Nitric oxide from perivascular nerves modulates cerebral arterial pH reactivity

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Lindauer, Ute, Alexander Kunz, Sigrid Schuh-Hofer, Johannes Vogt, Jens P. Dreier, and Ulrich Dirnagl. Nitric oxide from perivascular nerves modulates cerebral arterial pH reactivity. Am J Physiol Heart Circ Physiol 281: H1353–H1363, 2001.—In the isolated rat middle cerebral artery (MCA) we investigated the role of nitric oxide (NO)/cGMP in the vasodilatory response to extraluminal acidosis. Acidosis increased vessel diameter from 140 ± 27 μm (pH 7.4) to 187 ± 30 μm (pH 7.0, P < 0.01) and attenuated response to acidosis (9 ± 8 μm). Application of the NO-donors 3-morpholinosydnonimine (1 μM) or S-nitroso-N-acetylpenicillamine (1 μM), or of 8-bromoguanosine 3’,5’-cyclic monophosphate (8-BrcGMP, 100 μM) completely restored pH 7.0. NO synthase (NOS) inhibition by Nω-nitro-L-arginine (L-NNA, 10 μM) reduced baseline diameter (103 ± 20 μm, P < 0.01) and attenuated response to acidosis (9 ± 8 μm). The investigation of the source of NO and of the cellular compartment responsible for the basal NO level in the cerebral tissue within the cerebrovascular system is still of great importance. Many efforts are undertaken to increase this basal NO level to improve vascular reactivity under pathophysiological conditions. The most promising experimental approaches are the pharmacological induction of eNOS activity by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors [statins (13)] or the increase of eNOS activity by gene transfer (e.g., Ref. 2). However, to thoroughly assess the functional consequences of increased eNOS activity, we have at first to improve our knowledge on the specific role of eNOS and nNOS in cerebral vascular reactivity under physiological conditions.

The present study is the first to investigate the role of eNOS and nNOS in the NOS-dependent vasodilation to acidosis, using both pharmacological as well as nonpharmacological approaches in the same experimental setup. We examined whether NO originating from endothelial cells or perivascular nerves accounts for basal NO levels in isolated cerebral arteries and smooth muscle reactivity to extraluminal acidosis. In addition to pharmacological enzyme inhibition strategies, vasomotor endothelial cells or perivascular nerves accounts for basal NO levels in isolated cerebral arteries and smooth muscle reactivity to extraluminal acidosis. In addition to pharmacological enzyme inhibition strategies, vasomotor endothelial cells or perivascular nerves accounts for basal NO levels in isolated cerebral arteries and smooth muscle reactivity to extraluminal acidosis. In addition to pharmacological enzyme inhibition strategies, vasomotor endothelial cells or perivascular nerves accounts for basal NO levels in isolated cerebral arteries and smooth muscle reactivity to extraluminal acidosis. In addition to pharmacological enzyme inhibition strategies, vasomotor endothelial cells or perivascular nerves accounts for basal NO levels in isolated cerebral arteries and smooth muscle reactivity to extraluminal acidosis. In addition to pharmacological enzyme inhibition strategies, vasomotor endothelial cells or perivascular nerves accounts for basal NO levels in isolated cerebral arteries and smooth muscle reactivity to extraluminal acidosis.

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eration was induced by in vivo chronic dissection of the nasociliary nerve (NCN) and parasympathetic fibers (PSP) originating from the sphenopalatine ganglion 14 days before isolation of the arteries.

**MATERIALS AND METHODS**

Male Wistar rats (240–260 g) were anesthetized with halothane and then decapitated. The brain was rapidly removed from the skull and placed in cold (4°C) 3-[N-morpholino]propanesulfonic acid (MOPS)-buffered saline solution (with 1% dialyzed bovine serum albumin) containing (in mM) 144 NaCl, 3.0 KCl, 2.5 CaCl$_2$, 1.5 MgSO$_4$, 1.21 Na$_2$HPO$_4$, 0.02 EDTA, 2.0 pyruvate, 2.0 MOPS, and 5.0 glucose (11), pH 7.40 ± 0.02. Approximately 1 cm of the middle cerebral artery (MCA) was carefully dissected from the brain surface beginning at the circle of Willis. The surrounding parenchymal tissue was carefully removed, leaving the perivascular nerve endings within the adventitial layer intact as shown for NOS-positive fibers in Fig. 7A. The MCA was transferred into an organ chamber (arteriograph, Living Systems; Burlington, VT) containing cold (4°C) MOPS-buffered saline solution (with 1% dialyzed bovine serum albumin, as described above). Glass pipettes were inserted into each end of the artery, and a segment of the artery without branches, ~1 mm in length, was placed between the micropipettes. Two 10-0 nylon threads were used on each side to fix the MCA on the glass pipettes. The vessel was continuously perfused with an intraluminal flow of 100 μl/min of MOPS-buffered saline solution containing 1% bovine serum albumin (37°C) at a transmural pressure of 80 mmHg due to raised in- and outflow reservoirs. The extraluminal bath was then switched to MOPS-buffered saline solution without bovine serum albumin, heated to 37°C, and continuously exchanged at a rate of 20 ml/min (22).

