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-oxide (24±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 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.

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
NOs system has diverse vasodilator functions depending on the vessel size leading to superoxide production rather than nitric oxide in small vessels.\textsuperscript{24} There is therefore a need for determining the physiological role of eNOS derived H$_2$O$_2$ in FMD in cerebral arteries.

We hypothesized that eNOS produces physiologically relevant levels of free radicals leading to H$_2$O$_2$-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.\textsuperscript{19} Mice were euthanized by CO$_2$ 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$_2$ 1.6, MgSO$_4$ 1.17, NaHCO$_3$ 14.9, KH$_2$PO$_4$ 1.18, EDTA 0.026, glucose 10. In all experiments, the PSS was oxygenated by a gas mixture containing 12\% O$_2$, 5\% CO$_2$, and 83\% N$_2$, generating a P$_O_2$ of 150 mm Hg. 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 (\(\text{MT}_{\text{ref}}\))\textsuperscript{100}, where \(\text{D}_{\text{ref}}\) is the diameter before equilibration, \(\text{D}_{\text{max}}\) is the diameter after equilibration time, and \(\text{MT}_{\text{max}}\) is the maximal diameter obtained in Ca$^{2+}$-free solution. Similar levels of preconstrictions with phenylephrine (PE; 10 to 30 μmol/L; Table) were obtained before each experiment. FMD was induced using a flow control peristaltic pump (Living Systems Instrumentation) directly connected to the pressured myograph. A single cumulative FMD curve (0 to 10 μL/min, 2-μL step increase at constant internal pressure) was performed on each segment. Two minutes were allowed for each 2-μL step increase. Shear stress was calculated using the following equation: \(\text{S} = 4\pi Q / \pi r^2\) where \(r\) is the shear stress (dyn/cm$^2$), \(Q\) the flow rate through the lumen, and \(r\) 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}

We used (1) PEG-catalase (50 and 100 U/mL); (2) N-nitro-L-arginine (L-NNA; 10 μmol/L), an eNOS inhibitor; (3) pyruvate (3 mmol/L), a H$_2$O$_2$ scavenger;\textsuperscript{26} (4) silver diethyldithiocarbamate (DETC; 1 mmol/L), an eNOS inhibitor; (5) 2-phenyl-

**Fluorescence Studies**

Pressurized 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, Molecular Probes, Invitrogen, Burlington, Ontario, Canada) or 10 μmol/L of 4,5-diaminofluorescein diacetate (DAF-2, a nitric oxide-reacting fluorescent dye; Calbiochem, San Diego, Calif.\textsuperscript{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 acetoxymesters. 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 (\(\text{D}_{\text{max}}\)) was determined by changing the PSS to a Ca$^{2+}$-free PSS.\textsuperscript{19} Dilutions are expressed with the following equation: \((\text{D}_{\text{f}} - \text{D}_{\text{min}})/\text{D}_{\text{max}})*100\), where \(D_f\) is the diameter obtained at each flow value and \(D_{\text{min}}\) is the diameter of PE-induced constriction. One-way analysis of variance was performed to compare the effect of the different inhibitors on FMD curves and the increase in fluorescence intensity at a flow rate of 10 μL/min. Differences were considered to be statistically significant when the probability value was <0.05 (Scheffe’s F test).

| 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±3 | 10±2† |
| +BH$_4$ (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 mmol/L) | 25±12† | 49±4 | 4±2† |
| +DT-3 (25 nmol/L) | 7±2 | 39±6 | 11±1† |
| +BH$_4$ + PTIO | 15±4† | 39±7 | 20±4 |
| +BH$_4$ + PEG-catalase | 18±5† | 45±5 | 26±3 |
| +BH$_4$ + ODQ | 12±5 | 45±4 | 7±2† |
| +BH$_4$ + L-NNA | 17±4† | 44±3 | 11±2† |
| +BH$_4$ + PTIO + PEG-catalase | 21±9† | 47±5 | 9±2† |
| +Pyruvate (3 mmol/L) | 8±2 | 42±3 | 15±2† |
| +DETC (1 mmol/L) | 15±2† | 47±3 | 15±2† |
| +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 Dₘₐₓ and increased DAF-associated fluorescence by 959±47 a.u.
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 ±L/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).

