Cerebromicrovascular endothelial cells are resistant to L-glutamate

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1Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Winston-Salem, North Carolina; 2Department of Physiology, Faculty of Medicine, University of Szeged, Szeged, Hungary; and 3Department of Radiology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

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Domoki F, Kis B, Gáspár T, Bari F, Busija DW. Cerebromicrovascular endothelial cells are resistant to L-glutamate. Am J Physiol Regul Integr Comp Physiol 295: R1099–R1108, 2008. First published July 30, 2008; doi:10.1152/ajpregu.90430.2008.—Cerebral microvascular endothelial cells (CMVECs) have recently been implicated as targets of excitotoxic injury by L-glutamate (L-glut) or N-methyl-D-aspartate (NMDA) in vitro. However, high levels of L-glut do not compromise the function of the blood-brain barrier in vivo. We sought to determine whether primary cultures of rat and piglet CMVECs or cerebral microvascular pericytes (CMVPCs) are indeed sensitive to L-glut or NMDA. Viability was unaffected by 8-h exposure to 1–10 mM L-glut or NMDA in CMVECs or CMVPCs isolated from both species. Furthermore, neither 1 mM L-glut nor NMDA augmented cell death induced by 12-h oxygen-glucose deprivation in rat CMVECs or by 8-h medium withdrawal in CMVPCs. Additionally, transendothelial electrical resistance of rat CMVEC-astrocyte cocultures or piglet CMVEC cultures were not compromised by up to 24-h exposure to 1 mM L-glut or NMDA. The Ca2+ ionophore calcinein (5 μM), but not L-glut (1 mM), increased intracellular Ca2+ levels in rat CMVECs and CMVPCs assessed with fluo-4 AM fluorescence and confocal microscopy. CMVEC-dependent pial arteriolar vasodilation to hypercapnia and bradykinin was unaffected by intracarotid infusion of L-glut in anesthetized piglets by closed cranial window/intravital microscopy. We conclude that cerebral microvascular cells are insensitive and resistant to glutamatergic stimuli in accordance with their in vivo role as regulators of potentially neurotoxic amino acids across the blood-brain barrier.

N-methyl-D-aspartate; blood-brain barrier; cerebrovascular reactivity; glutamate excitotoxicity; intracellular calcium

L-GLUTAMATE (L-glut) is one of the most important excitatory amino acids in the central nervous system. Apart from its well-known neurotransmitter role, L-glut plays a critical role in ammonia removal from the brain. L-glut transported from the blood plasma takes up ammonia and leaves as L-glutamine (12). This balanced L-glutamate/L-glutamine countertransport is regulated by cerebral microvascular endothelial cells (CMVECs) forming the blood-brain barrier (BBB). Furthermore, CMVECs can pump L-glut from the brain to the blood, and thus they provide a functional interface for the regulated bidirectional transport of L-glut (18, 39). Human blood plasma L-glut levels range between 20 and 200 μM, and they may exceed 700 μM after ingestion of high amounts of L-glut under experimental conditions (4, 41). These plasma L-glut concentrations are not neurotoxic in vivo, although these levels are excitotoxic to neurons in vitro, suggesting that CMVECs are resistant to high L-glut levels and prevent the passive transport of L-glut into the brain.

Surprisingly, several recent reports have proposed that the L-glut levels that occur during cerebral ischemia can injure or kill CMVECs via excitotoxic mechanisms in vitro. CMVECs have been reported to possess various L-glut receptor subunits or L-glut binding sites in rat, piglet, or human CMVECs (1, 5, 27, 34, 36, 45). High concentrations of L-glut (1–3 mM), at least in vitro, are claimed to result in N-methyl-D-aspartate (NMDA) receptor activation with subsequent elevations in intracellular Ca2+ levels ([Ca2+]i) and subsequent production of reactive oxygen species leading to cell dysfunction/death of CMVECs (33, 35, 36, 45).