The vessel chamber was placed on an inverted microscope equipped with a video camera and monitor for online analysis of luminal diameters of the arteries. The camera was connected to a personal computer to store video images after each manipulation for later off-line analysis.

Before the experiments were started, the vessels were allowed to equilibrate for 1 h. Within this time, spontaneous tone developed, reducing resting diameter to ~80% compared with diameters measured directly after pressurizing. During the entire experiment, temperature of the extraluminal bath, perfusion inflow pressure, and flow rate were monitored and kept constant.

Acetylcholine was applied intraluminally by adding the drug into the reservoir of the inflow pipette; all other pharmacologically active substances were added to the reservoir of the extraluminal bath. Because of continuous circulation of the buffer solution, drug concentrations changed slowly in the organ chamber and needed 5–10 min to equilibrate.

Acidotic vasodilation was induced by changing the pH of the extraluminal bath to 7.00 ± 0.03 by adding HCl to the MOPS-buffered saline solution in the reservoir. Luminal diameters were registered after an equilibration period of 15 min. By changing the extraluminal bath to buffer solution with physiological pH values, vessels were allowed to return to baseline diameters.

**Experimental Design**

*Studies I–V.* The modulatory role of NO in the isolated cerebral artery was studied, which included the influence of NOS inhibition and subsequent vasodilator application (NO donors, cGMP, NO-independent vasodilators) on vessel response to extraluminal acidosis (see Fig. 1).

After a baseline response to acidosis was recorded, unspecific NOS inhibition was induced by adding 10 μM N^3^-nitro-L-arginine (L-NNA, Sigma) to the extraluminal bath for 30 min. The vessel response to acidosis was tested again. In studies I (n = 5) and II (n = 4), the NO donors 3-morpholinosydnonimine (SIN-1, 1 μM in MOPS buffer solution containing 10 μM L-NNA, Research Biochemicals International) or S-nitroso-N-acetylpenicillamine (SNAP, 1 μM in MOPS buffer solution containing 10 μM L-NNA, Research Biochemicals International), respectively, were then added to the extraluminal bath solution for 10 to 15 min to restore resting baseline diameter to pre-L-NNA levels. The response to extraluminal acidosis was tested again. In studies III (n = 4), IV (n = 3), and V (n = 3), the cell-permeable cGMP analog 8-bromoguanosine 3’,5’-cyclic monophosphate (8-BrcGMP, 100 μM in MOPS buffer solution containing 10 μM L-NNA; Sigma), the NO-independent vasodilators papaverine (20 μM in MOPS buffer solution containing 10 μM L-NNA; Sigma), or nifedipine (30 nM in MOPS buffer solution containing 10 μM L-NNA; Sigma), respectively, were used instead of the NO donors, SIN-1 or SNAP, to reestablish the pre-L-NNA resting vessel diameter.

*Study VI.* The modulatory role of cGMP in the isolated cerebral artery was studied, which included the influence of guanylyl cyclase inhibition and subsequent cGMP application on vessel response to extraluminal acidosis. After a baseline response to acidosis was recorded, soluble guanylyl cyclase inhibition and subsequent cGMP application was induced by adding 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) to the extraluminal bath for 30 min (5 μM ODQ dissolved in ethanol with a final concentration of 0.1% ethanol in MOPS buffer solution; Research Biochemicals International). The vessel response to acidosis was tested again (n = 6). In two of these arteries, the cell-permeable cGMP analog 8-BrcGMP (30 μM in MOPS buffer solution containing 5 μM ODQ; Sigma) was then added to the extraluminal bath solution for 10–15 min to restore resting baseline diameter to ODQ levels. The response to extraluminal acidosis was tested again.

*Studies VII–X.* The role of different NOS isoforms (neuronal vs. endothelial) was studied by testing the influence of inhibition of NOS via 7-nitroindazole (7-NI) application or chronic degeneration of perivascular nerve fibers (NCN and PSP) or of acute removal of vascular endothelium on vessel response to extraluminal acidosis.

