Mechanism of glutamate stimulation of CO production in cerebral microvessels

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In this study, we asked which vascular cells produce CO in response to glutamate and how is this production increased? Both endothelial cells and smooth muscle cells of the cerebral circulation express HO-2 (8, 21, 31) and have glutamate receptors (17, 26, 34). We (28) have shown that isolated cerebral vascular endothelial cells respond to glutamate by increasing CO production, but it was not known whether smooth muscle cells do so as well.

Rapid elevation of CO production in response to glutamate obviates the possibility of increased expression of HO-2 or induction of HO-1. Assuming that total cellular HO-2 does not change during the experimental time course, CO production could be controlled by regulation of substrate availability to HO-2 or the effective catalytic activity of HO-2. Catalytic activity includes the specific catalytic efficiency of the enzyme, as well as intracellular localization, to optimize substrate and cofactor proximity.

We used freshly isolated piglet cerebral microvessels and endothelial and smooth muscle cells in primary culture to test the hypothesis that glutamate increases CO production specifically in endothelial cells by increasing HO-2 catalytic activity. Results suggest that glutamate does increase HO-2 catalytic activity and that not only endothelial cells but also smooth muscle cells produce CO in response to glutamate. We also examined whether cytosolic Ca signaling and/or protein phosphorylation were involved.

MATERIALS AND METHODS

Experiments using animals were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Brains were removed from 1- to 3-day-old piglets under ketamine (33 mg/kg) and acepromazine (3.3 mg/kg) anesthesia.

Isolation of cerebral microvessels. Cerebral microvessels were isolated from the brains as described before (1, 28, 30). For experiments using freshly isolated microvessels, the isolation was accomplished in cold Krebs solution containing (in mM) 120 NaCl, 5 KCl, 0.62 MgSO4, 1.8 CaCl2, 10 HEPES, and 6 glucose (pH 7.4), whereas for culture of cells, the isolations of vessels from the cerebrums were done in cold carbon monoxide (CO) is an important cellular signaling molecule in the cerebral circulation. In newborn pigs, the constitutive enzyme that produces CO, heme oxygenase-2 (HO-2), has very high expression in cerebral microvessels. Of the vascular tissues examined, by far the greatest production of CO is by cerebral microvessels (19). The excitatory amino acid glutamate stimulates CO production by piglet cerebral microvessels (30). Furthermore, inhibition of HO reduces cerebral arteriolar dilation in response to glutamate and glutamatergic seizures (19, 25, 29). Therefore, glutamate increases activity of the HO-2 pathway, and the CO produced appears to cause subsequent dilation.
isolation solution containing medium 199, 0.015 M HEPES, 1 U/ml sodium hepamin, and antibiotic-antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B). The dura mater and attached vessels were removed from the tissue, and the tissue was washed three times with the isolation solution. Tissue was minced into tiny pieces by using two scalpels in isolation solution and was then transferred to a 40-ml Dounce homogenizer and homogenized with 10 strokes of a loose-fitting pestle. The homogenate was passed through a 300-µm nylon mesh screen, and the passage was then refiltered over a 60-µm nylon mesh screen. The screen was removed and placed in a 50-ml centrifuge tube containing isolation solution. Microvessels (60–300 µm) were washed off by agitation and scraping and then centrifuged at 1,200 rpm for 5 min. Experiments on freshly isolated cerebral microvessels began immediately after vessel collection with resuspension of the microvessels in Krebs solution.

**Primary culture of cerebral microvascular endothelial cells.**

Primary cultures of cerebral microvascular endothelial cells from newborn pig brain cortex were established as previously described (28) from vessels isolated as above. The isolated microvessels were resuspended in DMEM and were incubated in collagenase-dispase solution (1 mg/ml) for 2 h at 37°C. At the end of the incubation, the dispersed microvascular endothelial cells were separated by using Percoll density gradient centrifugation. Endothelial cells were resuspended in DMEM supplemented with 20% FBS, 2 mg/ml sodium bicarbonate, 1 U/ml heparin, 30 mg/ml endothelial cell growth supplement (ECGS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. Endothelial cells were plated on 6-well Costar plates coated with Matrigel and were grown in a 5% CO₂-air incubator at 37°C. The culture medium was changed frequently until the cells attained confluence. Confluent cells (after 6–7 days of cultivation) were used for passage to Cytodex microcarrier beads.