However, the attractive CMVEC excitotoxicity hypothesis is challenged by several lines of evidence. First, many studies have failed to find any functional glutamate receptors in ovine, bovine, rat, and human cerebral microvessels or cerebral arteries (2, 31, 44). Second, very high doses of L-glut can be ingested without any neurotoxic effects in humans (41, 43) or in experimental animals (42). Indeed, the BBB was found to be intact even after 24-h L-glut infusion in rats (20). Third, L-glut and NMDA dilate cerebral arteries in vivo (3, 30) but not in vitro (37, 44), suggesting that CMVECs do not respond directly to L-glut.

The present study was designed to study the effect of millimolar concentrations of L-glut and NMDA on CMVEC viability and function in two species. We extended our studies to include cerebral microvascular pericytes (CMVPCs). CMVPCs are common contaminating cells of primary CMVEC cultures, and it is possible that their potential sensitivity to L-glut might explain the conflicting results of previous studies. The following specific hypotheses were tested in vitro: 1) whether CMVECs and/or CMVPCs express the NMDA receptor subunit NR1 required for the expression of any functional NMDA receptors (6); 2) whether L-glut/NMDA affects viability in primary cultures of rat and piglet CMVECs and CMVPCs; 3) whether L-glut/NMDA enhances rat CMVEC injury induced by oxygen-glucose deprivation (OGD); 4) whether L-glut/NMDA affects transendothelial electrical resistance (TEER) in rat CMVEC-astrocyte cocultures as well as in piglet CMVPCs; and 5) whether L-glut/NMDA affects [Ca2+]i in rat CMVECs and CMVPCs. Additionally, we tested whether in vivo cerebrovascular reactivity to CMVEC-dependent stimuli such as hypercapnia and bradykinin is altered after intracarotid L-glut infusion in piglets.

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MATeRIALS AND METHODS

Materials

Cell culture plastics were purchased from Becton-Dickinson (San Jose, CA). DMEM, neurobasal medium, B27 supplement, 2-mercaptoethanol, and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY), and fetal bovine plasma-derived serum was purchased from Animal Technologies (Tyler, TX). Collagenase type II was obtained from Worthington (Lakewood, NJ), Percoll from Amersham Biosciences (Uppsala, Sweden), isoflurane (Forane) from Baxter (Deerfield, IL), and pentobarbital sodium (Nembutal) from Ovation (Deerfield, IL). CellTiter 96 AQueous One Solution Assay was purchased from Promega (Madison, WI). Fluoro-4 AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

Antibodies were obtained from the following sources: monoclonal mouse anti-β-actin from Sigma, monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase from Calbiochem (San Diego, CA), polyclonal rabbit anti-occludin and monoclonal mouse anti-zO-1 from Zymed Laboratories (San Francisco, CA), monoclonal mouse anti-NMDA receptor subunit 1 (NR1) antibody from Becton Dickinson, and anti-rabbit IgG and anti-mouse IgG from Jackson ImmunoResearch (West Grove, PA).

Cell Cultures

All animals were maintained and all procedures were carried out according to a protocol approved by the Animal Care and Use Committee of Wake Forest University Health Sciences. Rats were deeply anesthetized with 5% isoflurane in a 70:30 gas mixture of N2O and O2 and decapitated, while piglets were euthanized with pentobarbital sodium (80 mg/kg ip).

CMVEC culture. Rat and piglet CMVECs were isolated from Wistar rats (2 wk old) and newborn pigs (1-3 days old) as described in detail previously (8, 21, 24). Briefly, the brain cortices were freed from meninges, homogenized, and digested. The homogenate was redistributed in 20% BSA and centrifuged at 1,000 g for 20 min to yield cortical microvessels. The pellet microvessels were washed in DMEM and further digested, layered on a continuous 33% Percoll gradient, and centrifuged at 1,000 g for 10 min. The band of CMVECs was aspirated and washed twice in DMEM. The cells were then seeded onto collagen IV- and fibronectin-coated cell cultureware. The cell culture medium consisted of DMEM supplemented with 20% fetal bovine plasma-derived serum, 2 mM glutamine, 1 mg/ml basic fibroblast growth factor, 50 μg/ml endothelial cell growth supplement, 100 μg/ml heparin, 5 μg/ml vitamin C, and antibiotics. Confluent cultures [4-5 days in vitro (DIV)] consisted of >95% CMVECs, verified by positive immunohistochemistry for von Willebrand factor and by negative immunochemistry for glial fibrillary acidic protein (GFAP) and α-smooth muscle actin.

