Cerebral vascular endothelial heme oxygenase: expression, localization, and activation by glutamate

HELENA PARFENOVA, ROBERT A. NEFF III, JULIE S. ALONSO, BORIS V. SHLOPOV, CHAKLADER N. JAMAL, SVETLANA A. SARKISOVA, AND CHARLES W. LEFFLER. Cerebral vascular endothelial heme oxygenase: expression, localization, and activation by glutamate. Am J Physiol Cell Physiol 281: C1954–C1963, 2001.—Endogenous carbon monoxide (CO) contributes to vasodilator responses of cerebral microvessels in newborn pigs. We investigated the expression, intracellular localization, and activity of heme oxygenase (HO), the key enzyme in CO production, in quiescent cerebral microvascular endothelial cells (CMVEC) from newborn pigs. HO-1 and HO-2 isoforms were detected by RT-PCR, immunoblotting, and immunofluorescence. HO-1 and HO-2 are membrane-bound proteins that have a strong preference for the nuclear envelope and perinuclear area of the cytoplasm. Betamethasone (10^{-6} to 10^{-4} M for 48 h) was associated with upregulation of HO-2 protein by ~50% and inhibition of Cox-2 but did not alter HO-1 or endothelial nitric oxide synthase expression in CMVEC. In vivo betamethasone treatment of newborn pigs (0.2 and 5.0 mg/kg im for 48 h) upregulated HO-2 in cerebral microvessels by 30–60%. HO activity as measured by 14CO production from [14C]glycine-labeled endogenous heme was inhibited by chromium mesoporphyrin (10^{-6} to 10^{-4} M). L-Glutamate (0.3–1.0 mM) stimulated HO activity 1.5-fold. High-affinity specific binding sites for L-[3H]glutamate suggestive of the glutamate receptors were detected in CMVEC. Altogether, these data suggest that, in cerebral circulation of newborn pigs, endothelium-derived CO may contribute to basal vascular tone and to responses that involve glutamate receptor activation.

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pigs in vivo, excessive excitatory glutamatergic stimulation during seizures is associated with dilation of cerebral microvessels that is attenuated by HO inhibitors (26). Therefore, glutamic acid was selected as a physiologically relevant stimulus for HO activity.

METHODS

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

Cerebral microvessels and cell cultures. Brain cortex was obtained from ketamine- and acepromazine-anesthetized newborn pigs (1–5 days of age). Cerebral microvessels were collected by filtration of the tissue homogenate in medium 199 through 300- and 60-µm nylon mesh screens consecutively (23). Endothelial cells from cerebral microvessels were isolated and cultured in DMEM with 20% FBS, 30 µg/ml endothelial cell growth supplement, 1 U/ml heparin, and antibiotic/antimycotic mixture for 5–6 days until confluence as previously described (23). All experiments were performed on confluent quiescent cells in primary cultures. To achieve quiescence, cells were exposed to a serum-depleted medium (0.1% FBS) for 15–20 h before the experiment.

Betamethasone treatment. For in vitro treatment, endothelial cells (4–5 days, ~70% confluent) cultured in 20% FBS-DMEM were treated with betamethasone (0, 10⁻⁴, 10⁻⁵, and 10⁻⁴ M) for 32 h followed by overnight starvation (0.1% FBS-DMEM) in the presence of betamethasone (overall exposure to betamethasone, 48 h). Cells collected at the end of the incubation period were lysed in Laemmli sample buffer (total cell lysates) and used for detection of HO-1, HO-2, Cox-2, and eNOS by Western immunoblotting as described below. For in vivo treatment, three groups of newborn pigs were used (n = 4 in each group). Pigs received two intramuscular injections of saline (group I, control) or betamethasone (0.2 mg/kg (group II) and 5.0 mg/kg (group III)) over a 48-h period. The first injection was given at the onset of the treatment, and the second injection was given in 24 h. At the end of the treatment, the cerebral cortex was removed, and microvessels were isolated according to the standard procedure (as above). Microvessels were lysed in Laemmli sample buffer (10 min at 100°C) and used for detection of HO-2 by Western immunoblotting as described below.

