Potential role of MCP-1 in endothelial cell tight junction 'opening': signaling via Rho and Rho kinase

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Accepted 8 July 2003
Journal of Cell Science 116, 4615-4628 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00755

Summary

The expression of the monocyte chemoattractant protein-1 (MCP-1) receptor CCR2 by brain endothelial cells suggests that MCP-1 may have other functions than purely driving leukocyte migration into brain parenchyma during inflammation. This study examines one of these potential novel roles of MCP-1 regulation of endothelial permeability using primary cultures of mouse brain endothelial cells. MCP-1 induces reorganization of actin cytoskeleton (stress fiber formation) and redistribution of tight junction proteins, ZO-1, ZO-2 occludin and claudin-5, from the Triton X-100-soluble to the Triton X-100-insoluble fractions. These morphological changes are associated with a decrease in transendothelial electrical membrane resistance and an increase in [^{14}C]inulin permeability. MCP-1 did not induce these events in brain endothelial cells prepared from mice genotype *CCR2*$^{-/-}$. The Rho kinase inhibitor Y27632 and inhibition of Rho (C3 exoenzyme, and dominant negative mutant of Rho, RhoT19N) prevented MCP-1-induced stress fiber assembly, reorganization of tight junction proteins and alterations in endothelial permeability. In all, this suggests that a small GTPase Rho and Rho kinase have a pivotal role in MCP-1-induced junction disarrangement. These data are the first to strongly suggest that MCP-1, via CCR2 present on brain endothelial cells, contributes to increased brain endothelial permeability.

Key words: MCP-1, Tight junction, RhoA, Rho kinase, Brain endothelial permeability

Introduction

The blood-brain barrier (BBB) is formed by the cerebral endothelial cells and their linking tight junctions (TJs). At the functional level, these junctional complexes provide high transendothelial electrical resistance, typically 1500-2000 $\Omega.cm^2$, that help regulate the entry of blood-borne molecules into brain, and help preserve ionic homeostasis within the brain microenvironment (Wolburg and Risau, 1995; Rubin and Staddon, 1999; Huber et al., 2001a).

Tight junctions in the BBB are composed of an intricate combination of transmembrane integral proteins including claudins 1, 5 and 11, occludin and JAMs, and several cytoplasmic accessory proteins such as zonula occludens proteins, ZO-1, ZO-2, ZO-3, AF6, cingulin, 7H6 and atypical protein kinase C (Citi and Cordenonsi, 1998; Huber et al., 2001a). While transmembrane proteins, particularly claudins, are involved in forming the seal between adjacent cells, the accessory proteins are multidomain cytoplasmic molecules necessary for the formation of structural support for the TJ and are also involved in signal transduction (Citi and Cordenonsi, 1998; Denker and Nigam, 1998; Martin-Padura et al., 1998; Mitic and Anderson, 1998).

A variety of central nervous system (CNS) conditions alter BBB permeability in conditions such as stroke, brain tumors, multiple sclerosis, traumatic brain injury and epilepsy. At the cellular level, these alterations in endothelial cell permeability are manifested by intracellular gap formation and reorganization of both actin microfilament bundles (manifested as stress fiber formation) and endothelial junctional proteins (Lum and Malik, 1994; Garcia and Schaphorst, 1995; Tsukamoto and Nigam, 1997; Tsukamoto and Nigam, 1999; Farshori and Kachar, 1999). Some of the signal molecules involved in inducing such changes are: (i) myosin light chain kinase (MLCK) activated by Ca^{2+}/calmodulin and Rho/Rho kinase-dependent pathways; (ii) kinases such as protein kinase C (PKC, PKCζ and PKCλ) tyrosine kinase and serine kinase that phosphorylate occludin and ZO-1 proteins; and (iii) matrix metalloproteinases MMP-2 and MMP-9, which cleave occludin (Sakakibara et al., 1997; Fujimura et al., 1999; Gloor et al., 2001; van Hinsbergh and van Nieuw Amerongen, 2002).

