Cerebral microvascular nNOS responds to lowered oxygen tension through a bumetanide-sensitive cotransporter and sodium-calcium exchanger

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Bauser-Heaton HD, Song J, Bohlen HG. Cerebral microvascular nNOS responds to lowered oxygen tension through a bumetanide-sensitive cotransporter and sodium-calcium exchanger. Am J Physiol Heart Circ Physiol 294: H2166–H2173, 2008. First published March 7, 2008; doi:10.1152/ajpheart.01074.2007.—Na$^+$ cotransporters have a substantial role in neuronal damage during brain hypoxia. We proposed these cotransporters have beneficial roles in oxygen-sensing mechanisms that increase periarteriolar nitric oxide (NO) concentration ([NO]) during mild to moderate oxygen deprivation. Our prior studies have shown that cerebral neuronal NO synthase (nNOS) is essential for [NO] responses to decreased oxygen tension and that endothelial NO synthase (eNOS) is of little consequence. In this study, we explored the mechanisms of three specific cotransporters known to play a role in the hypoxic state: KB-R7943 for blockade of the Na$^+$/Ca$^{2+}$ exchanger, bumetanide for the Na$^+$/K$^+$-2Cl$^-$ cotransporter, and amiloride for Na$^+$/H$^+$ cotransporters. In vivo measurements of arteriolar diameter and [NO] at normal and locally reduced oxygen tension in the rat parietal cortex provided the functional analysis. As previously found for intestinal arterioles, bumetanide-sensitive cotransporters are primarily responsible for sensing reduced oxygen because the increased [NO] and dilation were suppressed. The Na$^+$/Ca$^{2+}$ exchanger facilitated increased NO formation because blockade also suppressed [NO] and dilatory responses to decreased oxygen. Amiloride-sensitive Na$^+$/H$^+$ cotransporters did not significantly contribute to the microvascular regulation. To confirm that nNOS rather than eNOS was primarily responsible for NO generation, eNOS was suppressed with the fusion protein cavinatin for the caveolae domain of eNOS. Although the resting [NO] decreased and arterioles constricted as eNOS was suppressed, most of the increased NO and dilatory response to oxygen were preserved because nNOS was functional. Therefore, nNOS activation secondary to Na$^+$/K$^+$-2Cl$^-$ cotransporter and Na$^+$/Ca$^{2+}$-exchanger functions are key to cerebral vascular oxygen responses.

Through the Na$^+$/K$^+$/2Cl$^-$ cotransporter (47). The Na$^+$/Ca$^{2+}$ exchanger apparently removes Na$^+$ while increasing intracellular Ca$^{2+}$ to activate endothelial NO synthase (eNOS) (47). In subsequent studies, we found that a very similar process involving the Na$^+$/K$^+$-2Cl$^-$ cotransporter and then the Na$^+$/Ca$^{2+}$ exchanger occurred to increase vessel wall NO concentration ([NO]) when oxygen tension was reduced in intestinal tissues (38, 48).

We questioned whether a similar process involving eNOS and these Na$^+$-handling systems might be globally present, such as in the brain microvasculature where oxygen tension is lowered by hypotension (4, 42). Our laboratory recently published data showing that the brain primarily uses neuronal NO synthase (nNOS) rather than eNOS to generate increased [NO] in response to decreased perivascular and tissue oxygen tension (4). These experiments were based on a recently available highly specific blocker of nNOS (25) and revealed that the brain nNOS system is responsive to reductions in oxygen tension, such as during hypotension and local reductions in oxygen availability. When the nNOS system was inhibited, much greater reductions in oxygen tension occurred during hypotension and less vasodilation occurred. It is not surprising that the brain would have a sensitive mechanism for sensing a decrease in oxygen availability given its high metabolic rate. Furthermore, nNOS-containing cerebral cells are known to respond to oxygen deprivation and hypoxia in part through opening Na$^+$ cotransporters (11, 12, 29, 31). However, for each of the references cited, the level of hypoxia would approach serious compromise and death of tissue. In the present study, we were much more concerned with comparatively small reductions in oxygen tension, such as those that would occur with modest, survivable reductions in oxygen availability, as we have shown occurs during arterial hypotension to ~60 mmHg (4).

