Neutrophil-derived Glutamate Regulates Vascular Endothelial Barrier Function*

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Endothelial barrier function is altered by the release of soluble polymorphonuclear leukocyte (PMN)-derived mediators during inflammatory states. However, endogenous pathways to describe such changes are only recently appreciated. Using an in vitro endothelial paracellular permeability model, cell-free supernatants from formylmethionylleucylphenylalanine-stimulated PMNs were observed to significantly alter endothelial permeability. Biophysical and biochemical analysis of PMN supernatants identified PMN-derived glutamate in modulating endothelial permeability. Furthermore, novel expression of metabotropic glutamate receptor 1 (mGluR1), mGluR4, and mGluR5 by human brain and dermal microvascular endothelial cells was demonstrated by reverse transcription-PCR, in situ hybridization, immunofluorescence, and Western blot analysis. Treatment of human brain endothelia with glutamate or selective, mGluR group I or III agonists resulted in a time-dependent loss of phosphorylated vasodilator-stimulated phosphoprotein (VASP) and significantly increased endothelial permeability. Glutamate-induced decreases in brain endothelial barrier function and phosphorylated VASP were significantly attenuated by pretreatment of human brain endothelia with selective mGluR antagonists. These observations were extended to an in vivo hypoxic mouse model in which pretreatment with mGluR antagonists significantly decreased fluorescein isothiocyanate-dextran flux across the blood-brain barrier. We conclude that activated human PMNs release glutamate and that endothelial expression of group I or III mGluRs function to decrease human brain endothelial VASP phosphorylation and barrier function. These results identify a novel pathway by which PMN-derived glutamate may regulate human endothelial barrier function.

Endothelial cells, which line the inner lumen of blood vessels, are the primary determinants of vascular barrier function. During episodes of infection, ischemic or traumatic injury, endothelial cells are primary targets for leukocytes and can result in altered barrier function (1). Under such pathological conditions, endothelial metabolism, gene expression, and cell surface protein expression may be altered by a myriad of soluble factors, including cytokines, bioactive lipids, and bacterial endotoxin (1). Similarly, vascular barrier function may be altered by the local release of soluble mediators from activated polymorphonuclear leukocytes (PMNs). For example, it is only recently appreciated that PMN-derived compounds, such as adenosine, may provide endogenous pathways to dampen changes in endothelial permeability during leukocyte extravasation (2). These same studies suggested the existence of other unknown PMN-derived compounds that might influence endothelial barrier properties.

Within the central nervous system (CNS), glutamate is the primary excitatory neurotransmitter. Four classes of glutamate receptors have been identified, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate, N-methyl-D-aspartate, and the more recently described metabotropic glutamate receptors (mGluRs) (3). mGluRs are single polypeptide chain receptors characterized by a seven-transmembrane-spanning structure and a long N-terminal extracellular domain important in Glu binding (4). Coupled to guanosine triphosphate-binding proteins, mGluRs form a family of at least eight subtypes, which are classified into three groups based on sequence homology, pharmacological profile of activation, and signal transduction pathways (5). Group I mGluRs (receptors 1 and 5) are coupled to phospholipase C activation and stimulate polyphosphoinositide hydrolysis via coupling to Gq/11 (5). In contrast, group II (receptors 2 and 3) and III (receptors 4, 6, 7 and 8) mGluRs are negatively coupled via G, to adenylate cyclase and decrease cAMP formation (5). Originally described within the CNS, recent evidence suggests that mGluRs are expressed by a wide variety of peripheral cell types outside the mammalian CNS (6). Although their role outside the CNS has yet to be defined, mGluRs are involved in various aspects of CNS physiology and pathology, including modulation of excitatory synaptic transmission, developmental plasticity, learning and memory processes, and neurodegeneration (5).

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The abbreviations used are: PMN, polymorphonuclear leukocyte; BBB, blood brain barrier; BMVEC, brain microvascular endothelial cells; mGluR, metabotropic glutamate receptor; HMVEC, human microvascular endothelial cells; VASP, vasodilator-stimulated phosphoprotein; HBSS, Hanks’ balanced salt solution; fMLF, formylmethionylleucylphenylalanine; CNS, central nervous system; HPLC, high performance liquid chromatography; FTIC, fluorescein isothiocyanate; DHPG, 3,5-dihydroxyphenylglycine; i-AP4, L-2-amino-4-phosphonobutyrate; PHCCC, N-phenyl-7-hydroxynaphthalenylpropyl)chromen-1a-carboxamide; CPPG, (RS)-N-cyclopropyl-4-phosphonophenylglycine; bp, base pairs; PBS, phosphate-buffered saline; Ab, antibody; RT, reverse transcriptase; EVH, Ena-VASP homology.

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injection of functional mGluRs on human endothelia has not been described.

In the present studies, we demonstrated that cell-free supernatants from FMLP-stimulated PMNs significantly altered endothelial permeability, suggesting the presence of a soluble PMN-derived mediator(s). Structural analysis of the bioactive, PMN-derived fractions identified glutamate as an active component in regulating human endothelial barrier function. Furthermore, we propose a novel pathway by which glutamate may regulate human vascular endothelial barrier function via its action on endothelial-expressed mGluRs.

