Functional role of astrocyte glutamate receptors and carbon monoxide in cerebral vasodilation response to glutamate

Helena Parfenova, Dilyara Tcheranova, Shyamali Basuroy, Alexander L. Fedinec, Jianxiong Liu, and Charles W. Leffler

Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee

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Parfenova H, Tcheranova D, Basuroy S, Fedinec AL, Liu J, Leffler CW. Functional role of astrocyte glutamate receptors and carbon monoxide in cerebral vasodilation response to glutamate. Am J Physiol Heart Circ Physiol 302: H2257–H2266, 2012. First published March 30, 2012; doi:10.1152/ajpheart.01011.2011.—In newborn pigs, vasodilation of pial arterioles in response to glutamate is mediated via carbon monoxide (CO), a gaseous messenger endogenously produced from heme degradation by a heme oxygenase (HO)-catalyzed reaction. We addressed the hypothesis that ionotropic glutamate receptors (iGluRs), including N-methyl-D-aspartic acid (NMDA)- and 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA)/kainate-type receptors, expressed in cortical astrocytes mediate glutamate-induced astrocyte HO activation that leads to cerebral vasodilation. Acute vasoactive effects of topical iGluR agonists were determined by intravital microscopy using closed cranial windows in anesthetized newborn pigs. iGluR agonists, including NMDA, (±)-1-aminoctylpentane-cis,1,3-dicarboxylic acid (cis-ACPD), AMPA, and kainate, produced pial arteriolar dilation. Topical l-2-aminoacidic acid, a gliotoxin that selectively disrupts glia limits, reduced vasodilation caused by iGluR agonists, but not by hypercapnia, bradykinin, or sodium nitroprusside. In freshly isolated and cultured cortical astrocytes constitutively expressing HO-2, iGluR agonists NMDA, cis-ACPD, AMPA, and kainate rapidly increased CO production two- to threefold. Astrocytes overexpressing inducible HO-1 had high baseline CO but were less sensitive to glutamate stimulation of CO production when compared with HO-2-expressing astrocytes. Glutamate-induced astrocyte HO-2-mediated CO production was inhibited by either the NMDA receptor antagonist (R)-3C4HPG or the AMPA/kainate receptor antagonist DNQX. Accordingly, either antagonist abolished pial arteriolar dilation in response to glutamate, NMDA, and AMPA, indicating functional interaction among various subtypes of astrocytic iGluRs in response to glutamate stimulation. Overall, these data indicate that the astrocyte component of the neurovascular unit is responsible for the vasodilation response of pial arterioles to topically applied glutamate via iGluRs that are functionally linked to activation of constitutive HO in newborn piglets.

cerebral circulation; newborns; piglets; heme oxygenase; astrocytes; N-methyl-D-aspartic acid; 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid; kainate

ESSENTIAL NONNEURONAL FUNCTIONS of the excitatory neurotransmitter glutamate include its vasodilator role in the cerebral vascular bed (2, 13). Pial arterioles, major resistance vessels in the cerebral circulation, respond to glutamate by dose-dependent vasodilation (17). Vascular control mechanisms are developmentally regulated. Mechanisms involved in regulation of cerebrovascular circulation are often different in newborn pigs, and presumably newborn infants, from those in older pigs, adult rodents, and adult humans (18). The mechanism of glutamate-induced dilation of pial arterioles in newborn piglets involves carbon monoxide (CO), a vasoactive gaseous messenger molecule endogenously produced in the brain via activity of constitutive heme oxygenase (HO-2) (17, 25). Vasodilator responses of cerebral vessels to glutamate are mediated via glutamate receptors (GluRs) comprising two major groups, ionotropic GluRs (iGluRs) and metabotropic GluRs (mGluRs). GluRs are expressed in the brain parenchyma (neurons and glia, e.g., see Refs. 1, 5, 7, 10, 12, 14, 29–31, 35, 40) and in cerebral vascular endothelium (24). The functional roles of nonneuronal GluRs in the brain are yet to be determined (11, 20). Astrocytes express functional iGluRs, including NMDA and 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA)/kainate types (5, 7, 12, 29, 33), as well as mGluRs (1, 30–32, 40). Astrocytic GluRs coupled to different signaling messengers, including Ca2+, cAMP, CGMP, and inositol trisphosphate, are important in regulating physiological neuronal and nonneuronal functions of astrocytes, including regulation of synaptic neurotransmitter release, neuronal activity, and production of vasoactive compounds [e.g., nitric oxide (NO) and arachidonic acid metabolites] by neurons and nonneuronal cells (2, 34, 35). Recently, astrocytes emerged as key players in regulating neurovascular coupling and cerebral blood flow (2, 13, 17). We found that the astrocytic component of the neurovascular unit is important in regulating endogenous production of CO, a potent vasodilator compound in the cerebral circulation of newborn piglets, juvenile pigs, and adult rats (6, 17). HO inhibitors blocked vasodilator responses of pial arterioles to glutamate and selected GluR agonists in newborn pigs in vivo (28), suggesting involvement of endogenously produced CO.

