Published in final edited form as:

Am J Physiol Heart Circ Physiol. 2007 November ; 293(5): H3193–H3200.

Carbon monoxide and Ca^{2+} -activated K^+ channels in cerebral arteriolar responses to glutamate and hypoxia in newborn pigs

Alie Kanu and Charles W. Leffler

Laboratory for Research in Neonatal Physiology, Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, U.S.A

Abstract

Large conductance calcium activated (K_{Ca}) channels regulate the physiological functions of many tissues, including cerebrovascular smooth muscle. L-glutamic acid (glutamate) is the principal excitatory neurotransmitter in the central nervous system and oxygen tension is a dominant local regulator of vascular tone. In vivo, glutamate and hypoxia dilate newborn pig cerebral arterioles and both dilations are blocked by inhibition of CO production. CO dilates cerebral arterioles by activating K_{Ca} channels. Therefore, the present study was designed to investigate effects of glutamate and hypoxia on cerebral CO production and the role of K_{Ca} channels in the cerebral arteriolar dilations to glutamate and hypoxia. In the presence of iberiotoxin or paxilline that block dilation to the K_{Ca} channel opener, NS1619, neither CO nor glutamate dilated pial arterioles. Conversely, neither paxilline nor iberiotoxin inhibited dilation to acute severe or moderate prolonged hypoxia. Both glutamate and hypoxia increased CSF CO concentration. Iberiotoxin that blocked dilation to glutamate did not attenuate the increase in CSF CO. The guanylyl cyclase inhibitor, 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ), that blocked dilation to sodium nitroprusside did not inhibit dilation to hypoxia. These data suggest that dilation of newborn pig pial arterioles to glutamate is mediated by activation of K_{Ca} channels consistent with the intermediary signal being CO. Surprisingly, although heme oxygenase (HO) inhibition attenuates dilation to hypoxia, hypoxia increases CSF CO concentration, and K_{Ca} channel antagonists block dilation to CO, neither K_{Ca} channel blockers nor ODQ altered dilation to hypoxia suggesting the contribution of the HO/CO system to hypoxia-induced dilation is not by stimulating vascular smooth muscle K_{Ca} channels or guanylyl cyclase.

Keywords

cerebrovascular circulation; vascular smooth muscle; paxilline; iberiotoxin

INTRODUCTION

Vascular tone in the cerebral circulation is regulated by numerous cellular mechanisms including modification of activity of potassium channels (6,7). Activation of potassium channels mediates relaxation of cerebral blood vessels in response to a diverse group of important stimuli. One such stimulus is carbon monoxide (CO), which is produced physiologically from the degradation of heme to biliverdin, iron, and CO by heme oxygenase (HO)(32), and can be produced in high enough concentration to cause vasodilation (10,21, 25,26). CO is a dilator of newborn cerebral arterioles in vivo and contributes to cerebral arteriolar dilation induced by excitatory amino acids in newborn pigs (13,26). In adult rabbits

and newborn pigs, HO inhibition reduces the elevation of cerebral blood flow during epileptic seizures but has no effect on cerebral hyperemia caused by hypercapnia, or in basal conditions (9,36). CO activates large conductance calcium-activated potassium (K_{Ca}) channels in intact newborn cerebral arteriole smooth muscle cells (18) and dilations to CO are blocked by inhibitors of K_{Ca} channels(26).

L-glutamic acid (glutamate) is a dilator of newborn cerebral arterioles (8) and the major excitatory neurotransmitter in the central nervous system (34). In the cerebral microcirculation of newborn pigs, glutamate stimulates CO production and causes CO-dependent vasodilation (43). Dilator responses to glutamate in vivo are blocked by the HO inhibitor, chromium mesoporphyrin (CrMP) (26,43).

Mechanisms involved in hypoxia-induced vasodilation remain poorly understood. Even less is known about the mechanisms that mediate vasodilation to hypoxia in the newborn cerebral circulation. Most data are consistent with a local response (7), but brainstem neuronal involvement in hypoxia-induced cerebral hyperemia, has also been reported (48). The HO/CO system seems to be involved because, similarly to glutamate, CrMP inhibits pial arteriolar dilations to hypoxia in piglets (26).

Therefore, experiments were designed and conducted to test the hypothesis that both hypoxia and glutamate increase cerebral CO production leading to K_{Ca} channel induced cerebrovascular dilation. The following results are consistent with the hypothesis when the stimulus is glutamate but do not support this direct action hypothesis when the dilator stimulus is hypoxia, suggesting the role of HO/CO system in hypoxia induced vasodilation is upstream from the mediator in the dilator pathway.

MATERIAL AND METHODS

All procedures that involved animals were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Newborn pigs (1 to 3 days old) were anesthetized with ketamine hydrochloride (33 mg/kg, i.m.) and acepromazine (3.3 mg/kg, i.m.) and maintained on α -chloralose (50 mg/kg, i.v.). Because ketamine appears to cause anesthesia by noncompetitive inhibition of NMDA receptors its use should be carefully considered in experiments that involve glutamatergic transmission. However, we have compared newborn piglets anesthetized with either ketamine or thiopental and could not detect any differences in glutamatergic seizure-induced cerebral hemodynamics, responses to topical glutamate, or even responses to topical NMDA (data not shown). Ketamine is very rapid in onset, but short acting, and piglets not administered α -chloralose recover from anesthesia in 20–40 min, long before experimentation begins in cranial window experiments. While we did experiment with thiopental, in piglets ketamine with acepromazine is clearly superior for rapid induction and depth of anesthesia. A catheter was inserted into a femoral artery to monitor blood pressure and heart rate and to collect blood for measurements of P_{CO_2} , P_{O_2} , and pH. A second catheter was placed in a femoral vein for anesthetic and fluid administration. The trachea was cannulated, and the animals were mechanically ventilated and supplemented with O_2 , if needed, to maintain arterial pH, P_{CO_2} , and P_{O_2} within the normal range. A heating pad was used to maintain the body temperature at 37.5–38.5°C, which was monitored with a rectal probe.

