Flow-Induced Dilation Is Mediated by Akt-Dependent Activation of Endothelial Nitric Oxide Synthase-Derived Hydrogen Peroxide in Mouse Cerebral Arteries

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Background and Purpose—Endothelial nitric oxide synthase produces superoxide under physiological conditions leading to hydrogen peroxide (H2O2) -dependent dilations to acetylcholine in isolated mouse cerebral arteries. The purpose of this study was to investigate whether H2O2 was involved in flow-mediated dilation (FMD).

Methods—Cerebral arteries were isolated from 12±2-week-old C57Bl/6 male mice. FMD (0 to 10 μL/min, 2-μL step increase at constant internal pressure) was induced in vessels preconstricted with phenylephrine (30 μmol/L). Simultaneously to diameter acquisition, H2O2 or nitric oxide production was detected by the fluorescent dyes CMH2CFDA or 4,5-diaminofluorescein diacetate, respectively. Results are expressed as mean±SEM of 6 to 8 mice.

Results—FMD (at 10 μL/min, 25±3% of maximal diameter) was prevented (P<0.05) by endothelium removal (6±1%) or endothelial nitric oxide synthase inhibition with N-nitro-L-arginine (11±1%) but not by the specific nitric oxide scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl3-oxide (23±3%). Addition of PEG-catalase and silver diethyl dithio-carbamate (superoxide dismutase inhibitor) reduced (P<0.05) FMD to 10±2% and 15±1%, respectively. Simultaneously to FMD, H2O2-associated rise in fluorescence (+133±19 a.u.) was prevented by N-nitro-L-arginine, PEG-catalase, and silver diethyl dithio-carbamate (+55±10, +64±4, and +50±10 a.u., respectively; P<0.05). Inhibition of FMD by PEG-catalase was fully restored by the addition of tetrahydrobiopterin, a cofactor of endothelial nitric oxide synthase (23±3%); this functional reversal in dilation was associated with the simultaneous increase in nitric oxide-associated fluorescence (+418±58 a.u., P<0.05), which was prevented by 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl3-oxide (+93±26 a.u.). Akt inhibition with triciribine prevented FMD and H2O2-associated rise in fluorescence (3±1% and +23±4% a.u., respectively; P<0.05), but not acetylcholine-induced dilation.

Conclusion—In healthy C57Bl/6 mouse cerebral arteries, Akt-dependent activation of endothelial nitric oxide synthase-derived H2O2 mediates flow-dependent dilation. (Stroke. 2009;40:1827-1833.)

Key Words: endothelium ■ nitric oxide ■ oxygen radicals ■ resistance arteries

Shear stress is one of the most important physiological stimuli for vasodilation and is greatly implicated in the regulation of vascular tone and vascular homeostasis by contributing to the maintenance of organ perfusion and vascular integrity.1,2 Several studies have evaluated the implication of endothelial nitric oxide synthase (eNOS) and cyclo-oxygenase (COX) derivatives in flow-mediated dilation (FMD).3–6 However, the implication of each endothelium-dependent relaxing factor (EDRF) varies among vascular beds and pathological states.3,7–11

The release of superoxide (O2•-) has been reported during FMD both in peripheral12,13 and cerebral arteries.14,15 Although an increase in reactive oxygen species (ROS) is traditionally considered as a pathological response, recent investigations show that ROS are implicated in the regulation of vascular function.12,16–18 It is unclear, however, if this concept applies to the physiological regulation of vascular tone and FMD. In a recent study, our group showed that hydrogen peroxide (H2O2) is an EDRF produced by eNOS activity after muscarinic receptor stimulation.19 H2O2 was also reported to induce endothelium-dependent and -independent dilation in healthy rat cerebral arteries18 and identified as an EDRF in FMD of human coronary arteries.1 These results led to the hypothesis that H2O2 could be physiologically implicated in the regulation of cerebral arteries.