The present study addressed the hypotheses that iGluRs expressed in cortical astrocytes: 1) mediate glutamate-induced CO production via HO-2 activation and 2) contribute to glutamate-mediated vasodilator responses of cerebral arterioles. To investigate the functional role of astrocyte iGluRs in newborn pigs, we combined the in vivo and in vitro approaches using intravital microscopy through a closed cranial window, freshly isolated astrocyte-enriched fractions of brain cortex, and cortical astrocytes in primary cultures.

METHODS

Protocols and procedures using animals were approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center.

Cranial windows and cerebral vascular reactivity. Newborn pigs (1–5 days old, 1.5–2.5 kg, either sex) were anesthetized initially with ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im) or xylazine (2 mg/kg im) and maintained on α-chloralose (30 mg/kg iv). In piglets, ketamine with acepromazine or xylazine is...
clearly superior for rapid induction and depth of preanaesthesia compared with thiopental. We did not find any discrepancies in cerebral vascular reactivity to glutamate or NMDA in piglets anesthetized with ketamine, a short-acting NMDA receptor (NMDAR) inhibitor, or thiopental (unpublished data).

Catheters were inserted in the femoral artery for monitoring arterial blood gases, pH, and blood pressure and in the femoral vein for the injection of drugs and fluids. The animals were intubated and artificially ventilated with room air to maintain physiological levels of arterial blood gases and pH. Body temperature was kept 37–38°C with a servo-controlled heating pad. The diameter of pial arterioles was determined by using a closed cranial window and intravital microscopy as described previously (17). The space under the window was filled with artificial cerebrospinal fluid (in mM): 3.0 KCl, 1.5 MgCl2, 1.5 CaCl2, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO3 equilibrated with 6% CO2:6% O2:88% N2 to pH 7.3–7.35 at 37°C.

To investigate the functional contribution of cortical astrocytes to vasodilation caused by agonists of GluRs, we used a selective glial toxin, l-2-aminoacidic acid (l-AAA; seeRefs. 8, 17, and 38). The concentration of l-AAA (2 mM) was selected based on evidence in literature and on our previous publications that provided evidence of selective morphological and functional damage to glial limitans (38, 39) and newborn piglets (9, 17). l-AAA (2 mM) dissolved in saline was applied to the brain surface under the cranial window for 5 h as we described previously (17). Pial arteriolar responses to glutamate, iGlur agonists, glial-dependent vasodilator ADP, endothelium-dependent vasodilators hypercapnia and bradykinin, and smooth muscle-dependent vasodilator sodium nitroprusside (SNP) were tested before and after exposure to l-AAA. To test vascular reactivity, two or three pial arterioles (60–80 μm) in each animal were selected for observation.

Isolation and culture of astrocytes. Astrocyte-enriched fractions of the brain cortex were prepared by consecutive filtration of the brain cortex homogenate through 300-, 60-, 30-, and 20-μm nylon mesh filters. Cerebral microvessels (300–600 μm) were collected on a 60-μm filter. The vessel-free brain parenchyma contains neurons and glial cells. Neurons were largely collected on the 30- and 20-μm filters as detected by immunostaining for the neuron-specific nuclear protein NeuN (Fig. 4A); the 20-μm filtrate was an astrocyte-enriched fraction of the cerebral cortex as detected by immunostaining for glial fibrillary acidic protein (GFAP) (Fig. 4B); neurons accounted for 10–15% of the cell population in this fraction.

The astrocyte-enriched fraction of the brain cortex parenchyma in astrocyte-supporting media (DMEM with 10 ng/ml epidermal growth factor, 20% fetal bovine serum, and antibiotic/antimycotic) was plated to 75-ml Costar flasks and grown for 10–14 days to confluence. Astrocytes were identified by immunostaining for GFAP and aquaporin-4, as described before (17). For the experiments involving detection of CO production, confluent astrocytes were replated to Cytodex-3 collagen-coated dextran-matrix beads (107 cells/ml beads) (GE Healthcare, Piscataway, NJ). Beads were stirred intermittently in Contes stirred bioreactors (Fisher Scientific, Pittsburgh, PA) overnight to promote cell attachment. The next day, the media containing nonattached cells was replaced, and cells were cultured with continuous stirring (20 rpm) for an additional 4–7 days until confluence. Cells were exposed to serum- and glucose-free media 15–20 h before the experimentation. All experiments were conducted in confluent quiescent astrocytes.