The first hypothesis evaluated was whether the Na$^+$/K$^+$-2Cl$^-$ cotransporter or Na$^+$/H$^+$ exchanger dominated as a potential oxygen-sensing mechanism around cerebral arterioles. From a past study (4), we found that reducing the bath oxygen tension over in vivo cerebral microvessels lowered the perivascular oxygen tension sufficiently to activate both increased [NO] and vasodilation. Both of these responses were suppressed by selective blockade of nNOS, which did not appreciably impair eNOS function because increased [NO] and dilation to increased blood flow were intact (4). Histochemistry studies by other investigators have indicated that nNOS is extensively located in perivascular neurons and neuronal support cells (14, 27, 34, 41, 45) with little expression in cerebral endothelial cells (35, 46). To pharmacologically inactivate...
Na$^+$ entry into nNOS- and eNOS-containing cells, bumetanide, a selective Na$^+-K^+-2Cl^-$ cotransporter blocker, was used. Bumetanide essentially eliminated the increase in [NO] and dilatation to reduced oxygen tension, whereas amiloride, a Na$^+/H^+$ exchanger blocker, had minor effects at a concentration known to minimize cerebral tissue damage during severe hypoxia (11, 24). The second hypothesis tested was that Na$^+$ entering NO-producing cells through the Na$^+-K^+-2Cl^-$ co-transporter activated the Na$^+/Ca^{2+}$ exchanger to increase the production of NO by NOS. In the brain, the Na$^+/Ca^{2+}$ exchanger is expressed in many isoforms, but the cerebral cortex is known to have primarily Na$^+/Ca^{2+}$ exchanger isofrom 1 (NCX1) (26). This exchanger is very effectively blocked by the commercially available agent known as KB-R7943 (3, 15). However, this agent may have some secondary effects on true Ca$^{2+}$ channels during prolonged exposure (40), which limited our exposure time to ~30 min. Our prior study (48) indicated that eNOS oxygen-sensitive mechanisms in intestinal arterioles were substantially suppressed by this approach, and much the same result occurred for cerebral vessels. To confirm that nNOS plays a large role in the oxygen-sensing mechanism in question (4), we chose to block eNOS and evaluate the remaining responses with nNOS active. We used a partial peptide of caveolin-1, cavtratin, which suppresses eNOS activity by binding to free eNOS in the caveolin scaffolding domain (5, 20). Using this approach, we tested the hypothesis that nNOS would still be functional after suppressing eNOS and much of the NO response to reduced oxygen tension would be preserved.

METHODS

All techniques and procedures were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Surgical preparation of the cerebral cortex. Adult male Sprague-Dawley rats of 300–349 g (Harlan Sprague Dawley, Indianapolis, IN) were anesthetized using tiopental sodium (200 mg/kg, Abbott, Chicago, IL). The solution was freshly made to a concentration of 49 mg/ml and injected into four subcutaneous sites along the upper and lower thighs. Throughout the experiment, additional anesthetic was administered intraperitoneally if needed as determined by corneal reflex and increases in arterial blood pressure in response to toe pinch. Body temperature was maintained at 37–38°C by placing the animal on an underbody heating pad held at 35–36°C as well as covering the animal with an insulation blanket if needed, as animals anesthetized with tiopental have a limited ability to thermoregulate. The trachea was intubated with a polyethylene tube (PE-250), and rats were ventilated (Harvard Apparatus, Holliston, MA) throughout the experiment at a control resting rate of 70 breaths/min. Tidal volume was adjusted by weight according to values given by Harvard Apparatus, increased to compensate for the known dead space, and adjusted to give both a stable and maximum arterial blood pressure and percent saturation of hemoglobin of oxygen in the foot pad vasculature of at least 90%, which was consistently ~5 saturation units below that of the ear vasculature. Ear measurements are not possible due to surgery near the ears and the brain tissue warming apparatus covered the ears.

The right femoral artery was cannulated using a polyethylene catheter (PE-50) filled with saline and heparin to monitor blood pressure throughout the experiment. Rats were given saline (0.5 ml h$^{-1}$·100 g$^{-1}$) to help maintain a very stable arterial pressure. Only those animals with a constant blood pressure above 100 mmHg were used for the experiments described in this study.