RNA isolation and RT-PCR. Total RNA was isolated from cells by guanidinium thiocyanate-phenol-chloroform using an RNA isolation Kit (Stratagene, La Jolla, CA). mRNA levels were assessed by RT-PCR. RNA was converted to cDNA by using reverse transcriptase (Stratagene) and random primers (100 ng/ml; Sigma, St. Louis, MO) for 35 min at 59°C, using reverse transcriptase (Stratagene) and random primers (100 ng/ml; Sigma, St. Louis, MO) for 35 min at 59°C, and extension at 69°C for 25 s. The thermal cycles were terminated by a final extension of 8 min at 96°C. To ensure the PCR specificity, a negative control without cDNA was included in each series of reactions. The PCR products were analyzed by 1.0% agarose gel electrophoresis, visualized with a SYBR Green I nucleic acid gel stain (dilution, 1:10,000; Molecular Probes, Eugene, OR), and scanned at 450 nm using a STORM 860 image detection system (Molecular Dynamics, Sunnyvale, CA) with Image Quant analysis software.

Cell fractioning. We detected HO distribution between cell fractions (total cell homogenate, membrane fractions, and cytosol). Cells were scraped in cold PBS and collected by centrifugation (10 min at 2,000 g). Cells were homogenized using a glass homogenizer (30–40 strokes) in a buffer containing 20 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, pH 7.6, and a mixture of protease inhibitors with a broad specificity (Sigma). Cells were fractionated according to a standard differential centrifugation protocol. Cell homogenate was centrifuged at 1,000 g for 5 min. Pellet P₁ (crude membrane fraction enriched with the cell nuclei) was collected, and the supernatant was centrifuged at 17,000 g for 30 min. Pellet P₂ (microsomal fraction) and supernatant S₂ (soluble cytosolic proteins and the plasma membrane fragments) were collected for Western immunoblotting. Cell fractions or whole cells were solubilized in Laemmli buffer (0.125 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2.5% SDS, 0.006% bromphenol blue, and 0.1 M dithiothreitol) for 10 min at 100°C. The protein amount was quantified by dot-blot staining with amido black as previously described (23).

Western blotting. Proteins (20–50 µg protein/lane) were separated by 9% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% BSA-0.1% Tween 20 and probed with polyclonal antibody to a human HO-2 synthetic peptide (at 1:50,000 dilution; SPA 897 from StressGen Biotechnologies, Victoria, Canada), or monoclonal antibody to a human HO-2 peptide (SPA 895 from StressGen, at 1:5,000 dilution), followed by peroxidase-conjugated donkey anti-rabbit IgG (dilution 1:10,000; Jackson ImmunoResearch, West Grove, PA). As positive controls, we used recombinant rat HO-1 and human HO-2 proteins (StressGen). In addition, membrane fractions from porcine spleen and testes were used as species-specific positive controls for HO-1 and HO-2 immunodetection. For COX-2 detection, the membranes were probed with anti-Cox-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by peroxidase-conjugated donkey anti-goat IgG (1:10,000; Jackson ImmunoResearch). For eNOS detection, monoclonal antibodies (PharMingen/Transduction Laboratories, San Diego, CA) were used (1:2,000) followed by peroxidase-conjugated donkey anti-mouse IgG (dilution 1:10,000; Jackson ImmunoResearch). To normalize the antigen expression to a major housekeeping gene product, the membranes were reprobed with monoclonal antibodies against a highly conserved region of actin (dilution 1:10,000; Roche Molecular Biochemicals, Indianapolis, IN) followed by peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch). Bands were visualized with the
Renatus chemiluminescence kit (NEN Life Science Products, Boston, MA) and quantified by digital densitometry using NIH Image 1.60.