Immunocytometry. Paraformaldehyde-fixed brain cortex slices (5 μm) were heat-treated for antigen unmasking; endogenous peroxidase was quenched with 3% hydrogen peroxide for 45 min, and nonspecific antigen binding was blocked with normal horse serum (30 min, room temperature). Immunostaining for GFAP was performed by the avidin-biotinylated enzyme complex technique (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) using monoclonal anti-porcine GFAP (US Biological, Swampscott, MA) as described elsewhere (17). Hematoxylin was used for counterstaining.

Immunofluorescence. Cerebral vessels excised from the brain cortex, smears of freshly isolated brain parenchyma, and cultured astrocytes were fixed with 3.7% paraformaldehyde in Dulbecco’s PBS (DPBS, pH 7.4), permeabilized by 0.1% Triton X-100-DPBS, and blocked with 5% BSA as described previously (17). For astrocyte detection, cerebral vessels and brain parenchyma were immunostained with monoclonal antibody/GFAP (1:30; US Biological) followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:100; Vector Laboratories). Aquaporin-4 immunostaining of astrocytes was performed using monoclonal antibodies from Abcam (Cambridge, MA). For neuron detection, smears of brain parenchyma were immunostained for neuronal nuclei (NeuN; Chemicon) (1:30) and visualized by rhodamine-conjugated anti-mouse IgG (Vector Laboratories) (1:100). For HO immunodetection, cultured astrocytes were incubated with a/HHO-2 or a/HHO-1 polyclonal antibodies (StressGen, Victoria, Canada) (dilution, 1:50) for 1 h at 37°C, followed by FITC-conjugated anti-rabbit IgG (Vector Laboratories) (1:100) for 1 h at 37°C. Cover slips were mounted on glass slides using anti-fade mounting medium (Vector Laboratories). Slides were viewed using a Nikon Diaphot microscope with fluorescein and rhodamine filters, and images were deconvolved using IPLab spectrum and Vaytech software for image collection and Adobe Photoshop (Adobe Systems) for digital processing.

Western immunoblotting. Cultured astrocytes were extracted with A/HHO-2 or A/HHO-1 polyclonal antibodies (StressGen, Victoria, Canada), or polyclonal antibody to a human HO-1 synthetic peptide (SPA 897; 1:5,000 dilution) (StressGen Biotechnologies, Victoria, Canada), or polyclonal antibody to a human HO-1 peptide (SPA 895; 1:5,000 dilution) (StressGen), followed by peroxidase-conjugated donkey anti-rabbit IgG (dilution 1:10,000) (Jackson Immunoresearch, West Grove, PA). Bands were visualized with Western Lightning Chemiluminescence Reagent (Perkin-Elmer Life Sciences, Waltham, MA).

CO detection by gas chromatography/mass spectrometry. Freshly isolated astrocytes or astrocytes cultured on Cytodex beads were incubated with glutamate and GluR agonists or antagonists in Krebs solution inside sealed amber vials (2.0 ml) that contained Krebs buffer and the internal standard of the isotopically labeled CO (13C18O) for 1 h at 37°C. The headspace gas CO concentration was determined by gas chromatography/mass spectrometry analysis as described previously (37). The components of the headspace gas were separated on a molecular sieve–coated capillary column (CP-Molsieve 5 Å column) (Varian, Palo Alto, CA), and the CO peak was quantitatively detected based on the peak areas, with the mass-to-charge ratios corresponding to 12C16O and 13C18O, respectively. The results were expressed as pico moles of CO released in the headspace gas per milligram of protein.

Statistical analysis. Values are presented as means ± SE of absolute values or percentage of control. Analysis of variance with repeated measures and the Tukey-Kramer multiple-comparisons test were used to confirm differences among and then between groups, respectively. A level of P < 0.05 was considered significant in all statistical tests.

Materials. GluR agonists and antagonists were from Tocris Biosciences (Ellisville, MO). Cobalt protoporphyrin (CoPP) was purchased from Frontier Scientific (Logan, UT). l-AAA and other reagents were from Sigma-Aldrich (St. Louis, MO).

RESULTS

Contribution of the glial component of the neurovascular unit to the vasodilation response of pial arterioles to the agonists of NMDA- and AMPA/kainate-type GluR. Cortical astrocytes identified by expression of the GFAP were in close contact with both surface pial arterioles and penetrat-
ing pial arterioles as detected by immunohistochemistry (Fig. 1A) and by indirect immunofluorescence of isolated pial arterioles (Fig. 1, B and C). We used a glia-specific toxin, l-AAA, to investigate the functional significance of cortical astrocytes in responses of pial arterioles to agonists of iGluRs. The specificity of l-AAA toxicity to glia limitans vs. vascular endothelial and smooth muscle cells was established in our previous in vivo and in vitro studies (9, 17). Confirming these findings, topical l-AAA (2 mM, 5 h) blocked the dilator response of pial arterioles to ADP, the astrocyte-dependent agonist (17, 38), and glutamate, but not to the smooth muscle-selective agent SNP (Fig. 2). l-AAA also did not reduce cerebral vascular responses to hypercapnia and bradykinin (Fig. 2) that cause endothelium-dependent vasodilation (9, 36). Topical application of saline, the l-AAA vehicle, to the brain surface of the anesthetized pigs for 5 h (time control) did not alter the dilator responses of pial arterioles to ADP, glutamate, hypercapnia, bradykinin, or SNP (Fig. 2). These data demonstrate that selective reductions in cerebral vascular responsiveness to glutamate after a 5-h exposure to l-AAA in anesthetized pigs are not due to a desensitization, time, or anesthesia factors.