The origin of O2•- that leads to the physiological formation of H2O2 is still unsettled. eNOS, however, is able to generate O2•- during enzymatic cycling.20,21 The eNOS-dependent generation of O2•- is nonetheless proposed to be functionally significant only in pathological conditions and related to the limited availability of l-arginine, the substrate of eNOS as well as of its essential cofactor tetrahydrobiopterin (BH4).22,23 In a recent study, Shimokawa’s group suggested that the
NOs system has diverse vasodilator functions depending on the vessel size leading to superoxide production rather than nitric oxide in small vessels. There is therefore a need for determining the physiological role of eNOS derived H\textsubscript{2}O\textsubscript{2} in FMD in cerebral arteries.

We hypothesized that eNOS produces physiologically relevant levels of free radicals leading to H\textsubscript{2}O\textsubscript{2}-dependent FMD in healthy mouse cerebral arteries.

Materials and Methods

Animals and Tissue Preparation

The procedures and protocols were performed in accordance with our institutional guidelines and the Guide for the Care and Use of Laboratory Animals of Canada. Experiments were conducted on cerebral arteries isolated from 3-month-old male C57BL/6 mice (29 ± 1 g, n = 66; Charles River Laboratories, Quebec, Canada) using a method previously described. Mice were euthanized by CO\textsubscript{2} inhalation and the brain was rapidly removed and placed in ice-cold physiological salt solution (PSS) of the following composition (mmol/L): NaCl 130, KCl 4.7, CaCl\textsubscript{2} 1.6, MgSO\textsubscript{4} 1.7, NaHCO\textsubscript{3} 14.9, KH\textsubscript{2}PO\textsubscript{4} 1.18, EDTA 0.026, glucose 10. In all experiments, the PSS was oxygenated by a gas mixture containing 12% O\textsubscript{2}, 5% CO\textsubscript{2}, and 83% N\textsubscript{2}, generating a pO\textsubscript{2} of 150 mmHg.

Cerebral arteries (anterior, posterior, and posterior communicating; internal diameter of 136±2 μm when pressurized at 60 mm Hg) were carefully isolated, cannulated at both ends, and pressurized at 60 mm Hg on a pressure myograph (Living Systems Instrumentation, Burlington, VT). The tips of the pipettes used in the study were calibrated and carefully checked to match the vessel inner diameter to minimize resistances.

Reactivity Studies

An equilibration period of 40 minutes was allowed before starting the experiment and the resulting myogenic tone was measured (\(D_{\text{max}} - D_{\text{after}}\))/\(D_{\text{max}}\)\(^{10}\), where \(D_{\text{after}}\) is the diameter before equilibration, \(D_{\text{max}}\) is the diameter after equilibration time, and \(D_{\text{min}}\) is the maximal diameter obtained in Ca\textsuperscript{2+}-free solution. Similar levels of preconstrictions with phenylephrine (PE; 10 to 30 μmol/L) were allowed for each 2-minute period.

ROS-reacting fluorescent dye; Molecular Probe, Invitrogen, Burlingame, Calif\textsuperscript{19,31} 30 minutes before the beginning of the experiment, with or without inhibitors. Vessels were then washed with PSS, preconstricted with PE, and dilated with flow (0 to 10 μL/min) or with a single dose of ACh (30 μmol/L) while recording simultaneously the changes in diameter and in fluorescence intensities of fluorescein retained intracellularly after cleavage of the acetate moieties. Fluorescence intensities at 492 to 495 nm (excitation) were measured at 520 nm with an IonOptix Acquire system for 3 minutes where \(r\) is the shear stress (dyn/cm\textsuperscript{2}), \(\gamma\) is the viscosity (0.8 cp), \(Q\) is the flow rate through the lumen, and \(r\) is the inside radius. In this study, the calculated shear stress was comparable to the physiological value of shear stress in arterioles of this size.\textsuperscript{25}