Immunofluorescence. Confluent quiescent endothelial cells on Matrigel-covered glass cover slips were fixed with 3.7% paraformaldehyde solution in PBS (pH 8.4; 15 min, room temperature) and permeabilized by 0.1% Triton X-100 solution in PBS (10 min, room temperature). The non-specific binding sites were blocked by 5% BSA (1 h at room temperature). Cells were incubated with the HO-1 or HO-2 isoform-specific primary polyclonal antibody (SPA-895 and OSA-200, respectively; StressGen) dissolved in PBS containing 0.5% BSA (dilution, 1:50) for 1 h at 37°C. To visualize antigen-antibody complexes, cells were incubated with FITC-conjugated anti-rabbit IgG (dilution 1:100; Vector Laboratories, Burlingame, CA) for 1 h at 37°C (25). For negative controls, cells were incubated with secondary antibody only. Coverslips were mounted on glass slides using antifade mounting medium (Vector Laboratories). Slides were viewed using an Image Deconvolution System consisting of a Nikon Diaphot microscope with a fluorescein filter coupled to a MacQuadra 950 computer system with a Power Mac processor 601. Three consequent images were taken from each slide. Images were deconvoluted using Vaytech software for deconvolution and IPLab Spectrum software for image collection in conjunction with a cooled charge-coupled device camera, Photometric model 250 CH. Digital processing of the images was done using Adobe Photoshop (Adobe Systems).

HO activity. HO activity was detected as 14CO release from [14C]heme-labeled cells using techniques we have developed. Confluent endothelial cells grown on Matrigel-covered 12-well plates were preloaded with [14C(U)]glycine (0.3 µCi/ml, 120 mCi/mmol; NEN Life Science Products) by overnight incubation in starvation medium (0.1% PBS-DMEM). Glycine via the β-aminoevulinate synthase pathway is used for heme synthesis, with eight of the carbon atoms of heme derived from the α-carbon of glycine molecules (27). To stop the heme labeling and to remove the nonbound tracer, cells were rinsed three times with PBS. Fresh artificial cerebrospinal fluid (aCSF; 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 to pH 7.4 was added to each well. HO catalyzes the first committed step in the degradation of the heme group to biliverdin by cleavage of the α-methene bridge; a methene bridge carbon released as 14CO is an indicator of HO activity. To quantify 14CO released from [14C]heme-labeled cells, we used the ability of red blood cells to immobilize CO by binding to hemoglobin with carboxyhemoglobin formation. Freshly isolated packed red blood cells were diluted with PBS (total volume, 200 µl) and placed in the Falcon filter inserts (0.45-µm pores). The inserts were installed in the wells with [14C]heme-prelabeled endothelial cells. The plate cover was tightly sealed, and cells were incubated with aCSF for 60 min in a 5% CO2-air incubator at 37°C. At the end of the incubation period, red blood cells were aspirated and transferred to scintillation vials to detect the total amount of immobilized 14C (14C RBC). To detect non-specific diffusion of 14C-labeled metabolites in the insert compartment (14C NSD), 200 µl of PBS were substituted for the red blood cell suspension. At the end of the experiment, cerebral microvascular endothelial cells (CMVEC) were lysed by 1% Triton X-100 solution and collected for detection of the total amount of incorporated [14C]glycine (14C CMVEC). 14CO production was calculated by subtracting non-specific diffusion using the following equation

\[
\text{14CO formation} = \frac{14C \text{ RBC} - 14C \text{ NSD}}{14C \text{ CMVEC} \times 100%}
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To determine the specificity of the assay system for 14CO formation by HO, we used CrMP, the enzyme inhibitor (31, 32). Cells were pretreated with 10–6 to 10–4 M CrMP for 30 min immediately before the experiment; CrMP was also included in the incubation media during the HO activity detection. To detect the effect of glutamic acid on HO activity, L-glutamic acid/monosodium salt (0.3 and 1.0 mM) alone or supplemented with 10–4 M CrMP was added to [14C]heme-labeled endothelial cells for the 1-h incubation period; 14CO production was detected as described above.