*Studies VII and VIII.* We investigated the contribution of 7-NI inhibitable NOS or of nNOS, mainly located in perivascular nerve fibers in the vessel wall (17), on basal NO levels in the isolated artery. After a baseline response to acidosis was recorded in *study VII* (n = 8), NOS inhibition was induced by adding 7-NI (100 μM, dissolved in ethanol, with a final concentration of 0.25% ethanol in MOPS buffer solution; Sigma) to the extraluminal bath for 15 min. After the vessel response to acidosis was tested, 8-BrcGMP (30 μM in MOPS buffer solution containing 100 μM 7-NI; Sigma) was added to the extraluminal bath solution for 15 min to restore resting baseline diameter to pre-7-NI levels. The response to extraluminal acidosis was tested again. In two additional arteries, the effect of up to 0.25% ethanol in MOPS buffer solution to extraluminal acidosis was tested, and no effect of the vehicle ethanol on the acidic vasodilation response was observed (vehicle control to *studies VI* and VII, data not shown).

The specificity of the NOS inhibitor 7-NI in in vitro as well as in vivo experiments has been discussed controversially (14, 27). Therefore, we tested the effect of 7-NI on the endothelium-dependent vasodilation to acetylcholine (100 μM in MOPS buffer solution; Sigma) in another four arteries.
Study VIII. nNOS-containing perivascular nerve fibers at the anterior portion of the circle of Willis and its branches including the MCA are mainly originating from the NCN and PSF from the sphenopalatine ganglion (7, 17, 36). Extracranial transsection of these nerve bundles at the ethmoidal foramen leads to a complete loss of nNOS-containing perivascular nerve fibers within 2 wk as shown by NADPH-diaphorase histochemistry (36). Therefore, in study VIIIb ($n=10$), 2 wk before the isolation of the MCA, the NCN and PSF were transsected according to the method described previously by Suzuki et al. (36). In brief, rats (200–220 g) were anesthetized with chloral hydrate (5%, 200 mg/kg body wt ip). The ethmoidal foramen at the nasal orbital wall ipsilaterally to the later isolated MCA was carefully prepared, and the NCN and PSF were thoroughly transsected. A sham-operated group (study VIIIa, $n=8$) served as a control. Responses of isolated ipsilateral MCAs to extraluminal acidosis ($n=10$ denervated group, $n=8$ sham group) as well as to 20 mM potassium (extraluminal) and 100 mM acetylcholine (intraluminal) ($n=4$ denervated group, $n=3$ sham group) were tested 12 to 16 days after nerve transsection. In four arteries of the denervated group, the effect of 8-BrcGMP (30 mM in MOPS buffer solution; Sigma) on the response to extraluminal acidosis was tested. NADPH diaphorase histochemistry was processed according to the protocol of Tomimoto et al. (39) to confirm degeneration of nNOS-containing perivascular nerve fibers in the transsection group compared with preserved NOS staining in the sham-operated group. In brief, additional animals ($n=2$ each) under deep pentobarbital anesthesia (100 mg/kg) were perfusion fixed with 2% paraformaldehyde and 0.1% picric acid in 20 mM phosphate-buffered saline at 4°C. The brain was removed and postfixed in 20 mM phosphate-buffered saline containing 20% sucrose at 4°C for 24 h. Subsequently the isolated artery was incubated in 20 mM phosphate-buffered saline containing 0.3% Triton X-100 (Sigma), 1 mg/ml NADPH, and 0.25 mg/ml nitroblue tetrazolium at 37°C for 45 min. The vessels were mounted on slides, coverslipped, and investigated under a conventional microscope.

Studies IX and X. To investigate the contribution of eNOS in the vasodilatory response of the isolated MCA to extraluminal acidosis, the endothelium was removed. After testing baseline vasodilation to intraluminal application of acetylcholine (100 mM in MOPS buffer solution; Sigma) to verify the integrity of endothelium-dependent vasoreactivity, the baseline response to acidosis was recorded. In study IX ($n=6$), the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, 0.3% in intraluminal buffer solution; Sigma) was then slowly perfused intraluminally for 2.5 min. In study X ($n=5$), 8 ml of air was passed through the lumen of the MCA over a period of 15 min with the intraluminal pressure never exceeding 80 mmHg. In both study groups, removal of the endothelium was confirmed by the absence of vasodilation during intraluminal application of acetylcholine. The response to extraluminal acidosis was tested again.

Fig. 1. Experimental paradigm. L-NNA, Nω-nitro-L-arginine; SIN-1, 3-morpholinosyndnonimine; SNAP, S-nitroso-N-acetylpenicillamine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one; 7-NI, 7-nitroindazole; PSF, parasympathetic fibers; NCN, nasociliary nerve; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate.
Data Analysis

Values are means ± SD. Comparisons were made using ANOVA, followed by multiple comparison procedures (Bonferroni’s method). In studies VIIIa and VIIIb, comparison was made using unpaired Student’s t-test (SigmaStat, Jandel). P values < 0.05 were considered statistically significant.

RESULTS

After spontaneous tone was developed (20% reduction of resting diameter compared with diameters measured directly after pressurizing) within the 1-h equilibration period after preparation, smooth muscle cell function was tested at the beginning of each experiment by increasing extraluminal potassium concentration to 20 mM. Only vessels were used for the experiments, which showed a potassium-induced dilation of more than 25%.