Cranial window placement and pial arteriolar monitoring

The scalp was surgically removed, a 2-cm-diameter parietocortical craniotomy performed, and the dura mater excised and reflected over the bone to prevent contact between the brain surface and the cut edge of the bone. A stainless steel-and-glass window was implanted into the hole and cemented sequentially with bone wax and dental acrylic. This window consisted of three

parts: a stainless steel ring, a circular glass coverslip, and three ports consisting of 17-gauge hypodermic needles attached to three precut holes in the stainless steel ring. The space under the window was filled with artificial cerebrospinal fluid (aCSF: 150 meq Na⁺/l, 3 meq K⁺/l, 2.5 meq Ca²⁺/l, 1.2 meq Mg²⁺/l, 132 meq Cl⁻/l, 3.7 mM glucose, 6 mM urea, 25 meq HCO₃⁻/l) which was warmed in a water bath to 37°C and bubbled with a mixture of N₂, O₂, and CO₂ to maintain pH at ~ 7.33 and Pco₂ and Po₂ at ~ 45mmHg which is within the physiological range (29,30). The fluid under the window was exchanged via needle ports on the sides of the window. Pial arterioles were observed with a dissecting microscope and were measured with a video micrometer coupled to a television camera mounted on the microscope and a video monitor. Arterioles were selected with a preference for approximately 60µm resulting in a bell shaped curve of sizes with a prominent single peak such that mean ± SE was 63.1±1.8µm with a lower 95% confidence limit of 59.5 and an upper confidence limit of 66.7µm.

Experimental design

After implantation of the cranial window, approximately 30 min were allowed before experimentation was begun. The baseline pial arteriolar diameter, along with heart rate, mean arterial blood pressure, and core temperature, were recorded. Three different combinations of treatment before and after paxilline were used in individual piglets in the first set of experiments: 1: NS1619; 2: hypoxia and isoproterenol, 3: CO, glutamate, and sodium nitroprusside (SNP). For experiments without or with iberiotoxin, combinations were SNP and glutamate or SNP and hypoxia. SNP and hypoxia was the treatment combination without and with 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one(ODQ)(2.5x10⁻⁴M). Treatment orders were constant rather than randomized, because responses to the separate treatments were not compared to each other but rather responses to each treatment were compared without and with paxilline, iberiotoxin, or ODQ. Between treatments, the area under the window was sufficiently irrigated with fresh aCSF to remove the previous stimulus. The pial arteriolar diameters were allowed to return to the baseline before the next stimulus or treatment was given.

In this first set of experiments all treatments as described below were administered for 10-min periods with the exception of hypoxia (5min) to minimize hypoxic stress. The maximal dilation was recorded as the response. In experiments with iberiotoxin and ODQ, pial arteriole diameter measurements were made for 5min. When CSF was collected for CO measurements, the collection was made at 7min of treatment. At the end of each tested response, arterial blood gases and pH, blood pressure, body temperature were measured and, with the exception of P_{O2} during hypoxia, were maintained within normal limits.

Pial arteriolar response to NS1619

Responses of pial arterioles to topical application of the benzimidazolone compound, NS1619, at 10⁻⁶M, an activator of K_{Ca} channels, were measured before and after topical application of 4 x 10⁻⁵ M paxilline, a selective K_{Ca} channel blocker. This test was done to confirm the efficacy of paxilline.

Pial arteriolar response to CO

Treatments with topical CO at concentrations of 10⁻⁷ M and 10⁻⁶ M were given before and during treatment with paxilline (4x10⁻⁵M topically). CO was purchased in a compressed gas cylinder of 100% CO. The initial stock solution (10⁻³M) was produced by saturation of water with CO using solubilities from the Handbook of Chemistry and Physics. Dilutions were produced in gas tight containers without a gaseous interface.

Pial arteriolar responses to Glutamate and SNP

Responses to topical application of 2×10^{-7} M SNP and glutamate (10^{-6} M and 10^{-5} M) were determined before and during treatment with paxilline (4×10^{-5} M) or iberiotoxin (10^{-6} M). CSF collections for CO determinations were made at the end of 7 min treatment or control.

Pial arteriolar responses to hypoxia

Acute hypoxia was produced by ventilation with 10% O₂ in N₂. Hypoxia was maintained for 5 min and the maximal dilation was recorded as the response. This treatment caused a decrease in PaO₂ to ~ 30 mm Hg within 5 min. Pial arteriolar diameters and arterial pressures were measured. For CSF CO determination, hypoxia was maintained for 7 min at which time CSF from under the window was collected.

In another group of piglets, hypoxia was given and measurements taken over 30 min of hypoxia (13% O₂). Hypoxia challenges were given to each piglet. One group then received paxilline and the other group vehicle during the second hypoxic challenge. We used 13% O₂ instead of 10% O₂ in these experiments because piglets deteriorate rapidly as ventilation with 10% O₂ is prolonged.

CO measurement

CO in a CSF was measured using GC-MS and ³¹CO as the internal standard as described before (22,23,26,43). Confirmation of accuracy of CO measurements in CSF under the cranial window was obtained by filling windows with known concentrations then collecting and measuring the CO in the CSF collected (Table 1).

