Glutamate-induced calcium signals stimulate CO production in piglet astrocytes

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Abstract

Glutamate-stimulated, astrocyte-derived carbon monoxide (CO) causes cerebral arteriole dilation by activating smooth muscle cell large-conductance Ca\(^{2+}\)-activated potassium (BK\(_{Ca}\)) channels. Here, we examined the hypothesis that glutamate activates heme oxygenase (HO)-2 and CO production via the \([Ca^{2+}]_i/Ca^{2+}\)-calmodulin signaling pathway in newborn pig astrocytes. The major findings are: 1) Glutamate stimulated Ca\(^{2+}\) transients and increased steady-state \([Ca^{2+}]_i\) in cerebral cortical astrocytes in primary culture. 2) In astrocytes permeabilized with ionomycin, elevation of \([Ca^{2+}]_i\) concentration-dependently increased CO production. 3) Glutamate did not affect CO production at any \([Ca^{2+}]_i\) when the \([Ca^{2+}]_i\) was held constant. 4) Thapsigargin, a sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase blocker, decreased basal CO production and blocked glutamate-induced increases in CO. 5) Calmidazolium, a calmodulin inhibitor, blocked CO production induced by glutamate and by \([Ca^{2+}]_i\) elevation. Taken together, our data are consistent with the hypothesis that glutamate elevates \([Ca^{2+}]_i\) in astrocytes, leading to Ca\(^{2+}\)-calmodulin dependent HO-2 activation, and CO production.
Key words: cerebral circulation, heme oxygenase, calmodulin, gasotransmitter, neurovascular coupling
Introduction

In the neurovascular unit, astrocytes act as intermediaries between neurons and arterioles to regulate vascular tone in response to neuronal activity. Release of glutamate from presynaptic neurons increases blood flow to match metabolic demands; such coupling is called functional hyperemia. Functional hyperemia has been extensively investigated and many astrocyte-derived vasoactive mediators, including ADP (40, 41), ATP (34, 36), epoxyeicosatrienoic acids (1-3), K⁺ (11, 13, 33), and prostacyclin (42) appear to be involved depending upon experimental models. We have shown that carbon monoxide (CO) is another critical regulator of cerebrovascular tone in newborn piglets (19, 24, 26, 31, 39).

CO dilates newborn pig pial arterioles (22, 25). CO causes vasodilation by stimulating Ca²⁺ sparks and large-conductance Ca²⁺-activated K⁺ (BKCa) channels and by increasing effective coupling between Ca²⁺ sparks and BKCa channels in arterial smooth muscle cells (17, 18, 25, 38). Physiologically, CO is produced by heme oxygenase (HO)-catabolized breakdown of heme to CO, iron, and biliverdin (27). Of three HO isoforms, only HO-2 is expressed under basal conditions in newborn piglet brain (31, 32). In piglets, endogenous HO-2 can produce sufficient CO to induce cerebral arteriole dilation (23). Our previous studies in piglets demonstrated that glutamate stimulates astrocytic CO production in primary-cultured astrocytes and in vivo (24, 26, 31). We also demonstrated that astrocytes release CO and astrocyte-derived CO causes glutamate-induced pial arteriolar dilation in vivo (24), supporting a regulatory role for astrocytic CO in neurovascular coupling in the newborn brain. Indeed, in brain
slices from newborn piglets, we demonstrated that glutamate regulates Ca$^{2+}$ signals in smooth muscle cells of arterioles through astrocyte- and HO-dependent mechanisms (26, 39).