Physiological Response

After equilibration, MCA diameter stabilized at 140.0 ± 26.8 μm at physiological pH values (pH 7.4), and extraluminal pH reduction to 7.0 led to a consistent and statistically significant (P < 0.01) diameter increase of 46.8 ± 21.7 μm (35.1 ± 18.5%), reaching vessel diameters of 186.8 ± 30.2 μm (n = 44).

Unspecific NOS Inhibition and Subsequent Vasodilator Application

Unspecific NOS inhibition with l-NNa reduced vessel diameters at physiological extraluminal buffer solutions to ~74% and reduced acidoic vasodilation to a diameter increase of only 8.7 ± 8.4 μm (from 102.7 ± 20.5 μm at pH 7.4 to 110.9 ± 26.4 μm at 7.0) (P < 0.01). Vasodilatory reaction to acidosis was, therefore, still present (P < 0.01) but markedly reduced to ~8% during NOS inhibition. Whereas we reestablished vessel diameter at pH 7.4 by adding the NO donors SIN-1 or SNAP or the cell-permeable cGMP analog 8-BrcGMP extraluminally during continued NOS inhibition, the vessel reactivity to acidosis was restored. In contrast, when MCA diameter was increased to pre-l- NNA values by adding the appropriate amount of the NO-independent vasodilators papaverine or nimodipine, the vasoreactivity to acidosis remained significantly reduced (diameter increase at pH 7.0 of only 3.7 ± 6.4 μm with papaverine or 9.0 ± 5.1 μm with nimodipine) (Fig. 2).

Soluble Guanylyl Cyclase Inhibition and Subsequent cGMP Application

Soluble guanylyl cyclase inhibition with ODQ reduced vessel diameters at physiological extraluminal buffer solutions to ~80% and reduced acidoic vasodilation to a nonsignificant diameter increase of 3.8 ± 4.8 μm (from 109.2 ± 7.9 μm at pH 7.4 to 113.0 ± 6.6 μm at 7.0) (P > 0.10). Vasodilatory reaction to acidosis was, therefore, abolished during soluble guanylyl cyclase inhibition. While reestablishing vessel diameter at pH 7.4 by the addition of the cell-permeable cGMP analog 8-BrcGMP extraluminally during continued guanylyl cyclase inhibition, the vessel reactivity to acidosis was completely restored (Fig. 3).

Source of Basal NO (nNOS Inhibition, Denervation of Perivascular Nerves, or Removal of Endothelium)

NOS inhibition with 7-NI reduced vessel diameters at physiological extraluminal buffer solutions slightly and not significantly to ~89%, and acidoic vasodilation was significantly attenuated to a diameter increase of only 13.7 ± 13.3 μm (from 126.5 ± 10.6 μm at pH 7.4 to 140.2 ± 11.3 μm at 7.0) (P < 0.05). Thus vasodilation to acidoic extraluminal buffer was significantly reduced to ~11% during NOS inhibition with 7-NI. Extraluminal addition of the cell-permeable cGMP analog 8-BrcGMP during further NOS inhibition with 7-NI restored vessel diameter at pH 7.4 and reestablished the pH-dependent vessel reactivity (50.6 ± 20.5 μm diameter increase due to reduced extraluminal pH) (Fig. 4).

To test the specificity of 7-NI for nNOS in our in vitro system of the isolated MCA, the vasodilation to the endothelium-dependent vasodilator acetylcholine was compared before and after 7-NI application. The acetylcholine-induced dilation of 53 ± 14 μm at baseline conditions was reduced to 20 ± 15 μm during NOS inhibition with 7-NI, suggesting an inhibitory effect of 7-NI on NOS located in endothelial cells.

To further clarify the isoform involved and the origin of NO responsible for vascular pH reactivity, nonpharmacological approaches were chosen. In one set of experiments, the NOS-containing perivascular neuronal source was removed, and in another set of experiments the endothelium was removed.

In study VIII, 2 wk after extracranial dissection of the NNC and PSF from the sphenopalatine ganglion, the vasodilation response to acidoic MOPS solution was nearly abolished compared with the sham-operated control group (P < 0.01), and extraluminal application of cGMP analog in the dissected study group significantly increased the vasodilation response to extraluminal acidosis (Fig. 5A). Vasodilatory reactivity to potassium (extraluminal, 20 mM, NO-independent vasodilator) or acetylcholine (intraluminal, endothelium-dependent vasodilator) did not differ between the dissected study group and the sham-operated study group (Fig. 5B), suggesting a specific role of nNOS from perivascular nerves during pH reactivity.