The head of the animal was secured in a small stereotaxis device, and the skull parietal bone was exposed by removing the overlying skin and periosteum. The bone surface was dried with a jet of room air to lessen bleeding from the bone vasculature. A high-speed drill (Fine Scientific Tools, Foster City, CA) was used to remove the parietal bone bilaterally just within the bone suture lines. To assure hemostasis, bone wax (Surgical Specialties, Reading, PA) was pressed into the cut parietal bone edge at sites of bleeding. The dura mater was exposed to air (Sigma, St. Louis, MO) to stop minor bleeding, and thrombin crystals were touched to any bleeding areas. The tip of a 30-gauge needle was used as a small scalpel to open the dura for exposure of arterioles over the brain cortex, and removal of the membrane was done using fine forceps. The brain was suffused with 5 ml/min of bicarbonate-buffered solution with Ca$^{2+}$ (7) that was equilibrated with 5% O$_2$-5% CO$_2$-90% N$_2$. These gas mixtures were chosen to replicate the typical cerebrospinal fluid gaseous composition of mammals and from past experience of our laboratory with rat cerebral microvascular preparations (8, 9, 21, 22, 42). Bathing media passed through a heating chamber (37.5°C) before flowing over the brain and into the bathing fluid chamber. The bathing fluid chamber was also heated (37.5°C) by an internally heated water perfusion system. Approximately 1.5 l/min of heated distilled water passed through the heating chamber and head support to insure a stable temperature.

Observation of the vasculature. After completion of the surgical procedure, the animal was moved to the stage of an intravital microscope (model BHMJ, Olympus, Hyde Park, NY) to visualize and capture images of the cerebral microvasculature. The brain surface was illuminated through a fiber optic bundle whose light source was a mercury arc lamp. Both infrared and ultraviolet light wavelengths were removed by appropriate filters. Visualization occurred through a ×20 Nikon water-immersion lens, and images were captured using a Video Scope camera (model CCD 200EI Videscope, Washington, DC) and an image-analysis system (Image 1, Universal Imaging, West Chester, PA). Inner arteriolar diameter measurements were made from stored digital images, and dimensions were chosen from calibration of the optical/image-analysis system with a stage micrometer marked in 10- and 100-μm units.

Perivascular NO measurements. [NO] emitted near microvessels was measured using carbon fiber microelectrodes adapted from methods described by Buerk et al. (13, 44) and Freidemann et al. (18). The carbon fiber was sealed to the glass envelope during pulling of the microelectrode and further stabilized with epoxy cement according to the methods of Freidemann et al. (18). The tip of the microelectrode was sharpened to ~10 μm or less in diameter. The electrode carbon tip was slightly electrolitically etched away after being sharpened to make a protected recess in which to deposit Nafion. The electrode tip was electroplated (+0.7 V, 15–20 min) with Nafion (Aldrich, Milwaukee, WI) to eliminate the potential undesired detection of charged molecules. Polarity of the NO microelectrode was chosen to replicate the typical cerebrospinal fluid gaseous composition of mammals and from past experience of our laboratory with rat cerebral microvascular preparations (8, 9, 21, 22, 42). Bathing media passed through a heating chamber (37.5°C) before flowing over the brain and into the bathing fluid chamber. The bathing fluid chamber was also heated (37.5°C) by an internally heated water perfusion system. Approximately 1.5 l/min of heated distilled water passed through the heating chamber and head support to insure a stable temperature.

The output voltage was monitored and recorded with a PowerLab analog-to-digital chart recorder system (AD Instruments, Colorado Springs, CO).
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Springs, CO). Typical NO microelectrodes would generate 1.5–2.5 pA/1,000 nM NO and had a linear current/[NO] relationship over the range tested. To establish a zero [NO] current during the experiment, the electrode was placed 200 µm above the brain surface. Vertical movement by ~50 µm from this reference point did not influence the reference voltage. This reference voltage was subtracted from each measured perivascular voltage and multiplied by the calibration factor. Electronic drift of the electrode “0” did occur and was compensated as needed by mathematically determining a virtual baseline over time using a presumed linear slope of the drift voltage. Although the electrode tip was small enough to measure [NO] on even the smallest arterioles, only the larger arterioles tolerated sustained touching of the electrode to the vessel surface without some degree of vasodilation over the extended measurement times required for this study. Therefore, only data from the larger arterioles that traverse across the brain surface will be presented for the different control and drug states explored.