**Primary culture of cerebral microvascular smooth muscle cells.**

Primary cultures of cerebral microvascular smooth muscle cells were grown as explants from cerebral microvessels isolated as above and described previously (1). The pellet was resuspended in culture medium consisting of DMEM containing 20% FBS, 2 mg/ml sodium bicarbonate, 1 U/ml sodium heparin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. Isolated microvessels were directly seeded onto Matrigel-coated 6-well plates. Smooth muscle cells that grew out from the ends of the vessels were grown to confluence under 5% CO₂-air at 37°C (12–14 days). These cells stain positively for α-smooth muscle actin, form hills and valleys characteristic of vascular smooth muscle, and demonstrate phenotypic characteristics of vascular smooth muscle ultrastructurally. At this age in primary cultures, these cells retain the contractile phenotype because they show the classical alignment of actin-myosin complexes and dense bodies—a smooth muscle contractile unit (1).

**Passage of endothelial and smooth muscle cells to Cytodex microcarrier beads.**

Procedures for passage and growth of endothelial and smooth muscle cells are the same with the exception that ECGS was maintained in endothelial cell cultures.

After 6–7 days of growth for endothelial cells and 12–14 days for vascular smooth muscle, cultured cells were dislodged with trypsin-PB (7–8 min, 37°C). Media and cells were transferred to cold DMEM with 20% FBS to inactivate the trypsin, and cells were precipitated by centrifugation (5 min, 1,200 rpm). Cells were passed to Cytodex 3 microcarrier beads (Pharmacia) (33). The average diameter of the beads is 175 µm for cell-covered beads. Cells were added to a suspension of Matrigel-coated beads at a density of 5 × 10⁵ cells/cm² of bead surface area and stirred intermittently (60 rpm, 2 min on and then 30 min off) overnight to promote attachment. The cultures were then continuously stirred (60 rpm 2–4 days) before being used for experiments. At this time, both endothelial cells and vascular smooth muscle were largely confluent on the beads.

**Experimental treatments.**

Treatments were begun by replacement of Krebs solution in the vial with fresh Krebs solution containing the experimental treatment. Glutamate was dissolved directly in Krebs solution, heme was prepared as heme-l-lysinate or heme (Sigma) and protected from light, and ionomycin, phorbol 12-myristate 13-acetate (PMA), tyrphostin-47, genistein, and phenylarsine oxide (PAO) were dissolved in DMSO and diluted a minimum of 100-fold with Krebs solution. Ca-free Krebs solution was prepared by replacing CaCl₂ with MgCl₂, with 4 mM EGTA and 5 × 10⁻⁶ M ionomycin. Orthovanadate and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) were dissolved in water and diluted with Krebs buffer.

**Measurement of CO production.**

For measurement of CO production, freshly isolated microvessels, microvascular endothelial cells, or microvascular smooth muscle cells grown on Cytodex beads were placed inside amber vials (2.0 ml) containing Krebs solution. All subsequent assay steps were carried out in the dark to prevent nonenzymatic photooxidative production of CO ex vivo due to the photodegradation of organic compounds. Krebs buffer in each vial was replaced with fresh Krebs or fresh Krebs containing the experimental treatment to begin incubation. The internal standard (see below) was injected into the bottom of the vial and the vial was immediately sealed with a rubberized Teflon-lined cap. Cerebral microvessels or beads carrying endothelial cells or vascular smooth muscle were incubated for 30 min at 37°C. Incubations were terminated by placing the samples in hot water (75°C) and CO production was determined immediately.