(n=4). These responses were reduced (P<0.05) by addition of PTIO (46±6% and 88±24 a.u., respectively).

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%).
of mouse arteries. It is known that eNOS is able to generate 
\( \text{O}_2^{*} \) during enzymatic cycling.\(^{20,21} \) The eNOS-dependent 
generation of \( \text{O}_2^{*} \) is proposed, however, to be only function-
ally significant in pathological conditions and related to the 
limited availability of eNOS cofactors.\(^{22,23} \) 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 pro-
duces 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\(^{35} \); this 
could be a reason for the preferential production of H\(_2\)O\(_2\), but 
itis 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.\(^{14} \) In 
our hands, apocynin failed to reduce FMD as well as the 
increase in H\(_2\)O\(_2\)-associate fluorescence, suggesting no in-
volvement in the activity of the NAD(P)H oxidase in the 
H\(_2\)O\(_2\)-dependent dilation of mouse cerebral arteries.

Our data, those of Rosenblum,\(^{15} \) and of Sobey’s group\(^{14} \) 
demonstrate that free radicals are involved in the physiolog-
ical regulation of the cerebrovascular tone. Free radicals, 
however, are commonly associated with endothelial dysfunc-
tion and cellular damages triggered by oxidative stress.\(^{34} \) 
Many physiological regulatory effects are mediated by H\(_2\)O\(_2\) 
and other ROS that are chemically derived from superoxide.\(^{36} \) 
NAD(P)H oxidase, for example, is essential for the regeneration of 
GSH from GSSG, reactivation of H\(_2\)O\(_2\)-inactivated catalase, 
and regeneration of thioredoxin.\(^{37} \) It is therefore essential 
to maintain adequate levels of NAD(P)H as well as the normal 
function of these enzymes. Hence, controlled production of 
free radicals has a physiological meaning, whereas unregu-
lated oxidative stress is deleterious.

The fact that PTIO addition blocked the rise in nitric 
oxide-associated fluorescence after the addition of det-
aNONOate demonstrates the specificity of DAF-2 dye for 
nitric oxide as previously discussed.\(^{19} \) DCF-DA is known to 
recognize all ROS species at high concentrations such as \( \text{O}_2^{*} \), 
OH\(^-\), and H\(_2\)O\(_2\) with a greater specificity for ONOO\(^-\).\(^{38} \) 
DCF-DA fluorescence signal was, however, abolished by 
PGE-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 phos-
phorylation of eNOS is essential for nitric oxide production.\(^{14} \) 
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 activa-
tion 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), prevent-
ing its use. In contrast to flow, muscarinic receptor activation 
directly promotes eNOS activation without requiring Akt-

**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 physiolog-
ically relevant signaling molecule in healthy mice.

Endothelium removal abolished FMD in cerebral artery 
consistent with previous reports.\(^{5,7,13,32} \) 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 demon-
stration 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\(^14 \) and peripheral\(^{19} \) arteries.

We\(^{19} \) and others\(^{1,34} \) previously reported that H\(_2\)O\(_2\) is an 
EDRF derived from eNOS activity after Ach-induced dilation

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**Figure 5.** Effect of removal of the endothelium (-Endo) and tricir-
biné, an inhibitor of Akt (TB; 1 µmol/L) on (A) FMD (10 µL/min) and 
(B) endothelium-dependent ACh-induced (30 µmol/L) di-
lation of pressurized cerebral arteries isolated from C57Bl/6 mice. 
Effect of TB on H\(_2\)O\(_2\)-associated rise in fluorescence induced by 
(C) FMD (10 µL/min) and (D) ACh (30 µmol/L). *P<0.05 com-
pared with control; †P<0.05 compared with control; "n" numbers are 
in the Table). *P<0.05 compared with FMD.
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.19 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.18,39

FMD was not completely blocked by L-NNA with no additive effects of indomethacin, suggesting the involvement of another EDRF. Previous studies3,7 suggested the role of an EDRF during FMD. 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.19 Like in our previous report,19 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,40 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.31–43

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 C57BI/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.

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Disclosures
None.

References


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