Glutamate-induced calcium signals stimulate CO production in piglet astrocytes

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Glutamate-stimulated, astrocyte-derived carbon monoxide (CO) appears to be involved in CO production by astrocytes. In cortical neurons, Ca2+ binds to CaM, which binds and activates HO-2. Similarly, in astrocytes, Ca2+ binds to CaM, which binds and activates HO-2. However, in astrocytes, it was still unclear if CaM is involved in glutamate-induced CO production. Before 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, Ca2+ binds to CaM, which binds and activates HO-2. 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 [Ca2+]i, and through CaM stimulates 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 [Ca2+]i, and through CaM stimulates HO-2 catalytic activity and blocks glutamate stimulation of CO production (20).

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 im) and acepromazine (3.3 mg/kg im). 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 was 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 perivas-
cullar astrocyte processes (30). Freshly isolated astrocytes identified by immunostaining for GFAP and aquaporin-4 account for >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 Ca2+ concentration modification. Free intracellular Ca2+ concentration ([Ca2+]i) was modified by treating cells with the Ca2+ ionophore ionomycin (20 μM; Sigma, St. Louis, MO) in Krebs solution (in mM: 10 HEPES, 6 glucose, 5 KCl, 120 NaCl, 5 EGTA, and MgSO4·7H2O). The final [Ca2+]i, was held at 50, 100, 200, 500, 1,000, and 1,500 nM. The [Ca2+]i, was calculated using WEBMAXC software (Stanford University; http://www.stanford.edu/~captton/webmaxc5.htm). Ionomycin was dissolved in extracellular solution from a stock solution (10 mM in dimethyl sulfoxide).

[Ca2+]i measurement using fura 2-AM. Primary cultured astrocytes were seeded on poly-D-lysine-treated cover slip-bottomed petri dishes (BD Biosciences; Discovery Labware, Lincoln Park, NJ) in 1-glutamine-free and serum-free (DMEM with antibiotic/antimycotic and 10 ng/ml EGF) media for 24 h. Astrocytes were incubated with 3 μM fura 2-AM (Invitrogen, Carlsbad, CA) and 0.05% Pluronic F-129 (Invitrogen) in Krebs solution at room temperature for 30 min. Cells were then washed two times with Krebs and allowed to equilibrate for another 20 min in Krebs solution. Fura 2 was excited alternately at 340 and 380 nm using a PC-driven hyperswitch (Ionoptix, Milton, MA). Background-corrected signals were collected every 1 s 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 (1 part astrocytes to 17 parts Krebs), and 1.7 ml of the diluted astrocytes were placed into vials. Astrocytes grown on beads were placed on Cytodex beads was diluted with Krebs solution to a total of 1.7 ml in each vial. 13C18O (31CO; 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 mass-to-charge ratio 28 and 31. The results are expressed as picomoles of CO produced per 1 mg protein in 1 h. Protein was measured using a method developed by Henkel and Bieger (15).

Effects of chromium mesoporphyrin on CO production by freshly isolated astrocytes from newborn piglets. The astrocytes were pre-treated with chromium mesoporphyrin (CrMP, 20 μM), an inhibitor of HO, in amber vials without caps on for 20 min incubation at 37°C. Next, the astrocytes were further treated with glutamate (100 μM) in capped-amber vials for 1 h incubation at 37°C before the CO measurements. The appropriate controls were also conducted.

Statistics. All data are expressed as means ± SE. 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−1·h−1 (n = 29; Fig. 1A). Glutamate (1–1,000 μM) stimulated CO production in a concentration-dependent manner with a half-maximal effective concentration (EC50) 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).

To determine the effect of intracellular Ca2+ on astrocytic CO production, we clamped astrocytic [Ca2+]i constant by permeabilizing astrocytes with 20 μM ionomycin. [Ca2+]i increased CO production in a concentration-dependent manner with an EC50 of 86.6 ± 8.1 nM (n = 12; Fig. 1C).

Glutamate elevates [Ca2+]i in primary cultured astrocytes. We detected fura 2 fluorescence changes (ΔF340/380) in re-

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Glutamate stimulates astrocytic CO production in a [Ca^{2+}]-dependent manner. [Ca^{2+}] was clamped between 0 and 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 Fig. 3, the ratios of CO production with glutamate (100 μM) over CO production at the same [Ca^{2+}], without exogenous glutamate are shown. A ratio of one 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).

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

**DISCUSSION**

The major findings in newborn pig astrocytes are: 1) glutamate stimulates Ca\(^{2+}\) transients and increases 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 increases CO production, 3) glutamate stimulates ER Ca\(^{2+}\) release, leading to intracellular Ca\(^{2+}\) transients and an elevation of global [Ca\(^{2+}\)]\(_{i}\), 4) when [Ca\(^{2+}\)]\(_{i}\) is clamped constant, glutamate fails to stimulate CO production, 5) thapsigargin, an ER Ca\(^{2+}\)-ATPase blocker, reduces basal CO production and blocks glutamate-induced CO production, and 6) calmidazolium, a CaM blocker, inhibits CO production stimulated by glutamate and by [Ca\(^{2+}\)]\(_{i}\) elevation. Our data indicate that glutamate activates release of Ca\(^{2+}\) from
ER and elevates \([\text{Ca}^{2+}]\), in astrocytes, leading to \(\text{Ca}^{2+}\)-CaM dependent HO-2 activation and CO production. Therefore, \(\text{Ca}^{2+}\) and CaM lead to increased CO production in astrocytes in response to glutamate. In smooth muscle, CO decreases cytosolic \([\text{Ca}^{2+}]\).