A variety of the factors are postulated to be involved in altering BBB permeability depending on the type of BBB pathology; for example, downregulation of energy metabolism, mobilization of intracellular Ca^{2+}, generation of reactive oxygen species (ROS), vascular endothelial growth factor (VEGF) and activation of MMPs (Rosenberg et al., 1996; Rosenberg et al., 1998; Vouret-Craviari et al., 1998; Lum and Roebuck, 2001; Wang et al., 2001). During an inflammatory response, proinflammatory mediators also significantly contribute to BBB breakdown (Wojciak-Stothard et al., 1998; Abbott, 2000). However, the increase in microvascular permeability is also closely correlated with leukocyte extravasation and some of the regulators of leukocyte trafficking, such as ICAM-1, may also regulate permeability

(Etienne et al., 1998). These findings suggest that there may be new potential regulators of BBB permeability, among which are chemokines, molecules that are important regulators of the transmigration of leukocytes across the BBB.

Chemoattractant cytokines, known as chemokines, are a superfamily of structurally related pro-inflammatory peptides (~70-90 amino acids) that mediate cell-specific, directed migration of leukocytes into tissues at sites of inflammation. Biochemically, they are divided into four subfamilies (C, CC, C-X-C and C-XXX-C) that also reflect functional differences (Murphy, 1994; Rollins, 1997; Yoshie et al., 1997). All chemokines mediate their effects by binding to seven-transmembrane G protein-coupled receptors. Most chemokines and chemokine receptors are selectively expressed on different leukocyte subsets but are also found in a number of non-hematopoietic cells including endothelial cells. In the CNS, chemokines are expressed by glial, neuronal and endothelial cells (Ransohoff et al., 1993; Horuk, 1997; Andjelkovic et al., 1999a; Andjelkovic et al., 1999b; Andjelkovic and Pachter, 2000; Boddeke, 1999; Mennicken et al., 1999).

During CNS inflammation one of the most commonly expressed chemokines is monocyte chemoattractant protein-1 (MCP-1, CCL2), a member of the CC subfamily. There is strong evidence that MCP-1 is involved in the recruitment of both monocytes/macrophages and activated lymphocytes into the CNS (Hulkower et al., 1993; Glabinski et al., 1996; Lahrtz et al., 1998; Miller and Meucci, 1999). However, a growing body of evidence implicates MCP-1 and its receptor CCR2 in a variety of functions beyond its 'conventional role' as a host defense protein. MCP-1 is an angiogenic factor and is involved in development of the CNS by affecting glial cell proliferation and migration (Salcedo et al., 2000; Andjelkovic et al., 2002; Banisadr, 2002; Rezaie et al., 2002). Also, intracerebral injection of MCP-1 has been shown to alter the BBB permeability during monocyte infiltration into brain (Bell et al., 1996**)**. The current study focuses on how MCP-1, via its receptor CCR2 on brain endothelial cells, may alter permeability, as well as elucidating the potential signal molecules involved in that process.

Materials and Methods

Mouse brain microvascular endothelial cells (mBMEC)

Four- to 6-week-old CD-1 and C57BL/6 × 129Sv mice, genotype *CCR2*$^{+/+}$ and *CCR2*$^{-/-}$, were used for mBMEC preparation. Briefly, the brain was minced in Hanks balanced salt solution (HBSS, Invitrogen Corp, CA) and homogenized gently in a Dounce type homogenizer. The microvessels were then cleaned from myelin using a 18% dextran solution (Dextran, USB, OH), and separated from erythrocytes using a Percoll (Pharmacia, NJ) gradient. The microvessels were digested in HBSS solution containing 1 mg/ml collagenase/dispase (Roche, IN) for 40 minutes at 37°C. Primary cultures of mBMEC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated fetal calf serum, 20 mM Hepes, 2 mM glutamine, antibiotic/antimycotic (all purchased from Invitrogen Corp., CA), 2.5 μg/ml heparin (Sigma, MO), endothelial cell growth supplement (BD Bioscience, NJ) and grown in six-well plates coated with collagen type IV (BD Bioscience, NJ).