Pial arterioles responded to glutamate (10^{-4} M) by vasodilation ~30% above the baseline diameter (Fig. 3). NMDAR agonists, NMDA (10^{-4} M) and cis-ACPD (10^{-4} M), caused ~20% dilation of pial arterioles (Fig. 3). Agonists of AMPA/kainate receptors, AMPA (10^{-4} M) and kainic acid (10^{-4} M), had a potent vasodilator effect (40—60% above the baseline diameter). The glial toxin l-AAA (2 mM, 5 h) blocked or greatly reduced the dilation of pial arterioles in response to glutamate, NMDA, cis-ACPD, AMPA, and kainate (70—90% inhibition, Fig. 3). Overall, these data indicate the functional importance of the glial component of the neurovascular unit in pial arteriolar responses to the agonists of NMDAR and AMPA/kainate types of iGluRs.

Fig. 1. Immunodetection of astrocytes in brain cortex slices (A) and in isolated pial cerebral arterioles (B and C). A: glial fibrillar acidic protein (GFAP) immunohistochemistry of the brain cortex; arrows indicate glial cells in close contact with pial arterioles; arrowheads indicate cortical astrocytes; counterstaining, hematoxylin. B: GFAP immunofluorescence in isolated pial arterioles. C: GFAP-positive astrocytes on the surface of isolated pial arterioles.

![Fig. 2](http://example.com/f2.png)

Fig. 2. Effects of l-2-amino adipic acid (l-AAA) on selected cerebral vasodilator responses. l-AAA (2 mM) was applied to the brain surface for 5 h. The pial arteriolar responses to topically applied glutamate (10^{-4} M), astrocyte-dependent vasodilator ADP (10^{-4} M), endothelium-dependent vasodilator stimuli hypercapnia (PaCO2, 75–80 mmHg) and bradykinin (BK, 10^{-6} M), and smooth muscle-dependent vasodilator sodium nitroprusside (SNP, 10^{-6} M) were measured before and after the treatment with l-AAA (n = 6 animals). The time control group represents cerebral vascular reactivity following a 5-h exposure to saline, the l-AAA vehicle (n = 5 animals). Values are means ± SE. *P < 0.05 compared with the control values.
Effects of iGluR agonists on CO production by freshly isolated and cultured brain cortex astrocytes. Our previous findings demonstrate that astrocyte-dependent vasodilation of cerebral arterioles in response to glutamate is mediated via endogenously produced CO (17). We therefore proceeded to investigate the involvement of iGluRs in the regulation of CO production by freshly isolated brain cortex astrocytes that presumably preserve the physiological properties of the in vivo preparations (12). The neuron-depleted astrocyte-enriched brain cortex fractions were obtained by sequential filtration of the vessel-free brain parenchyma through 300-, 60-, 30-, and 20-μm mesh filters. Neurons identified by the neuronal specific nuclear protein, NeuN (19), were retained on the 300- and 60-μm filters (Fig. 4A), whereas the 20-μm filtrate was enriched with GFAP-positive astrocytes (Fig. 4B). Agonists of NMDARs, NMDA, cis-ACPD, and quinolinic acid (10^{-4} M, 1 h) rapidly increased astrocytic CO production two- to fourfold over the basal level (Fig. 5). Agonists of non-NMDAR types of iGluRs, AMPA, kainate, and l-quisqualic acid (10^{-4} M, 1 h) also increased CO production three- to fourfold (Fig. 5). These data indicate that iGluR activation leads to an acute increase in CO production by freshly isolated astrocytes. Because the cross-contamination of the astrocyte-enriched preparations of the brain cortex with some neuronal cells expressing GluRs cannot be completely excluded (Fig. 4), we repeated the experiments in primary cultures of cortical astrocytes.