**MATERIALS AND METHODS**

**Endothelial Cell Culture**—Human brain (BMVEC; Cell Systems Corp., Kirkland, WA) and dermal (HMVEC; Cascade Biologics, Portland, OR) microvascular endothelial cells were obtained as primary cultures and passaged as previously described (2, 7). For preparation of experimental HMVEC or BMVEC monolayers, confluent endothelial cells (passage <10) were on permeable polycarbonate inserts or 100-mm Petri dishes coated with 0.1% gelatin, as described previously (2). Endothelial cell purity was assessed by phase microscopic "cobblestone" appearance and uptake of fluorescent acetylated low density lipoprotein.

**Human Neutrophil Isolation**—PMNs were freshly isolated from whole blood obtained by venipuncture from human volunteers and anticoagulated with citrated/dextran (dilution of FITC-dextran. Paracellular flux was calculated by linear regression of sample fluorescence. Consistent with previous reports, control experiments demonstrated decreased permeability to [3H]ouabain and [3H]8-bromo-cAMP (11) and increased permeability with thymulin and hydrogen peroxide (data not shown) (12).

Paracellular flux was also determined across monolayers treated with glutamate, 3,5-dihydroxyphenylglycine (DHPG; selective group I mGluR agonist), L-2-amino-4-phosphobutyrate (1-AP; selective group II mGluR agonist), or glutamate (1 mM) plus N-phenyl-7-hydroxy-3-trifluoromethylchromen-4-carboxamide (PHCCC; selective group I mGluR antagonist), or (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG; selective group III mGluR antagonist) (Tocris Cookson Inc., Ellisville, MO).

**High Performance Liquid Chromatography**—A Hewlett-Packard HPLC (Model 1050) with a HP 1100 diode array detector was used with a C18 reverse phase HPLC column (5 μm, 4.6 × 250 mm, Phenomenex, Torrance CA). Activated PMN-derived samples were eluted (1-ml fractions), evaporated to dryness, reconstituted in HBSS, and assayed for influence on paracellular permeability.

Dabsylated glutamate and glutamine were measured as previously described (13). Briefly, 1 g of dabsyl-Cl (Sigma) was recrystallized by boiling in 10 ml of acetone for 5 min and then cooled. The solution was filtered (0.45 μm, nylon filter) and crystallized overnight at 4 °C. Dabsylated amino acids were prepared by dissolving 1 mg of L-glutamic acid or glutamine (Sigma) in 100 μl of 0.1 M NaHCO3 buffer, pH 9.0, and then adding 100 μl of dabsyl-Cl solution (2 nmol/ml acetone). The mixture was then heated for 10 min at 70 °C, dried (Savant Automatic SpeedVac System, Holbrook, NY), and re-dissolved in 20 μl of 70% (v/v) ethanol.

1-L-Glutamic acid and glutamine were measured in cell-free supernatants using a NaHPO4 (0.1 M), Na2EDTA (0.1 mM), pH 6.38, mobile phase (1.2 ml/min). Absorbance was measured at 460 and 275 nm. UV absorption spectra were obtained at chromatographic peaks. 1-L-Glutamic acid and glutamine were identified by their chromatographic behavior (e.g. retention time, UV absorption spectra, co-elution with standards) (13).

**Mass Spectroscopy**—For structural elucidation of L-glutamic acid and glutamine, bioactive materials eluted by HPLC were injected into a liquid chromatograph in tandem with a mass spectrometer (LC/MS) using a 2.1 × 100-mm ODS Hypersil column (Hewlett Packard). The LC/MS was run at 0.5 ml/min. Buffers were A = water with 0.1% formic acid and B = acetonitrile with 0.1% formic acid, with the gradient set at 20.5% B to 75% B in 35 min using buffer A as a diluent. The mass spectrometer used was a Platform II (Micromass Instruments, Beverly, MA) operated using atmospheric pressure chemical ionization and operated such that scans of opposite polarity were obtained on alternating scans (alternating positive/negative ion mode). The instrument was scanned from mass 100 to 600 in 1 s, with a 0.1-s interscan time. In this manner, full positive and negative ion mass spectra were obtained from a single chromatographic analysis.