Perivascular O₂ measurements. Perivascular oxygen tension was measured using the same electrodes and Keithley electrometer as mentioned in Perivasular NO measurements and polarized at a voltage of ~0.7 V relative to a silver-silver chloride ground. Microelectrodes were calibrated using oxygen concentrations equivalent to 0, 40, and 144 mmHg oxygen tension at 37–38°C to obtain a certain number of millivolts output per mmHg of oxygen tension. Oxygen microelectrodes generate a linear current/concentration relationship. A zero oxygen tension value during experiments was obtained by complete occlusion of a small arteriole or venule with the tip of the oxygen-sensing microelectrode. Measurements of perivascular oxygen during the experiment were obtained by placing the tip of the electrode against the outer wall of the vessel. Subtraction of the “0” oxygen tension voltage from the perivascular voltage multiplied by the calibration factor yielded the tissue oxygen tension (in mmHg).

Determination of NO generation and the inner arteriolar diameter response to lowered oxygen tension. To determine the response of the microvasculature to decreased oxygen tension, the bath oxygen tension was lowered from 40–45 mmHg to ~10–15 mmHg at a constant PCO₂ of ~40 mmHg. Bath oxygen saturation was changed from 90% N₂-5% CO₂-5% O₂ to 95% N₂-5% CO₂-0% O₂ to manipulate the bath oxygen tension in terms of altered mean arterial blood pressure. All microvascular measurements were taken after a period of stable oxygen tension and NO were reached, which was usually ~5–10 min following the change in oxygenation of the bath media.

Involvement of the Na⁺/Ca²⁺ exchanger in oxygen-sensing mechanisms. A Na⁺/Ca²⁺ exchanger blocker, KB-R7943, was used throughout these series of experiments (3, 15). KB-R7943 was applied to the cerebral microvasculature at a concentration of 50 µM by slow infusion through a micropipette directed at a small area of the arteriole under investigation for 30 min (47). This approach was used because the drug was quite expensive and longer exposure times may interfere with true Ca²⁺ channels (40). Following the 30-min treatment, [NO] and diameter measurements at both normal (40–45 mmHg) and low (10–15 mmHg) bath oxygen tension were repeated. These data were compared with those obtained for identical conditions prior to KB-R7943.

Effect of Na⁺ transporter inhibitors bumetanide and amiloride on the microvascular response to decreased oxygen tension. Involvement of both the Na⁺/H⁺ exchanger as well as the Na⁺-K⁺-2Cl− cotransporter in the oxygen-sensing mechanisms of the brain were explored using their respective blockers, amiloride and bumetanide, added to the bathing media. Separate animals were used for each drug. Perivascular NO and inner vessel diameter were measured at 40–45 mmHg oxygen as well as 10–15 mmHg oxygen before and after the addition of either 10µM amiloride or 10µM bumetanide to the bathing media. Both drugs were exposed for a minimum of 30 min before postdrug measurements of NO and inner arteriolar diameter were obtained with the drugs still in the media. We used 10µM bumetanide in the media because despite the fact this drug is effective as a blocker at a free plasma concentration of 5 µM (19) in mice, the compound binds to proteins in general. This likely explains why 5 µM in a tissue bath over living tissue was variably effective in our hands but 10 µM was consistently effective (47). The use of 10 µM amiloride was as high a concentration as we were comfortable. Amiloride under ideal conditions has a half-maximal blocking concentration of <0.5 µM at normal extracellular Na⁺ concentrations and has little affinity for the Na⁺/Ca²⁺ exchanger at up to the >100 µM range, as reviewed by Teiwes and Toto (43). However, in practice over solid blood perfused tissues, we have found that 10 µM was required in the bath to be sure of an effect in the intestinal vasculature (47). In the present study, this concentration was ineffective for the brain tissue of the cortical surface. We undoubtedly could have found some effect at higher concentrations, but such effects might be due to nonspecific effect on other Na⁺ channels or exchangers.