![Image of freshly isolated cortical astrocytes](http://www.ajpheart.org)

**Fig. 4.** Collection of freshly isolated cortical astrocytes. The brain cortex homogenate was filtered consecutively through 300-, 60-, 30-, and 20-μm nylon mesh filters. Cerebral vessels (300–60 μm) were separated from the brain parenchyma (a 60-μm filtrate). Immunodetection of neurons and glial cells in vessel-free brain parenchyma was performed by co-immunostaining using anti-neuron-specific nuclear protein (NeuN, red) and anti-GFAP (green) antibodies. A: neuron-enriched fraction collected on a 30-μm mesh filter. B: astrocyte-enriched fraction (freshly isolated cortical astrocytes) collected as a 20-μm filtrate of the vessel-free brain parenchyma. Bar, 20 μm.
Astrocytes in primary cultures were identified by expression of aquaporin-4, an astrocyte-specific water channel (Fig. 6A). For the experiments involving CO detection, astrocytes were replated on Cytodex beads (Fig. 6, B and C). NMDA, cis-ACPD, AMPA, and kainate, as well as glutamate (10^{-4} M, 1 h), increased astrocytic CO production two- to threefold over the basal level (Fig. 7). These data indicate that astrocytes in primary cultures fully retain the physiological property of brain cortex astrocytes to respond to glutamate by acute stimulation of endogenous CO production via activation of iGluRs.

**Expression and functional significance of HO isoforms in cortical astrocytes in responses to glutamate stimulation.** Next, we investigated the contribution of HO isoforms to iGluR-mediated astrocytic CO production. In quiescent astrocytes in primary cultures, HO-2 is the predominant HO isoform, although HO-1 protein is also detectable (Fig. 8). CoPP is a potent HO-1 inducer in cerebral vessels and brain parenchyma in vitro and in vivo (22, 23, 27). In cultured astrocytes treated with 20 μM CoPP for 20 h, HO-1 expression was greatly induced (>10-fold), whereas HO-2 expression was not altered (Fig. 8). Both HO-2 and HO-1 are localized in the cytoplasmic compartment of glial cells and in the nuclear envelope; in some cells, HO-1 is also detected in the nucleoli (Fig. 8). We compared the contributions of astrocytic HO-2 and HO-1 with overall CO production under the baseline conditions, and when the excess of the HO substrate heme was provided to unmask the enzyme catalytic activity. HO-1-overexpressing astrocytes had a greater baseline CO production (2-fold higher than hemin-stimulated activity in control cells that predominantly express HO-2; Fig. 9). However, elevation of the enzyme activity was significantly less than could be anticipated for a 10-fold increase in the HO-1 protein. This discrepancy may indicate that the apparent enzymatic activity of HO-1 is significantly less than that of HO-2. Furthermore, HO-1-overexpressing astrocytes had little or no capacity to respond to glutamate by rapidly increasing the enzyme activity (glutamate stimulation of CO production, 2.6 ± 0.1- and 1.4 ± 0.2-fold in control and CoPP-treated astrocytes, respectively). Moreover, when the excess heme substrate was provided to fully unmask the HO activity, glutamate had little or no additive effect on CO production in HO-1-overexpressing, in contrast to HO-2-expressing, astrocytes (Fig. 9). Overall, these data suggest that HO-2 is the major catalytically active isoform in unstimulated astrocytes. Furthermore, HO-2 catalytic activity is rapidly elevated by glutamate via an iGluR-mediated posttranslational mechanism. In contrast, HO-1 activity is upregulated via long-term transcriptional/translational pathways that increase HO-1 expression and functional significance of HO isoforms in cortical astrocytes in responses to glutamate stimulation.

**Fig. 6.** Cortical astrocytes cultured on Costar plates (A) and Cytodex beads (B and C). A: immunostaining for aquaporin-4, an astrocyte-specific marker. B and C: phase-contrast images of astrocytes on Cytodex beads.

**Fig. 7.** Agonists of iGluRs increase CO production by cortical astrocytes in primary cultures. Cortical astrocytes on Cytodex beads were incubated with glutamate (Glu), agonists of NMDAR, NMDA and cis-ACPD, or agonists of AMPA/kainate receptors (non-NMDAR), AMPA and kainic acid (glutamate and iGluR agonists, 10^{-4} M), for 1 h at 37°C. The CO level in the headspace gas was detected by gas chromatography/mass spectrometry (n = 6 experiments). Ctr, control. Values are means ± SE. *P < 0.05 compared with the baseline values.
protein rather than by immediate posttranslational modifications of the enzyme activity that involve iGluRs.

Functional interplay between distinct iGluR receptors, astrocyte CO production, and CO-mediated cerebral vascular responses to glutamate. We used a ligand displacement approach to investigate the functional contribution of different GluRs to glutamate-stimulated CO production. CO production was detected in cultured astrocytes exposed to \(10^{-4}\) M glutamate alone or in the presence of iGluR antagonists \(10^{-4}\) M for 1 h at 37°C. The NMDAR antagonist (R)-4C3HPG and the AMPA/kainate receptor antagonist DNQX completely eliminated glutamate-induced stimulation of CO production (Fig. 10). These data indicate functional interplay between distinct iGluRs in glutamate stimulation of endogenous CO production.