RT-PCR

Mouse brain endothelial cells were plated in 35 mm culture dish and grown to 95% confluence. Total RNA was prepared from the culture (2×10^5 cells) using the RNAgents, Total RNA Isolation System (Promega, WI) according to the manufacturer's instructions. Aliquots (1 μg) of RNA were reverse transcribed (RT) with the Gibco-BRL cDNA synthesis kit (Invitrogen Corp., CA). A mix of equal amounts of mouse primers for CCR1, 2, 3, 4, 5 and GADPH (Biosource International Inc., CA) were used to prime PCR. A total of 28 cycles for CCR2 were applied. The PCR cycles involved 1 minute denaturation at 94°C, 4 minutes annealing at 55°C and 3 minutes extension at 72°C, except for the first cycle in which there was 2 minutes denaturation and the last cycle in which there was 10 minutes elongation. The PCR products were resolved using electrophoresis in a 2% agarose gel in 1× TBE buffer (Tris-HCl/EDTA/boric acid, pH 8). The gel was stained with ethidium bromide and photographed.

Chemotaxis assay

This assay was performed in a 96-well microchemotaxis chamber Neuroprobe filter apparatus (Neuroprobe, MA). Briefly, serum-free medium for BMEC with different concentrations of MCP-1 (0.1-1000 nM) was placed in the lower well of the 96-well microchemotaxis chamber. Suspension of mBMEC (10^4 cells), previously left in serum free medium for 6 hours, was added to the upper filter surface, having a pore size of 5 μm. After 3 hours, the filter was stained with Leukostat (Fisher Scientific, PA), and the number of endothelial cells adherent to the filter counted. The results were expressed as the mean number of counted or migrated cells per 10 fields at 10× magnification. In a separate set of experiments, MCP-1 (100 nM) was added to the suspension of mBMEC just prior to adding the suspension to the upper filter surface, thereby inhibiting the creation of a chemotactic gradient. To examine the effect of inhibiting MCP-1, a polyclonal neutralizing anti-MCP-1 antibody (1 μg/ml; R&D System, MN) was added to the lower chamber together with MCP-1. Samples were taken in triplicate. All experiments were performed 5 times.

Cell treatment and transfection

To test the effect of MCP-1 on brain endothelial permeability, confluent monolayers of BMEC were treated with 100 nM mouse recombinant MCP-1 (PreproTech, NJ) for 15 minutes and 2 hours. To determine expression of CCR2 receptors, mBMEC were also treated with proinflammatory cytokine IL-1β (PreproTech, NJ) for 6, 12 and 24 hours at a concentration of 10 ng/ml. To block the activity of Rho kinase, PKC, mitogen-activated protein (MAP) kinase ERK1/2, phosphatidylinositol 3-kinase (PI 3-kinase), calmodulin and protein lipase C (PLC), confluent monolayers of mBMEC were pretreated with the inhibitors Y27632 (10 μM), Ro-37840 (1 μM), SB203580 (10 μM), PD95209 (30 μM), LY29402 (20 μM), W7 hydrochloride (5 μM) and U7322 (50 μM) (all purchased from Calbiochem, CA), respectively, for 30 minutes at 37°C and then treated with recombinant murine MCP-1 in the presence of inhibitors for 15 minutes or 2 hours.

To specifically inhibit the activity of RhoA proteins, fusion protein *Clostridium botulinum* C3 exoenzyme and dominant negative mutant T19NRho were introduced to mBMEC. Briefly, confluent cultures of mBMEC were pretreated with 5 μg/ml C3 exoenzyme for 18 hours or transiently transfected with plasmid 1 μg pCMVRhoT19N (Upstate Biotechnology, NY) in Opti-MEM serum-deprived medium supplemented with Lipofectin 10 μg/ml (Invitrogen Corp., NY), followed by washing with serum-free DMEM. Transfection efficiency was around 40% as evaluated by western blot analysis. The cells were then exposed to 100 nM MCP-1 for 15 minutes and 2 hours and processed for immunofluorescence or western blot analysis.

cDNA array

Mouse brain endothelial cells were plated in 60 mm culture dishes and grown to 95% confluence. Total RNA was prepared from the culture (1×10^6 cells) using Trizol reagents (Invitrogen Corp., CA)

according to the manufacturer's instructions. Aliquots (5 μg) of RNA were reverse transcribed (RT) with the MMLV reverse transciptase (Promega, WI). The array used in this study was G-protein pathway finder microarray from SuperArray Inc., MD. Preparation of biotin-labeled cDNA and hybridization were performed as outlined by the manufacturer. For visualization, a chemiluminescent alkaline phosphatase substrate was used. Different patterns of gene expression were analyzed by scanning densitometry using a software package provided by SuperArray, Inc.