**RT-PCR and Sequencing of Human Endothelial mGluRs**—RT-PCR analysis of BMVEC and HMVEC mRNA levels was performed using DNaese-treated total RNA as previously described (14) and primers (Sigma-Genosys, The Woodlands, TX) specific for mGluR1 (forward primer 5′-GGG GCT GT-3′ and reverse primer 5′-ATG GAA GGG CTA CCA GGC-3′) and reverse primer 5′-TCT GTA CCC GGT AGT CAT GC-3′, 194-bp fragment), mGluR3 (forward primer 5′-CTT GTT TTT TTA GAC TGT TA-3′ and reverse primer 5′-CAG TAT ATA CAG TCC TCA AA-3′, 132-bp fragment), mGluR4 (forward primer 5′-GCA CAA GAT CCA GCA AA-3′ and reverse primer 5′-AAG AAG GAG GGC GAA ACA A-3′, 661-bp fragment), mGluR5 (forward primer 5′-CCG TGT TCA CAC ACA CAC AA-3′ and reverse primer 5′-CCC TAG AGC AAA GCA GTT GG-3′, 253-bp fragment), mGluR6 (forward primer 5′-GTA CAC TCA GGA TTT GCT CT-3′ and reverse primer 5′-CGG GAT ACG ATC CTC AGT GC-3′, 592-bp fragment), mGluR7 (forward primer 5′-ATT TTT GGA TCT CTA AC-3′ and reverse primer 5′-ATG AAA AGG TTA GTT TTA-3′, 661-bp fragment), mGluR8 (forward primer 5′-GCC CGC CCA GAT CGA ATT GG-3′, 502-bp fragment). All PCR reactions were then visualized on a 1.8% agarose gel containing 20 μg/ml ethidium bromide. Bands were digitized using an electrophoresis documentation and analysis system and analyzed by onedimensional analysis software (Kodak Digital Science). Net band intensity (background subtracted intensity) was normalized to values for β-actin and plotted as relative units. Water samples or RNA samples not amplified were sampled (Promega, Madison WI) according to the manufacturer’s protocol. DNA was amplified using the dNTPs and PCR buffer (Invitrogen) (20 mg Tris-Cl, pH 8.4, 50 mM KCl), 1.5 μM MgCl2, 200 μM of each dNTP, and 2.5 units of Taq DNA polymerase (Invitrogen). Reactions were heated to 94 °C for 5 min before adding 20 pmol of each primer. Each primer set was then amplified at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s for the indicated number of cycles followed by a 10-min extension at 72 °C. The PCR reactions were then visualized on a 1.8% agarose gel containing 0.6 μg/ml ethidium bromide. Bands were digitized using an electrophoresis documentation and analysis system and analyzed by onedimensional analysis software (Kodak Digital Science). Net band intensity (background subtracted intensity) was normalized to values for β-actin and plotted as relative units. Water samples or RNA samples not amplified were considered negative or no DNA was present during PCR (data not shown).

**Human Endothelial mGluR mRNA Sequencing and in Situ Hybridization**—PCR products for human endothelial mGluR1, mGluR4, and mGluR5 were gel-purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA), ligated into a pGEM-T vector, and transformed using the pGEM-T Easy vector system (Promega) according to the manufacturer’s protocol. DNA was amplified using the dNTPs and PCR buffer (Invitrogen) (20 mg Tris-Cl, pH 8.4, 50 mM KCl), 1.5 μM MgCl2, 200 μM of each dNTP, and 2.5 units of Taq DNA polymerase (Invitrogen). Reactions were heated to 94 °C for 5 min before adding 20 pmol of each primer. Each primer set was then amplified at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s for the indicated number of cycles followed by a 10-min extension at 72 °C. The PCR reactions were then visualized on a 1.8% agarose gel containing 0.6 μg/ml ethidium bromide. Bands were digitized using an electrophoresis documentation and analysis system and analyzed by onedimensional analysis software (Kodak Digital Science). Net band intensity (background subtracted intensity) was normalized to values for β-actin and plotted as relative units. Water samples or RNA samples not amplified were considered negative or no DNA was present during PCR (data not shown).
instructions. The predicted nucleotide sequence for each mGluR was confirmed by sequencing (Harvard sequencing core) several colonies from each mGluR ligation (data not shown). For probe production, EcoRI-digested fragments were gel-purified using a Qiagen gel extraction kit (Qiagen) and labeled with biotin-16-DUTP, and the probes were separated on a denaturing gel.

For mGluR analysis by in situ hybridization, human BMVEC or HMVEC were grown to confluence on LabTech tissue culture microscope slides (Nalge Nunc International, Rochester, NY). After aspirating the media, the cells were fixed with 4% paraformaldehyde, PBS (10 min) and washed in PBS/MgCl₂ (5 mM). All materials were kept RNase-free throughout the procedure. Before hybridization, the cells were hydrated in 0.2× Tris-Cl, pH 7.4, and 0.1 M glycine for 10 min and then changed to 50% formamide, 2× SSC (SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at 65°C for 15 min. Although the cells were being hydrated, the probe (80–100 liters probe), 4 μl of Escherichia coli tRNA (Sigma), and 4 μl of salmon sperm DNA (Sigma) were melted in 10–30 liters of 100% formamide (Sigma) at 90°C for 10 min.

An equal volume of hybridization mix was added for a final concentration of 50% formamide, 2× SSC, 0.2% bovine serum albumin, 10 mM vanadyl sulfate-ribonucleoside complex (Invitrogen), 10% dextran sulfate, and 1 g/ml each E. coli tRNA and salmon sperm DNA. The final concentration of the probe was 80–100 ng/30 μl hybridization. The probe and hybridization mix were added to the tissue culture slides, the coverslips were placed, and the mixture was incubated at 37°C (4°C in a closed, 2× SSC-sealed chamber). After hybridization, the cells were washed with 2× SSC, 50% formamide for 30 min at 37°C, then in 1× SSC, 50% formamide for 30 min at 37°C, and twice in 1× SSC at room temperature for 30 min.

The cells were incubated in 4× SSC-1% bovine serum albumin with avidin-FITC (2 μg/ml) for 30 min and washed 3 times in 2× SSC at room temperature on a rotating shaker. The cells were mounted in antifade mounting medium and covered and viewed on a Leica confocal scanning microscope. Control BMVEC and HMVEC were incubated in RNase A (100 μg/ml in 2× SSC for 1 h at 37°C) to determine the specificity of the probe for RNA. After incubation in RNase A, the cells were hybridized as described above and incubated with avidin-FITC, washed, and viewed by confocal microscopy. A second negative control preparation consisting of BMVEC and HMVEC hybridized with a porcine mannose binding lectin cDNA probe, washed, then reacted with FITC-avidin and viewed on a confocal microscope. All in situ hybridization studies were done in triplicate.