Radioligand binding. To determine glutamate binding with endothelial membranes, confluent endothelial cells were incubated overnight with glutamine-free DMEM-0.1% FBS. Cells were washed with cold PBS, mechanically detached in ice-cold PBS containing protease/phosphatase inhibitor cocktail (1:50; Sigma), and collected by centrifugation at 2,000 rpm for 10 min at 4°C. Cells were disrupted on ice by sonication (3 pulses for 20 s each) in a buffer containing 20 mM HEPES-NaOH/10 mM EDTA (pH 7.4) and centrifuged at 1,000 g for 5 min at 4°C. The P1 pellet (crude membrane fraction) was resuspended in 20 mM HEPES-NaOH containing 2 mM MgCl2 and 2 mM CaCl2 (pH 7.4; buffer A) and was used immediately in ligand-binding experiments. The supernatant S1 was centrifuged at 17,000 g for 30 min, and the P2 pellet (microsomal fraction) was collected. The P2 pellet resuspended in the buffer A was used immediately in ligand-binding experiments. The membrane protein was quantified by Bradford assay with BSA as the standard.

Ligand-binding assays were performed using L-[G-3H]glutamic acid ([3H]Glu, 42 Ci/mmol; Amersham Pharmacia Bio-tech, Piscataway, NJ). In high-affinity binding experiments, 50 µl of membrane suspension (30–50 µg protein) were incubated on ice for 1 h with 20–60 nM [3H]Glu in the absence and presence of 1 mM glutamate (total and nonspecific binding, respectively) in the assay buffer A (total volume, 100 µl). The reaction was terminated by dilution with ice-cold buffer A and rapid filtration through GF/C glass fiber filters (Whatman Paper, Gaithersburg, MD). The filters were washed three times with 5 ml of ice-cold buffer A. The filters were air-dried, placed in vials containing Opti Fluor scintillation cocktail (Packard Instruments, Meriden, CT), and left overnight. The bound radioactivity was detected in a beta scintillation counter. The binding experiments were performed three times in duplicate.

Materials. Cell culture reagents were purchased from Life Technologies (Gaithersburg, MD) and Amersham Pharmacia Biotech. Matrigel (growth factor reduced) was from Becton-Dickinson (Bedford, MA). CrMP was purchased from Porphyrin Products (Logan, UT). All other reagents were from Sigma, unless otherwise indicated.

RESULTS

HO-1 and HO-2 expression in quiescent endothelial cells. HO-1 and HO-2 expression was detected by RT-PCR and Western immunoblotting. The amplification of mRNA with designed HO isoform-specific primers yielded distinct bands of the expected length for porcine HO-1 (355 bp) and HO-2 (213 bp). The HO-2 RT-PCR product also yielded a second band of larger size (355 bp). It is possible that the HO-2 primers also partially recognize the HO-3 sequence because of ~80% homology between these isoforms (18).
Both HO-1 and HO-2 (mRNA and protein) were expressed in quiescent endothelial cells from cerebral microvessels of newborn pigs (Fig. 1, A and B), whereas only HO-2 was immunodetected in freshly isolated cerebral microvessels and in the brain cortex tissue (Fig. 1B). In CMVEC, HO was found exclusively in association with the cell membranes (Fig. 2). HO-1 and HO-2 proteins were distributed between crude membrane fraction P1 containing cell nuclei (1,000 g for 5 min) and microsomal fraction P2 (17,000 g for 30 min; Fig. 2). No HO expression was detected in supernatant S2 (Fig. 2), which contains soluble cytosolic proteins and the plasma membrane fragments.