Effect of eNOS blockade through the caveolin-1 peptide derivative cavtratin. To determine the role of eNOS in the generation of increased NO in response to changes in oxygen tension, a specific eNOS blocker, cavtratin, was used. This protein has been described in several studies and has been used in both in vivo and in vitro preparations (20, 23). The peptide contains portions of the sequence of caveolin-1, the eNOS scaffolding protein. The peptide complexes with active eNOS to block the generation of NO. The generation by CPC Scientific (San Jose, CA) to a purification of 95.1% of the peptide sequence DGIWKASFTTFTVTKYWFYR was fused with an internalization sequence of antennapedia (5, 20, 23). Control measurements of NO and inner arteriolar diameter were taken as previously described in both control (40–45 mmHg) oxygen tension and lower (10–15 mmHg) oxygen tension.

To test for eNOS suppression by cavtratin, both diameter and [NO] responses to elevated blood flow were used. Collateral occlusion of a parallel arteriole was done at 40–45 mmHg bath oxygen tension (control conditions) to measure the increase in NO in response to increased blood flow in the vessel of interest, as used previously in this laboratory (4). Occlusion was done with a blunted micropipette tip with pressure application to a vessel in hemodynamic parallel while measurements of the paired arteriole were obtained. This procedure forces the open arteriole to perfuse about twice as many small arterioles that are shared with the paired collateral arteriole. Occlusion was done before and after the application of cavtratin at a concentration of 100 µM for 1 h in static solution over the preparation. Loss of vasodilation and increased [NO] during elevated blood flow confirmed that cavtratin had suppressed eNOS. An unanticipated problem with cavtratin in high concentration was that it appeared to decrease the NO sensitivity of the NO microelectrode if left in contact with the electrode for 1 h. Therefore, the electrode tip was removed from the cavtratin solution and not replaced over tissue until the peptide solution had been washed away. Even with bulk removal of the cavtratin solution, responses to elevated blood flow were suppressed for over 1 h, the longest time period routinely required during the study.

Glutamate (5 µM) was used before and after the application of cavtratin, bumetanide, and KB-R7943 to assure that nNOS was still functional. Glutamate activates N-methyl-D-aspartate (NMDA) receptors of neurons, astrocytes, and glial cells, which allows nNOS to uncouple from its scaffolding protein and begin NO generation (37, 42a). If an increase in [NO] and vasodilation occurred in response to glutamate after cavtratin application, we were confident that nNOS remained functional.

Statistical methods. For repeated measurements of oxygen tension, NO, and vessel diameter under control and blockade conditions, two-way ANOVA was used as well as the Tukey least-significant-difference test for specific comparisons. All analyses were performed with Statistica software (Statsoft, Tulsa, OK).
RESULTS

Vascular responses after blockade of Na\(^+\) cotransporters with amiloride and bumetanide. To evaluate the mechanism of Na\(^+\) entering the cell in response to sensing decreased oxygen tension, 10 \(\mu\)M amiloride or 10 \(\mu\)M bumetanide was applied to the cortical microvessels to evaluate the Na\(^+\)/H\(^+\) antiporter or Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, respectively. Following application of amiloride to the brain surface of four rats, the vasodilation and increased NO generation at decreased bath oxygen tension were unaffected. Under control conditions, the vessels had a resting diameter of 45.1 ± 5.0 \(\mu\)m and [NO] of 1,143 ± 132 \(nM\) compared with 55.9 ± 6.8 \(\mu\)m and 1,619 ± 183 \(nM\) at reduced oxygen tension. During amiloride exposure, the resting diameter of larger arterioles traversing over the brain surface of 45.6 ± 4.9 \(\mu\)m and [NO] of 1,034 ± 121 \(nM\) were unchanged from control conditions and the vasodilation to 60.0 ± 6.7 \(\mu\)m and elevated [NO] of 1,648±177 \(nM\) at lowered oxygen tension was equivalent to events during control conditions. By comparison, bumetanide had major effects on vascular responses to reduced oxygen tension as shown in Fig. 1 based on experiments on an average of 2 large and 2 small arterioles per animal in 10 rats. The resting [NO] of large arterioles at normal bath oxygen tension was not altered by bumetanide, but [NO] did not increase during low oxygen tension as occurred during control conditions (Fig. 1, bottom). Bumetanide caused a ~20\% dilation of intermediate transferring arterioles at rest during normal bath oxygen tension but ~16\% constriction of the smaller arterioles that penetrated the brain surface, as shown in Fig. 1, top. Neither large nor small arterioles dilated additionally from their resting status to lowered oxygen tension after bumetanide exposure. This was a particularly important observation for smaller arterioles since they were constricted at rest after bumetanide exposure. As shown in Fig. 2 for separately studied rats (9 small and 8 large arterioles, 4 rats), larger arterioles were not maximally dilated by bumetanide and could have dilated if the appropriate signal had been present. During bumetanide exposure, both large and small arterioles dilated normally in response to glutamate at 2.5–10 \(\mu\)M. Also note that in this separate group of rats exposed to bumetanide, larger arterioles dilated and smaller arterioles constricted in response, in support of the observations on the animals used to generate the data shown in Fig. 1.