Prior to this study, the mechanism by which glutamate stimulates astrocytic CO production was not known. Data acquired from other cell types, such as neurons, cerebral microvessels and endothelial cells, suggest that calmodulin (CaM) could be involved in CO production by astrocytes. In cortical neurons, Ca$^{2+}$ binds to CaM, which binds and activates HO-2 (6). Similarly, in cerebral microvessels, calmidazolium, a CaM inhibitor, decreases HO-2 catalytic activity and blocks glutamate stimulation of CO production (20). However, in astrocytes, it was still unclear if CaM is involved in glutamate-induced CO production. Therefore, we sought to examine the hypothesis that in piglet astrocytes glutamate elevates [Ca$^{2+}$], and through CaM stimulates HO-2, leading to CO generation.

**Methods**

*Piglet astrocyte isolation and culture.* Procedures were approved by the University of Tennessee Health Science Center Animal Care and Use Committee. Newborn pigs (1–3 days old, 1–2.5 kg; Nichols Hog Farm; Olive Branch, MS) were anesthetized with ketamine hydrochloride (33 mg/kg, i.m.) and acepromazine (3.3 mg/kg, i.m.). The piglet brains were then removed and placed in ice-cold DMEM with antibiotic/antimycotic (100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B). Collection of astrocyte-enriched brain cortex isolates was accomplished by gentle homogenization of the piglet cortex in DMEM (1:10) followed by
sequential filtration through 300–, 60–, and 20–µm nylon mesh filters (24). Cerebral vessels and microvessels were retained on the 300– and 60–µm filters, and neurons were retained on the 20–µm filter. The 20–µm filtrate is an astrocyte-enriched fraction of cerebral cortex. Following homogenization, serial filtration, and centrifugation, pelleted brain filtrate was either used immediately for the experiments (freshly isolated astrocytes) or suspended in astrocyte growth-supporting media (DMEM with antibiotic/antimycotic, 10 ng/ml epidermal growth factor (EGF), and 20% FBS) grown in 75-ml flasks for 10–14 days (primary-cultured astrocytes). Confluent piglet astrocytes then were dislodged with trypsin-EDTA and mixed with Cytodex microcarrier beads (175 µm diameter, denatured collagen chemically coupled to a matrix of cross-linked dextran; GE Healthcare; Piscataway, NJ), transferred into spinner flasks, and stirred intermittently (20 rpm for 30 min on and 3 h off) at 37°C and in an air mixture containing 5% CO₂. After 12 h, the beads were stirred continuously (20 rpm) for 4–6 days before experiments. The astrocytes in primary culture were identified by immunostaining for glial fibrillary acidic protein (GFAP) and aquaporin-4, the major water channel expressed in brain perivascular astrocyte processes (30). Freshly isolated astrocytes identified by immunostaining for GFAP and aquaporin-4 account for over 90% of the vessel-free astrocyte-enriched fraction of brain parenchyma purified by consecutive filtration through 300-, 60- and 20-µm nylon mesh filters. Such staining of primary cultures of these isolates indicates that these procedures produce pure astrocyte cultures. To minimize the effects of culture on astrocytes, for measurement of intracellular Ca²⁺ signals, we only used primary cultured astrocytes. For CO
measurements, we also used freshly isolated astrocytes to compare with responses of astrocytes in culture.

*Intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) modification.* Free [Ca$^{2+}$]$_i$ was modified by treating cells with the Ca$^{2+}$ ionophore ionomycin (20 µM; Sigma; St Louis, MO) in Krebs solution (in mM: 10 Hepes, 6 glucose, 5 KCl, 120 NaCl, 5 EGTA, and MgSO$_4$•7H$_2$O). The final [Ca$^{2+}$]$_i$ was held at 50, 100, 200, 500, 1000, and 1500 nM. The [Ca$^{2+}$]$_i$ was calculated using WEBMAXC software (Stanford University, CA; http://www.stanford.edu/~cpatton/webmaxcS.htm). Ionomycin was dissolved in extracellular solution from a stock solution (10 mM in DMSO).