Effects of betamethasone on HO-1 and HO-2 expression in quiescent endothelial cells. To investigate whether the expression of HO-1 and/or HO-2 isoforms is under control of glucocorticoids, cultured cells were treated with betamethasone (10^{-6} to 10^{-4} M) for 48 h, including an overnight starvation period. Because inducible Cox-2 is under control of glucocorticoid hormones (13), Cox-2 expression was used as a positive control for the effectiveness of the betamethasone treatment, whereas constitutive eNOS expression was used as a negative control. The experimental treatment did not affect endothelial cell count and was not cytotoxic. Betamethasone (10^{-6} to 10^{-4} M) dose-dependently inhibited Cox-2 expression two- to threefold (Fig. 3C), although Cox-2 remained immunodetectable, even in endothelial cells exposed to 10^{-4} M betamethasone for 48 h. Expression of eNOS was not altered upon betamethasone treatment (Fig. 3D), as expected for a negative control. Prolonged treatment of CMVEC with betamethasone (10^{-6} to 10^{-4} M for 48 h) did not alter the inducible isoform HO-1 expression (Fig. 3A). However constitutive HO-2 expression was upregulated by ~50% in cells treated with 10^{-6} to 10^{-4} M betamethasone (Fig. 3B).
Effects of betamethasone on HO-2 expression in cerebral microvessels in vivo. To investigate whether glucocorticoids affect HO expression in the cerebral microcirculation in vivo, newborn pigs were treated with two doses of betamethasone (0.2 and 5.0 mg/kg) administered intramuscularly two times over a 48-h period. We found that HO-2 expression in cerebral microvessels was increased by 30–60% in piglets treated with both low and high doses of betamethasone (Fig. 4).

HO-1 and HO-2 localization in quiescent endothelial cells. HO localization was investigated using indirect immunofluorescence with isoform-specific antibodies. For these purposes, we used antibodies raised against a purified recombinant (HO-1) or native (HO-2) protein (StressGen) followed by FITC-conjugated anti-rabbit IgG (dilution, 1:100). In quiescent endothelial cells, HO-2 has multiple localization sites that include the nuclear envelope, the perinuclear zone of the cytoplasm, and a wide area of the cytoplasm around the nucleus (Fig. 5B). In the cytoplasm, a "lace"-like HO-2 immunostaining pattern reveals its possible association with the endoplasmic reticulum. HO-1 localization is limited mainly to the nuclear envelope, although some HO-1 immunofluorescence is also observed in the perinuclear region (Fig. 5A). No immunostaining for HO-1 and HO-2 was detected within the nucleus, at the cell periphery, or the cell-cell contact zones (Fig. 5, A and B). No fluorescence was observed in cells treated by FITC-conjugated second antibody only (Fig. 5C), confirming the specificity of immunostaining.

HO activity in quiescent endothelial cells. HO activity was detected as 14CO formation from [14C]heme-labeled endothelial cells; red blood cells were used as a biological trap to immobilize the 14CO released from CMVEC. After overnight incubation with [14C]glycine (0.3 μCi/ml), 5–7% of the tracer was incorporated in CMVEC; endothelial heme is expected to be fully labeled with 14C atoms. The amount of endothelial cell-derived 14C-labeled products, including 14CO, produced
during 1 h of incubation under basal conditions and immobilized by red blood cells (14C RBC), was ~0.5–0.7% of the [14C]glycine uptake by CMVEC. In CMVEC treated with the HO inhibitor CrMP (10^{-6} to 10^{-4} M), a dose-dependent decrease in the amount of 14C RBC formation by 50–80% was observed (Fig. 6A). These data indicate that, indeed, 14C RBC is represented largely by the HO-derived 14CO. We found that glutamic acid (0.3–1.0 mM) dose-dependently stimulated endothelial 14CO production by 1.5- to 2-fold; the stimulation was abolished in CrMP-treated cells (Fig. 6B).

**Glutamate radioligand binding with endothelial membrane fractions.** Stimulation of HO activity by glutamate may indicate the presence of glutamate receptors in CMVEC. To directly detect endothelial glutamate receptors, we investigated the binding of [3H]Glu, the glutamate receptor ligand, with the membrane fractions of CMVEC. We focused on high-affinity glutamate binding sites that are detectable in the presence of 20–60 nM radioligand. Nonspecific binding was detected in the presence of cold glutamate (1 mM) and was subtracted from total radioligand binding. We detected a significant amount of high-affinity [3H]Glu binding sites in endothelial cell membranes (Fig. 7). Specific binding of [3H]Glu was estimated as 80–90% of the total radioligand binding. Both membrane fractions (crude membrane fraction P1 and microsomal fraction P2) demonstrated equal distribution of high-