Statistical analysis

Values are presented as means $\pm$ SE. For comparisons among groups, results were subjected to a one-way ANOVA for repeated measures with Tukey's post hoc test to isolate differences between groups. Paired t tests were used to compare two groups. A level of $P < 0.05$ was considered significant.

RESULTS

There were no significant changes in arterial blood gas levels, pH, pressure or body temperature, when the values were compared at the beginning and end of the experiments.

Effects of NS1619 on newborn pig pial arterioles

NS1619, that opens K_{Ca} channels, caused dilation of pial arterioles that was abolished by paxilline, a K_{Ca} channel blocker (Fig. 1).

Effects of K_{Ca} channel blockers in pial arteriolar diameter

Pial arterioles previously treated with glutamate, following washout and return to resting diameters, constricted in response to paxilline and iberiotoxin (Fig. 2,3). Conversely, in groups of piglets in which pial arterioles had not had previous exogenous glutamate exposure neither paxilline nor iberiotoxin caused constriction (Fig. 4,5).

Effects of Paxilline on vasodilation in response to CO and isoproterenol

As shown in Figure 2, CO-induced dilation was abolished by paxilline (Fig. 2) as shown before for another K_{Ca} channel blocker, iberiotoxin (25), but paxilline did not affect dilation to isoproterenol (10^{-7} M) (increase in pial arteriolar diameter, $22 \pm 3\%$ and $22 \pm 6\%$, without and with paxilline, respectively).

Effects of paxilline and iberiotoxin on dilations to glutamate and SNP

Both glutamate and SNP dilated newborn pig cerebral arterioles in vivo (Fig. 3). Paxilline and iberiotoxin blocked the dilation to glutamate but did not affect the dilation to SNP.

Effects of paxilline and iberiotoxin on pial arteriolar dilations to hypoxia

Hypoxia accomplished by ventilation with 10% O₂ for 5min caused dilation of pial arterioles (Fig 4). PaO₂, PaCO₂ and arterial pH before hypoxia were 103±4, 32±1, 7.40±0.01mm Hg, and after 5min of 10% O₂ were 33±2, 36±2, 7.35±0.02mm Hg. The hypoxia-induced increase of pial arteriolar diameter was unaltered by paxilline or iberiotoxin.

The effects of prolonged hypoxia (13% oxygen) on diameters of pial arterioles of newborn pigs are shown in Fig 5. PaO₂, PaCO₂, and pH were 91±4, 38±2, 7.40±0.02 before and 51±2, 30±2, 7.40±0.08 mm Hg after 30min ventilation with 13% O₂. Dilation to hypoxia progressed over about the first 15 min and then dilations were sustained for the next 15 min. Following return to normoxia, repeated ventilation with 13% O₂ for 30min produced dilation identical to the first hypoxic challenge (Fig. 5A). Similarly, when paxilline was included during the second hypoxic challenge, dilation to hypoxia was unaltered (Fig 5B).

Effects of ODQ on dilation to hypoxia—Because HO inhibition inhibits dilation to hypoxia, K_{Ca} channel inhibitors had no effect on the dilation, and CO may stimulate guanylyl cyclase and increase cGMP, we examined the effect of ODQ on dilation of pial arterioles to hypoxia. Efficacy of ODQ was demonstrated by blocking dilation to SNP (Fig. 6). ODQ did not alter pial arteriolar dilation to hypoxia (Fig. 6).

Effects of glutamate and hypoxia on CSF CO concentration—As shown before (43), glutamate increases CO concentration in CSF collected from under the cranial window (Fig. 7). Iberiotoxin, that blocked dilation to glutamate (Fig. 3), did not change the increase in CSF CO concentration to glutamate (Fig. 7). Hypoxia also increased CSF CO concentration prior to and following iberiotoxin (Fig. 7), although dilation to hypoxia was not blocked by iberiotoxin or paxilline (Fig. 4). Effects of PPOH and ABT (will be fig. 6) on pial arteriolar dilations to hypoxia. Effects of glutamate and hypoxia on aCSF concentration of CO (will be fig. 7)

DISCUSSION

The new findings of the present study of newborn pigs are: 1) the selective K_{Ca} channel blockers, paxilline and iberiotoxin, block the dilation of pial arterioles to glutamate but not the NO releasing molecule, SNP, as shown before (1,2), nor to the beta adrenergic agonist, isoproterenol, 2) neither paxilline nor iberiotoxin alters dilation to either short severe hypoxia or longer-term moderate hypoxia (paxilline only), 3) ODQ does not inhibit dilation to hypoxia, and 4) hypoxia increases cerebral production of CO similarly to glutamate and these increases in CO are not affected by iberiotoxin that blocks the dilation of glutamate.

The efficacy of paxilline was confirmed with NS1619 a vasodilator that opens K_{Ca} channels at micromolar concentrations, which do not activate voltage-gated or ATP-dependent K⁺ channels nor block L-type Ca²⁺ channels (14,44). The concentration of paxilline was chosen as the minimal concentration used previously by others. Paxilline is as selective and can be more effective than the more commonly employed K_{Ca} channel blocker, iberiotoxin (15). The increased efficacy of paxilline can be related to reduced affinity of specific K_{Ca} channels for iberiotoxin, particularly when complexed with β-subunits, diffusion limitations, and peptide adhesion sites of equipment. Nevertheless, in the present experiments using paxilline and iberiotoxin in identical protocols produced the same results in each case. Further, since

paxilline and iberiotoxin did not alter the dilation to SNP or isoproterenol, it appears that the loss of responses to NS1619 and CO (26 and present study) are selective rather than a paxilline or iberiotoxin-induced generalized depression in pial arteriolar responsiveness.