A saturated solution of the isotopically labeled CO (¹³C₁₆O; isotopic purity >99%) was used as an internal standard for quantitative measurements by gas chromatography/mass spectrometry (GC/MS) (19, 30).

GC/MS analysis of the headspace gas was performed by using a Hewlett-Packard 5970 mass-selective ion detector interfaced to a Hewlett Packard 5890A gas chromatograph. The separation of CO from other gases was carried out on a Varian-5A mole sieve capillary column (30 m, 0.32 mm ID) with a linear temperature gradient from 35 to 65°C at 5 degrees/min. Helium was the carrier gas at a column head pressure of 4.0 psi. Aliquots (100 µl) of the headspace gas were injected by using a gas-tight syringe into the splitless injector having a temperature of 120°C. Ions at mass-to-charge ratio (m/z) 28 and 29 corresponding to ¹²C₁₆O and ¹³C₁₆O, respectively, were recorded via selective ion monitoring. The amount of CO in samples was calculated from the ratio of peak areas of m/z 28 and 29. The results are ex-
pressed as picomoles of CO released into the headspace gas per 100 µg protein in 30 min. Protein was measured by the Bradford method.

**Statistical analysis.** Values are presented as means ± SE. Results were subjected to repeated-measures ANOVA with Tukey’s post hoc test to isolate differences between groups. A level of \( P < 0.05 \) was considered significant.

**RESULTS**

In freshly isolated cerebral microvessels, treatment with glutamate dose dependently increased CO production from endogenous substrate (Fig. 1). The increase of CO production from endogenous substrate was more than threefold at 1 mM glutamate.

We then examined whether both of the component cell types of the microvascular wall respond to glutamate by increasing CO production. At baseline, the CO production by endothelial and smooth muscle cells were similar (Fig. 2). Glutamate stimulated CO production by both endothelial and smooth muscle cells. Additional control experiments were performed in which all of the treatments were incubated in the vials without microvessels and no CO production was detected. In mammalian cells, the only known source of CO is heme metabolism by HO.

To determine whether glutamate increased CO production by increasing the catalytic activity of HO, we measured CO production when exogenous substrate was provided (Fig. 3). Glutamate increased the conversion of exogenous heme to CO in both endothelial cells and vascular smooth muscle cells. The results from intact microvessels were similar to those from cultured cells. In microvessels, glutamate (10^{-5} M) and exogenous heme (5 \times 10^{-6} M) increased CO production from 22 ± 1 to 48 ± 5 and 49 ± 5 pmol CO·100 µg protein^{-1}·30 min^{-1}, respectively. In the presence of the exogenous heme substrate, glutamate further stimulated CO production by cerebral microvessels (69 ± 4 pmol CO·100 µg protein^{-1}·30 min^{-1}) \((n = 8)\).

Protein phosphorylation is a common cellular mechanism for altering enzyme activity posttranscriptionally. To test the hypothesis that tyrosine phosphorylation is involved in the mechanism of glutamate stimulation of CO production, we used two inhibitors of PTK and two inhibitors of protein tyrosine phosphatases (PTP). Inhibition of PTK with either genistein or tyrphostin-47 inhibited basal CO production and blocked glutamate-stimulated increases in CO production by cerebral microvessels (Fig. 4). Furthermore, inhibition of PTP with PAO or orthovanadate increased CO production similarly to glutamate (10^{-5} M) (Fig. 5). In the presence of PTP inhibitors, glutamate-induced stimulation of CO production was not further increased in the presence of PAO but was further increased in the...
presence of orthovanadate. Nevertheless, either glutamate or inhibition of PTP with either inhibitor markedly increased CO production over the basal level.

To begin to address the question of whether the mechanism responsible for glutamate-induced stimulation of CO production might involve protein kinases C (PKC), we used the PKC stimulator PMA and the PKC inhibitor H-7. PMA did stimulate CO production. However, in the absence and presence of PMA, glutamate increased CO production by the same amount (Fig. 6). Furthermore, H-7 had no effect on glutamate stimulation of CO production (Fig. 7). These data do not suggest that glutamate increases CO production via a PKC-mediated mechanism.