![Fig. 4](http://ajpcell.physiology.org/download/doi/10.1152/ajpcell.00089.2001/fig2.png)

**Fig. 4.** In vivo effects of betamethasone treatment on HO-2 expression in cerebral microvessels in newborn pigs. Newborn pigs (n = 12) were given two intramuscular injections of betamethasone (group II, 0.2 mg/kg, n = 4; group III, 5.0 mg/kg, n = 4) during a 48 h-period. Group I (control, n = 4) received intramuscular saline. Freshly isolated cerebral microvessels were resolved by SDS-PAGE (50 μg protein/lane). Top: HO-2 immunodetection in cerebral microvessels isolated from control and betamethasone-treated animals. The results were quantified by densitometry and normalized to the actin amount. Bottom: HO-2-to-actin ratio (%control); values are means ± SE for each treatment group. *P < 0.05 compared with the control group.

![Fig. 5](http://ajpcell.physiology.org/download/doi/10.1152/ajpcell.00089.2001/fig3.png)

**Fig. 5.** Intracellular localization of HO by indirect immunofluorescence in cerebral microvascular endothelial cells. Quiescent cells were immunostained with anti-HO-1 antiserum raised against a purified recombinant rat HO-1 protein (A; dilution, 1:50) or HO-2 antiserum raised against a purified native rat HO-2 protein (B; dilution, 1:50) followed by FITC-conjugated anti-rabbit IgG (dilution, 1:100). For negative control staining, primary antibodies were avoided (C).

![Fig. 6](http://ajpcell.physiology.org/download/doi/10.1152/ajpcell.00089.2001/fig4.png)

**Fig. 6.** Dose-dependent stimulation of HO activity by glutamate. A: HO activity of CMVEC (0.2 mg/ml) following the addition of 0.3 mM glutamate for 30 min (n = 6). B: HO activity of CMVEC (0.2 mg/ml) treated with 10^{-6} M CrMP (n = 6). Data are means ± SE of three experiments.
affinity specific [3H]Glu binding sites (1–6 pmol/mg protein; Fig. 7, A and B).

DISCUSSION

Here, we present data demonstrating that cultured endothelial cells from cerebral microvessels of newborn pigs express functionally active HO. Under basal conditions, HO-1 and HO-2 were detected by RT-PCR, Western immunoblotting, and immunofluorescence. Expression of HO-2 was selectively upregulated by betamethasone treatment in vitro and in vivo. Endothelial HO activity was detected as CrMP-inhibited 14CO production from [14C]glycine-labeled endogenous heme. We demonstrate, for the first time, that the glutamate receptor agonist, glutamic acid, stimulates HO activity in endothelial cells, indicating possible relationships between HO and glutamate receptors. Using radioligand binding studies, we directly detected the presence of high-affinity specific [3H]glutamate binding sites on endothelial cell membranes that are suggestive of glutamate receptors.

CO, a product of the HO pathway of heme degradation, has been found to function as a messenger molecule with neuronal and vascular functions (18, 29, 30). In the systemic and cerebral circulations, CO acts as a vasodilator that plays important roles in regulation of blood flow (10, 11, 15, 30, 35). In the cerebral circulation of newborn pigs, CO is a potent vasodilator, and
endogenously produced CO is important in maintaining cerebral vascular tone (14, 15). HO, the key enzyme in CO formation, has very high activity in brain tissue compared with other organs (18). HO is represented by two major isoforms that are encoded by distinct genes located on different chromosomes. HO-2, a constitutive isoform, is expressed in different tissues under basal conditions, whereas HO-1 (Hsp32) is rapidly induced in response to a variety of stimuli, including heat shock and growth factors (18). HO-1 and HO-2 proteins share only ~40% similarity in the protein structure. HO isoform-specific antibodies selectively distinguish between the two isoforms and are widely used to investigate HO distribution in different organs, tissues, and cells in vivo and in vitro. In the brain, HO-2 expressed in neurons has been linked to the regulation of neuronal functions and cerebral blood flow (29). In glial cells, HO-2 is poorly expressed, whereas HO-1 expression is observed exclusively under induced conditions (3, 18).