To study the eNOS activity, cerebral arteries were incubated in oxygenated PSS (37°C) containing either 5 μmol/L of 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCF-DA, a ROS-reacting fluorescent dye; Molecular Probe, Invitrogen, Burlington, Ontario, Canada)\textsuperscript{29} or 10 μmol/L of 4,5-diaminofluorescein diacetate (DAF-2, a nitric oxide-reacting fluorescent dye; Calbiochem, San Diego, Calif\textsuperscript{30,31}) 30 minutes before the beginning of the experiment, with or without inhibitors. Vessels were then washed with PSS, preconstricted with PE, and dilated with flow (0 to 10 μL/min) or with a single dose of ACh (30 μmol/L) while recording simultaneously the changes in diameter and in fluorescence intensities of fluorescein retained intracellularly after cleavage of the acetate moieties. Fluorescence intensities at 492 to 495 nm (excitation) were measured at 520 nm with an IonOptix Acquire system (IonOptix, Milton, Mass). Before each experiment, basal fluorescence intensity was recorded. Results represent differences between stimulated and basal intensity.

Statistics

\(n\) refers to the number of animals used in each protocol. Continuous variables are expressed as mean ± SEM. The maximal diameter (\(D_{\text{max}}\)) was determined by changing the PSS to a Ca\textsuperscript{2+}-free PSS\textsuperscript{19} Dilutions are expressed with the following equation: \((D_{\text{max}} - D_{\text{after}})/D_{\text{max}}\)\(^{10}\), where \(D_{\text{after}}\) is the diameter at the end of equilibration time, and \(D_{\text{max}}\) is the maximal diameter obtained in Ca\textsuperscript{2+}-free solution. Similar levels of preconstrictions with phenylephrine (PE; 10 to 30 μmol/L) were allowed for each 2-minute period. All drugs were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada), except for triciribine and DT-3, which were purchased from Calbiochem EMD Chemicals Inc (San Diego, Calif). All drugs were directly added to the bath chamber (extraluminal) and the final concentration of ethanol or DMSO never exceeded 0.1%.

| Table. Myogenic Tone (MT), Contraction With PE (10 or 30 μmol/L), and Maximal Dilation to FMD (10 μL/min) of Cerebral Arteries Isolated From C57Bl/6 Male Mice* |
|----------------------------------|--------|--------|--------|
| Groups                           | MT (%) | PE (%) | FMD (%) |
| Control conditions               | 8 ± 1  | 40 ± 2 | 25 ± 3  |
| +L-NNA (10 μmol/L)               | 21 ± 4† | 46 ± 5 | 11 ± 1† |
| +PEG-catalase (50 U/mL)          | 15 ± 3† | 43 ± 3 | 16 ± 1† |
| +PEG-catalase (100 U/mL)         | 19 ± 7† | 46 ± 5 | 10 ± 2† |
| +BH4 (1 mmol/L)                  | 16 ± 3 | 42 ± 4 | 28 ± 3  |
| +l-arginine (5 μmol/L)           | 12 ± 8  | 49 ± 5 | 24 ± 1  |
| +PTIO (100 μmol/L)               | 8 ± 2   | 40 ± 4 | 26 ± 2  |
| +ODQ (1 mol/L)                   | 25 ± 12† | 49 ± 4 | 4 ± 2†  |
| +DT-3 (25 nmol/L)                | 7 ± 2   | 39 ± 6 | 11 ± 1† |
| +BH4 + PTIO                      | 15 ± 4† | 39 ± 7 | 20 ± 4  |
| +BH4 + PEG-catalase              | 18 ± 5† | 45 ± 5 | 26 ± 3  |
| +BH4 + ODQ                       | 12 ± 5  | 45 ± 4 | 7 ± 2†  |
| +BH4 + L-NNA                     | 17 ± 4† | 44 ± 3 | 11 ± 2† |
| +BH4 + PEG-catalase + PTIO       | 21 ± 9† | 47 ± 5 | 9 ± 2†  |
| +Pyruvate (3 mmol/L)             | 8 ± 2   | 42 ± 3 | 15 ± 2† |
| +DETC (1 mol/L)                  | 15 ± 2† | 47 ± 3 | 15 ± 1† |
| +L-NNA + indomethacin (10 μmol/L) | 8 ± 3 | 36 ± 3 | 10 ± 3† |
| +PEG-catalase + PTIO             | 7 ± 2   | 39 ± 4 | 14 ± 3† |
| +Triciribine (1 μmol/L)          | 11 ± 3  | 45 ± 5 | 3 ± 1†  |
| +Apocynin (10 μmol/L)            | 12 ± 5  | 45 ± 4 | 33 ± 4  |
| −Endothelium                    | 12 ± 5  | 39 ± 9 | 6 ± 1†  |

Note: Data are expressed as mean ± SEM.