Immunofluorescence

mBMEC were fixed in 4% paraformaldehyde for 20 minutes at 20°C then preincubated with blocking solution of 5% normal goat serum, 0.05% Tween and phosphate-buffered saline (PBS). Cells were then incubated overnight in primary antibody [mouse anti-occludin, anti-ZO-1, anti-claudin-5 (Zymed Laboratories Inc., CA), mouse anti-ZO-2 (BD Bioscience, KY) rabbit anti-CCR2 antibody (Santa Cruz CA)] at 4°C. Reactions were visualized by fluorescein-conjugated anti-mouse or anti-rabbit antibody (Vector Lab, CA). Actin filament rearrangement was monitored using Phalloidin staining which specifically labels F-actin. mBMEC monolayers were fixed in 4% paraformaldehyde for 20 minutes at room temperature then washed 3 times with PBS (pH 7.4). The endothelial cells were then permeabilized in 0.1% Triton X-100/PBS and incubated with 0.1 μg/ml of Alexa 488-conjugated Phalloidin (Molecular Probes, OR) for 1 hour. All samples were viewed on the confocal laser scanning microscope LSM 510 Zeiss, objective 40× 1.3 NA.

Triton X-100 extraction of TJ proteins

mBMEC were subjected to an extraction protocol modified from that of Fey and colleges (Fey et al., 1984). Briefly confluent monolayers of mBMEC were overlaid with extraction buffer [0.5% Triton X-100, 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 300 mM sucrose, plus proteinase inhibitor mixture containing phenylmethylsulfonyl floride, iodoacetamide, benzamidine (each 1 μM), aprotinin, leupeptin, pepstatin A and antipain (each 20 μg/ml, Roche, IN)] for 20 minutes at 4°C on a gently rocking platform. The soluble supernatant was collected and this fraction was defined as the Triton X-100-soluble fraction. The residue of cells with well preserved nuclei and cytoskeleton fibers adherent to the culture vessels was gently washed twice with Tris-buffered saline (TBS) with the protease inhibitors and then lysed with the radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, 140 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM phenylmethylsulfonyl floride and 1 μg/ml aprotinin; Sigma, MO). The extract was collected and this fraction was defined as the Triton X-100-insoluble fraction.

Western blotting

Protein concentrations in the resulting extraction and residual fraction were calculated using a Pierce protein assay kit (Pierce, IL). Equal amounts of protein samples were loaded and separated using 7.5% and 15% SDS-polyacrylamide gel electrophoresis, and then transferred to Trans-Blot nitrocellulose membrane (BioRad, CA). Immunoblotting was performed with mouse anti-occludin, anti-ZO-1, anti-claudin-5, anti-ZO-2 antibody, 1:200 dilution, rabbit anti-Rho antibody, 1:500 dilution: (Upstate Biotechnology, NY) and rabbit anti-CCR2-antibody 1:200 dilution. Immunoblots were then exposed to secondary anti-mouse or anti-rabbit HRP-conjugated antibody (BioRad, CA) and visualized using a chemiluminescent HRP substrate kit (Pierce, IL). The relative densities/volumes of the bands on the film negatives were measured using the 1.61 NIH image software package.

Rho activation assay

The affinity precipitation of lysed mBMEC with agarose-bound recombinant Rhotekin protein (Upstate Biotechnology, NY) and western blotting analysis were performed to determine activation of Rho proteins after treatment of mBMEC with MCP-1. Briefly, confluent mBMEC or cells pretreated with Y27632, C3 exoenzyme, or transiently transfected with T19NRho were incubated with recombinant mouse MCP-1 for 10, 20, 30, 60 or 120 minutes and lysed. The affinity precipitation was performed according to the manufacturer's instructions (Upstate Biotechnology, NY). After agarose bead removal, samples were resuspended in buffer and processed for western blot using a rabbit polyclonal anti-Rho antibody.

