellular. Therefore, another question concerns how exogenous NADPH enters the cell to fuel NADPH oxidase. NADPH is a relatively large and charged molecule that hardly crosses cell membranes. However, the observation that NADPH increases NADPH oxidase–dependent ROS production both in vitro and in vivo (present study and others) suggests that this nucleotide, either directly or indirectly, triggers NADPH oxidase activation.

Another question concerns the mechanisms by which NADPH activates the oxidase. If NADPH oxidase is basally active and assuming that substrate availability is rate limiting, then NADPH could increase its activity by providing more substrate. On the other hand, if NADPH oxidase is not active in the basal state, then it must be assumed that NADPH leads to the assembly and activation of the enzyme. Some studies suggest that vascular NADPH oxidase is basally active. However, it remains to be established whether this is also the case in our experimental preparation. If NADPH oxidase is basally active, our finding that gp91ds-tat does not affect resting CBF would indicate that its ROS output is not sufficient to influence CBF. Irrespective of the mechanisms of activation, the observation that exogenous NADPH increases CBF, in part, through NADPH oxidase–mediated mechanisms suggests that the availability of NADPH may regulate the activity of the enzyme and thus, may play a role in CBF regulation.

The results of the present study are in agreement with those of in demonstrating that a component of the CBF response induced by NADPH is dependent on NADPH oxidase. However, at variance with those studies, we found that l-NNA also attenuated the increase in CBF, a finding consistent with the hypothesis that NO contributes to the CBF increase. Although the fact that 7-NI did not alter the response is consistent with the hypothesis that eNOS, rather than nNOS, is involved in the mechanisms of the vasodilation, further data are needed to establish this point more firmly. In gp91-nulls, a small (<10%) residual response was present after l-NNA, the significance of which is unclear. If indeed NO is involved in the response, its role could be at least 2-fold. One possibility is that the component of the response mediated by NO is distinct from that initiated by NADPH oxidase. In this scenario, NO and ROS would be independent effectors of the vascular changes. Another possibility is that NO reacts with superoxide produced by NADPH oxidase to generate peroxynitrite, which in turn, mediates the vasodilation. In this case, NO and ROS would act in concert to produce the vasodilation through a common effector, peroxynitrite. However, we have no data in support of these possibilities. Irrespective of the mechanisms of the involvement of NO, our observation that catalase did not alter the CBF response to NADPH supports the hypothesis that H2O2 is not involved.

In conclusion, we have demonstrated that the increase in CBF produced by NADPH is in part mediated by NADPH oxidase. We also found that NOS is also involved in the mechanisms of the vasodilation. Although these findings suggest that NADPH oxidase may be active in the cerebral circulation, they also indicate the mechanisms of the vasodi-
loration produced by NADPH are complex, involving both NADPH oxidase–dependent and –independent factors.

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References

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