CMVPC culture. Rat and piglet CMVPCs were obtained by prolonged culture of primary CMVECs as described previously (21). Briefly, to promote CMVPC proliferation the culture medium on 4-5 DIV CMVEC cultures was switched to DMEM supplemented with 10% FBS and antibiotics, and then the cells were passed onto uncoated dishes. CMVPCs were characterized by their large size and branched morphology, positive immunostaining for α-smooth muscle actin, absence of von Willebrand factor, and GFAP staining. CMVPCs were used for experiments at 13-14 DIV.

Astrocyte culture. Rat cerebral astrocyte cultures were prepared from neonatal Wistar rats as described in detail elsewhere (21). The culture medium (DMEM supplemented with 10% FBS and antibiotics) was changed every 3 days. The cells were used after passage 2 in the astrocyte-CMVEC coculture experiments.

Neuronal culture. Rat cerebral cortical neurons were isolated from embryonic day 18 Sprague-Dawley rat fetuses as described in detail elsewhere (23). The culture medium [neurobasal medium supplemented with B27 (2%), l-glutamine (0.5 mM), 2-mercaptoethanol (55 μM), and KCl (25 mM)] was changed every 3 days. The experiments were performed on 8 DIV cultures.

Study of l-glut/NMDA on CMVEC/CMVPC Culture Viability

l-glut/NMDA treatments. Rat and piglet 4-5 DIV CMVECs and 13 DIV CMVPCs in 96-well plates were exposed to l-glut and NMDA (0.1-10 mM) for 8 h at 37°C in 5% CO2. l-Glut and NMDA were dissolved either in culture medium or in Earle’s balanced salt solution (EBSS) supplemented with glucose (5.5 mM) to combine medium deprivation with the l-glut/NMDA challenge. After the l-glut/NMDA treatments, the solutions were replaced with regular culture medium supplemented with Ara-C (10 μM) for 1 h, after which cellular viability was determined.

To combine l-glut/NMDA treatment with OGD, rat CMVECs were rinsed and then exposed to 1 mM l-glut/NMDA (0.1-1 mM) in glucose-free EBSS, and the cultures were placed in a ShelaLab Bactron Anaerobic Chamber (Sheldon Manufacturing, Cornelius, OR) filled with anaerobic mixed gas (AMG; 5% CO2, 5% H2, 90% N2) at 37°C for 12 h. The 5% H2 in the AMG removes remaining traces of oxygen via a catalyst, which typically results in <0.1% O2 in the chamber. After OGD, the EBSS was replaced with the regular culture medium supplemented with 10 μM Ara-C for 12 h, and then cellular viability was determined.

Neuronal excitotoxicity. Neuronal cultures in 96-well plates were exposed to l-glut or NMDA (0.2 mM) dissolved in culture medium for 1 h at 37°C in the 5% CO2 incubator at 8 DIV as described previously (22). Viability was determined 1 day after the excitotoxic insult.

Quantification of cellular viability. Viability was determined with the tetrazolium-based CellTiter 96 AQueous One Solution assay according to the manufacturer’s instructions. Twenty microliters of assay solution was added directly to culture wells and incubated for 1 h at 37°C, followed by measurement of absorbance at wavelength (λabs) = 492 nm with a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany). Results were compared with appropriate sister cultures, and cell viability was expressed as a percentage of the corresponding untreated control culture.