It was not clear if the dilation of larger arterioles and constriction of smaller arterioles after bumetanide and their mutual lack of dilation at reduced bath oxygen tension had relevant physiological effects. To evaluate such effects in terms of tissue oxygenation, periarteriolar and perivenular oxygen tensions were measured in arterioles that perfused the smallest arterioles about to enter the brain tissue and the small collecting venules that ascended from the brain tissue to the surface. This data set was based on results from four arterioles and four venules in four rats. In every case, a red blood cell passing through the arteriole selected would have emerged into the venule studied. The selection process for venules was done by transient occlusion of the arteriole with the tip of the oxygen microelectrode to find nearby venules with dramatically slowed blood flow. Under control conditions, as shown in Fig. 3, the averaged periarteriolar oxygen tension was 57 mmHg for the arterioles and decreased ~10 mmHg in the presence of lower bath oxygen tension. Following the application of bumetanide, periarteriolar oxygen tension was lowered by 10–15 mmHg at normal bath oxygen tension and further decreased another 10 mmHg in response to low bath oxygen tension for a net reduction of 20–25 mmHg, or to about half of the normal resting oxygen tension. Perivenular oxygen tension, which likely reflects tissue oxygenation at the capillary level, was decreased during the reduction of bath oxygen tension under control conditions. After bumetanide application, the resting perivenular oxygen tension at the normal bath oxygen tension was as low as during control conditions at low bath oxygen tension and decreased slightly more when the bathing oxygen tension was lowered.

Effect of KB-R7943 on the generation of NO and increased inner vessel diameter in response to decreased oxygen tension. The experiments performed in this study demonstrated the significant contribution of the Na\(^+\)/Ca\(^{2+}\) exchanger in the mechanism to sense decreased oxygenation. In this experiment, six larger arterioles of six animals were exposed to the specific Na\(^+\)/Ca\(^{2+}\) blocker KB-R7943 through a micropipette directed at the vessel wall, as described in METHODS. As shown in Fig. 4, under normal conditions, both the inner arteriolar diameter and [NO] increased in response to decreased bath oxygen tension. Following exposure of the brain microvasculature to KB-

![Graph](image-url)