*Ca$^{2+}$]$_i$ measurement using fura-2-AM.* Primary cultured astrocytes were seeded onto poly-D-lysine-treated coverslip-bottomed Petri dishes (BD Biosciences; Discovery Labware, Lincoln Park, NJ) in L-glutamine-free and serum-free media (DMEM with antibiotic/antimycotic and 10 ng/ml EGF) for 24 h. Astrocytes were incubated with 3 µM fura-2-AM (Invitrogen, Carlsbad, CA) and 0.05% Pluronic F-129 (Invitrogen, Carlsbad, CA) in Krebs solution at room temperature for 30 min. Cells were then washed twice with Krebs and allowed to equilibrate for another 20 min in Krebs solution. Fura-2 was alternately excited at 340 and 380 nm using a PC-driven hyperswitch (Ionoptix, Milton, MA). Background corrected ratios were collected every 1s at 510 nm using an integrating charge-coupled device camera (Dage-MTI, Ionoptix) (9).

*CO measurement.* Freshly isolated astrocytes or astrocytes grown on Cytodex beads were placed in amber vials for CO measurements. Freshly isolated astrocytes were diluted with Krebs solution at 1:17 (one part astrocytes to 17 parts Krebs) and 1.7 ml of the diluted astrocytes were placed into vials. Astrocytes grown on beads were
placed in L-glutamine-free and serum-free DMEM overnight and then 1 ml of beads was
diluted with Krebs solution to a total of 1.7 ml in each vial. $^{13}$C$^{18}$O ($^{31}$CO; ISOTEC–
Sigma–Aldrich; St Louis, MO) was added to each vial as the internal standard. After 1 h
incubation at 37°C, incubations were terminated by placing the samples in hot water
(75°C) for 4 min. Immediately, 80 µl of headspace gas were collected for CO detection
by gas chromatography/mass spectrometry (GC/MS) using a Varian Saturn 3 GC/MS
as described before (20, 21). The amount of CO in samples was calculated from the
ratio of peak areas of m/z 28 and 31. The results are expressed as picomoles of CO
produced per 1 mg protein in an hour. Protein was measured using a method developed
by Henkel and Bieger (15).

**Effects of chromium mesoporphyrin (CrMP) on CO production by freshly
isolated astrocytes from newborn piglets.** The astrocytes were pretreated with CrMP (20
µM), an inhibitor of HO, in amber vials without caps on for twenty minutes incubation at
37°C. Then, the astrocytes were further treated with glutamate (100 µM) in capped-
amber vials for 1 hr incubation at 37°C prior to the CO measurements. The appropriate
controls were also conducted.

**Statistics.** All data are expressed as means ± SEM. The statistical significance ($P$
< 0.05) for multiple comparisons was assessed using one-way ANOVA followed by the
Student-Newman-Keuls test for multiple comparisons and Student's t-test for comparing
paired and unpaired data.
Results

Glutamate stimulates astrocytic CO production. In astrocytes isolated from newborn pigs, the basal CO production was ~180 pmol/mg protein/h (n = 29; Fig. 1A). Glutamate (1-1000 µM) stimulated CO production in a concentration-dependent manner with a half maximal effective concentration (EC$_{50}$) of 1.3 ± 0.3 µM. There was no significant difference in CO production between the freshly isolated astrocytes and the astrocytes in primary culture.

Astrocytic CO production was also elevated by an exogenous HO substrate, hemin. Hemin concentration-dependently stimulated CO production in isolated astrocytes (n = 4; Fig. 1B). At 10 µM, hemin induced a 3.5-fold increase of CO production (n = 4; Fig. 1B).

In order to determine the effect of intracellular Ca$^{2+}$ on astrocytic CO production, we clamped astrocytic [Ca$^{2+}$]$_i$, constant by permeabilizing astrocytes with 20 µM ionomycin. [Ca$^{2+}$]$_i$ increased CO production in a concentration-dependent manner with an EC$_{50}$ of 86.6 ± 8.1 nM (n = 12; Fig. 1C).