Immunofluorescence—Confluent human BMVEC and HMVEC grown on coverslips were washed in PBS, fixed in 1% paraformaldehyde, 100 mM cacodylate buffer for 10 min at room temperature and washed again. The coverslips were incubated with rabbit anti-mGluR1 (Upstate Biotechnology, Lake Placid, NY), mGluR4, or mGluR5 (Chemicon International, Temecula, CA) polyclonal Ab for 1 h at room temperature. After washing, the slips were incubated with FITC-conjugated goat anti-rabbit IgG (Sigma) or rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR). The coverslips were washed (×3; 10 min each) in PBS, incubated with 1% anti-fade mounting medium (Molecular Probes), and analyzed with a Zeiss confocal microscope as previously described (15). Controls incubated with FITC-conjugated goat anti-rabbit IgG only were processed as above, omitting the primary antibody to determine nonspecific binding. Additionally, some coverslips were incubated with an irrelevant, isotype control rabbit anti-rabbit mannan binding lectin polyclonal Ab. All analyses were conducted at the same pinhole, voltage, and laser settings. This experiment was performed three times (n = 3).

Immunoprecipitation and Western Blotting—Confluent human BMVEC were labeled with biotin and lysed, and the cell debris was removed by centrifugation (16). Cell lysates were pre-cleared with 50 μl of preequilibrated protein G-Sepharose (Amersham Biosciences). Overnight immunoprecipitation of mGluR was performed by the addition of anti-mGluR1, mGluR4, and mGluR5 polyclonal Ab (10 μg/ml) or control Ab directed against cAMP response element-binding protein (Upstate Biotechnology) and 50 μl of preequilibrated protein G-Sepharose. Washed immunoprecipitates were boiled in Laemmli buffer plus 20 mM dithiothreitol, separated by SDS-PAGE, transferred to nitrocellulose (Bio-Rad) and blocked overnight in blocking buffer. Biotinylated protein G-Sepharose was added to streptavidin-conjugated mouse and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Western Blot of Human Endothelial Vasodilator-stimulated Phosphoprotein (VASP)—Confluent BMVEC 100-mm Petri dishes were treated with indicated concentrations of glutamate, DHPG (10 μM), and 1-AP4 (10 μM) for 0–50 min. As a positive control, separate BMVEC dishes were treated with forskolin (10 μM) for 15 min. The dishes were washed with HBSS and then scraped with Laemmli buffer plus 20 mM dithiothreitol. BMVEC lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and the membranes were blocked with 10% nonfat dry milk. The membranes were then incubated with murine anti-human VASP mAb (1 μg/ml; BD Transduction Laboratories, Lexington, KY) for 1 h at 20°C. The membranes were then washed 5 times with PBS/Tri buffer and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Sigma) for 1 h at 20°C. Labeled bands were detected by ECL.

Brain Blood Barrier (BBB) Permeability in Hypoxic Mice—Alterations in BBB permeability in vivo were determined in a hypoxic mouse model (17). As guided by previous in vitro studies using mGluR agonists and antagonists in rodent brain microvessel endothelial cell cultures (Taconic, Germantown, NY) were injected intraperitoneally with PBS or 2 mg/kg DHPG, 1-AP4, PHCCC, or CPPG. The mice were then gavaged with 4.4-kDa FITC-dextran (60 mg/100 gm of body weight) and immediately placed into a hypoxia chamber (8% O₂) or back into their cage (21% O₂) for 4 h. The mice were then sacrificed, and the brains were removed and weighed. After sonication and centrifugation to remove cellular debris, the fluorescence intensity (excitation, 485 nm; emission, 530 nm) of each brain sample was measured (Cytofluor 2300, Millipore Corp., Bedford, MA) and normalized to the serum fluorescence and expressed as a permeability index. Control tissues derived from mice not administered FITC-dextran were used as background controls for fluorescence. The PMN marker myeloperoxidase was quantified as previously described (20) in brain homogenates. This protocol was in accordance with NIH guidelines for use of live animals and was approved by the Institutional Animal Care and Use Committee at Brigham and Women’s Hospital.

Statistical Analysis—All data presented represent the mean and S.E. for n determinations. Data analyses were performed using Sigma Stat (Jandel Scientific, San Rafael, CA). A p value of <0.05 was considered significant. Transendothelial FITC-dextran flux across endothelial monolayers was normalized to untreated cells and analyzed by one- or two-way analysis of variance. All pairwise multiple comparisons were made using the Student-Newman-Keuls test. FITC-dextran flux across the BBB in hypoxic mice is presented as a permeability index calculated as the ratio of measured brain to serum fluorescence.

RESULTS

Soluble Supernatants Derived from Activated PMNs Regulate Endothelial Barrier Function—We have previously demonstrated that upon activation, PMNs release AMP and adenosine, which through activation of surface adenosine receptors provide a sealing mechanism during PMN transendothelial migration (2). Here, we pursued the existence of additional pathways for regulation of barrier function by PMNs. Initial studies were undertaken to screen supernatants derived from fMLF (10⁻⁸ M)-stimulated PMNs. As shown in Fig. 1A, the activation of the activated PMN supernatants significantly decreased transendothelial FITC-dextran flux compared with untreated, control cells (Fig. 1, panel A). Because we have previously demonstrated no apparent endothelial uptake of FITC-dextran, no influence of fMLF (10⁻⁸–10⁻¹⁰ M) on FITC-dextran transendothelial flux, and no quenching of FITC-dextran fluorescence by activated PMN supernatants (2), we concluded that a PMN-derived soluble mediator(s) decreases endothelial permeability.