Study of l-glut/NMDA on CMVEC TEER

To induce in vitro BBB-like phenotype in rat CMVECs, cells were seeded onto collagen type IV- and fibronectin-coated Transwell inserts (diameter 24 mm, pore size 3 μm; Corning, Midland, MI) and placed into six-well plates containing confluent layers of astrocytes as described previously (21). TEER was measured with an EVOM resistance meter (World Precision Instruments, Sarasota, FL); the resistance of cell-free inserts was subtracted from the measured values, after which they were normalized to the surface of the CMVEC monolayer (Ω·cm2). TEER was determined daily, and then at 4 DIV l-glut or NMDA (1–1 mM) was applied on either the luminal or the astrocyte side. TEER was also determined 0.5–24 h after l-glut/NMDA treatment. Since values were not significantly different, data were combined as presented in RESULTS. Piglet CMVECs on the inserts were cultured in the absence of a glial feeder layer, and l-glut or NMDA (both 1 mM) was simultaneously applied to both sides of the inserts. TEER measurements in piglet CMVEC cultures were performed as described for rat CMVEC cultures.

Western Blotting for Ocludin, ZO-1, and NMDA Receptor Subunit NR1

Proteins from CMVECs and CMVPCs cultured in 35-mm dishes were harvested by scraping in ice-cold NP-40 lysis buffer supplemented with proteinase inhibitors (1 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin) and a phosphatase inhibitor cocktail (in mM: 1 EDTA, 1 sodium orthovanadate, 10 mM NaF, 10 mM β-glycerophosphate) and were solubilized in sodium dodecyl sulfate sample buffer. Equal amounts of protein were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Blots were probed with antibodies against Ocludin, ZO-1, NR1, NR2A, NR2B, and α-tubulin and analyzed by densitometry.
1 sodium pyrophosphate, and 1 sodium fluoride, with 10 μg/ml benzamidine). Samples collected from the cerebral cortex and freshly isolated cortical microvessels were homogenized and used to study the expression of NR1 subunit. For each sample, equal amounts of protein were separated by 4–20% SDS-PAGE and transferred onto a polyvinylidene difluoride sheet (Polyscreen PVDF; Perkin Elmer Life Sciences, Boston, MA); for occludin and ZO-1 or nicotinellose (for NR1 subunit). Membranes were incubated in a blocking buffer (Tris-buffered saline, 0.1% Tween 20, and 5% skim milk powder for occludin or 1% BSA for ZO-1 and NR1 subunit) for 1 h at room temperature, followed by incubation with polyclonal rabbit anti-
occludin (1:5,000), monoclonal mouse anti-ZO-1 (1:5,000), or monocl
on al mouse anti-NR1 (1:5,000) antibodies overnight at 4°C. The membranes were then washed three times in Tris-buffered saline with 0.1% Tween 20 and incubated for 1 h in the blocking buffer with anti-rabbit IgG (1:50,000) or anti-mouse IgG (1:50,000) conjugated to horseradish peroxidase. The final reaction products were visualized with enhanced chemiluminescence (SuperSignal West Pico; Pierce, Rockford, IL) and recorded on X-ray film.

**Measurement of Intracellular Free Calcium Levels**

Changes of [Ca^{2+}]i were monitored with the Ca^{2+} indicator dye fluo-4 AM. Rat CMVEC cultures on glass coverslips were loaded with 2 μM fluo-4 AM and 1 μM Pluronic F-127 in PBS containing 1 mg/ml glucose in the dark for 1 h at 21°C and were then washed three times with PBS. Stained neurons were examined with an inverted confocal microscope connected to a Zeiss LSM 510 laser scanning confocal system with a Zeiss C-Apochromat ×63/numerical aperture 1.2 water-immersion objective (Carl Zeiss, Jena, Germany). i-Glut (1 mM) or calcimycin (5 μM) was applied topically for 3 min per dose. When pial arteriolar responses to hypercapnia and bradykinin were then repeatedly determined. Pial arteriolar diameters were determined each minute during the stimulation with hypercapnia or bradykinin. Cerebrovascular reactivity was expressed as percent change from baseline diameter, and the average of 3 min periods (5th–7th min for hypercapnia) is presented in RESULTS.