Glutamate elevates [Ca$^{2+}$]$_i$ in primary cultured astrocytes. We detected fura-2 fluorescence changes (ΔF340/380) in response to glutamate to determine the effects of glutamate on astrocytic [Ca$^{2+}$]$_i$ (Fig. 2). Glutamate concentration-dependently (10-1000 µM) elevated [Ca$^{2+}$]$_i$ (Fig. 2C). Glutamate increased the steady-state [Ca$^{2+}$]$_i$ with an EC$_{50}$ of 83.0 ± 1.8 µM (n = 11). The lower concentrations of glutamate (10 µM and 30 µM) stimulated [Ca$^{2+}$]$_i$ transients (n=11, Fig. 2A), which reached the maximum level in 3-5 min, returning to an elevated steady-state [Ca$^{2+}$]$_i$. To study the mechanism by which glutamate elevates [Ca$^{2+}$]$_i$, we used thapsigargin, an endoplasmic reticulum (ER) Ca$^{2+}$
ATPase blocker, to deplete the intracellular Ca\(^{2+}\) stores. Thapsigargin (500 nM) induced a small elevation in steady-state [Ca\(^{2+}\)], and blocked glutamate-induced Ca\(^{2+}\) signaling (n = 9; Fig. 2C). The thapsigargin-induced [Ca\(^{2+}\)] elevation suggests ER Ca\(^{2+}\) ATPase functions to reduce [Ca\(^{2+}\)]. These data demonstrate that glutamate stimulates ER Ca\(^{2+}\) release, leading to [Ca\(^{2+}\)] elevations in piglet astrocytes.

Glutamate stimulates astrocytic CO production in a [Ca\(^{2+}\)]-dependent manner. [Ca\(^{2+}\)] was clamped between 0 – 1.5 µM by permeabilizing freshly-isolated astrocytes with ionomycin to determine the effect of [Ca\(^{2+}\)] elevation on astrocytic CO production stimulated by glutamate. In figure 3, the ratio of CO production with glutamate (100 µM) over CO production at the same [Ca\(^{2+}\)], without exogenous glutamate are shown. A ratio of 1 is no effect. Glutamate failed to increase CO production when Ca\(^{2+}\) was held constant (Fig. 3A). In contrast to glutamate (Fig. 3A), hemin increased the CO production at all levels of [Ca\(^{2+}\)] (n = 5; Fig. 3B).

Chromium mesoporphyrin (CrMP) was used to inhibit HO-2 in freshly isolated astrocytes from newborn piglets. CrMP (20 µM) reduced basal CO production and blocked elevation of CO production caused by 100uM glutamate (n=4, Fig. 4).

**Thapsigargin inhibits CO production by astrocytes in primary culture.** To study the regulatory role of intracellular Ca\(^{2+}\) stores in CO production, astrocytes were treated with thapsigargin (2 µM) to deplete intracellular Ca\(^{2+}\) stores. Thapsigargin reduced basal CO production and blocked the CO increase stimulated by glutamate (n = 6; Fig. 5A).

The combination of hemin and glutamate induced a larger CO production than that induced by glutamate or hemin alone (n = 6; Fig. 5). Thapsigargin reduced the CO production induced by the combination of hemin and glutamate to about the CO level
caused by hemin alone (n = 6; Fig. 5B), indicating that thapsigargin only blocked glutamate-induced CO production without altering hemin-increased CO production.

_Calmidazolium inhibits glutamate- and [Ca\textsuperscript{2+}]\textsubscript{i}-induced astrocytic CO production._

Calmidazolium (40 µM), a calmodulin (CaM) blocker, did not change basal CO production (n = 10 in Fig. 6A and in Fig. 6B). However, calmidazolium abolished the glutamate-induced CO increase (n = 10; Fig. 5A). In ionomycin-permeabilized astrocytes, calmidazolium blocked CO production induced by [Ca\textsuperscript{2+}]\textsubscript{i} higher than 200 nM but had no effect on CO production induced by [Ca\textsuperscript{2+}]\textsubscript{i} lower than 50 nM (n = 6, Fig. 6B).