Inhibition of K_{Ca} channels with paxilline or iberiotoxin did not alter basal pial arteriolar diameters in any group in the present study except piglets that had previously received topical application of glutamate. Since dilation to glutamate was blocked by both iberiotoxin and paxilline this dilation clearly involves K_{Ca} channels. However, removal of glutamate indicated that the dilation of pial arterioles was completely reversible suggesting termination of the K_{Ca} channel activity elevation. The consistent constriction produced by K_{Ca} channel inhibition even 30 min or more following removal of the glutamate suggest topical exogenous glutamate stimulation changes the contribution of K_{Ca} channels to regulation of vascular tone. The increased contribution could be explained by either an increase of constrictor that is counterbalanced by increased K_{Ca} activity or prolonged activation of K_{Ca} channels from the initial signaling pathway with compensatory elevation of a constrictor influence. Neuronal activation, which could be caused by glutamate, was not measured in the present experiments. It is conceivable that initial topical glutamate altered baseline neuronal activity. These data emphasize the potential for complex compensatory mechanisms to contribute to the establishment of final vascular tone and that simply reversal of a single response upon removal of a stimulus does not guarantee that original conditions are achieved.

Evidence for an involvement of K^+ channel activation in CO-induced vasodilation has arisen from various studies employing intact circulation in vivo, isolated vessels and patch-clamp techniques. For example, CO administration produced dose-dependent increases of piglet pial arteriolar diameters in vivo that were abolished in the presence of TEA and iberiotoxin (26). Furthermore, CO has a direct effect on the vascular smooth muscle K_{Ca} channels (49,50,52) increasing the open probability of the K_{Ca} channels (17).

Topical application of agonists and antagonists to the brain surface impacts brain and vascular cells. Of particular note, in addition to vascular smooth muscle, astrocytes and endothelial cells have K_{Ca} channels. CO can dilate cerebral arterioles by activating K_{Ca} channels on the smooth muscle, specifically by binding to heme attached to the alpha subunit of the K_{Ca} channel, increasing its Ca sensitivity, and thereby increasing spark to STOC coupling (17,18). This mechanism can be observed in isolated cerebrovascular smooth muscles and intact pressurized arterioles (17), and is the most likely explanation for the ability of K_{Ca} channel inhibitors to abolish cerebral arteriole dilation to CO in situ (present study, 26). Nevertheless, activation of K_{Ca} channels on astrocytes, neurons, and/or endothelium by CO may contribute to CO-induced cerebrovascular dilation by enhancing production of dilatory signals to the vascular smooth muscle. Therefore, while CO can cause changes in isolated myocytes consistent with dilation, multiple additional signaling mechanisms could contribute to the dilation in the intact brain and the relative contributions of these mechanisms could be stimulus specific. However, the observation that pial arteriolar dilation to topical CO is abolished by K_{Ca} channel inhibition results in the extrapolation that a stimulus producing cerebrovascular dilation using CO as the transmitter would also be blocked by K_{Ca} channel inhibition.

In the cerebral microcirculation glutamate is a vasodilator (35,43). The increased neuronal activity caused by glutamate requires increased cerebral blood flow which involves glutamate stimulation of CO production from heme by HO-2 in newborn pigs (23,26). In the present study, blockade of dilation to glutamate by paxilline and iberiotoxin is consistent with a mechanism involving CO as a messenger, since CO-induced dilation is mediated by K_{Ca} channels. Further, as reported previously (43) topical application of glutamate to the brain surface increased CO accumulation in the CSF. Inhibition of the glutamate-induced dilation with iberiotoxin did not affect the increase in CSF CO. These data further suggest glutamate

increases CO production and that CO then causes the dilation by activating K_{Ca} channels, rather than K_{Ca} channel activation causing the increase in CO.

Although both glutamate and hypoxia increase cerebral production of CO, inhibition of the K_{Ca} channel that serves as the receptor for the direct effect of CO on cerebral arteriolar smooth muscle cells (18) blocks only the dilation to glutamate, not to hypoxia. This apparent inconsistency has important conceptual connotation. If CO produced by brain cells was delivered to target cells by release and transport in the CSF, CSF CO concentrations during stimulation would be sufficient to produce equivalent dilation when applied topically to the brain surface. However, this is not the case. For example, although glutamate ($10^{-5}M$) nearly doubles CSF CO concentration, the resultant CSF concentration is less than $10^{-7}M$ (Fig. 7). The dilation to $10^{-5}M$ glutamate (Fig 3) is similar to that produced by a CO concentration of $10^{-6}M$ (Fig. 2). Because the CSF CO concentration is insufficient to account for the dilation, it appears reasonable to propose that, while CSF CO concentration under the window reflects changes in production by cells at the brain surface, concentrations at the sites of production are higher where CO can function as a precision gasotransmitter. Although both glutamate and hypoxia increase CSF CO concentration and produce dilation that is blocked by HO inhibition, available data implicate astrocytes as the signaling cells in the case of glutamate (28) and endothelium in the case of hypoxia (27). Because K_{Ca} channel inhibition blocks dilation to glutamate, but not to hypoxia, CO produced by astrocytes in response to glutamate may dilate adjacent arterioles by activating the smooth muscle K_{Ca} channels while CO produced in endothelium may be involved in an endothelium-derived signal for hypoxic vasodilation.