Next, we investigated the functional significance of astrocyte CO-mediated interactions between NMDAR and AMPA/kainate receptors in cerebral vasodilatation to glutamate. To this point, cerebral vasodilator effects of glutamate, NMDA, and AMPA alone \(10^{-4}\) M or in the presence of iGluR inhibitors \(10^{-4}\) M were investigated using the cranial window microscopy. In the first set of the experiments, we found that dilation of pial arterioles to glutamate and iGluR agonists was completely reproducible during the second application, indicating that there is no GluR desensitization (Fig. 11). In the second set of experiments, the NMDAR antagonist D-AP5 \(10^{-4}\) M was applied under the cranial window 10 min before and kept on the brain surface during the repeated application of glutamate, NMDA, or AMPA \(10^{-4}\) M. D-AP5 itself did not cause any changes in the baseline pial arteriolar diameter (58 ± 6 and 57 ± 5 μm before and after topical application) but completely prevented the responses of pial arterioles to NMDA as anticipated. Moreover, D-AP5 completely blocked the responses of pial arterioles to glutamate and AMPA (Fig. 11). The AMPA/kainate receptor antagonist DNQX \(10^{-4}\) M also did not have vasoactive effects (baseline arteriolar diameter, 57 ± 4 and 53 ± 5 μm before and after topical application), but completely prevented pial arteriolar dilation not only to AMPA, but also to NMDA and glutamate (Fig. 11). In contrast, vasodilator responses to SNP \(10^{-4}\) M were not reduced by either iGluR antagonist (Fig. 11), indicating that general cerebral vascular responsiveness was unchanged. Cerebral vasodilator responses to glutamate were completely restored after iGluR antagonists were washed off the brain surface (data not shown).

DISCUSSION

Cortical astrocytes (glia limitans), via the production of endogenous gaseous vasodilator CO, are critical in providing increased cerebral blood flow in response to glutamate (17). Much about the mechanisms of glial regulation of glutamatergic cerebrovascular responses remains uncertain. The present study utilized in vivo and in vitro approaches to address the hypothesis that physiological functions of glial iGluRs involve stimulation of astrocytic CO production that produces cerebral vasodilation and increased cerebral blood flow. We report here novel findings that multiple astrocytic NMDA and AMPA/kainate iGluRs are functionally linked to posttranslational activation of constitutive HO-2 in newborn piglets.
Astrocytes that account for the majority of cells in human brain are important structural and functional components of the neurovascular unit (11). Pial arterioles, major resistance vessels of the brain, as well as penetrating cerebral arterioles are in close contact with astrocytes and astrocytic foot processes. Recent data demonstrate the importance of astrocytes in regulating cerebral blood flow in response to neurotransmitter stimulation (2). Glutamate, the major excitatory neurotransmitter in the brain, exerts its effects via GluRs expressed in the neuronal, glial, and endothelial components of the neurovascular unit. Astrocytes express a variety of GluRs that modulate neuronal excitability and participate in neurovascular coupling by linking neuronal activity to cerebral blood flow. However, the specific functions of astrocyte GluRs remain largely unknown (10–12).

L-AAA is a selective glial toxin that has been successfully used to investigate the functional contribution of cortical astrocytes (glia limitans) to cerebral vascular responses (17, 38, 39). Prolonged treatment with topical L-AAA placed under the cranial window inhibited the vasodilator response to ADP that indicates involvement of glia limitans in newborn pigs (17) and in adult rats (38, 39). In rats, an endothelial component, via a NO-dependent mechanism, also partially contributes to cerebral vasodilation to ADP (39, 41). In newborn pigs, NO-dependent cerebral vascular responses are not fully developed, whereas NO dependency increases with age (18). In newborn piglets, L-AAA did not reduce dilation of pial arterioles to isoproterenol or SNP, direct vascular smooth muscle-dependent dilators (Refs. 9 and 17 and the present data). Further confirming the selectivity of the toxin, endothelial-dependent responses in newborn piglets were not influenced by L-AAA: 1) the responses of pial arterioles to bradykinin and hypercapnia that require intact endothelium were not reduced (present data); and 2) viability of endothelial cells from newborn pigs was not decreased, whereas cultured astrocytes were severely damaged by exposure to L-AAA (17).

We used L-AAA to investigate the functional importance of glia limitans in responses of cerebral arterioles to glutamate and iGluR agonists. The present data demonstrate that dilation of pial arterioles in response to glutamate and iGluR agonists, including agonists of NMDARs (NMDA, cis-ACPD) and non-NMDARs (AMPA/kainate), was completely abolished or greatly reduced by the glial toxin L-AAA, indicating functional contribution of the glial component of the neurovascular unit to the glutamatergic stimulation of pial arterioles. These data indicate that glial cells are among the target cells for glutamatergic stimulation and suggest that iGluRs contribute to the astrocyte-mediated dilation of pial arterioles in response to glutamate.