Fig. 1. Effect of bumetanide on cerebral arteriolar diameter (top) and nitric oxide (NO) concentration ([NO]) responses (bottom) to decreased oxygen tension. During normal conditions, cerebral larger and smaller arterioles responded to decreased oxygen tension with a significant increase in arteriolar diameter and [NO]. Following blockade with 10 \(\mu\)M bumetanide, larger arterioles were somewhat enlarged at rest but were unable to dilate appreciably more at decreased oxygen tension. In addition, these arterioles were unable to increase their [NO] in response to decreased oxygen availability. All small arterioles that penetrated the brain cortex were significantly constricted at rest after bumetanide and failed to dilate normally at reduced oxygen tension. Both large and small arterioles were capable of normal dilation to glutamate, as is shown in Fig. 2. Bumetanide results are based on experiments on an average of 2 large and 2 small arterioles per animal in 10 rats. \(*P < 0.05\) vs. control conditions prior to any perturbation or blockade; \(\#P < 0.05\) vs. the vascular response prior to drug application.
Effect of cavtratin on the generation of NO and increased vessel diameter. Following application of cavtratin to suppress active eNOS, basal [NO] decreased from a control of 616.8 ± 42.6 to 368.5 ± 46.2 nM (Fig. 5, bottom). However, the actual increase in [NO] from the new baseline during nNOS stimulation through glutamate and low oxygen tension was ~70% of normal. Surprisingly, the inner vessel diameter following cavtratin application at rest did not change despite the decline in [NO] (Fig. 5, top). The vasodilation to glutamate and low oxygen tension were unaffected by cavtratin blockade of eNOS, presumably because nNOS continued to be functional to these stimuli. However, the dilation and increased [NO] during high flow conditions were suppressed by cavtratin, as would be expected since the flow-dependent NO mechanisms of eNOS function should be suppressed.

DISCUSSION

The purpose of this study was to determine if Na⁺ and Ca²⁺ transport mechanisms would activate primarily nNOS or eNOS in in vivo cerebral tissues during a decline in oxygen tension. The general set of mechanisms for eNOS has been demonstrated in in vivo intestinal arterioles by Zani and Bohlen (47). As found in the present study at reduced oxygen tension, suppression of the Na⁺/H⁺ exchanger with amiloride had only a minor effect on vasodilation and increased [NO] generation of cerebral arterioles, but suppression of the Na⁺-K⁺-2Cl⁻ cotransporter with bumetanide or blockade of the Na⁺/Ca²⁺ exchanger with KB-R7943 were both effective to prevent increased periarteriolar [NO] and compensatory vasodilatation. In the brain vasculature, eNOS has very little role in the oxygen-sensing process because suppression with the scaffolding domain blocker cavtratin (20) did not eliminate increased [NO] and vasodilation to reduced oxygen tension (Fig. 5). nNOS was functional after cavtratin blockade because the arterioles increased both [NO] and vessel diameter in response to glutamate, which activated NMDA receptors of nNOS-containing neurons and neuronal support cells (37, 42a). nNOS has been found in limited amounts in cerebral endothelial cells and is primarily localized in cells near microvessels (35, 46). Based on NO and diameter responses with Na⁺/Ca²⁺ exchanger suppression, nNOS in the brain tissue and eNOS of intestinal arterioles share common oxygen-sensing mechanisms but in very different cell populations.
Even though we propose that a Na+/H1001 for Ca2+/H1001 exchange process is one means to activate NO regulation of cerebral arterioles at mildly to moderately reduced oxygen tension, this same process is cytotoxic in extreme conditions of oxygen deprivation. Studies of brain tissue have shown that blockade of the Na+/H1001–K+–2Cl– cotransporter and, to a lesser extent, the Na+/H1001/Ca2+/H1001 exchanger provided protection to the brain from cellular edema following ischemia (28, 39) and damage of cultured neural and glial cells after hypoxia (2, 17, 26, 30, 36). Kintner et al. (32) and Lenart et al. (33) have shown that the pharmacological blockade of either Na+ entry through the Na+/K+–2Cl– cotransporter or Na+ and Ca2+ exchange by the Na+/Ca2+ exchanger limited the increase in intracellular and mitochondrial Ca2+ accumulation by cultured astrocytes. The present results with these same blockade agents indicated that nNOS-containing cells of the brain use the Na+–driven Ca2+ accumulation during mild reductions in oxygen availability to increase NO production and cause arteriolar dilation (Figs. 1 and 4). Furthermore, neither bumetanide nor KB-R7943 blockade compromised the relative glutamate-induced vasodilation, shown in Fig. 2, which we have previously found was almost entirely dependent on nNOS generation of NO (4). In effect, Na+ and Ca2+ transport mechanisms that can lead to tissue damage during severe cerebral hypoxia are highly beneficial to increase NO generation by the brain tissue during mild to moderate reductions of oxygen tension.