Hypoxia stimulates the production by endothelium, astrocytes, and neurons of a wide variety of vasodilator metabolites, including potassium and hydrogen ions, prostaglandins, excitatory amino acids, NO, endothelial- derived hyperpolarizing factor, and adenosine that can work in concert to produce the final dilatory response (19,37,39,40). The present results indicate that neither the initial onset of dilation to hypoxia nor the maintenance of the dilation requires functional K_{Ca} channels in newborn pigs. Further, the lack of effect of paxilline or iberiotoxin on responses to hypoxia coupled with blockade of glutamate-induced dilation suggests hypoxic stimulation of glutamatergic signaling plays a minimal role in dilation to hypoxia in the newborn cerebral vasculature.

In response to 10% O_2 ventilation, pial arterioles dilate rapidly over the first 2 min, with only a slight further dilation between 2 and 5 min (data not shown). Five min of hypoxia produces minimal changes in arterial pressure. With longer hypoxia periods using 10% O_2 , arterial pressure will drop without support. Although 5 min are clearly sufficient for activation of peripheral reflex pathways, it is probably insufficient to involve numerous humoral regulators such as vasopressin and opioids (27). Therefore, to sustain a longer period of hypoxia, 13% O_2 in the inspired air was used. Similarly to 5min of severe hypoxia, cerebrovascular dilation to 30 min of moderate hypoxemia was totally unaffected by inhibition of K_{Ca} channels. While, when K_{Ca} channel activity is blocked, the influence of other dilator pathways could be accentuated masking a normal contribution of K_{Ca} channels, this possibility appears unlikely because HO inhibition blocks dilation to hypoxia(26)

In newborn pigs, in contrast to the K_{Ca} channel blocker, a HO inhibitor did reduce the increase of pial arteriolar diameter in response to hypoxia (26). Further, while blockade of neither of the most appreciated mediators of CO-induced vascular responses, K_{Ca} channels and cGMP, altered hypoxia-induced cerebral vasodilation, hypoxia did increase CSF CO concentration. The correlation of the CO elevation with response blockade by K_{Ca} channel inhibition in the case of glutamate but not hypoxia emphasizes the point that CSF CO concentration indicates brain cells on the surface produced CO in response to a stimulus but do not indicate which cells

or whether the producing cells are in sufficient proximity to the vascular smooth muscle to signal dilation.

The ability of K_{Ca} channel inhibitors to block CO-induced dilation, but not hypoxia-induced dilation, might suggest that the contribution of HO to hypoxia-induced dilation is indirect. HO-2 has been proposed to be a cellular O_2 sensor (27), suggesting that the sensing of hypoxia may involve HO/CO, but the dilation in response may not. Recently, O_2 has been reported to stimulate K_{Ca} channels by activating HO-2, leading to the generation of CO, the downstream channel activator (51). The presence of HO-2 in the K_{Ca} channel complex provides a molecular explanation for the observation that HO inhibition results in carotid body excitation (42). Hypoxic depression of K_{Ca} channel activity in neurons of the central nervous system may also contribute to the excitotoxicity that results from increased neuronal excitability (31). Nevertheless, the present data suggest lack of K_{Ca} channel involvement in pial arteriolar dilation to hypoxia in newborn pigs.

Results of the present report are consistent in many respects with those of another group using the same model (1,2), but also different with regards to iberiotoxin effects on responses to both hypoxia and glutamate. Both these previous studies and the present one find no effect of K_{Ca} channel inhibition on dilation to SNP. However, when hypoxia equivalent to the 5 min hypoxia level in the present study was administered for 10 min, Armstead detected about a 20% reduction in hypoxia-induced vasodilation following iberiotoxin. Although the precise timing of the measurements differ between the studies, it is difficult to detect any differences between this study and the present one to explain our total lack of effect of iberiotoxin at a higher concentration on responses to hypoxia given for 5 min at the same P_{O_2} . Our data with iberiotoxin are identical to those using another highly selective and potent K_{Ca} channel inhibitor, which also had no effect on hypoxia-induced dilation. Also, Phillip and Armstead found only about 50% inhibition of the dilator response to glutamate with iberiotoxin that abolished dilation to NS1619 (41). Experimental design and methods appear identical leaving no obvious explanation for the greater efficacy of K_{Ca} channel blockers, iberiotoxin and paxilline, on glutamate-induced dilation in the present study. Consistent between both groups is the conclusion that the contribution of K_{Ca} channels to glutamate-induced dilation is greater than to dilation to moderately severe acute hypoxia.

As reported before (1,2) the present results indicate that the mechanism by which NO dilates piglet pial arterioles does not involve K_{Ca} channels because dilation to SNP was unaltered by paxilline or iberiotoxin. NO-induced dilation is associated with increased levels of cGMP in vascular smooth muscles cells (16,37,39) and ODQ blocked dilation to SNP. It has also been suggested that the effects of CO on cerebral blood flow may be mediated by NO (33). However, the ability of K_{Ca} channel inhibition to block dilation to CO but not to NO indicates distinct mechanisms and argues strongly against the dilation to either of these gaseous messengers being mediated by the other. Similarly, dilation to the beta adrenergic agonist, isoproterenol, was not affected by paxilline, indicating that cAMP induced cerebrovascular dilation does not involve K_{Ca} channels.