**Discussion**

The major findings in newborn pig astrocytes are: 1) Glutamate stimulates Ca\textsuperscript{2+} transients and increases steady-state [Ca\textsuperscript{2+}]\textsubscript{i} in cerebral cortical astrocytes in primary culture. 2) In astrocytes permeabilized with ionomycin, elevation of [Ca\textsuperscript{2+}]\textsubscript{i} concentration-dependently increases CO production. 3) Glutamate stimulates ER Ca\textsuperscript{2+} release, leading to intracellular Ca\textsuperscript{2+} transients and an elevation of global [Ca\textsuperscript{2+}]\textsubscript{i}. 4) When [Ca\textsuperscript{2+}]\textsubscript{i} is clamped constant, glutamate fails to stimulate CO production. 5) Thapsigargin, an ER Ca\textsuperscript{2+} ATPase blocker, reduces basal CO production and blocks glutamate-induced CO production. 6) Calmidazolium, a CaM blocker, inhibits CO production stimulated by glutamate and by [Ca\textsuperscript{2+}]\textsubscript{i} elevation. Our data indicate that glutamate activates release of Ca\textsuperscript{2+} from ER and elevates [Ca\textsuperscript{2+}]\textsubscript{i} in astrocytes leading to Ca\textsuperscript{2+}-calmodulin dependent HO-2 activation and CO production. Therefore, Ca\textsuperscript{2+} and CaM lead to increased CO production in astrocytes in response to glutamate. In smooth muscle, CO decreases cytosolic [Ca\textsuperscript{2+}].
In the current paper, glutamate induces CO production in both freshly isolated astrocytes and astrocytes in primary culture. In cultured astrocytes and in brain slices, glutamate stimulates astrocytic CO release that activates BK\textsubscript{Ca} channels in smooth muscle cells (26), indicating that astrocytes in culture function similarly to the astrocytes in brain slice preparation with respect to glutamate-induced CO production.

Glutamate is the dominant excitatory neurotransmitter in the brain. Release of glutamate from presynaptic neurons increases blood flow to match the increased metabolic demands of stimulated neurons (14). Glutamate causes neonatal cerebral vasodilation in an astrocyte-dependent manner (24). Under in vitro conditions, glutamate-stimulated astrocytic CO regulates pial arteriolar diameter (39). CO causes vasodilation by activating Ca\textsuperscript{2+} sparks and BK\textsubscript{Ca} channels in arterial smooth muscle cells (39). Glutamate-stimulated astrocytic CO is a pathway for astrocytic regulation of neurovascular coupling.