To identify potentially novel, barrier-influencing molecules, supernatants derived from activated PMNs (fMLF 10⁻⁸ M, 1 h) were fractionated by HPLC, concentrated 20-fold, and screened for influences on HMVEC permeability. As shown in Fig. 1B, this analysis revealed two major biologically active fractions eluting in the time periods of 2–3 min (fraction 3, 85 ± 9% decrease in permeability) and 8–9 min (fraction 9, 82 ± 11% decrease in permeability), respectively. Further evaluation of fraction 9 identified it at 5'-AMP (based on retention time, UV spectra, and co-elution with standards, data not shown), as we have studied in the past (2). Isolation and further purification of fraction 3 revealed this fraction to be stable to extremes in temperature and pH and of low molecular mass (<1 kDa) with a dominant UV chromophore at 199 nm, likely indicative of a saturated amine linkage (21).
Glutamate and mGluR Agonists Alter in Vitro Endothelial Permeability

Identification of Fraction 3 as Glutamate—We next employed mass spectroscopy to obtain structural insight into fraction 3. As shown in Fig. 2A, in the positive ion mode, HPLC-purified fraction 3 revealed a dominant ion at m/z 147 and a [M + H]⁺ fragment at m/z 148, providing evidence for M = 147 kDa. By contrast, negative ion mass spectra (Fig. 2A) revealed a dominant peak at m/z 146 [M – H]⁻, providing evidence for M = 147. These structural characteristics along with the λ_max of 199 are consistent with an amino acid, of which both glutamic acid and glutamine fit. HPLC experiments to define fraction 3 using internal standards of glutamine and glutamate did not provide the level of resolution necessary to distinguish between these amino acids. We thus resorted to derivatization of fraction 3 with dabsyl chloride (13). As shown in Fig. 2B, this approach allowed us to distinguish between glutamate and glutamine based on retention time and revealed that purified, derivatized fraction 3 eluted with authentic glutamate, thus glutamine based on retention time and revealed that purified, approach allowed us to distinguish between glutamate and mGluRs in the regulation of human endothelial barrier function and define distinct differences between HMVEC and BMVEC for such responses.

Selective analog agonists and antagonists were used to verify these results. As shown in Fig. 3B, group I (DHPG) or III (t-AP4) mGluR agonists (1 μM) significantly increased BMVEC paracellular flux compared with untreated cells (p < 0.01). Similar treatment of human BMVEC with the selective group I (PHCCC) or III (CPPG) mGluR antagonists (1 μM) significantly attenuated glutamate (1 μM)-mediated increases in paracellular permeability. These data suggest a novel role for glutamate and mGluRs in the regulation of human endothelial barrier function and define distinct differences between HMVEC and BMVEC for such responses.

Human Endothelia Express mGluRs—Based on the above findings, we screened endothelia for the existence of mGluRs. Although evidence is rapidly accumulating that mGluRs have been expressed by a variety of cell types (5), functional mGluRs have not been described on human endothelia. Initial studies by RT-PCR revealed that endothelia express only mGluR1, mGluR4, and mGluR5 mRNA (BMVEC data shown in Fig. 4A). Detectable transcripts for mGluR2, mGluR3, mGluR6, mGluR7, or mGluR8 were not evident, even at high PCR cycle numbers (>35 cycles). These data were confirmed by in situ hybridization, with both BMVEC and HMVEC staining positively for mGluR1, mGluR4, and mGluR5 (Fig. 4, lower panels A–C, respectively). BMVEC and HMVEC hybridized with a control probe (porcine mannose binding lectin) revealed no detectable binding and, as such, served as a negative control (Fig. 4D). To assess the relative levels of mGluRs in BMVEC and HMVEC, we employed semi-quantitative RT-PCR of mGluR1, mGluR4, and mGluR5 relative to β-actin. As shown in Fig. 4, E and F, this relative comparison revealed that although both BMVEC and HMVEC express mGluR1, -4, and -5, HMVEC express relatively more mGluR1 and -5 compared with BMVEC and each expresses approximately equal amounts of mGluR4 (see Fig. 4F).

Having demonstrated that human endothelia express mGluR mRNA, mGluR cell surface protein expression was investigated by immunofluorescence and by immunoprecipitation of biotinylated surface protein. Human endothelial mGluR expression was also confirmed by immunofluorescence. Consistent with our in situ hybridization results, non-permeabilized BMVEC and HMVEC stained positively for mGluR1, mGluR4, and mGluR5 (Fig. 4, panels A, C, and E, respectively), in which a surface distribution of mGluR was evident with dominant staining at the edge of the plasma membrane. BMVEC and HMVEC incubated with an irrelevant, isotype control rabbit anti-rat mannose binding lectin polyclonal Ab (Fig. 5, panel G) or FITC-conjugated goat anti-rabbit IgG only (Fig. 5, panel F) revealed no demonstrable staining and, thus, served as a negative control. In each case, rhodamine phalloidin staining was used to generally localize cells (Fig. 5, B, D, F, H, and J).