The mechanism of glutamate-induced dilation of pial arterioles involves CO, a vasoactive gaseous messenger molecule endogenously produced in the brain via activity of constitutive HO-2 (17). This conclusion is based on the following observations: 1) HO-2 is the major isoform of HO in the brain under physiological conditions (18, 25), 2) glutamate acutely in-
creases brain CO production in vivo (Refs. 18 and 25 and the present data), and 3) pharmacological inhibitors of HO block the vasodilator responses of pial arterioles to glutamate (28). The potential contribution of inducible astrocytic HO-1 to glutamatergic stimulation has not been addressed yet.

To investigate whether astrocytic iGluRs are functionally linked to HO activation, we used freshly isolated astrocytes and astrocytes in primary cultures. These two major preparations were used to potentially avoid complications due to the fact that the cells could be phenotypically and/or physiologically transformed in culture. Freshly isolated and cultured astrocytes have the advantages and limitations regarding physiological applicability. Freshly isolated astrocytes preserve the physiological properties of the in vivo preparations (11). However, cross-contamination of the astrocyte-enriched preparations with neuronal cells expressing GluRs cannot be completely excluded. On the other hand, cultured astrocytes are completely neuron-free due to the culture conditions unfavorable to neuronal survival. However, we cannot completely exclude the possibility of phenotypic or physiological transformation of the astrocytes even in primary cultures. Nevertheless, our data demonstrate that both freshly isolated and cultured brain cortex astrocytes have identical responsiveness to iGluR receptor agonists/antagonists. The astrocyte responsiveness is in agreement with the glia-mediated cerebrovascular responses in vivo. Therefore, the dual approach strengthens our conclusions on the applicability of primary cultured astrocytes to the in vivo studies of the mechanisms of the physiological cellular responsiveness.

Although astrocytes account for the majority of cells in the brain (21), we further enriched the vessel-free fraction of brain parenchyma by consecutive filtration through 30- and 20-μm nylon mesh filters. A 20-μm filtrate was identified by GFP+ astrocytes, whereas larger neurons identified by the neuronal nuclear protein NeuN were retained on 30- and 20-μm filters. In the brain under normal conditions, HO is expressed as a constitutive HO-2 isoform, whereas inducible HO-1 is not detectable (23, 25). Our novel data reveal a functional link between glutamate, iGluRs, and endogenous CO production via constitutive HO-2. Freshly isolated cortical astrocytes rapidly respond to the agonists of iGluRs (NMDAR agonists, NMDA, cis-ACPD, and quinolinic acid; and to non-NMDAR agonists, AMPA, kainic acid, and L-quisqualic acid) by a two- to fourfold increase in CO production.

To further confirm that GluRs are functionally linked to HO activation in astrocytes, rather than in neurons or capillary vascular cells that may contaminate the astrocyte-enriched fraction of the brain cortex, we used cortical astrocytes in primary cultures. Astrocytes immunoidentified by the expression of GFAP and aquaporin-4 accounted for over 98% of the cell population. Similar to freshly isolated cells, cultured astrocytes responded to the iGluR agonists (NMDA, ACPD, AMPA, and kainic acid) by a 2.5- to 3-fold rapid increase in CO production within 1 h of the stimulation. In another approach, we compared the effectiveness of various iGluR antagonists in competing with the HO activation by glutamate. Antagonists of iGluRs, (R)-4C3HPG (NMDAR antagonist) and DNQX (AMPA/kainate receptor antagonist), individually completely blocked cerebral vasodilatation to glutamate and prevented glutamate stimulation of CO production. These data demonstrate that, although different iGluRs are functionally linked to HO activation, blocking either receptor completely eliminates the stimulatory response. This finding suggests that both NMDA and AMPA/kainate astrocyte receptors must be simultaneously activated to produce CO and initiate CO-mediated pial arteriolar vasodilation to glutamate. Overall, the potential implication of these data is that there is a common link between different receptors and an effector that is responsible for the stimulation of HO activity.

The effects of GluR ligands in vivo do not appear to allow simple, straightforward extrapolation from the individual capabilities of the neurons, astrocytes, and vascular smooth muscle and endothelial cells in vitro. Astrocyte injury blocks dilation to glutamate and iGluR agonists (present data). The neurotoxin tetrodotoxin blocks dilation to NMDA, but not to glutamate, suggesting neuronal NMDAR initiate responses to NMDA (17). We reported previously that dilations to the...
agonists of NMDA, AMPA, and kainate receptors were blocked completely by HO inhibitors (28). Also, data in Fig. 10 show individual inhibition of NMDA and AMPA/kainate receptors each completely blocks the elevation of astrocyte CO production caused by glutamate. In the intact vessel, in vivo, endothelial cells would respond to iGluR agonists by increasing CO production (24), and dilation to CO is itself endothelial-dependent (18). Therefore, overall, data seem to suggest that NMDA topically applied to the intact neurovascular unit activates perivascular neurons that may stimulate astrocyte AMPA/kainate receptors that increase astrocyte CO production. Astrocyte CO increases Ca\(^{2+}\) sparks and Ca\(^{2+}\) sensitivity of vascular smooth muscle large-conductance Ca\(^{2+}\)-sensitive K\(^{+}\) channels, increasing Ca\(^{2+}\) spark-to-BK channel coupling, membrane hyperpolarization, a reduction in smooth muscle cell voltage-dependent Ca\(^{2+}\) channel activity, a reduction in global intracellular Ca\(^{2+}\) concentration, and arteriolar vasodilation (18).