*MT, PE, and FMD are expressed as the percentage of the maximal diameter.

†P<0.05 compared with control conditions.
Results

Implication of Hydrogen Peroxide and Endothelial Nitric Oxide Synthase in Flow-Mediated Dilation

Flow-mediated dilations of cerebral arteries were reduced by nitric oxide synthase inhibition with L-NNA (10 μmol/L; Figure 1A; Table). The nitric oxide scavenger PTIO (100 μmol/L), however, had no impact on the dilation induced by flow (Figure 1A; Table). The cell-permeable PEG-catalase (50 and 100 U/mL) reduced FMD in a dose-dependent manner (Figure 1; Table). The likely involvement of H₂O₂ was further confirmed by the inhibitory effect of pyruvate (3 mmol/L), a H₂O₂ scavenger, and DETC (1 mmol/L), a superoxide dismutase inhibitor (Figure 1B; Table).

To confirm these pharmacological data, H₂O₂ production was assessed in pressurized vessels after incorporation of the fluorescent ROS-reactive dye, DCF-DA.¹⁹,²⁹,³⁰ FMD was associated with an increase in fluorescence intensity: ROS-dependent signals (Figure 2A, C) were abolished by L-NNA, PEG-catalase, DETC, and pyruvate, demonstrating the specificity of the dye for H₂O₂ in our experimental conditions. Apocynin, a NAD(P)H oxidase inhibitor, neither reduced FMD (Table) nor DCF-DA fluorescence (Figure 2C).

Nitric oxide production was assessed after incorporation of the fluorescent nitric oxide-reactive dye, DAF-2.¹⁹ FMD was not associated with an increase in fluorescence intensity, which was unaffected by the addition of PEG-catalase and DETC (Figure 2B, D).

In contrast, addition of the nitric oxide donor detaNONOate (0.1 μmol/L) induced a potent dilation of 73±5% of D_max and increased DAF-associated fluorescence by 959±47 a.u.
These responses were reduced ($P<0.05$) by addition of PTIO (46±6% and 88±24 a.u., respectively).

**Effects of Tetrahydrobiopterin on Endothelial Nitric Oxide Synthase Activity and Nitric Oxide Production**

Addition of BH$_4$ (1 mmol/L) did not alter FMD (Figure 3A; Table) but led to the production of nitric oxide as revealed by the appearance of a strong DAF-2-associated fluorescence that was prevented by PTIO and L-NNA (Figure 2B, D). Although PTIO or PEG-catalase alone did not alter FMD in the presence of BH$_4$ (Table), the dilation to flow was prevented by combining PTIO and PEG-catalase (Table). This was associated with a reduction in both nitric oxide- and H$_2$O$_2$-associated fluorescence from 418±58 and 571±94 a.u. to 45±4 and 58±20 a.u., respectively ($P<0.05$).

In contrast to BH$_4$, L-arginine (5 mmol/L) neither affected FMD (10 μL/min; Table) nor nitric oxide-associated fluorescence (26±4 a.u.).

**Vasodilator Mechanism of Action of Hydrogen Peroxide**

ODQ (1 μmol/L), an inhibitor of the sGC, reduced the dilation triggered by flow as efficiently as endothelial denudation (Figures 3B and 4; Table). ODQ, however, did not prevent H$_2$O$_2$-associated rise in fluorescence (Figure 3C). In the presence of BH$_4$, ODQ still reduced FMD (Figure 3A). Cell-permeant G protein kinase inhibition with DT-3 reduced FMD, but not the H$_2$O$_2$-associated rise in fluorescence (Figure 3B, C).