As shown in Fig. 5K, streptavadin blot analysis of biotinylated human BMVEC immunoprecipitates under reduced conditions revealed 140-, 102-, and 140-kDa bands consistent with the known molecular mass of human mGluR1, mGluR4, and
mGluR5, respectively (22, 23). Taken together, these data confirm that human endothelia express group I and III mGluR mRNA and protein.

**Glutamate-elicted Changes in Endothelial VASP Phosphorylation**—We next attempted to gain insight into the mechanism(s) of glutamate-regulated barrier function. It was recently shown that the Ena-VASP homology 1 (EVH1) domain of the Homer protein family (Vesl-1s, Vesl-1L, and Vesl-2) interacts with mGluR1 and mGluR5 via the specific mGluR peptide sequence (TPPSPF) found in the N terminus (24). VASP is an

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**Figure 2. Identification of PMN-derived glutamate and glutamine.** Panel A, HPLC purified fraction 3 was analyzed by liquid chromatography in tandem with a mass spectrometer as described under “Materials and Methods.” Shown here are representative mass spectral tracings in the positive and negative ion mode. Panel B, representative HPLC chromatogram (UV absorbance (Abs.) 460 nm) of dabsylated fraction 3 (dashed line) and co-elution with dabsylated glutamate but not dabsylated glutamine (solid line). Also shown is free dabsyl chloride. Panel C, PMNs (10⁷/ml) suspended in HBSS were activated with fMLF (10⁻⁸ m). PMN suspensions were sampled at various time points during 1 h of activation and dabsylated, and glutamate concentrations were determined using HPLC. Data are from 3 donors and are expressed as the mean concentration ± S.E.
**mGluR and Endothelial Permeability**

Fig. 3. Glutamate alters endothelial permeability in vitro.

Panel A, HMVEC, BMVEC, or intestinal epithelial (T84) monolayers grown on permeable supports were exposed to glutamate (0.1–100 \( \mu M \)) and examined for FITC-dextran paracellular permeability. Data are normalized to vehicle controls within groups and represent means ± S.E. from three separate experiments (the double asterisk indicates significantly increased compared with control (\( **, p < 0.005 \)), whereas the single asterisk indicates significantly decreased compared with control (\( *, p < 0.025 \)). Panel B, human BMVEC were exposed to selective, group I (DHPG) or III (L-AP4) mGluR agonists (1 \( \mu M \)) or selective, group I (PHCCC) or III (CPPG) mGluR antagonists (1 \( \mu M \)) in the presence of glutamate (1 \( \mu M \)) and assessed for paracellular permeability (basal permeability, shown with horizontal dotted line). Data are derived from nine monolayers in each condition. Results are expressed as means ± S.E. (the double asterisk indicates significantly increased compared with base-line control (\( **, p < 0.05 \)), whereas the single asterisk indicates significantly decreased compared with glutamate alone (\( *, p < 0.025 \)).

EVH1 domain-containing protein that serves as a negative regulator of actin dynamics (25), and we have recently shown that VASP phosphorylation is critical to cyclic nucleotide-induced promotion of endothelial barrier function (26). In addition, VASP has been implicated in the regulation of BMVEC cell-cell contacts and BBB permeability through inhibition of cell retraction (27). Therefore, using glutamate concentrations that most influence HMVEC (10 \( \mu M \)) and BMVEC (0.1 \( \mu M \)) barrier function (see Fig. 3A), we investigated the influence of glutamate on VASP phosphorylation in vitro. As shown in Fig. 6A, Western blot analysis of HMVEC lysates after exposure to glutamate (10 \( \mu M \)), a concentration that promotes HMVEC barrier (see Fig. 3A), revealed dominant VASP phosphorylation (phosphorylation of serine at position 157 leads to a marked shift in apparent molecular mass of VASP by SDS-PAGE from 46 to 50 kDa) (28). Similarly, exposure of BMVEC to glutamate (0.1 \( \mu M \)), a concentration that increases endothelial permeability (see Fig. 3A), resulted in a decreased basal VASP phosphorylation.

As an extension of these findings, we determined whether pharmacologic inhibition of glutamate-regulated VASP phosphorylation manifest as changes in permeability. Phosphorylation of VASP in the serine 157 position is predominantly dependent on protein kinase A (29), and therefore, as shown in Fig. 6B, the protein kinase A inhibitor H89 (10 \( \mu M \)) inhibited glutamate-induced VASP phosphorylation and significantly dampened the increase in barrier associated with glutamate exposure (\( p < 0.01 \) by analysis of variance). Dephosphorylation of VASP is predominantly via protein phosphatase 2A (30), and therefore, as shown in Fig. 6C, okadaic acid (10 \( \mu M \)) was used to inhibit basal dephosphorylation in BMVEC (Fig. 6A) and assessed for permeability changes. As can be seen, these conditions resulted in a loss of glutamate-regulated barrier function in BMVEC (\( p < 0.05 \) by analysis of variance). Taken together, such data indicate that glutamate-regulated VASP phosphorylation parallels changes associated with endothelial permeability.