Elevations of cytosolic Ca could affect substrate availability or HO-2 catalytic activity. Therefore, to determine whether the mechanism by which glutamate increases CO production might involve Ca signaling, we measured CO production by microvessels that were given treatments expected to markedly alter cytosolic Ca. The increase in CO production caused by glutamate was the same in 1.8 and 0 mM Ca with ionomycin and was not increased by ionomycin in the presence of normal extracellular Ca (Fig. 8).

DISCUSSION

The new findings in the present study of the newborn cerebral microvasculature are that 1) endothelial cells and smooth muscle cells both increase production of CO in response to glutamate, 2) glutamate increases the CO production from exogenous heme, 3) glutamate stimulation of CO production involves protein tyrosine phosphorylation, 4) PKC does not appear to be involved in glutamatergic stimulation of CO production, and 5) glutamate induction of CO production does not appear to involve Ca signaling.

It is unlikely that the effects of the tyrosine kinase and phosphatase inhibitors are unrelated to the desired action because we used two different PTK inhibitors and two different PTP inhibitors and the results
were virtually identical. Questions related to whether the tyrosine phosphorylation effect on HO-2 activity is
direct or via another enzyme that alters HO-2 activity,
phosphorylation sites, and kinases and/or phosphatases
involved must be pursued in future experiments.
A previous report indicated that HO-2 catalytic activity
in rat and mouse neuronal cultures could be increased
by serine phosphorylation (10), but we could not detect
an effect of PMA or H-7 on glutamate stimulation of
CO production in piglet cerebral microvessels. PMA
was used as a stimulator of PKC and H-7 as an inhibi-
tor of PKC for the present experiments, because we
previously showed efficacy of these pharmacologi-
cal tools in our system. Thus prostacyclin production
by piglet cerebral microvascular endothelial cells was
stimulated by PMA, and this stimulation was mark-
edly inhibited by H-7. Furthermore, in the present
experiments, PMA did increase CO production, al-
though it did not effect stimulation by glutamate. Thus
it seems reasonable to assume that PMA and H-7 did
affect PKC in the present experiments, although nei-
ther altered the response to glutamate. It remains
possible that if a PKC species were involved that is not
sensitive to PMA or H-7, PKC could play a role.

We could detect no effect of treatments intended to
alter cytosolic Ca on glutamate stimulation of CO pro-
duction. Although cytosolic Ca was not measured in
the present experiments, we are confident that the
controls altered cytosolic Ca as desired. First, iono-
mycin treatment with Ca-replete medium did increase
CO production. Second, we previously measured cere-
bral microvascular smooth muscle (data not shown)
and endothelial (27) Ca changes caused by ionomycin,
stimulates CO production by microvessels freshly isolated from the newborn pig cerebral cortex (30) and by cultured cerebral microvascular cells (28). These data suggest that cerebral microvessels express glutamate receptors that are functionally linked to HO (26).

The physiologically relevant concentration of brain and brain vasculature glutamate in vivo is difficult to estimate. Arterial and brain extracellular fluid concentration is only 1–3 \times 10^{-5} \text{ M}, but the intracellular concentration may be >10^{-3} \text{ M} (2, 22). The predominant source of extracellular glutamate outside the synapse is glial nonvesicular transport from the cystine-glutamate antiporter (3). Cerebral microvessels sit on or are surrounded by glia. Thus it appears to be conceivable that the concentration at receptors on endothelial cells and vascular smooth muscle could be in the 10^{-5}–10^{-3} \text{ M} range in which glutamate produced dose-dependent increases in CO production.

In conclusion, glutamate increases CO production by intact cerebral microvessels and cerebral microvascular endothelial and smooth muscle cells in primary culture. This stimulation is produced, at least in part, by increasing HO-2 catalytic activity via a mechanism that involves tyrosine phosphorylation.

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