**Determination of Serum i-Glutamate Levels**

To approximate the concentration of i-glut exposed to CMVECs in the pial vessels, after the completion of the cerebrovascular reactivity experiments the i-glut infusion was continued (200 mM i-glut, 100 μl/min) for 10 min. The cranial window was then removed rapidly, and venous blood was obtained from the superior sagittal sinus. The anesthetized piglets were then euthanized with KCl administration (2 M, 5 ml iv). Before the first infusion of i-glut, blood samples were also obtained from the femoral catheter to determine baseline i-glut levels. The blood samples were allowed to clot, and the serum samples were stored at −80°C. Serum i-glut levels were determined with the Amplex Red Glutamme Acid/Glutamate Oxidase Assay Kit (Invitrogen, Eugene, OR) according to the manufacturer’s directions and a FLUOSTar OPTIMA microplate reader (λex = 550 nm and λem = 590 nm).

**Statistical Analysis**

All cell culture experiments were performed in duplicate. Statistical analysis was performed with SigmaStat (SPSS, Chicago, IL). Data are presented as means ± SE. Differences between groups were assessed by one-way ANOVA, one-way repeated-measures ANOVA, or two-way repeated-measures ANOVA where appropriate. For pairwise comparisons, the Tukey post hoc test was used. P < 0.05 was considered to be statistically significant.

**RESULTS**

The isolation and cell culture methods used in the present study yielded confluent rat and piglet CMVEC cultures after 3 DIV with a low percentage of contaminating CMVPCs (Fig. 1, A and B). Prolonged culturing of CMVEC cultures with FBS-containing culture medium resulted in a virtually pure CMVEC culture. Confluent CMVEC cultures may superficially resemble CMVCs (Fig. 1, C and D); however, in addition to morphological differences the prominent expression of ZO-1 and occludin was missing in CMVCs (Fig. 1E). Neither CMVEC nor CMVEC cultures from rats or piglets expressed the NMDA receptor subunit NR1 (Fig. 2). Furthermore, samples obtained from freshly isolated cerebrocortical microvessel preparations, but not from the cerebral cortices, were also negative for NR1 expression (Fig. 2).

Rat CMVEC viability was unaffected even by prolonged exposure (8 h) of very large doses (10 mM) of i-glut and...
NMDA (Fig. 3A). In addition, neither l-glut nor NMDA worsened rat CMVEC cell death induced by OGD (Fig. 3B). In contrast, brief (1 h) exposure of smaller (0.2 mM) doses of l-glut or NMDA resulted in significant cell death of cultured rat cortical neurons (Fig. 3C). Similar to rat CMVECs, piglet CMVECs were also resistant to l-glut/NMDA exposure (Fig. 3D).

TEER values increased daily after isolation in both rat and piglet CMVEC cultures (Fig. 4, A and B) until 4–5 DIV. l-Glut (Fig. 4, C and D) or NMDA (Fig. 4, E and F) did not deteriorate TEER in CMVECs isolated from either species.

Rat and piglet CMVPC viability was also unchanged by 8-h exposure to l-glut and NMDA (Fig. 5). Although CMVPC viability was significantly decreased by 8-h incubation in EBSS (medium withdrawal), neither l-glut nor NMDA induced additional cell loss in these cultures (Fig. 5). l-Glut did not affect $[\text{Ca}^{2+}]_i$ in either rat CMVECs or CMVPCs, although the positive control calcimycin resulted in large $[\text{Ca}^{2+}]_i$ increases in both cell types (Fig. 6).