In conclusion, the present study shows that K_{Ca} channels account for the relaxation induced in pial arterioles by CO and glutamate in newborn pigs. As glutamate increases CO production (43 and present study) and CO activates K_{Ca} channels (17) it is reasonable to propose that CO is the messenger by which glutamate dilates piglet cerebral arterioles. Conversely, inhibition of K_{Ca} channels did not alter cerebrovasodilation to hypoxia, SNP or isoproterenol. These data suggest dilation to hypoxia is not caused by hypoxia increasing CO that activates K_{Ca} channels. The present results also suggest lack of involvement of guanylyl cyclase. Previous data show inhibition of CO production attenuates dilation to hypoxia and the present data show hypoxia

increases cerebral CO production. We speculate that HO/CO is acting as an oxygen sensor rather than dilator mechanism in this instance.

Acknowledgements

This research was supported by the National Heart, Lung, and Blood Institute/National Institutes of Health (NHLBI/NIH). Dr. Kanu is supported by a postdoctoral training grant from NLHBI/NIH. We thank Gregg Short for preparing the figures, Ed Umstot and Alex Fedinec for measuring CO in solutions and CSF, and Michelle Lester and Stephanie Sherman for clerical assistance.

References

1. Armstead WM. Contribution of K_{Ca} channel activation to hypoxic cerebrovasodilation does not involve NO. *Brain Res* 1998;799:44–48. [PubMed: 9666071]
2. Armstead WM. Role of activation of calcium-sensitive K^+ channels in NO- and hypoxia-induced pial artery dilation. *Am J Physiol Heart Circ Physiol* 1997;272:H1785–H1790.
3. Armstead WM. Opioids and nitric oxide contribute to hypoxia-induced pial arterial vasodilation in newborn pigs. *Am J Physiol Heart Circ Physiol* 1995;268:H226–H232.
4. Baranano DE, Snyder SH. Neural roles for heme oxygenase: contrasts to nitric oxide synthase. *Proc Natl Acad Sci USA* 2001;98:10996–11002. [PubMed: 11572959]
5. Brian JEJ, Bryan RM, Steenberg ML, Eichler MY, Johnson TD, Swafford MW, Suresh MS. Permissive role of NO in α_2 -adrenergic-mediated dilations in rat cerebral arteries. *Am J Physiol Heart Circ Physiol* 1995;269:H1171–H1174.
6. Brian JEJ, Faraci FM, Heistad DD. Recent insights into the regulation of cerebral circulation. *Clin Exp Pharmacol Physiol* 1996;23:446–457.
7. Busija D, Heistad DD. Factors involved in physiological regulation of the cerebral circulation. *Rev Physiol Biochem Pharmacol* 1984;101:161–211. [PubMed: 6441228]
8. Busija DW, Leffler CW. Dilator effects of amino acid neurotransmitters on piglet pial arterioles. *Am J Physiol Heart Circ Physiol* 1989;257:H1200–H1203.
9. Carratu P, Pourcyrus M, Fedinec A, Leffler CW, Parfenova H. Endogenous heme oxygenase prevents impairment of cerebral vascular functions caused by seizures. *Am J Physiol Heart Circ Physiol* 2003;285:H1148–H1157. [PubMed: 12915392]
10. Christodoulides N, Durante W, Kroll MH, Schafer AI. Vascular smooth muscle cell heme oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. *Circulation* 1995;91:2306–2309. [PubMed: 7729015]
11. Domoki F, Perciaccante JV, Shimizu K, Puskar M, Busija DW, Bari F. N-methyl-D-aspartate-induced vasodilation is mediated by endothelium-independent nitric oxide release in piglets. *Am J Physiol Heart Circ Physiol* 2002;282:H1404–H1409. [PubMed: 11893577]
12. Durante W, Kroll MH, Christodoulides N, Peyton KJ, Schafer AI. Nitric oxide induces heme oxygenase-1 expression and carbon monoxide production in vascular smooth muscle cells. *Circ Res* 1997;80:557–564.
13. Fiumana E, Parfenova H, Jaggar JH, Leffler CW. Carbon monoxide mediates vasodilator effects of glutamate in isolated pressurized cerebral arterioles of newborn pigs. *Am J Physiol Heart Circ Physiol* 2003;284:H1073–H1079. [PubMed: 12666665]
14. Hu S, Kim HS, Fink CA. Differential effects of BK_{Ca} channel openers NS004 and NS1608 in porcine coronary arterial cells. *Eur J Pharmacol* 1995;294:357–360. [PubMed: 8788454]
15. Hu H, Shao LR, Chavoshy S, Gu N, Trieb M, Behrens R, Laake P, Pongs O, Knaus HG, Ottersen OP, Storm JF. Presynaptic Ca^{2+} -activated K^+ channels glutamatergic hippocampal terminals and their role in spike repolarization and regulation of transmitter release. *J Neurosci* 2001;21(24):9585–97. [PubMed: 11739569]
16. Iadecola C, Pelligrino DA, Moskowitz MA. Nitric oxide synthase inhibition and cerebrovascular regulation. *J Cerebral Blood Flow and Metab* 1994;14:175–192.
17. Jaggar JH, Leffler CW, Cheranov SY, Tcheranova D, E S, Cheng X. Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca^{2+} sparks to Ca^{2+} -activated K^+ channels. *Circ Res* 2002;91:610–617. [PubMed: 12364389]