After removal of the endothelium by intraluminal application of the detergent CHAPS in study IX, vessel diameter at pH 7.4 increased by 28% (P < 0.05). The effectiveness of endothelium removal was tested by acetylcholine application, leading to a 40–50% diameter increase during physiological conditions, which was completely abolished by CHAPS as well as air application in studies IX and X. During extraluminal application of acidoic buffer solution in endothelium-denuded vessels in study IX, the diameters still significantly increased by 28.5 ± 6.9 μm (P < 0.05) compared with...
the elevated baseline. The vasodilator response to pH 7.0 after CHAPS was therefore reduced due to baseline diameter increase (Fig. 6). In study X, in which the vascular endothelium was carefully removed by transient air application, resting diameter at pH 7.4 did not change. The response to pH 7.0 was totally preserved and did not differ between the condition with intact endothelium and the condition in which the endothelium was removed (Fig. 6).

**Histochemical Confirmation of Chronic Denervation of NOS-Containing Perivascular Nerves**

Under physiological conditions (sham operation), high densities of NOS-containing perivascular nerves were detected in the adventitial layer of the MCA (NADH diaphorase staining). Transsection of NCN and PSF 14 days before the experiments led to a complete loss of these NOS-containing nerve endings. Figure 7 shows the typical finding 2 wk after NCN and PSF transsection compared with nontranssected animals.

**DISCUSSION**

There are five key findings of this study. First, the NOS inhibition-induced attenuation of the vasodilator response of the isolated rat MCA to extraluminal acidosis can be fully restored by reestablishing basal NO levels. Second, this permissive role of NO during acidosis-induced vasodilation is to a large degree mediated by a NO-dependent basal cGMP concentration in the vessel. Third, this basal NO-cGMP level in the isolated MCA is mainly produced by NO from the neuronal isoform of the constitutive NOS. Fourth, the endothelium plays no role in pH-dependent diameter changes in the isolated cerebral artery. Finally, the nNOS, providing basal NO, is located in perivascular nerve endings in the adventitial layer of the vessel. NOS-containing nerve endings at the MCA are mostly originating from NCN and PSF.

The model of isolated cerebral arteries allows investigation of mediators or modulators of cerebrovascular reactivity without parenchymal influences. The cannula...
lated and perfused isolated cerebral artery develops spontaneous tone in response to physiological intraluminal pressure without pharmacological intervention (11, 22), and the intact adventitial layer provides a physiological neurotransmitter and neuromodulator tonus from perivascular nerve endings (21). In our model, we used MOPS-buffered physiological salt solution for the extraluminal bath as well as the intraluminal perfusion. Albumin (1%) was added intraluminally to maintain the integrity of the endothelial cells and luminal matrix (11). Changes in pH were induced

Fig. 3. Effect of extraluminal application of 8-Br-cGMP (30 μM, study VI) on soluble guanylyl cyclase inhibition by ODQ (5 μM) on resting MCA diameter at pH 7.4 and vasodilator response to extraluminal acidosis. Soluble guanylyl cyclase inhibition by ODQ reduced resting diameter at pH 7.4 (P > 0.05) and abolished diameter increase at pH 7.0. 8-Br-cGMP application during soluble guanylyl cyclase inhibition reestablished resting diameter at pH 7.4 and completely restored vasodilator response to pH 7.0. *P < 0.05, significant differences in vessel diameters at pH 7.0.

Fig. 4. Effect of extraluminal application of 8-Br-cGMP (30 μM, study VII) on NOS inhibition by 7-NI (100 μM)-induced attenuation of resting MCA diameter at pH 7.4 and vasodilator response to extraluminal acidosis. NOS inhibition by 7-NI reduced resting diameter at pH 7.4 (P > 0.05) as well as diameter increase at pH 7.0 (P < 0.05, not indicated). 8-Br-cGMP application during NOS inhibition by 7-NI reestablished resting diameter at pH 7.4 and completely restored vasodilator response to pH 7.0. *P < 0.05, significant differences in vessel diameters at pH 7.4; #P < 0.05, significant differences in vessel diameters at pH 7.0.