**mGluR Blockade Attenuates BBB Permeability after in Vivo Hypoxia**—To further confirm these in vitro findings, the influence of mGluR therapy on BBB permeability was investigated in an in vivo hypoxic mouse model (17). As shown in Fig. 7A, a comparison of mice exposed to normoxia (21% O\(_2\), 4 h) and hypoxia (8% O\(_2\), 4 h) revealed a 9 ± 0.4-fold increase in BBB permeability in hypoxia and paralleled the accumulation of PMNs within brain tissue (measured as myeloperoxidase activity, mean 5.5 ± 0.6-fold increase in mice exposed to hypoxia). To examine the potential role of glutamate under these circumstances, mice were subjected to hypoxia and co-administered mGluR-selective antagonists or agonists. Pretreatment of mice with a selective, mGluR group I (PHCCC, 2 mg/kg) or III (CPPG, 2 mg/kg) receptor antagonist significantly decreased measured brain FITC-dextran after 4 h of hypoxia compared with untreated mice (\( p < 0.01 \)). Interestingly, pretreatment (2 mg/kg) of mice with a selective, group I (DHPG) or III (L-AP4) mGluR receptor agonist resulted in a significant increase in brain-associated FITC-dextran after 4 h of hypoxia or normoxia compared with untreated mice. These data suggest that PMNs accumulate within brain tissue during hypoxia and mGluRs may mediate hypoxia-induced increases in BBB permeability.
Furthermore, these data suggest that mGluR therapy may represent a novel, therapeutic strategy for regulation of BBB function.

**DISCUSSION**

PMN-derived mediators released during inflammation have the potential to alter endothelial barrier function. However, the nature of these mediators and their mechanism(s) of action have only recently been studied. We demonstrate here that fMLF-stimulated PMNs release glutamate and that such extracellular glutamate alters human microvascular endothelial permeability in vitro. Additionally, we present the previously unappreciated finding that human endothelia express functional mGluR1, mGluR4, and mGluR5. Treatment of human brain endothelia with glutamate or selective, group I or III mGluR agonists decreases endothelial VASP phosphorylation and increases transendothelial flux. Our in vitro observations were extended to an in vivo hypoxic mouse model in which FITC-dextran flux across the BBB was significantly decreased by pretreatment with selective, group I or III mGluR antagonists. Together, these data suggest a novel role for glutamate and mGluRs in regulating human endothelial VASP and barrier function.

PMN transmigration across endothelial surfaces occurs as both physiologic (e.g. PMN movement from the bone marrow) and pathophysiologic (e.g. PMN recruitment to during inflammation) events, and for this reason, endogenous pathways likely exist to regulate the barrier properties of the vasculature. Biophysical and biochemical characterization of one such bioactivity identified PMN-derived glutamate. Extracellular glutamate is best known as the predominant excitatory neurotransmitter in the vertebrate nervous system, providing a multitude of roles in neuronal function (5). Consistent with these findings, others have implicated PMN in the generation of glutamate. For instance, in a whole cell metabolic assay, Curi et al. (32) demonstrate that rat PMNs can use glutamine to generate large amounts of intracellular glutamate and extracellular glutamate can inhibit glutamine utilization by PMNs (31, 32). Although we do not presently know the mechanism(s) of glutamate release from PMNs, it is likely to occur through well characterized pathways such as glutamate transporter reversal (33). Consistent with previous reports (2, 34), our present studies also identified the release and bioactivity of 5′-AMP, a compound that promotes endothelial barrier and functions to reseal the vasculature during transmigration (2, 29, 30, 31). As such, it is possible that 5′-AMP and glutamate cooperatively regulate endothelial barrier function. For exam-
An unexpected aspect of this work revealed differential glutamate responses between BMVEC and HMVEC. Indeed, although both human BMVEC and HMVEC express similar mGluR subtypes, extracellular exposure to equimolar glutamate/selective mGluR agonist concentrations resulted in differential responses (i.e., HMVEC and BAEC responded with significantly decreased permeability, although BMVEC responded with increased permeability). At present, we do not know the underlying mechanism(s) of this differential response. Based on the findings that glutamate phosphorylates VASP in HMVEC and dephosphorylates VASP in BMVEC, it is possible that mGluR G-protein receptor coupling is different, with the likely possibility that the relative difference in expression of mGluR1 in HMVEC over BMVEC (see Fig. 4F) may more dominantly couple to G_{i} (i.e., elevation in intracellular cAMP) in response to glutamate and in BMVEC may be relative more G_{q} coupled (decrease in intracellular cAMP). It is equally possible that post-receptor events could explain these differences. A rapidly evolving idea suggests that endothelial cells from different anatomic locales may be phenotypically distinct (51) and that cerebral endothelial cells may be quite different.

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**FIG. 5. Human endothelia express mGluRs.** Human BMVEC mGluR expression was determined by immunofluorescence. Non-permeabilized BMVEC and HMVEC stained positively for mGluR1, mGluR4, and mGluR5 (Panels A, C, and E, respectively). No fluorescence was observed in control cells also incubated with an irrelevant, isotype control rabbit anti-rat mannose binding lectin polyclonal antibody (panel G) or FITC-conjugated goat anti-rabbit IgG only (panel I). Phalloidin cytoskeletal staining is shown in panels B, D, F, H, and J. This figure is representative of three experiments. Panel K represents an avidin blot after immunoprecipitation of surface-biotinylated BMVEC using antibodies directed against mGluR1, 4, or 5, as indicated. A control (Ctl) immunoprecipitation using anti-cAMP response element-binding protein is also shown. This figure is representative of two experiments. Std, standards.

ple, Boeck et al. (35) recently reported that extracellular glutamate activates hydrolysis of ADP and AMP and such activity is attributable to regulated expression and activity of ectonucleotidase on cerebellar granule cells (35). Although this influence was predominantly mediated by ionotropic and not metabotropic glutamate receptors, this example nonetheless provides for the possibility that these two mediators may act cooperatively.