Our present data indicate that glutamate stimulates intracellular Ca\textsuperscript{2+} transients and increases astrocyte steady state [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 2). An [Ca\textsuperscript{2+}]\textsubscript{i} elevation can result from extracellular Ca\textsuperscript{2+} influx, intracellular Ca\textsuperscript{2+} release, or both. Glutamate is the physiological glutamate receptor agonist and activates all glutamate receptor subtypes, including both ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). In adult rodent astrocytes, stimulation of mGluRs and iGluRs activate intracellular Ca\textsuperscript{2+} oscillations (4, 5, 11, 37). In rat brain slices electrical field stimulation leads to Ca\textsuperscript{2+} waves spreading from the astrocytic soma to the endfeet (11). In the present study, we observed that glutamate causes Ca\textsuperscript{2+} transients at 10 and 30 μM, but does not induce Ca\textsuperscript{2+} transients at 100 and 1000 μM where [Ca\textsuperscript{2+}]\textsubscript{i} steadily rises.
In astrocytes, $[\text{Ca}^{2+}]_i$ can be elevated by intracellular $\text{Ca}^{2+}$ release from intracellular $\text{Ca}^{2+}$ stores via RyRs and IP$_3$Rs and/or extracellular $\text{Ca}^{2+}$ influx via $\text{Ca}^{2+}$ channels (7). In addition, mitochondria can be another source of intracellular $\text{Ca}^{2+}$ release. However, here we have shown that thapsigargin pretreatment abolishes glutamate-induced $[\text{Ca}^{2+}]_i$ elevations, indicating that extracellular $\text{Ca}^{2+}$ influx or mitochondrial $\text{Ca}^{2+}$ release are unlikely to be the primary source of $\text{Ca}^{2+}$. In adult rat, $\text{Ca}^{2+}$ waves/transients in perivascular endfeet (12) are induced by IP$_3$ receptors, but not ryanodine receptors (37). In piglet astrocytes, IP$_3$Rs may also contribute to $\text{Ca}^{2+}$ transients. Therefore, intracellular released by IP$_3$Rs might be a major source of glutamate-induced $\text{Ca}^{2+}$ elevation in piglet astrocytes. Since we were not measuring microdomain/localized intracellular $\text{Ca}^{2+}$ signals, our measurement reflects global $[\text{Ca}^{2+}]_i$ signals. Higher glutamate concentrations (>30 $\mu$M) may activate many the IP$_3$Rs, leading to a sustained total $\text{Ca}^{2+}$ elevation. Conceivably, lower glutamate concentrations may induce local $\text{Ca}^{2+}$ transients.

CO production has been measured in neurons (16), vascular smooth muscle cells (21, 29), endothelial cells (21), and astrocytes (this study). In the cerebral vasculature, glutamate stimulates CO production in astrocytes (26) and endothelial cells (21). Glutamate is an excitatory neurotransmitter. It has been shown to activate HO-2 activity in cultured cortical neurons (6). However, our evidence using the cranial window preparation (in vivo) shows that topical glutamate activates astrocyte HO to make CO to activate BK$_{\text{Ca}}$ channels in vascular smooth muscle, leading to a vasodilation (24). In contrast, after glia toxin, topical glutamate no longer causes an increase in CO or cause dilation (24). In line with this evidence, we have previously shown in vitro that glutamate-induced BK$_{\text{Ca}}$ channel activation is HO-dependent. However, glutamate does
not activate BK$_{\text{Ca}}$ channels of isolated smooth muscle cells without astrocytes (26).

Therefore, in piglets, astrocytes use CO as a signal to vascular smooth muscle cells. In this study, the CO production by piglet astrocytes was reduced by CrMP, an HO blocker, at baseline and in response to glutamate stimulation, supporting the involvement of HO-2 in CO production by astrocytes (Fig. 4). In the brain, HO-2 is expressed in neurons (35), glia (10, 28), and cerebral microvessels (23, 28). We have previously shown in wild type (HO-2$^{+/+}$) mice that glutamate stimulates CO production by astrocytes (26). In contrast, glutamate has no effect on CO production by HO-2$^{-/-}$ astrocytes, indicating that HO-2 is the enzyme involved in glutamate-induced astrocytic CO production. In astrocytes, [Ca$^{2+}$] elevation increases CO production in a concentration-dependent manner with an EC$_{50}$ of 87 nM (Fig. 1 and 3B). When [Ca$^{2+}$] was clamped constant between 0 and 1.5 µM, glutamate failed to increase astrocytic CO production (Fig. 3). These data show that [Ca$^{2+}$] elevation is essential for glutamate-induced astrocytic CO production.