**Endothelial Regulation of Flow-Mediated Dilation**

FMD was strongly reduced by L-NNA with no additive effects of indomethacin, suggesting no involvement of COX derivatives during FMD of mouse cerebral arteries (Figure 4). FMD and its associated increase in H$_2$O$_2$ fluorescence were abolished by Akt inhibition with triciribine (Figure 5A, C; Table). In contrast, triciribine only limited ACh-induced endothelium-dependent dilation (30 μmol/L) and its associated increase in H$_2$O$_2$ fluorescence (Figure 5B, D).

**Impact of Endothelial Nitric Oxide Synthase Activity on Myogenic Tone**

L-NNA, PEG-catalase, DETC, and ODQ increased myogenic tone, whereas Akt inhibition and PTIO had no effect (Table). The rise in myogenic tone induced by L-NNA was prevented by COX-1/2 inhibition, suggesting that addition of L-NNA reveals a COX-derived contracting factor (Table).

**Exogenous Hydrogen Peroxide Dilation**

Exogenous H$_2$O$_2$ (10 μmol/L, n=4) induced a large dilation (65±4%). Addition of L-NNA or BH$_4$ did not affect this response (60±6% and 52±7%, respectively). Addition of ODQ, however, prevented H$_2$O$_2$-induced dilation (6±2%).
H$_2$O$_2$-Induces FMD in Mouse Cerebral Arteries

Discussion

The main finding of the present study is that FMD is mediated by H$_2$O$_2$ derived from eNOS activity in pressurized cerebral arteries isolated from young and healthy C57Bl/6 mice. FMD was sensitive to eNOS inhibition and H$_2$O$_2$ scavengers and associated with an increase in H$_2$O$_2$-associated fluorescence. In addition, Akt-dependent activation of eNOS is mandatory for FMD, unlike for ACh-induced dilation. These results support the concept that eNOS-derived H$_2$O$_2$ is a physiologically relevant signaling molecule in healthy mice.

Endothelium removal abolished FMD in cerebral artery consistent with previous reports. Inhibition of eNOS by L-NNA limited the dilation similarly to H$_2$O$_2$ scavengers, whereas PTIO, a nitric oxide scavenger, had no impact. These results strongly suggest that H$_2$O$_2$ originates from stimulated eNOS activity and is responsible for FMD in mouse cerebral arteries. This hypothesis is supported by the absence of H$_2$O$_2$-associated fluorescence in cerebral arteries incubated in the presence of L-NNA and PEG-catalase and by the demonstration that FMD is not associated with an increase in nitric oxide-associated fluorescence. These data are in line with recent work showing that H$_2$O$_2$ is an important signaling molecule in cerebral and peripheral arteries.

We previously reported that H$_2$O$_2$ is an EDRF derived from eNOS activity after Ach-induced dilation of mouse arteries. It is known that eNOS is able to generate O$_2$ during enzymatic cycling. The eNOS-dependent generation of O$_2$ is proposed, however, to be only functionally significant in pathological conditions and related to the limited availability of eNOS cofactors. In this study, the addition of BH$_4$, but not L-arginine, induced an increase in nitric oxide-associated fluorescence, but neither BH$_4$ nor L-arginine had significant effect on the extent of FMD. This increase in nitric oxide-associated fluorescence intensity was blocked by PTIO and L-NNA, confirming that eNOS produces nitric oxide in the presence of an excess of BH$_4$. The level of BH$_4$ is significantly lower in the brain than in other mouse organs as previously reported by Heales’ group; this could be a reason for the preferential production of H$_2$O$_2$, but it remains to be demonstrated. The NAD(P)H oxidase is known to be a source of superoxide and H$_2$O$_2$ during endothelium-dependent dilation of rat cerebral arteries.

Our data, those of Rosenblum, and of Sobey’s group demonstrate that free radicals are involved in the physiological regulation of the cerebrovascular tone. Free radicals, however, are commonly associated with endothelial dysfunction and cellular damages triggered by oxidative stress. Many physiological regulatory effects are mediated by H$_2$O$_2$ and other ROS that are chemically derived from superoxide.