Transendothelial electrical resistance (TEER)

Electrical resistance across endothelial cell monolayers was measured by Millicell ERS (World Precision Instruments, FL). In these sets of experiments, mBMEC were plated in Transwell culture dishes, 0.4 μm pore size (Corning Inc., NY). MCP-1 (100 nM) was placed in the lower and upper compartment of the Transwell dual chamber system. TEER was measured for between 15 minutes and 2 hours. The resistance of blank filters was subtracted for calculation of final TEER values ($\Omega.cm^2$). All experiments were carried out in triplicate. The results are expressed as means ± s.e.m. of 5 independent experiments.

mBMEC monolayer permeability

The effects of MCP-1 on the permeability of endothelial monolayers cultured on Transwell, 0.4 μm pore size filters (Corning Inc., NY) was examined using [^{14}C]inulin (New England Nuclear, MA), a tracer that crosses the endothelium by passive diffusion (Kazakoff et al., 1995). The permeability experiments were initiated by the addition of 0.2 μCi of the isotope to the apical or donor chamber which contained 0.4 ml of DMEM (Invitrogen Corp., CA). The basal or receiving chamber contained 1.2 ml of DMEM. 0.2 ml of medium from the basal chamber was sampled and replaced with fresh DMEM at 15-minute intervals from 0 to 120 minutes. Scintillation fluid was added to the samples and radioactivity counted using a Beckman 3801 liquid scintillation counter (Fullerton, CA). The permeability (P; cm/minute) of the monolayer during any time interval (T) was calculated using the following equation:

$$P = \frac{[C(B)_T - C(B)] \times V(B) \times 2}{[C(A)_T + C(A)_{T+15}] \times A \times T},$$

where C(B) and $C(B)_T$ are, respectively, the concentrations of isotope in the basal chamber at the start and at the end of the time interval (in dpm/ml), and V(B) is the volume of the basal chamber (in ml). C(A) and $C(A)_T$ are, respectively, the concentrations of isotope in the apical or donor chamber at the start and at the end of the time interval (in dpm/ml) and $[C(A)_T + C(A)_{T+15}]/2$ is the average concentration over the time interval. T is the duration of the time interval in minutes, while A is the area of the filter (cm^2). The results are expressed as means±s.e.m. of 3 independent experiments.

Statistics

All results are expressed as means±s.e.m. One-way analyses of variance were used to compare the mean responses among the experimental groups. Dunnett's test was used to determine significance between groups.