In this study, thapsigargin induced a small elevation in [Ca$^{2+}$] and reduced HO-2 activity and CO production. These data suggest that sarcoplasmic reticulum intracellular store may be important in HO-2 activation and CO production in astrocytes: a local calcium elevation generated by sarcoplasmic reticulum-mediated calcium release that is higher than global Ca$^{2+}$ may be necessary to stimulate HO-2 to generate CO. Thapsigargin depletes sarcoplasmic reticulum calcium, removing the local calcium elevation, leading to a reduction in HO-2 activity, and a decrease in CO production. However, the direct evidence is still lacking. Future investigation will be needed to investigate this hypothesis.
How $[\text{Ca}^{2+}]_i$ activates HO-2 and thus CO production in astrocytes was not known. Present results demonstrate that the Ca-CaM complex is involved. CaM immunoreactivity has been found both in neurons and in glial cells (8). In the present study, calmidazolium, a CaM blocker, blocked glutamate- and $[\text{Ca}^{2+}]_i$-induced CO production (Fig. 6), supporting the hypothesis that Ca-CaM mediates astrocytic CO production. In line with this hypothesis, in HO-2$^{-/-}$ mice, glutamate did not increase CO production by astrocytes (26), indicating that Ca-CaM may bind to HO-2. Indeed, HO-2 but not HO-1 has a CaM binding site (6). Taken together, data suggest that CaM is essential for glutamate-stimulated CO elevation.

In summary, we propose that brain glutamate activates GluRs on astrocytes, causing an elevation of astrocytic $\text{Ca}^{2+}$ that combines with CaM to stimulate HO-2, producing CO. CO of astrocytic origin increases vascular smooth muscle $\text{BK}_{\text{Ca}}$ channel activity by elevating $\text{Ca}^{2+}$ sparks and increasing spark-to-$\text{BK}_{\text{Ca}}$ channel coupling, causing vascular smooth muscle cell hyperpolarization that inhibits voltage-gated $\text{Ca}^{2+}$ channels leading to a decrease in cytosolic $\text{Ca}^{2+}$ and vasodilation.
Acknowledgments

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References

1. Alkayed NJ, Birks EK, Hudetz AG, Roman RJ, Henderson L, and Harder DR.
Inhibition of brain P-450 arachidonic acid epoxygenase decreases baseline cerebral

2. Alkayed NJ, Birks EK, Narayanan J, Petrie KA, Kohler-Cabot AE, and Harder DR.
Role of P-450 arachidonic acid epoxygenase in the response of cerebral blood flow to

3. Alkayed NJ, Narayanan J, Gebremedhin D, Medhora M, Roman RJ, and Harder
DR. Molecular characterization of an arachidonic acid epoxygenase in rat brain

4. Beck A, Nieden RZ, Schneider HP, and Deitmer JW. Calcium release from
intracellular stores in rodent astrocytes and neurons in situ. *Cell Calcium* 35: 47-58,
2004.

Volterra A. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes.

6. Boehning D, Sedaghat L, Sedlak TW, and Snyder SH. Heme oxygenase-2 is

7. Butt AM. Neurotransmitter-mediated calcium signalling in oligodendrocyte

8. Caceres A, Bender P, Snavely L, Rebhun LI, and Steward O. Distribution and
subcellular localization of calmodulin in adult and developing brain tissue. *Neuroscience*


differences in mechanisms mediating eNOS-dependent pial arteriolar dilation in rats.

41. Xu HL, Ye S, Baughman VL, Feinstein DL, and Pelligrino DA. The role of the glia
limitans in ADP-induced pial arteriolar relaxation in intact and ovariectomized female

42. Zonta M, Sebelin A, Gobbo S, Fellin T, Pozzan T, and Carmignoto G. Glutamate-
mediated cytosolic calcium oscillations regulate a pulsatile prostaglandin release from
Figure legends

Fig. 1. Effects of glutamate, hemin, and intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) on carbon monoxide (CO) production by freshly isolated astrocytes from newborn pigs. Astrocytic CO production is stimulated by glutamate (A, the numbers of experiments were, from left to the right, 29, 5, 11, 20, and 5, respectively), hemin (B; n = 4), and [Ca\textsuperscript{2+}]\textsubscript{i} elevation (in astrocytes permeabilized with 20 µM ionomycin; C; n = 12). Values are means ± SEM. *P < 0.05 compared with control.