The fact that PTIO addition blocked the rise in nitric oxide-associated fluorescence after the addition of dethioperoxidate demonstrates the specificity of DAF-2 dye for nitric oxide as previously discussed. DCF-DA is known to recognize all ROS species at high concentrations such as O$_2$-, OH-, and H$_2$O$_2$ with a greater specificity for ONOO$. DCF-DA fluorescence signal was, however, abolished by PEG-catalase, pyruvate, and DETC, but not PTIO (data not showed), demonstrating greater selectivity of the dye for H$_2$O$_2$ than nitric oxide or its derived reactive nitrogen species. Hence, in these healthy arteries, and in our experimental conditions, peroxynitrites are not produced.

The mechanism of eNOS activation by flow is not fully understood. It has been proposed that Akt-dependent phosphorylation of eNOS is essential for nitric oxide production. Our study demonstrates that FMD is abolished by Akt inhibition and this was associated with a reduction in H$_2$O$_2$-associated fluorescence. Thus, shear stress-dependent activation of Akt is responsible for eNOS activation. To support this hypothesis, we used another Akt inhibitor (LY294002). LY294002, however, is also a PI3-K inhibitor and reduced the precontraction induced by PE (data not shown), preventing its use. In contrast to flow, muscarinic receptor activation directly promotes eNOS activation without requiring Akt-dependent phosphorylation of eNOS.
dependent activation. Importantly, neither the dilation nor H₂O₂-associated fluorescence was abolished by Akt inhibition after muscarinic receptor stimulation. Akt inhibition reduced, nonetheless, ACh-dependent dilation, suggesting that there is a basal level of Akt activity influencing eNOS cycling.

Addition of ODQ to block the sGC abrogated FMD without affecting H₂O₂-associated fluorescence. Furthermore, exogenous H₂O₂-induced dilation was unaffected by L-NNA or BH₄, but prevented by ODQ, which is in agreement with our previous report. To further validate the involvement of the cGMP pathway in FMD, we used a cell-permeant G protein kinase inhibitor, FMD dilation, but not H₂O₂ production, was abolished by DT-3, confirming the implication of cGMP in H₂O₂-dependent FMD. In the presence of BH₄, FMD was also abolished by ODQ, confirming that H₂O₂ shares with nitric oxide a similar vasodilator pathway. FMD was not completely blocked by L-NNA with no additive effects of indomethacin, suggesting the involvement of another EDRF. Previous studies suggested the role of an EDRF. This factor may account for the residual response obtained in the presence of L-NNA. It is also interesting that COX inhibition had no effect on FMD. This is another difference with ACh-induced endothelium dependent dilation of cerebral arteries in which prostacyclin significantly contributes to this response. Like in our previous report, myogenic tone was increased by L-NNA but reduced by combining indomethacin, suggesting that in the absence of nitric oxide, a COX-derived contracting factor is produced or its effect revealed. We observed the same response in renal arteries, which suggests that both constrictors and dilators coexist to regulate tone. In age and in the presence of risk factor for cardiovascular diseases, the expression of these contracting factors dominate as a consequence of a reduced nitric oxide synthase activity.

In conclusion, our results suggest that flow triggers Akt-dependent eNOS activation leading to a dilation essentially induced by H₂O₂ in pressurized cerebral arteries isolated from healthy and young C57Bl/6 mice. Like nitric oxide, H₂O₂ activates the sGC. Our results highlight the multifaceted function of eNOS in its mechanisms of regulation of vascular function.

Sources of Funding
This work has been supported in part by the Foundation of Montreal Heart Institute, the Heart and Stroke Foundation of Quebec, and the Canadian Institute for Health Research (MOP87388). A.H. holds the Frederick Banting and Charles Best Canada Graduate Scholarships–Doctoral Award in association with the Canadian Institute for Health Research.

Disclosures
None.

References


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*Stroke*. 2009;40:1827-1833; originally published online March 12, 2009; doi: 10.1161/STROKEAHA.108.536805

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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