In addition to neurons, HO-2 was detected in cerebral microvessels (4, 15, 18), indicating that CO may also have autocrine/paracrine functions in regulating blood flow to the brain. In the systemic circulation, isolated resistance vessels dilate in response to heme and contract in response to the HO inhibitor CrMP, indicating that CO of vascular origin could regulate vascular tone independently of the influences of the central nervous system and of circulating hormones (10, 11).

We addressed the hypothesis that vascular endothelium is a source of CO in the cerebral microcirculation. Primary cultures of CMVEC used in our experiments consist of not less than 95% of cells that positively stain for von Willebrand factor (6, 25). No glial fibrillary acidic protein has been immunodetected, indicating that contamination with glial cells is low (6). To detect the components of the HO/CO system, we used several approaches: 1) HO detection (as mRNA and protein), 2) effect of glucocorticoids on HO expression, 3) HO intracellular localization by immunofluorescence, 4) HO activity as CO production from endogenous heme, and 5) effect of glutamate on CO production. Our data demonstrate that in quiescent endothelial cells from cerebral microvessels, HO is represented at least by two isoforms, HO-2 (constitutive) and HO-1 (inducible). HO-2 expression in endothelial cells from rat aorta and human umbilical vein has been reported (8, 20, 35). Inducible HO-1 is expressed in quiescent endothelial cells but not in cerebral microvessels, as demonstrated in the present study and previously (15). HO-1 (mRNA and protein) expression under basal conditions also has been detected in endothelium from rat aorta (8) and from human umbilical vein (9). Serum is one of the factors that upregulates HO-1 expression (18). It is likely that 15–20 h of starvation in the presence of 0.1% serum are not sufficient to downregulate HO-1 expression in cultured cells. Expression of inducible gene products is under control of glucocorticoid hormones (5, 13). We investigated the effect of prolonged treatment with betamethasone on HO expression in endothelial cells in vitro and in cerebral microvessels in vivo. Betamethasone (10⁻⁶ to 10⁻⁴ M) did not decrease HO-1 expression in cultured endothelial cells after 48 h of treatment. Similarly, betamethasone did not affect expression of eNOS, which is constitutively expressed in endothelial cells. In contrast, expression of glucocorticoid-sensitive inducible Cox-2 in CMVEC was effectively inhibited by betamethasone treatment. It is likely that HO-1, which is also known as Hsp32, is easily upregulated and is present in cultured cells despite their prolonged exposure to low serum media and glucocorticoid. Furthermore, even the low level of serum used for 24 h may have been sufficient to cause HO-1 expression. The lack of inhibition of HO-1 by glucocorticoids also has been reported for rat brain (18). On the contrary, expression of HO-2 in endothelial cells was upregulated by betamethasone. Isoform-selective increases in HO-2 mRNA and protein expression by adrenal glucocorticoids have been demonstrated in developing and adult rat brain and testes and in HeLa cells (17–19, 33). The presence of a glucocorticoid response element in the untranslated HO-2 sequence accounts for upregulation of HO-2 expression (17). We found that in vivo treatment of newborn piglets with betamethasone (0.2 and 5.0 mg/kg) also resulted in upregulation of HO-2 protein expression in cerebral microvessels. These results indicate that glucocorticoid hormones that are widely used in neonatal practice for management of premature babies have a potential to effect CO-mediated vascular responses in neonatal cerebral circulation.