18. Jaggar JH, Li A, Parfenova H, Liu J, Umstot ES, Dopico AM, Leffler CW. Heme is a carbon monoxide receptor for large-conductance Ca^{2+} -activated K^{+} channels. *Circ Res* 2005;97:805–812. [PubMed: 16166559]
19. Kitazono T, Faraci FM, Taguchi H, Heistad DD. Role of potassium channels in cerebral blood vessels. *Stroke* 1995;26:1713–1723. [PubMed: 7660420]
20. Kontos HA. Nitric oxide and nitrosothiols in cerebrovascular and neuronal regulation. *Stroke* 1993;24:1155–1158.
21. Kozma F, Johnson RA, Nasjletti A. Role of carbon monoxide in heme induced vasodilation. *Eur J Pharmacol* 1997;323:R1–R2. [PubMed: 9128852]
22. Leffler CW, Balabanova L, Fedinec AL, Parfenova H. Nitric oxide increases carbon monoxide production by piglet cerebral microvessels. *Am J Physiol Heart Circ Physiol* 2005;289:H1442–1447. [PubMed: 15964921]
23. Leffler CW, Balabanova L, Fedinec AL, Waters CM, Parfenova H. Mechanism of glutamate stimulation of CO production in cerebral microvessels. *Am J Physiol Heart Circ Physiol* 2003;285:H74–80. [PubMed: 12623781]
24. Leffler CW, Fedinec AL, Parfenova H, Jaggar JH. Permissive contributions of NO and prostacyclin in CO-induced cerebrovascular dilation in piglets. *Am J Physiol Heart Circ Physiol* 2005;289:H432–438. [PubMed: 15708959]
25. Leffler CW, Nasjletti A, Johnson RA, Fedinec AL. Contributions of prostacyclin and nitric oxide to carbon monoxide-induced cerebrovascular dilation in piglets. *Am J Physiol Heart Circ Physiol* 2001;280:H1490–1495. [PubMed: 11247758]
26. Leffler CW, Nasjletti A, Yu C, Johnson RA, Fedinec AL, Walker N. Carbon monoxide and cerebral microvascular tone in newborn pigs. *Am J Physiol Heart Circ Physiol* 1999;276:H1641–1646.
27. Leffler CW, Smith JS, Edrington JL, Zuckerman SL, Parfenova H. Mechanisms of hypoxia-induced cerebrovascular dilation in the newborn pig. *Am J Physiol Heart Circ Physiol* 1997;272:H1323–1332.
28. Leffler CW, Parfenova H, Fedinec AL, Basuroy S, Tcheranov D. Contributions of astrocytes and CO to pial arteriolar dilation to glutamate in newborn pigs. *Am J Physiol Heart Circ Physiol* 2006;291:H2897–904. [PubMed: 16891404]
29. Levasseur JE, Wei EP, Raper AJ, Kontos AA, Patterson JL. Detailed description of a cranial window technique for acute and chronic experiments. *Stroke* 1975;6:308–317. [PubMed: 1154467]
30. Lips J, de Haan Bouma GJ, Holman R, Dongen E, Kalkman CJ. Continuous monitoring of cerebrospinal fluid oxygen tension in relation to motor evoked potentials during spinal cord ischemia in pigs. *Anesthesiology* 2005;102:340–345. [PubMed: 15681949]
31. Liu H, Moczydlowski E, Haddad GG. O_2 deprivation inhibits Ca^{2+} -activated K^{+} channels via cytosolic factors in mice neocortical neurons. *J Clin Invest* 1999;104:577–588. [PubMed: 10487772]
32. Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Ann Rev Pharmacol Toxicol* 1997;37:517–554. [PubMed: 9131263]
33. Meilin S, Rogatsky GG, Thom SR, Zarchin N, Gugginheimer-Furman E, Mayevsky A. Effects of carbon monoxide on the brain may be mediated by nitric oxide. *J Appl Physiol* 1996;81:1078–1083. [PubMed: 8889737]
34. Meldrum BS. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr* 2000;130:1007S–1015S. [PubMed: 10736372]
35. Meng W, Tobin JR, Busija D. Glutamate-induced cerebral vasodilation is mediated by nitric oxide through NMDA receptors. *Stroke* 1995;26:857–863. [PubMed: 7740580]
36. Montecot C, Seylaz J, Pinard E. Carbon monoxide regulates cerebral blood flow in epileptic seizures but not in hypercapnia. *Neuroreport* 1998;9:2341–2346. [PubMed: 9694225]
37. Pearce WJ. Mechanisms of hypoxic cerebral vasodilation. *Pharmac Ther* 1995;65:75–91.
38. Pearce WJ, Ashwall S, Cuevas J. Direct effects of graded hypoxia on intact and denuded rabbit cranial arteries. *Am J Physiol Heart Circ Physiol* 1989;257:H824–H878.
39. Pelligrino DA, Wang Q, Koenig HM, Albrecht RF. Role of nitric oxide, adenosine, N-methyl-D-aspartate receptors, and neuronal activation in hypoxia-induced pial arteriolar dilation in rats. *Brain Res* 1995;704:61–70. [PubMed: 8750962]