Results

Expression of functional active CCR2 receptors by mouse brain endothelial cells

Peripheral endothelial cells constitutively express CCR2

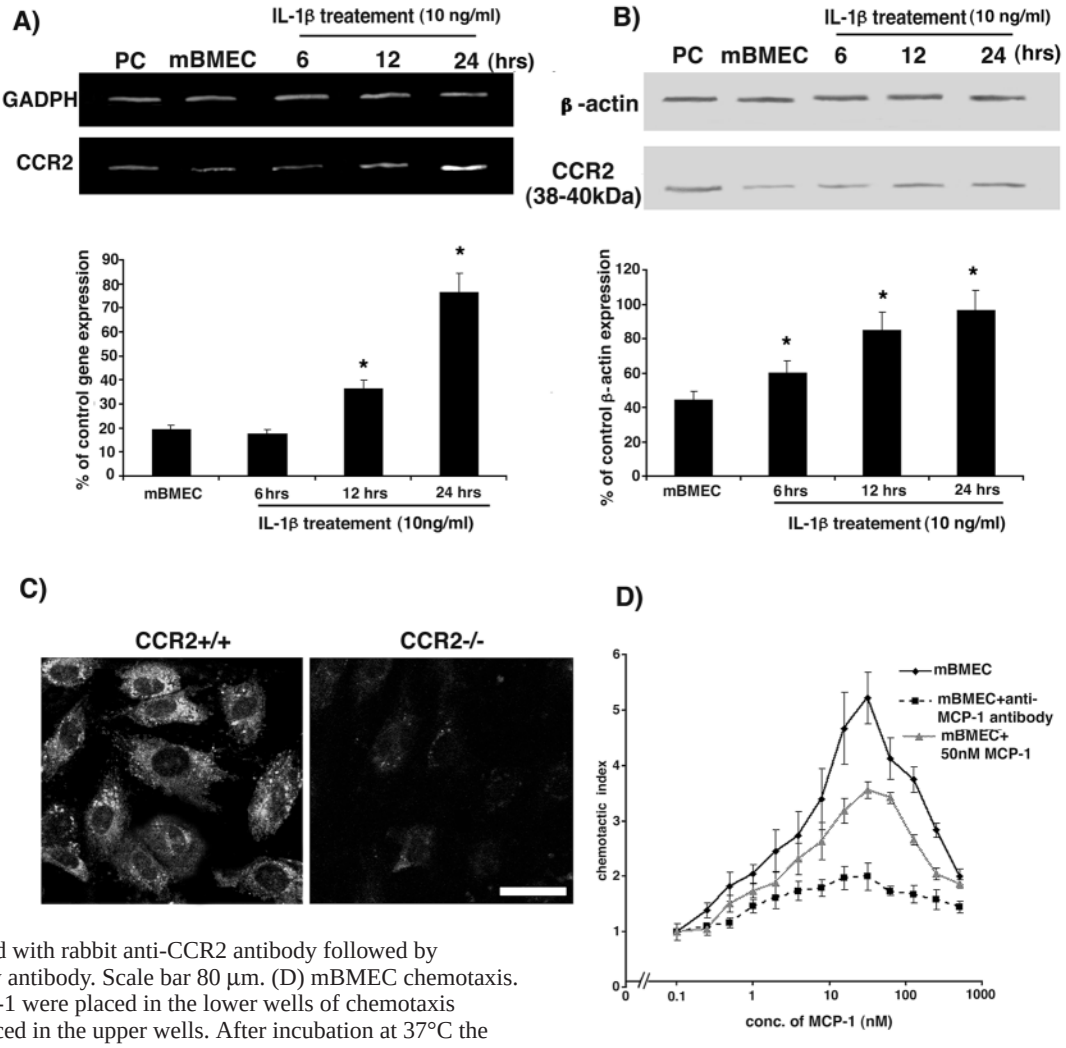

Fig. 1. mBMEC express the CCR2 receptor. (A) Expression of CCR2 mRNA in quiescent and IL-1β-treated cells examined by RT-PCR. GADPH, glyceraldehyde phosphate dehydrogenase; PC, positive control: a mixture of cDNA provided by the supplier of the chemokine receptors and GADPH primers. Volumes of CCR2 bands were expressed as percentage of control gene (GADPH) bands amplified in same PCR reactions. Each bar represents the mean±s.e.m. of three independent experiments. Asterisks indicated significant differences (P<0.001) from corresponding control levels. (B) Western blot analysis of CCR2 receptors. Cells were either quiescent mBMEC in first passage or were treated with 10 ng/ml IL-1β for 6, 12 and 24 hours. PC, positive control: lysate of murine peritoneal macrophages. Results are presented as means±s.e.m. of three independent experiments; * P<0.01. (C) $CCR2^{+/+}$ or $CCR2^{-/-}$ mBMEC were incubated with rabbit anti-CCR2 antibody followed by fluorescein-conjugated secondary antibody. Scale bar 80 μm. (D) mBMEC chemotaxis. Different concentrations of MCP-1 were placed in the lower wells of chemotaxis chambers and mBMEC were placed in the upper wells. After incubation at 37°C the cells on the Neuroprobes filter were counted. Chemotaxis index represents the ratio of migrating endothelial cells in the presence of MCP-1 and in the absence of MCP-1 (control medium). In a separate set of experiments, MCP-1 was also added to a suspension of mBMEC or anti-MCP-1 antibody (1 μg/ml) was added to the lower chamber.