Fig. 5. Effect of chronic denervation of neuronal NOS (nNOS)-containing perivascular nerve fibers in the adventitial layer of the MCA, originating from the NCN and PSF from the sphenopalatine ganglion due to extracranial transection of these nerve bundles at the ethmoidal foramen 14 days before experiments. A: resting MCA diameter at pH 7.4 was not changed in denervated arteries (PSF/NCN cut) compared with arteries from sham-operated animals. In contrast, vasodilator response to extraluminal acidosis was nearly abolished after PSF/NCN dissection. Extraluminal application of cGMP (30 μM) significantly increased vasodilator response to extraluminal acidosis in the denervated group, reaching dilations comparable to the sham-operated group. *P < 0.05, significant difference in vessel diameters at pH 7.0. B: endothelium-dependent (intraluminal application of 100 μM acetylcholine) as well as NO-independent (extraluminal application of 20 mM K+) vasodilations were unchanged in the PSF/NCN dissection group compared with the sham-operated group.
by the addition of small amounts of HCl to the buffer solution, because MOPS-buffered saline contains no bicarbonate and is, therefore, characterized by CO$_2$-free or at least hypocarbic conditions. Under physiological conditions in the in vivo system, a change in extracellular pH is associated with alterations in CO$_2$ and/or HCO$_3^-$. These molecules may have independent effects on vascular tone. Nevertheless, it has been shown that in the cerebral vasculature a change in CO$_2$ with a maintained extracellular pH has generally little effect on vascular tension in vivo (24) as well as in vitro (38). These findings suggest that the vascular response to CO$_2$ in the brain is mediated by a change in the proton concentration (24, 30). Compared with the reduction of vascular tone reached by using bicarbonate-buffered saline solution, a similar reduction has been shown to occur during lowered extracellular pH using artificial buffer solutions such as HEPES or Tris (3). In the present study, vasoreactivity in the physiological range was tested by changes of extracellular potassium concentrations or pH changes in the extraluminal bath and led to similar diameter changes compared with those shown by others (22, 28).

Relaxation or constriction of cerebrovascular smooth muscle cells occurs due to decreased or increased intracellular Ca$^{2+}$ concentrations, in part by closing or opening of voltage-gated Ca$^{2+}$ channels due to hyper- or hypopolarization of the cells. Hypercapnic acidosis, but not normocapnic acidosis, is associated with hyperpolarization (16), suggesting a difference in the mechanisms of action between hypercapnic and normocapnic extracellular acidification. In contrast to experimental models using normocapnic acidification, isolated cerebral arteries studied in bicarbonate-free buffered saline solutions also showed hyperpolarization accompanied by vasodilation during acidification (9), suggesting identical mechanisms of action during physiological hypercapnic acidosis and the HCO$_3^-$/CO$_2$-free, MOPS-buffered in vitro system.

The role of NO in the vascular response to acidosis in the isolated artery has not been investigated in detail so far. In the present study, we have shown that NOS inhibition in the isolated artery led to a significant reduction of resting diameter. Analogous results have been described during NOS inhibition in studies measuring regional cerebral blood flow (rCBF) in the cerebral cortex in vivo (10) or resting basilar artery diameter in vitro (43), indicating a basal NO tonus produced directly by the vascular compartment. In addition, we have shown that vasodilation to extraluminal acidosis was nearly abolished during NOS inhibition. The same results have been shown for the reactivity of the basi-

Fig. 6. Effect of removal of the endothelium on resting diameter at pH 7.4 and vasodilator response to extraluminal acidosis. Transient intraluminal application of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (0.3% in intraluminal buffer solution for 2.5 min, study IX) increased vessel diameter at pH 7.4 $\#P < 0.05$ (A), whereas transient intraluminal application of air (8 ml for 15 min, study X) had no effect on resting diameter at pH 7.4 (B). Vasodilator response to pH 7.0 was preserved (significant diameter increase in endothelium-denuded vessels, $P < 0.05$, not indicated). Compared with the baseline response, vasodilator response after CHAPS was significantly reduced ($P < 0.05$, not indicated), whereas the response was completely preserved after transient intraluminal application of air. Acetylcholine (intraluminal application, 100 $\mu$M-induced vasodilatation of 40–50% during baseline conditions (endothelium intact, pH 7.4) was totally abolished after removal of endothelium by CHAPS (study IX, A) as well as air (study X, B). *$P < 0.05$, significant differences in vessel diameters at pH 7.0 or during acetylcholine application compared with resting diameter.

Fig. 7. NADPH diaphorase histochemistry shows a typical example of confirmation of chronic denervation of NOS-containing perivascular nerves. A: under physiological conditions (sham operation), high densities of NOS-containing perivascular nerves were detected in the adventitial layer of the MCA. B: extracranial transection of the NCN and PSF from the sphenopalatine ganglion at the ethmoidal foramen 14 days before the experiments led to a complete loss of these NADPH diaphorase positive nerve endings.
lar artery to acidosis (43). Intracerebral arterioles (38–55 μm diameter), however, have been shown to dilate to pH 6.8 buffer solution independent of NO (29). These inconsistent findings suggest a difference in the NO dependency of the reactivity of intracerebral arterioles compared with the MCA itself. In addition, it has been shown that hypercapnic cerebral blood flow (CBF) response is not uniformly dependent on NO, and vascular reactivity to high arterial partial pressure of CO₂ is completely independent of the bioradical NO (18). Ngai and Winn (29) used buffer solution with lower pH value in their study, and this may, in addition, explain the differences in the NO dependency between the results by Ngai and Winn and our study.