Intracellular distribution of HO isoforms in vascular endothelial cells had not been reported previously. Both HO-1 and HO-2 proteins were detected by immunofluorescent staining in quiescent endothelial cells from cerebral microvessels. The nuclear envelope and the perinuclear zone of the cytoplasm are the major localization sites for HO-1 and HO-2. In addition, HO-2 is extensively distributed in the area of the cytoplasm surrounding the nucleus; the lace-like pattern of immunostaining indicates possible association of HO-2 with the endoplasmic reticulum. No HO-1 or HO-2 immunostaining was observed within the cell nucleus, at the cell periphery, or in the cell-cell contact areas. Although recent knowledge of intracellular compartmentalization of HO remains very limited, electron immunocytochemistry data demonstrated association of HO-2 with the nuclear outer membrane and the endoplasmic reticulum (but not the Golgi complex) in rat kidney epithelial cells (7). Upon cell fractioning, we found HO-1 and HO-2 immunodetectable proteins in association with the crude membrane fraction enriched with cell nuclei and in the microsomal fraction that, among others, includes endoplasmic reticulum membranes. No HO-1 and HO-2 proteins were detected in the high-speed membrane fraction enriched with plasma membranes or in the cytosol. Together, these data demonstrate that HO-1 and HO-2 are membrane-bound proteins localized in the nuclear envelope, perinuclear zone, and endoplasmic reticulum areas of endothelial cells.

We detected HO activity in cultured endothelial cells with endogenous heme labeled with [¹⁴C]glycine. Gly-
cine is a substrate for heme synthesis, with eight of the carbon atoms of heme derived from the α-carbon of glycine molecules (27). HO cleaves heme at the $^{14}$C-glutamate-derived site, thus forming $^{14}$CO. Red blood cells were used as biological traps to immobilize endothelial-derived $^{14}$CO, resulting in carboxyhemoglobin formation; CrMP, an HO inhibitor, was used to show that the source of captured CO was HO-cleaved $^{14}$CO. $^{14}$CO production was dose-dependently inhibited by CrMP at concentrations that effectively inhibit the purified enzyme ($10^{-5}$ to $10^{-4}$ M). Overall, CrMP-inhibited $^{14}$CO production by endothelial cells was estimated as 0.5–0.7% of the total $^{14}$Cglucose incorporation. Because at the present time no HO isoform-specific inhibitors are available, there are no functional tools to distinguish between contributions of HO-1 and HO-2 isoforms to endothelial CO production.

Vasodilation of cerebral arterioles and the increase in cerebral blood flow in response to glutamatergic seizures is inhibited by HO inhibitors (21, 26). Close relationships between CO and glutamatergic transmission in neural control of cardiovascular responses also have been reported in conscious rats (28). These data suggest that glutamate may stimulate CO production by brain tissue. Therefore, we investigated whether CMVEC respond to glutamatergic stimulation by increasing CO production. Exposure of endothelial cells to glutamate (0.3–1.0 mM) indeed increased CO production from endogenous heme 1.5- to 2-fold; CrMP effectively inhibited glutamate-induced CO production. These data may indicate that glutamate receptors functionally coupled to HO or heme delivery might be expressed in endothelial cells from cerebral microvessels.

Using radioligand binding studies, we identified the presence of high-affinity binding sites for $[^3]$Hglutamate (20–60 nM) in membrane fractions of endothelial cells. Cultured endothelial cells exhibited high specificity for the radioligand. Not less than 80% of total $[^3]$Hglutamate was displaced by an excess of nonlabeled glutamate. The concentration of specific $[^3]$Hglutamate binding sites was ~1–6 pmol/mg protein. These data are suggestive of glutamate receptors being expressed in CMVEC. The data on glutamate receptors in endothelial cells are very limited and controversial. The presence of ionotropic and metabotropic glutamate receptors has been reported in cultured endothelial cells from rat brain (12). However, in other studies, glutamate receptors were not detected in rat and human cerebrovascular endothelial cells in situ or in primary culture (22) or in ovine and bovine cerebral microvessels (1, 34). Another possibility is that the binding of glutamate may occur at a glutamate/aspartate transporter site rather than at a receptor. Further studies are in progress to identify the subclasses of the glutamate receptors and/or glutamate transporter that are functionally coupled to CO formation in endothelial cells from cerebral microvessels of newborn pigs.

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