mRNA and protein and they respond chemotactically to an MCP-1 gradient (Boring et al., 1998; Weber et al., 1999; Salcedo et al., 2000). A few recent studies also indicate that isolated brain microvessels possess a high affinity binding site for MCP-1, for example, K_d <2 nM. Furthermore, exposure of mouse brain microvessels to high concentrations of MCP-1 transiently downregulates the binding site for MCP-1 on the surface of endothelial cells (Andjelkovic et al., 1999b; Andjelkovic and Pachter, 2000; Dzenko et al., 2001). Although these studies suggest that brain endothelial cells express CCR2, identification of this receptor has not yet been confirmed. To elucidate whether or not quiescent brain endothelial cells express a functional, active CCR2 receptor, we investigated expression of CCR2 mRNA and protein on mouse brain endothelial cells, as well as their chemotactic response towards a MCP-1 gradient. As shown in Fig. 1, quiescent mBMEC constitutively express CCR2 mRNA, evaluated by RT-PCR (Fig. 1A) and CCR2 protein as determined by western blot analysis (Fig. 1B) and immunocytochemistry (Fig. 1C). The addition of the proinflammatory cytokine IL-1β at 10 ng/ml for 6, 12 and 24 hours, significantly upregulated CCR2 mRNA and protein surface expression (Fig. 1A,B). In other systems, MCP-1 induces signaling and chemotaxis of leukocytes that is characterized by a bell-shaped dose-response curve with the optimal concentration of MCP-1 for chemotaxis at 1 nM (Uguccioni et al., 1995; Dunzendorfer et al., 2001). In our system, adding different concentrations of MCP-1 to the bottom wells of the microchemotaxis chamber to create a MCP-1 gradient, elicited mBMEC chemotaxis with a maximum chemotactic index, or ratio of stimulated migration/spontaneous migration, at 25 nM with a specific, bell-shaped dose-response curve for MCP-1 (Fig. 1D). Adding MCP-1-neutralizing antibody to the bottom along with MCP-1 blocked the MCP-1-induced chemotaxis. The presence of 100 nM MCP-1 in both the suspension of mBMEC added to the top chamber well and the bottom well, as in the absence of MCP-1 gradient, still induced chemotaxis of mBMEC but with a lower index than in the presence of MCP-1 gradient (Fig. 1D). These data demonstrated that the migration induced by MCP-1 is both chemotactic (directed) and chemokinetic