Fig. 2. Effect of glutamate on the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in piglet astrocytes in primary culture. A, a representative trace of glutamate-induced elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. B, a representative trace of thapsigargin blockage of glutamate-stimulated [Ca\textsuperscript{2+}]\textsubscript{i} elevation. C, group data of panel A and B (n = 9). ∆F (340/380), the ratio changes of Fura-2. Values are means ± SEM. *P < 0.05 compared with control.

Fig. 3. Effects of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) on glutamate-stimulated CO production and heme oxygenase (HO)-2 activity. [Ca\textsuperscript{2+}]\textsubscript{i} was controlled by permeabilizing the cells with ionomycin (20 µM) and regulating medium Ca\textsuperscript{2+}. A, Effects of [Ca\textsuperscript{2+}]\textsubscript{i} clamped constant at different levels on glutamate-induced CO production in freshly isolated astrocytes. To cancel out the intracellular Ca\textsuperscript{2+}-induced CO changes and isolate just the glutamate-induced changes, the y-axis
is the ratio of CO production with and without glutamate (100 µM, n=8). In the astrocytes with constant [Ca\(^{2+}\)], glutamate did not change CO production, the ratio is ~1. Without ionomycin, glutamate increases intracellular Ca\(^{2+}\) concentration (Fig. 2). CO production increased in response to glutamate (last bar, [Ca\(^{2+}\)]\(_e\), extracellular Ca\(^{2+}\) concentration). B, Effects of [Ca\(^{2+}\)] on HO-2 activity in freshly isolated astrocytes. [Ca\(^{2+}\)] elevation increased CO production (n = 6). Elevation of [Ca\(^{2+}\)] increased CO production from exogenous substrate (hemin 10 µM, n=6). Values are means ± SEM. *P < 0.05 compared with control.

Fig. 4. Effects of chromium mesoporphyrin (CrMP), an inhibitor of HO, on CO production by freshly isolated astrocytes from newborn piglets. CrMP (20 uM) reduced basal CO production and blocked elevation of CO production caused by glutamate (100uM, n=4). Values are means ± SEM. *P < 0.05 compared with the basal CO production in the first bar. † P < 0.05 compared with CO production caused by glutamate alone.

Fig. 5. Effects of thapsigargin on glutamate- (100 µM) and hemin- (10 µM) induced CO production by astrocytes in primary culture. Thapsigargin (2 µM) blocked CO production stimulated by glutamate (A; n = 6) but not by hemin (B; n = 6). Values are means ± SEM. *P < 0.05 compared with control. † P < 0.05 compared with glutamate. ‡ p<0.05 compared with thapsigargin alone.
Fig. 6. Effect of calmidazolium on astrocytic CO production by freshly isolated astrocytes from newborn pigs. Calmidazolium (CZCl, 40 µM) blocked glutamate (10 µM) stimulation of CO production (A; n = 10). CZCl inhibited CO production at all [Ca^{2+}]_i above 0.1 µM (B; n = 6). Values are means ± SEM. *P < 0.05 compared with control. † P < 0.05 compared with glutamate.
Figure 2

A

Glutamate (μM)

1.0 ΔF(340/380)

500 s

B

Thapsigargin

Glutamate (μM)

0.2 ΔF (340/380)

500 s

C

ΔFura Ratio (340/380)

Control

Thapsigargin

Glutamate (μM)
Figure 3

A

Increase of CO production stimulated by glutamate

(Glutamate/Control)

[Ca^{2+}]_i (μM)

[Ca^{2+}]_o (μM)

B

CO production (pmol/mg protein)

[Ca^{2+}]_i (μM)

Hemin

Control
Figure 4

Graph showing CO production (pmol/mg protein) with Control and CrMP conditions.

Glutamate

Bars indicate statistical significance with * and † symbols.