In the present study, we have shown that the acidic vasodilation was solely dependent on basal NO concentration because NOS inhibition nearly abolished vasodilation response to extraluminal acidic buffer solution. Reestablishing the vascular tone by adding NO donors in the extraluminal bath completely restored pH-dependent reactivity of the isolated MCA. This NO effect seems to be mediated by cGMP, because cGMP application during NOS inhibition as well as soluble guanylyl cyclase inhibition was equally effective in restoring pH reactivity. The NO-independent vasodilators papaverin or nimodipine had no effect on reduced vasoreactivity with extraluminal acidosis during NOS inhibition, suggesting a specific NO-cGMP-mediated mechanism. In accordance with our findings are data by You et al. (43) demonstrating that high CO₂ elicits vasodilation of the isolated basilar artery by a mechanism that depends on basal NO levels but which is independent of increased NO production by elevated NO activity. In addition, no increase of basal cGMP concentration was observed during CO₂-induced vasodilation (43). In a recent study by Wang et al. (41), an only transient increase in cGMP production within the first minute of hypercapnic acidosis has been shown in neuronal cells in vitro. The cGMP concentration then returned to baseline despite ongoing acidosis, suggesting that this transient increase does not account for vascular reaction in intact tissue.

To our knowledge the present study is the first to show the modulatory action of NO during acidotic vasodilation in the rat isolated MCA. The permissive effect of NO-cGMP has been shown for the hypercapnic rCBF response in vivo in the cerebral cortex of rats (20). Kontos and Wei (25), however, did not confirm the permissive role of NO via cGMP in the cat cerebral circulation. The authors suggest that arginine analogs inhibit hypercapnic vasodilation by blocking ATP-sensitive K⁺ channels independently of cGMP, and that these ATP-sensitive K⁺ channels may have an arginine site that influences their function in contrast to the proposed modulation by NO, either directly or via cGMP.

There are various potential cellular sources for the basal NO level in the cerebrovascular environment. Inhibition of the neuronal isoform of NOS by 7-NI in vivo led to a significant reduction of hypercapnic rCBF responses in a permissive way (31). Analogous results have been shown in the present study for the reaction of the isolated MCA to extraluminal acidosis. However, the specificity of the NOS inhibitor 7-NI in in vitro as well as in vivo experiments has been discussed controversially (14, 27). We, therefore, tested the effect of 7-NI on the vasodilator response to acetylcholine in our model. Acetylcholine has been shown to induce dilatation at least in part via NO production in vascular endothelium (12). The acetylcholine response was reduced by ~50% during 7-NI application, pointing to a partial inhibition of NOS within the vascular endothelium, and a possible role of NOS in endothelial cells during acidic vasodilation cannot be excluded. Besides morphological findings (35), recently Benyó et al. (4) provided strong functional evidence that the neuronal isoform of NOS is also present in cerebrovascular endothelial cells. They demonstrated a partial inhibition of vasodilation to acetylcholine during 7-NI inhibition in the isolated rat basilar artery, whereas the response to bradykinin, a specific eNOS-dependent vasodilator, was unchanged. Therefore, it can be suggested that nNOS is also involved in the endothelium-dependent vasodilatation to acetylcholine, and 7-NI may indeed act as a specific inhibitor of nNOS in the in vitro system. The possibility that acetylcholine stimulates NO release from perivascular nerves can be excluded in their study as well as in our study, because removal of the endothelium completely abolishes relaxation to acetylcholine.

To further identify the cellular source of NO and to exclude the possibility of an involvement of nNOS located in endothelial cells, we performed further experiments independent of pharmacological NOS inhibition. Removal of the endothelium in vivo as well as in vitro had no effect on hypercapnic or acidotic vessel responses (38, 42, 43), suggesting a more specific role for nNOS distinct from endothelial cells in the pH-dependent vasoreactivity. In the present study, removal of the endothelium with transient intraluminal application of the detergent CHAPS increased vessel diameters at physiological pH 7.4 by 26%. Besides a possible vasodilator tone due to NO production, vascular endothelial cells have the ability to produce a variety of vasoconstrictors, such as thromboxane and endothelins. To test whether the observed increase in vessel diameters after endothelium removal by CHAPS, in our study, suggests a stronger influence of constriction factors rather than vasodilating factors on vascular smooth muscle cells or whether the vessels were simply damaged by the denudation procedure, we very carefully added air intravascularly for a short time period in another group of arteries. This procedure did not change resting diameter at physiological pH. Both procedures totally abolished the endothelium-dependent vasodilation to acetylcholine. After removal of the endothelium by application of air, the vessel response to acidic extraluminal environment was unchanged compared with the reaction under physiological conditions. Because the observed increase in resting diameter after removal of the endothelium with CHAPS has not been shown by others and is not consistent with our
study group using air, the diameter increase after CHAPS application may indeed be caused by a functional impairment of the smooth muscle cell layer itself, and the reactivity to acidosis cannot be properly interpreted in this study group. However, because of our findings using air to carefully remove the endothelium without diameter increases after the procedure, an endothelial component in the NO-dependent response to acidosis can be excluded.