40. Phillis, JW. Cerebral vascular responses to hypoxia, hyper and hypocapnia and hypotension. Boca Raton, FL: CRC; 1993. p. 249-268.
41. Philip S, Armstead WM. NMDA dilates pial arteries by K_{ATP} and K_{Ca} channel activation. *Brain Res Bul* 2004;63:127–131.
42. Prabhakar NR, Dinerman JL, Agani FH, Snyder SH. Carbon monoxide: a role in carotid body chemoreception. *Proc Natl Acad Sci USA* 1995;92:1994–1997. [PubMed: 7892214]
43. Robinson JS, Fedinec AL, Leffler CW. Role of carbon monoxide in glutamate receptor-induced dilation of newborn pig pial arterioles. *Am J Physiol Heart Circ Physiol* 2002;282:H2371–2376. [PubMed: 12003848]
44. Siemer C, Bushfield M, Newgreen D, Grissmer S. Effects of NS1608 on MaxiK channels in smooth muscle cells from urinary bladder. *J Membr Biol* 2000;173:57–66. [PubMed: 10612692]
45. Sobey CG, Faraci FM. Effect of nitric oxide and potassium channel agonists and inhibitors on basilar artery diameter. *Am J Physiol Heart Circ Physiol* 1997;272:837–847.
46. St'astny F, Schwendt M, Lisy V, Jezova D. Main subunits of ionotropic glutamate receptor are expressed in isolated rat brain microvessels. *Neurol Res* 2002;24:93–96. [PubMed: 11783759]
47. Toda N, Okamura T. Nitroxidergic nerve: regulation of vascular tone and blood flow in the brain. *J Hypertens* 1996;14:423–434. [PubMed: 8761890]
48. Underwood MD, Iadecola C, Reis DJ. Lesions of the rostral ventrolateral medulla reduce the cerebrovascular response to hypoxia. *Brain Res* 1994;635:217–223. [PubMed: 8173958]
49. Wang R, Wu L. The chemical modification of K_{Ca} channels by carbon monoxide in vascular smooth muscle cells. *J Biol Chem* 1997;272:8222–8226. [PubMed: 9079640]
50. Wang R, Wu L, Wang Z. The direct effect of carbon monoxide on K_{Ca} channels in vascular smooth muscle cells. *Pfluegers Arch* 1997;434:285–291. [PubMed: 9178628]
51. Williams SE, Wootton P, Mason HS, Bould J, Iles DE, Riccardi D, Peers C, Kemp PJ. Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* 2004;306:2093–2097. [PubMed: 15528406]
52. Xi Q, Tcheranova D, Parfenova H, Horowitz B, Leffler CW, Jaggar JH. Carbon monoxide activates K_{Ca} channels in newborn arteriole smooth muscle cells by increasing apparent Ca^{2+} sensitivity of α -subunits. *Am J Physiol Heart Circ Physiol* 2004;286:H610–618. [PubMed: 14563665]
53. Zakhary R, Gaine SP, Dinerman JL, Ruat M, Flavahan NA, Snyder SH. Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation. *Proc Natl Acad Sci USA* 1996;93:795–798. [PubMed: 8570637]

Figure 1. Effect of NS1619 (10^{-6} M) on pial arterioles before and in the presence of paxilline (PAX) (4×10^{-5} M). Mean $\pm$ SEM. N=8. * $P < 0.05$ compared to control.

Figure 2.

Effect of CO on newborn pig pial arterioles before and in the presence of paxilline (4×10^{-5} M). Each point represents the mean $\pm$ SE of 6 experiments. * $P < 0.05$ compared to zero concentration of CO. † $P < 0.05$ compared to no paxilline.

Figure 3. Effects of glutamate and sodium nitroprusside (SNP) ($2 \times 10^{-7} \text{M}$) on diameters of newborn pig pial arterioles without and with (A) paxilline (PAX, $4 \times 10^{-5} \text{M}$) or (B) iberiotoxin (IBTX, 10^{-6}M). Mean $\pm$ SEM. N=8 PAX and IBTX. *P < 0.05 compared to zero (0) concentration. † P<0.05 compared to without PAX or IBTX.

Figure 3.

Effects of glutamate and sodium nitroprusside (SNP) ($2 \times 10^{-7} \text{M}$) on diameters of newborn pig pial arterioles without and with (A) paxilline (PAX, $4 \times 10^{-5} \text{M}$) or (B) iberiotoxin (IBTX, 10^{-6}M). Mean $\pm$ SEM. N=8 PAX and IBTX. *P < 0.05 compared to zero (0) concentration. † P<0.05 compared to without PAX or IBTX.

Figure 4.

Effects of hypoxia on diameters of newborn pig pial arterioles before and during topical application of (A) paxilline (PAX, 4×10^{-5} M) or (B) iberiotoxin (IBTX, 10^{-6} M). Mean $\pm$ SEM. N=8 PAX and N=10 IBTX. * P < 0.05 compared to normoxia.

A: Vehicle

B: Paxillin

Figure 5.

Effect of prolonged hypoxia (13% oxygen) before and in the presence of paxilline vehicle (A) or paxilline (4×10^{-5} M) (B) on diameters of newborn pig pial arterioles. Open bars are the initial hypoxia challenge and the filled bars are the second with vehicle (A) or paxilline (B). Values are means $\pm$ SEM; $n=8$ in each group. * $P < 0.05$ compared with normoxia.

Figure 6. Effect of SNP and hypoxia on diameters of newborn pig pial arterioles before and in the presence of ODQ (2.5×10^{-4} M). N=6. *P<0.05 compared to preceding and succeeding controls.

Figure 7.

Effect of A. glutamate (10^{-5} M) and B. hypoxia (10% O_2 ventilation) on the concentration of CO in CSF under the cranial window before and in the presence of iberiotoxin (IBTX 10^{-6} M). N=8 glutamate and N=8 hypoxia. *P<0.05 compared to control.

Table 1

Carbon monoxide (CO) concentrations calculated to be in solution and concentrations measured in aCSF collected following placement of each concentration under cranial windows implanted in newborn pigs.

Concentration calculated (nM)	Concentration measured (nM)
0	49±6
100	136± 12
1000	1004±89
10,000	13,936±1052

n= collections at each concentration from 9 piglets.