Our previous data demonstrate that cortical astrocytes are required for CO-dependent pial arteriolar dilation leading to increased cerebral blood flow in response to glutamatergic stimulation. We now present data indicating that astrocytic CO production in response to glutamate involves iGluR-mediated activation of constitutive HO-2. Posttranslational activation of HO-2 in response to glutamatergic stimulation has been previously described in neurons and cerebral vascular cells (3, 4, 15, 16, 25). HO-2 activation occurs via complex cell-specific regulatory mechanisms that involve protein phosphorylation, Ca\(^{2+}\) influx, and Ca\(^{2+}\)/calmodulin-dependent molecular events (25). Protein kinase C (PKC)- and casein kinase (CK-2)-mediated phosphorylation of HO-2 accompanied by Ca\(^{2+}\)/calmodulin-dependent modifications of the enzyme have been demonstrated in neurons (3, 4). In piglet cerebral vascular endothelial cells, iGluRs are functionally linked to HO-2 activation via protein tyrosine kinase-mediated posttranslational modifications of the enzyme by glutamate without involvement of PKC, CK-2, or Ca\(^{2+}\)/calmodulin (15, 16, 25). In cortical astrocytes, Ca\(^{2+}\)/calmodulin-dependent mechanisms are involved in rapid regulation of HO-2 activity by glutamate (37). However, the details of these cell- and signal-specific mechanisms and events leave much to be resolved.

We also present novel data demonstrating that astrocytic HO-1 is not activated by glutamate and, therefore, is not functionally involved in the cerebrovascular dilation response to glutamatergic stimulation. Although astrocytes with overexpressed HO-1 have greatly enhanced baseline HO activity, they were not responsive to glutamate stimulation even when excess hemin substrate was provided. These results suggest that, in contrast to HO-2, HO-1 is regulated mainly via long-term transcriptional/translational pathways. Astrocytic HO-2 is the primary source of physiological vascular responses to excitatory amino acids involving iGluR stimulation leading to an immediate rise in CO production, pial arteriolar dilation, and increased cerebral blood flow.

While data are consistent with the hypothesis that AMPA and kainate act on astrocyte iGluRs to increase CO production, data on neuronal dependence as well as HO dependence of NMDA remind us that alternative possibilities exist. Neurons, astrocytes, and endothelial cells express iGluR that could be intermediate targets of GluR agonists that then stimulate astrocytic CO production in vivo. For example, we showed before that NO stimulates CO production by freshly isolated cerebral microvessels and cultured endothelial and vascular smooth muscle cells (18). Therefore, it is possible that iGluR agonists could stimulate NO production by neurons or endothelium that causes the elevation of astrocyte CO production. This is certainly a possibility in the case of NMDA because tetrodotoxin blocks dilation to NMDA, but not to glutamate itself (17, 18), suggesting an intermediate event. Even though glutamate-induced dilation is not affected by tetrodotoxin, an intermediate event involving, for example, endothelial signals remains possible. Excluding endothelial cells is difficult because dilation of pial arterioles in vivo to CO itself is endothelial-dependent (18). However, in the present study, we show that iGluR antagonists block glutamate-induced CO production by astrocytes seemingly arguing against an intermediate event involving other cell types.

Overall, our data indicate that the astrocyte component of the neurovascular unit is responsible for vasodilation of pial arterioles in response to glutamatergic stimuli via iGluRs functionally linked to posttranslational activation of constitutive HO-2 in newborn piglets. Astrocytic iGluRs act as the transducers of glutamatergic stimulation in the neurovascular unit to cerebral vasodilator responses. Specifically, astrocytic NMDARs and the AMPA/kainate type of GluR are functionally linked to HO-2-mediated CO-dependent vasodilator responses of pial arterioles to glutamatergic stimulation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.P. and C.W.L. conception and design of research; H.P., D.T., A.L.F., and C.W.L. analyzed data; H.P., D.T., S.B., J.L., and C.W.L. interpreted results of experiments; H.P. and S.B. prepared figures; H.P. drafted manuscript; H.P. and C.W.L. edited and revised manuscript; H.P., D.T., S.B., A.L.F., and C.W.L. approved final version of manuscript; D.T., S.B., A.L.F., and J.L. performed experiments.

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