Although previously described on rat cerebral and cardiac endothelia (36, 37), we demonstrate for the first time that diverse human endothelia express functional mGluRs. Specifically, RT-PCR and in situ hybridization demonstrated that both human brain and dermal microvascular endothelia express group I (mGluR1 and mGluR5) and group III (mGluR4) mGluR mRNA, with relative expression levels for HMVEC mGluR5 ≈ mGluR1 > mGluR4, and for BMVEC, mGluR5 ≈ mGluR4 > mGluR1. Human endothelial mGluR1, mGluR4, and mGluR5 cell surface protein expression was confirmed by immunofluorescence and immunoprecipitation of surface labeled cells. At present, the exact mechanism(s) by which glutamate regulates vascular permeability is unknown. Gluta-

mate is known to activate a variety of signaling pathways, including calmodulin kinase II (38), protein kinase C (39), nitric-oxide synthetase (40), nuclear factor xB (41), p38 (42), c-Jun N-terminal kinase (43), and extracellular signal-regulated kinases 1 and 2 (44). Additionally, evidence is accumulating that glutamate-induced alterations in cytoskeletal dynamics are mediated by mGluRs. For example, group I mGluRs (receptors 1 and 5) are coupled to phospholipase C and stimulate polyphosphoinositide hydrolysis via coupling to G_{q/11} (5) and have been shown to phosphorylate the focal tyrosine kinase in a protein kinase C-dependent manner (45). Group III mGluRs (receptors 4, 6, 7, and 8) are negatively coupled via G_{i/o}, to adenylate cyclase and decrease cAMP formation (5). Our observation that treatment with the group III mGluR agonist, t-AP4, increased human brain endothelial permeability is consistent with previous reports demonstrating an inverse correlation between endothelial barrier function and intracellular cAMP levels (46, 47). Stimulation of mGluRs has also been linked to glutamate-induced activation of Ca^{2+}/calmodulin-dependent protein kinase II, which in turn regulates phosphorylation of the intermediate filament protein, vimentin (38). This is an unlikely possibility, since previous studies with human endothelial cells suggest that glutamate does not directly elevate intracellular Ca^{2+} (48). In the present study, the influence of glutamate and selective, group I (DHPG) or III (t-AP4) mGluR agonists on VASP phosphorylation was elucidated. VASP phosphorylation acts as a negative regulator of actin dynamics (25) and has been suggested to regulate BMVEC cell-cell contacts and BBB permeability by inhibiting cell retraction (27). Treatment of human BMVEC with glutamate, DHPG, or t-AP4 significantly attenuated VASP phosphorylation, as demonstrated by a VASP mobility shift from 50 to 46 kDa by Western blot (49). VASP is composed of a central proline-rich domain, and highly homologous N- and C-terminal domains termed EVH domains 1 and 2 (EVH1 and EVH2), respectively (50). Although a direct interaction between VASP and mGluRs has not yet been reported, the EVH1 domain of the Homer protein family (Vesl-1s, Vesl-1L, and Vesl-2) is known to interact with mGluR1 and mGluR5 via binding of the N-terminal Homer EVH1 domain to a specific mGluR peptide sequence (TPPSPF) (24). Although further studies are necessary to determine whether the EVH1 domain of VASP directly interacts with mGluRs, these data do nonetheless indicate that mGluRs may influence endothelial barrier function via a VASP-dependent mechanism.
distinct from most peripheral vascular endothelia (52). It is possible, for example, that microvascular endothelial cells express distinct isoforms of adenylyl cyclase compared with macrovascular endothelial cells and that such differential expression of adenylyl cyclase may explain agonist selectivity for endothelial permeability in a regionally specific manner (46).
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In addition to our observation that PMN-derived glutamate may alter endothelial barrier function, it should be noted that neuronal release of glutamate into the extracellular space after CNS hypoxia plays a key role in mediating ischemic brain damage by causing direct neurotoxicity (53) and increasing BBB permeability (54). We thus investigated the influence of mGluR therapy in regulating BBB permeability in an in vivo hypoxic mouse model. Initial studies indicated a relationship between PMN accumulation and increases in BBB permeability, providing at least the possibility that PMN-derived glutamate might be a pathophysiologically relevant mediator in vivo. Extensions of these experiments indicated that pretreatment of mice with a selective, group I (PHCC) or III (CPPG) mGluR receptor antagonist significantly decreased BBB permeability after 4 h of hypoxia compared with untreated mice. In contrast, mice pre-exposed to selective, group I (DHGP) or III (t-AP4) mGluR receptor agonist resulted in a small but significant increase in BBB permeability after 4 h of hypoxia or normoxia compared with untreated mice. These data suggest that glutamate-induced increases in BBB permeability after hypoxia are mediated at least in part by group I and III mGluRs.

Taken together, these results suggest a previously unappreciated pathway by which glutamate derived from activated PMNs may regulate human vascular endothelial barrier function via its action on endothelial expressed mGluRs. Globally, such findings suggest that mGluR therapy may thus represent a novel therapeutic strategy for regulation of microvascular permeability in diverse tissues.

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