In the in vivo experiments, mean arterial blood pressure under baseline conditions was $60 \pm 4$ mmHg before and $58 \pm 4$ mmHg after l-glut infusion, and blood pressure values did not change significantly during the hypercapnia/bradykinin challenges. Baseline arteriolar diameters were not significantly different: the values were $93 \pm 10$ $\mu$m before versus $99 \pm 9$ $\mu$m after l-glut infusion. We did not observe any changes in the morphology of arterioles or brain swelling after l-glut...
infusion. Baseline serum l-glut concentration was 230 ± 24 μM, which was increased significantly to 586 ± 105 μM (P < 0.05) by the intracarotid infusion of l-glut in serum samples obtained from the superior sagittal sinus. Because the sagittal sinus collects blood from both the infused and noninfused hemispheres, L-Glut levels to the infused cortex were 1 mM.

During hypercapnia exhaled PCO2 was significantly elevated from 36.0 ± 0.8 to 75.6 ± 1.8 mmHg before (P < 0.05) and from 35.9 ± 1.0 to 75.2 ± 1.4 mmHg after (P < 0.05) l-glut infusion. Hypercapnia-induced pial arteriolar vasodilation was unaltered before and after l-glut transfusion (Fig. 7). Bradykinin-induced dose-dependent pial arteriolar vasodilation was also repeatable after l-glut infusion (Fig. 7).

DISCUSSION

The major findings of the present study are as follows. 1) Very high (10 mM) concentrations of l-glut and NMDA do not significantly affect the viability of young (4–5 DIV) primary CMVEC cultures obtained from two species. 2) l-Glut and NMDA do not enhance cellular injury in CMVECs induced by OGD. 3) l-Glut and NMDA do not deteriorate TEER, indicating an intact in vitro BBB. 4) l-Glut does not increase [Ca2+]i in cultured rat CMVECs, indicating the insensitivity of these cells to glutamatergic stimulation. 5) l-Glut does not affect CMVEC-dependent cerebrovascular responses in piglets. CMVPCs are also insensitive and resistant to l-glut or NMDA. Our present findings do not support the hypothesis that CMVECs or CMVPCs are subject to excitotoxic injury by l-glut/NMDA as suggested by some previous in vitro data (1, 5, 33, 35, 36, 45).

There is virtually no in vivo evidence demonstrating that l-glut/NMDA directly injures CMVECs or BBB integrity. Conversely, there are numerous studies suggesting the remarkable resistance of CMVECs to l-glut. Peripheral administration of very high doses of l-glut (typically 4 g/kg ip) given to neonatal/young rodents induced neurotoxic lesions confined to areas in the immediate vicinity of circumventricular organs lacking the BBB formed by CMVECs (for a review of early findings see Ref. 25). These high doses of l-glut may cause some BBB dysfunction through hyperosmolarity since equiosmolar administration of NaCl resulted in the same degree of BBB dysfunction in rats (29). Furthermore, mice (including pregnant females and their fetuses, lactating females and their sucklings, and weanlings) were shown to be resistant against the neurotoxic effect of extreme l-glut intakes (up to 42 g/kg) if they were given ad libitum access to water, thus preventing hyperosmolar stress (42). In accordance with this conclusion, even 24 h of isotonic l-glut infusion failed to cause brain edema in rats (20).

In contrast, small amounts of l-glut are neurotoxic when administered directly into the brain parenchyma (38). Intracerebroventricular infusion of NMDA (7) or administration of l-glut to the pial surface (28) in rats results in BBB dysfunction and brain edema that could be attenuated with MK801, an NMDA receptor antagonist. However, this BBB dysfunction is likely secondary to neurotoxicity. Nag (32) found that BBB
breakdown in the spinal cord always followed the excitotoxic neuronal lesion after intrathecal NMDA infusion.