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 \(\text{BK}_{\text{Ca}}\) channels in smooth muscle cells (26), indicating that astrocytes in culture function similarly to the astrocytes in brain slice preparations with respect to glutamate-induced CO production.

Glutamate is the dominant excitatory neurotransmitter in the brain. Release of glutamate from presynaptic neurons increase 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 \(\text{Ca}^{2+}\) sparks and \(\text{BK}_{\text{Ca}}\) channels in arterial smooth muscle cells (39). Glutamate-stimulated astrocytic CO is a pathway for astrocyte regulation of neurovascular coupling.

Our present data indicate that glutamate stimulates intracellular \(\text{Ca}^{2+}\) transients and increases astrocyte steady-state \([\text{Ca}^{2+}]\) (21). An \([\text{Ca}^{2+}]\) elevation can result from extracellular \(\text{Ca}^{2+}\) influx, intracellular \(\text{Ca}^{2+}\) 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 activates intracellular \(\text{Ca}^{2+}\) oscillations (4, 5, 11, 37). In rat brain slices, electrical field stimulation leads to \(\text{Ca}^{2+}\) waves spreading from the astrocytic soma to the end feet (11). In the present study, we observed that glutamate causes \(\text{Ca}^{2+}\) transients at 10 and 30 \(\mu\text{M}\) but does not induce \(\text{Ca}^{2+}\) transients at 100 and 1,000 \(\mu\text{M}\), where \([\text{Ca}^{2+}]\) steadily rises. In astrocytes, \([\text{Ca}^{2+}]\) can be elevated by intracellular \(\text{Ca}^{2+}\) release from intracellular \(\text{Ca}^{2+}\) stores via ryanodine receptors and inositol triphosphate receptors (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 \([\text{Ca}^{2+}]\) elevations, indicating that extracellular \(\text{Ca}^{2+}\) influx or mitochondrial \(\text{Ca}^{2+}\) release is unlikely to be the primary source of \(\text{Ca}^{2+}\). In adult rat, \(\text{Ca}^{2+}\) waves/transients in perivascular end feet (12) are induced by IP\(_3\)Rs, but not ryanodine receptors (37). In piglet astrocytes, IP\(_3\)Rs may also contribute to \(\text{Ca}^{2+}\) transients. Therefore, intracellular \(\text{Ca}^{2+}\) release by IP\(_3\)Rs might be a major source of glutamate-induced \(\text{Ca}^{2+}\) elevation in piglet astrocytes. Because we were not measuring microdomain/localized intracellular \(\text{Ca}^{2+}\) signals, our measurement reflects global \([\text{Ca}^{2+}]\) signals. Higher glutamate concentrations (>30 \(\mu\text{M}\)) may activate many of 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 \(\text{BK}_{\text{Ca}}\) channels in vascular smooth muscle, leading to a vasodilatation (24). In contrast, after glia toxin, topical glutamate no longer causes an increase in CO or causes dilatation (24). In line with this evidence, we have previously shown in vitro that glutamate-induced \(\text{BK}_{\text{Ca}}\) channel activation is HO-dependent. However, glutamate does not activate \(\text{BK}_{\text{Ca}}\) 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 (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, \([\text{Ca}^{2+}]\) elevation increases CO production in a concentration-dependent manner with an \(EC_{50}\) of 87 nM (Figs. 1 and 3B). When \([\text{Ca}^{2+}]\) was clamped constant between 0 and 1.5 \(\mu\text{M}\), glutamate failed to increase astrocytic CO production (Fig. 3). These data show that \([\text{Ca}^{2+}]\) elevation is essential for glutamate-induced astrocytic CO production.

In this study, thapsigargin induced a small elevation in \([\text{Ca}^{2+}]\) 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 \(\text{Ca}^{2+}\) elevation generated by sarcoplasmic reticulum-mediated \(\text{Ca}^{2+}\) release that is higher than global \(\text{Ca}^{2+}\) may be necessary to stimulate HO-2 to generate CO. Thapsigargin depletes sarcoplasmic reticulum \(\text{Ca}^{2+}\), removing the local \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]\) activates HO-2 and thus CO production in astrocytes was not known. The present results demonstrate that the Ca\(^{2+}\)-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 \([\text{Ca}^{2+}]\)\(^{-}\)-induced CO production (Fig. 6), supporting the hypothesis that Ca\(^{2+}\)-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\(^{2+}\)-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 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-BK\(_{\text{Ca}}\) channel coupling, causing vascular smooth muscle cell hyperpolarization that inhibits voltage-gated Ca\(^{2+}\) channels, leading to a decrease in cytosolic Ca\(^{2+}\) and vasodilation.
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DISCLOSURES

No conflicts of interest are declared by the authors.

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