Because 7-NI has been shown to partially inhibit NOS from vascular endothelium in our in vitro system in addition to its specific effect on extra-endothelial nNOS, we studied the specific role of NOS-positive perivascular nerves (17), the main compartment containing nNOS in the isolated artery. The presence of NOS-positive nerves surrounding the MCA in the sham-operated group, and the efficacy of denervation was examined in whole mount preparations processed for NADPH-diaphorase histochemistry, a procedure that stains NOS-containing neurons (17). Transsection of the NCN and the PSF originating from the sphenopalatine ganglion at the ethmoidal foramen led to a complete loss of NOS-positive neuronal endings surrounding the MCA. This result demonstrates the main source of nNOS in the NCN and the PSF originating from the sphenopalatine ganglion. In these denervated arteries, reactivity to pH changes was nearly abolished compared with arteries harvested from sham-operated animals, whereas the NO-independent vasodilation to 20 mM potassium as well as the endothelium-dependent vasodilation to acetylcholine was unchanged. cGMP application in denervated arteries reestablished the reactivity to acidosis. These results further support our hypothesis that the basal NO concentration in the MCA is indeed provided by NOS-containing perivascular nerves originating from the sphenopalatine ganglion at the ethmoidal foramen. It has been shown that in vivo the rCBF response to hypercapnia was only slightly and not significantly reduced from 2.23 ± 0.3%/mmHg to 1.78 ± 0.5%/mmHg, when NCN and PSF were chronically transected (33) or no influence at all was observed (19). Both large arteries and microvessels contribute to reductions in vascular resistance during hypercapnia in vivo. The different vascular compartments investigated may explain the discrepancy between these in vivo studies in which microcirculatory changes in MCA territory of the parietal cortex were examined and the present in vitro study of the MCA itself. Because NCN and PSF fibers only project to large cerebral arteries in the anterior and MCA territory, the lack of influence of NCN and PSF denervation in microcirculation studies is not unexpected. The denervation-induced changes in large cerebral arteries may be compensated by the downstream smaller vessels with a net result of no alteration in blood flow. In addition, basal NO in the intact cerebral tissue is not only provided by perivascular nerve endings originating from the sphenopalatine ganglion and the trigeminal system. nNOS is constitutively expressed in neurons and astrocytes. nNOS-positive interneurons have been shown in a small percent in deeper lamina of the somatosensory cortex (8) and to a larger extent, surrounding larger cerebral arteries as well as cerebral microvessels (17).

The functional significance of the present study is, therefore, on the one hand, the role of basally released NO from perivascular nerves in the reactivity of large cerebral arteries to extraluminal acidosis in the context of systemic hypercapnia. Within the blood flow response to hypercapnia in vivo, NOS-containing nerve fibers from extracerebral origin provide only part of the basal NO level in the parenchymal tissue, as shown by the unchanged microcirculatory blood flow response after NCN and PSF dissection. On the other hand, however, the present study provides for the first time evidence that the reactivity of large cerebral arteries can be significantly impaired by a reduction of the basal NO level in the direct vicinity of the artery itself, provided by perivascular nerve fibers. This impairment may play a significant role during pathophysiological conditions as focal ischemia or brain trauma, conditions that are accompanied by tissue acidosis independent of systemic hypercapnia.

The molecular mechanism of acidosis-induced vasodilation and the role of the basal NO level in the vicinity of cerebral arteries, providing physiological smooth muscle cell function and pH-dependent vascular reactivity, are not clear so far. Hypercapnic and normocapnic acidosis (37) as well as acidosis in HCO₃⁻/CO₂-free systems (3) are associated with intracellular acidosis in vascular tissues. Nevertheless, there is evidence that changes in intracellular pH are not responsible for smooth muscle cell relaxation during extracellular pH reduction (1). Thus the question arises how the information of increased extracellular proton concentration is transduced into vasodilation. Hypo- or hypopolarizing K⁺ channel activity has been shown to be modulated by NO or cGMP, and by the extracellular proton concentration (5, 6, 25, 34). Recent findings provide strong evidence for a contribution of ATP-sensitive potassium channels in the vasodilation of cerebral arteries to acidosis (15, 23, 25). The results concerning a role for K⁺ channels in hypercapnia-induced cerebrovasodilation, however, are inconclusive, because others did not find evidence for a role of K⁺ channels in hypercapnia-induced cerebrovasodilation under physiological conditions (32, 40). Further experiments have to be performed to solve this discrepancy.

In conclusion, our findings have shown that acidotic vasodilation in the isolated cerebral artery depends on a basal NO concentration in a modulatory sense. This basal NO level is provided by constitutive nNOS located in perivascular nerves. The endothelium plays no role in the vasodilation to extracellular acidosis.

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