Although L-glut does not appear to cause direct damage to the BBB, there is a possibility that L-glut affects CMVECs involved in cerebrovascular control. Our in vivo data provide novel evidence that CMVECs of pial arterioles involved in regulation of cerebral blood flow are also unaffected by high plasma L-glut levels. We tested hypercapnia- and bradykinin-induced arteriolar vasodilation, since in a previous study (9) we showed that intracarotid infusion of phorbol 12,13-dibutyrate causing oxidative stress in CMVECs attenuated hypercapnia- and bradykinin-induced responses by 70%. In the present study, we found intact CMVEC-dependent vasodilation to hypercapnia or bradykinin, suggesting that high levels of L-glut did not affect CMVEC function.

Only in vitro studies lend support to the hypothesis that L-glut may directly affect and exert toxicity to CMVECs. However, even the mere expression of L-glut receptor subtypes in cultured CMVECs is controversial. The presence of L-glut receptors, or rather their mRNAs, is likely affected by culture conditions and time spent in culture since CMVECs are known to dedifferentiate rather quickly. Glutamate receptors have also been found in freshly isolated rat brain microvessel preparations (26, 40). Unfortunately, astrocytic processes tightly adhering to the basal lamina of isolated capillaries were recognized that could not be separated by enzymatic digestion with either papain or collagenase. Therefore, the possibility of these tissues as sources of the mRNA signal in the isolated capillaries could not be excluded (40). However, in the present study the primary microvessel preparations appeared to be devoid of any NR1 immunopositivity, suggesting that functional NMDA receptor expression was virtually nonexistent in the cortical microvasculature.

Krizbai et al. (27) found the mRNA of numerous L-glut receptor subunits for NMDA and AMPA and metabotropic L-glut receptors (mGluRs) in rat CMVEC cultures, albeit the mRNA
levels were quite small compared with the brain homogenate used as controls. In contrast, a very detailed study by Morley et al. (31) was unable to find any NMDA or AMPA receptor expression in rat or human CMVEC cultures assessed at both mRNA and protein levels. Some mRNA expression of kainate receptor subunits was shown in rat CMVECs only, but the CMVECs were unresponsive to kainate stimulation. Thus the authors concluded that CMVECs in these species at least do not express functional ionotropic L-glut receptors. Sharp et al. (35) refuted this conclusion by demonstrating NMDA receptor 1 mRNA and protein expression in immortalized human brain endothelial cells by using human specific primer sets instead of the rat primers used by Morley et al. Recently Andras et al. (1) found expression of NMDA and AMPA receptor subunits in rat CMVECs. In addition, mGluR1, -2, and -5 were found in human CMVECs (5). In piglet CMVECs, the presence of NMDA receptors was suggested by [3H]L-glut binding assays (34); however, these binding sites may not be ionotropic receptors but rather the well-known L-glut transporters of CMVECs. We restricted our efforts to seek L-glut receptor expression in CMVECs to the study of NMDA receptor subunit NR1 expression, since most of the positive studies demonstrating L-glut excitotoxicity emphasized the role of NMDA receptors in the mechanism of CMVEC injury (1, 33–36, 45). NR1 subunits are ubiquitous components of any functional NMDA receptors (6); thus the lack of NR1 immunopositivity in our CMVEC and CMVPC cultures is consistent with the insensitivity and resistance of these cells to the high doses of L-glut/ NMDA shown in the present study. Since the primary microvessel preparations also lacked NR1 expression, the possibility of our cultures losing their NMDA receptors because of some unknown culture conditions is unlikely. It is still possible that some minor, possibly asymmetric, NMDA receptor expression exists in CMVECs in vivo, but we are unaware of any data showing L-glut receptors on CMVECs despite their abundant distribution in the brain.

Two early reports claimed direct L-glut toxicity to rat brain and retinal endothelial cell cultures, but unfortunately both have been published only as abstracts (10, 19). L-Glutamine was praised as a growth supplement in rat CMVEC cultures by the same authors in a full paper that did not mention L-glut toxicity (11). More recently, L-glut (1–3 mM, 30 min–3 h) has been suggested to induce apoptotic cell death in murine and piglet CMVECs caused by oxidative stress, and the protective role of inducible hemoxygenase-2 activity was emphasized (33, 45). However, Andras et al. (1) found no decrease in CMVEC viability with the same dose of L-glut (1 mM, 24 h) in rat CMVECs, and we confirmed this finding in both rat and piglet CMVEC cultures in the present study. In addition, we demonstrated that L-glut and NMDA do not worsen OGD-induced CMVEC death.