We (4) demonstrated that most of the increase in [NO] was from nNOS during mild to moderate reductions in brain oxygen availability associated with systemic hypotension. This was shown with a highly specific blockade of nNOS that suppressed [NO] and dilation responses during reduced oxygen tension. eNOS function was intact because dilation and increased [NO] responses to elevated blood flow were substantially intact. The data shown in Fig. 5 indicate that the increase in cerebral perivascular [NO] with reduced oxygen availability did not require an intact eNOS system. Cavitratin suppresses active eNOS by binding to its caveolin scaffolding domain, caveolin-1 (5, 20). As shown in Fig. 5, cavitratin dramatically decreased the basal [NO], and the high-flow protocol did not
increase the [NO] or vessel diameter (Fig. 5), as occurred for the same arterioles during control conditions. However, glutamate to release nNOS from its scaffolding protein through stimulation of the NMDA receptor and lowered oxygen availability both caused a relatively normal absolute increase in vessel diameter and [NO], indicating that nNOS was nearly fully functional (Fig. 5). We have shown that responses to both of these challenges were strongly suppressed by nNOS blockade (4). What was unusual about the data shown in Fig. 5 was that vessel diameter at rest was not influenced by cavitratin despite the reduced [NO]. We presume that other important dilatory mechanisms have quickly offset the loss of NO from an eNOS source. The pharmacological tests shown in Fig. 5 to suppress eNOS, as well as the nNOS blockade experiments referenced from our prior study (4), predict that mechanisms that activate nNOS rather than eNOS are very important to cerebral oxygen sensing.

While bumetanide blockade of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter was highly effective to eliminate the increase in [NO] during reduced oxygen tension (Fig. 1), it did cause an unexplained dilation of the larger arterioles yet substantial constriction of the smaller arterioles (Figs. 1 and 2). Larger arterioles were not maximally dilated at rest by bumetanide because their \(\sim 17\%\) dilation was only \(\sim 50\%\) of the typical dilation during 10 \(\mu\)M glutamate exposure, a near-maximal dilatory stimulus, shown in Fig. 2. We propose that the larger arterioles diluted under bumetanide exposure because of the substantial constriction of small arterioles, as shown in Figs. 1 and 2 for different sets of animals studied under identical conditions. As evidence that constriction of the small arterioles limited oxygenation of the cortex, the resting oxygen tension beside arterioles was reduced by \(\sim 21\%\) and that by venules by \(\sim 34\%\), with the latter likely best reflecting tissue oxygenation (Fig. 3). At lowered bath oxygen content, much lower than normal periarteriolar and venular oxygen tensions were reached. These observations indicate that the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter mechanism normally has a role to both maintain oxygenation near arterioles and in general brain tissue during control conditions and becomes even more important during stressful conditions that limit oxygen availability.

After establishing that Na\(^+\) entry through the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter was a major component of the increased NO generation at normal and reduced oxygen tension, we confirmed that the Na\(^+\)/Ca\(^2+\) exchanger was required for the activation of NOS. For this purpose, KB-7943 was used to block the exchanger (3, 15). After the exchanger had been blocked (Fig. 4), the [NO] at rest was reduced, and there was no attempt to elevate [NO] or vessel diameter during reduced oxygen availability. As mentioned above, we have evidence that the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter actively contributed to the resting [NO] and that the Na\(^+\)/Ca\(^2+\) exchanger may be active at rest. This would explain the decline in [NO] after Na\(^+\)/Ca\(^2+\) exchanger blockade at rest. The complete absence of both increased [NO] and vasodilation at reduced oxygen tension after blockade shown in Fig. 4 would argue that the Na\(^+\)/Ca\(^2+\) exchanger is a major component of the mechanism to couple Na\(^+\)-dependent oxygen-sensing mechanisms to NO generation by nNOS-containing cells. However, as discussed earlier, the overall mechanism involving Na\(^+\) entry into cells with subsequent exchange for Ca\(^2+\) when carried to the extreme in hypoxia is detrimental to brain tissues (28, 39). Consequently, even though NO generation from nNOS linked to tissue oxygenation may contribute an important component of brain microvasculature regulation during mild conditions of oxygen stress, at high levels of activation, this process is potentially lethal to the very cells it normally serves so well.

**GRANTS**

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