L-Glut may not kill CMVECs in vitro or in vivo but may induce endothelial dysfunction manifested in deteriorating in vitro BBB characteristics. Collard et al. (5) studied the effect of polymor-
phonuclear leukocytes on human CMVECs, and they identified L-glut acting on mGluRs as one of the leukocyte-released mediators involved in BBB dysfunction. However, an incredibly low dose (1/9262M) of L-glut that is 20–100 times less than the plasma levels of L-glut was reported to adversely affect the BBB shown by increased FITC-dextran permeability (5); therefore the interpretation of this finding is problematic. Sharp et al. (35) used 1 mM L-glut or NMDA to diminish TEER in immortalized human CMVEC cultures, and this effect was blocked by MK801, Ca2+/H11001 channel blockers, and antioxidants, suggesting NMDA receptor-mediated oxidative stress as the mechanism of CMVEC dysfunction. However, in that study, 0.1 mML-glut was not effective to reduce TEER values, although this dose should also excite NMDA receptors. Andras et al. (1) had similar preservation of TEER by MK801 after 1 mM L-glut exposure in rat CMVECs. The efficacy of MK801 may not be related to NMDA receptor blockade, however, since it was also effective in preventing decrease in TEER by hypoxia in pig CMVEC cultures (14).

We did not observe a decrease in TEER in L-glut/NMDA-treated CMVEC cultures, although the TEER values were similar to those in the previous positive studies, and we used young occludin- and ZO-1-immunopositive confluent primary CMVEC cultures, indicating the presence of BBB forming tight junctions (17). There were several differences in methodology between the studies. For instance, in the recent paper by Andras et al. (1) rat CMVECs were incubated with hydrocortisone, 8-4-chlorophenylthio)-cAMP, and the cAMP phosphodiesterase-4-specific inhibitor RO-201724 for 24 h before exposure to L-glut. This treatment can change the phenotype of CMVECs and may have sensitized them to L-glut. Furthermore, Andras et al. reported a 20% drop of TEER even in control cultures after 24 h, in sharp contrast to our study, where TEER was maintained or even increased after 24-h incubation with L-glut or NMDA. The involvement of NMDA receptor activation in the observed L-glut effects reported in previous papers (1, 36) is not supported by our negative findings showing that L-glut, but not calcymicin, fails to increase [Ca2+]i in rat CMVECs, although L-glut results in a prompt increase in [Ca2+]i in cultured rat neurons with the same technique (13).

As a novel approach to the controversy on CMVEC excitotoxicity, we studied the effect of L-glut on CMVPC cultures, since the study of Parfenova et al. (33) used 20% FBS-supplemented culture medium and prolonged culturing, which in our experience promote CMVPC proliferation (21). Indeed, the morphology of cultures shown in that study (33) do not show much resemblance to the characteristic features of CMVEC cultures (Fig. 1, A and B) but rather look like the cells in our CMVPC cultures (Fig. 1, C and D). However, we were still unable to induce cytotoxicity with L-glut and NMDA in CMVPCs as well, despite the fact that CMVPCs in vivo were claimed to possess mGluRs (15).

Perspectives and Significance

The present study provides evidence that cultured CMVECs and CMVPCs from two species are as resistant to supposedly...
excitotoxic concentrations of l-glut in vitro as found previously in vivo, thus challenging recent in vitro findings. We also show that high l-glut levels do not affect CMVEC-dependent cerebral vascular reactivity and CMVECs do not possess functional NMDA receptors. Our data are consistent with the physiological role of cerebral reactivity and CMVECs do not possess functional NMDA receptors. We also show that

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