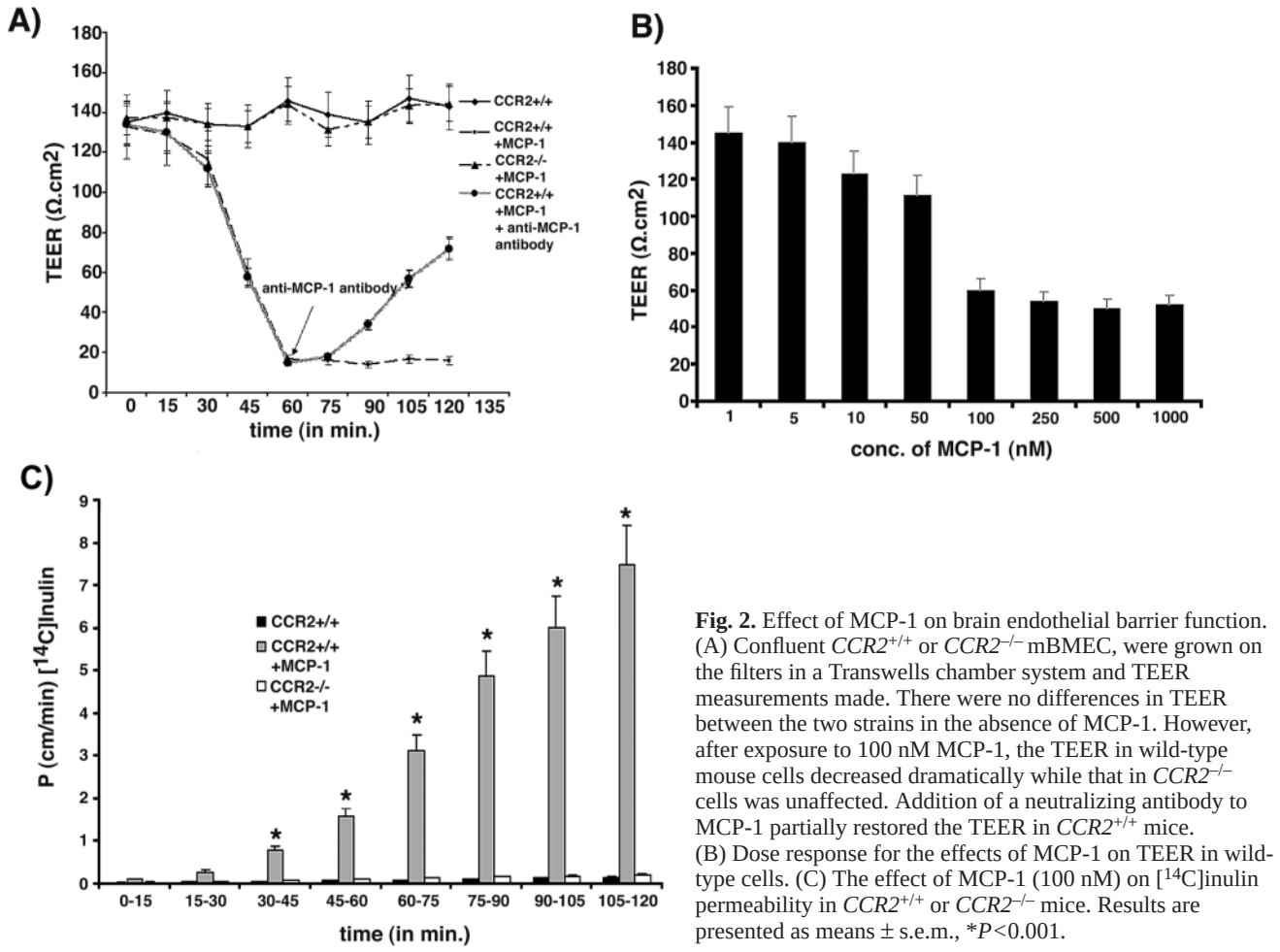

Fig. 2. Effect of MCP-1 on brain endothelial barrier function. (A) Confluent *CCR2*$^{+/+}$ or *CCR2*$^{-/-}$ mBMEC, were grown on the filters in a Transwells chamber system and TEER measurements made. There were no differences in TEER between the two strains in the absence of MCP-1. However, after exposure to 100 nM MCP-1, the TEER in wild-type mouse cells decreased dramatically while that in *CCR2*$^{-/-}$ cells was unaffected. Addition of a neutralizing antibody to MCP-1 partially restored the TEER in *CCR2*$^{+/+}$ mice. (B) Dose response for the effects of MCP-1 on TEER in wild-type cells. (C) The effect of MCP-1 (100 nM) on [^{14}C]inulin permeability in *CCR2*$^{+/+}$ or *CCR2*$^{-/-}$ mice. Results are presented as means ± s.e.m., *P<0.001.

(nondirected) and indicates that mBMEC express functional active CCR2 receptor on their surface.

Alteration in brain endothelial permeability by MCP-1

In order to elucidate whether or not MCP-1 can regulate brain endothelial permeability, two types of assay were performed: measurement of TEER, and evaluation of permeability coefficient for passage of [^{14}C]inulin through the mBMEC monolayer (Fig. 2). MCP-1 decreased TEER of mBMEC monolayer in a concentration- and time-dependent manner. The lowest MCP-1 concentration that decreased TEER was 20 nM and the most pronounced decrease in mean TEER was recorded across monolayers exposed to 100 nM MCP-1 (Fig. 2B). The effect of MCP-1 on brain endothelial barrier function was also time dependent. During the first 60 minutes of exposure to 100 nM MCP-1, the mean TEER of these monolayers dramatically decreased from 118±12 to 19±2 Ω.cm^2. TEER of monolayers was partially restored by adding a neutralizing antibody to MCP-1 after exposure to MCP-1. This indicates that barrier modification initiated by exposure to 100 nM MCP-1 is reversible. To test the hypothesis that alteration of mBMEC transendothelial electrical resistance is CCR2 dependent, MCP-1 was applied to mBMEC isolated from *CCR2*$^{-/-}$ mice. Our results clearly showed that MCP-1 could not induce any alteration of TEER in monolayers of *CCR2*$^{-/-}$ mBMEC (Fig. 2A).

The time-dependent reduction in TEER with MCP-1 was associated with an increased monolayer permeability to [^{14}C]inulin. As shown in Fig. 2C, MCP-1 induced significant increments in [^{14}C]inulin permeability throughout the 2-hour study period, from 0.1±0.013 to 7.5±0.9 cm/minute, compared to the control group of mBMEC monolayer exposed to medium without MCP-1. The absence of the CCR2 on the brain endothelial cells completely abolished this effect. Taken together, these results strongly suggest that MCP-1 increases mBMEC permeability through its interaction with the CCR2 receptor.

Effect of MCP-1 on actin cytoskleton and the TJ complex of brain endothelial cells

To determine the morphological basis for increase in permeability of MCP-1, the distribution of actin filaments and junctional proteins were monitored in response to 100 nM MCP-1. Quiescent mBMEC (Fig. 3 control) showed the marginal position of the ring-like bundle of F-actin filaments and a few stress fibers aligned with the major axis of the cell. Cells were well-spread and displayed so-called cobblestone morphology. The distribution patterns of actin filaments were

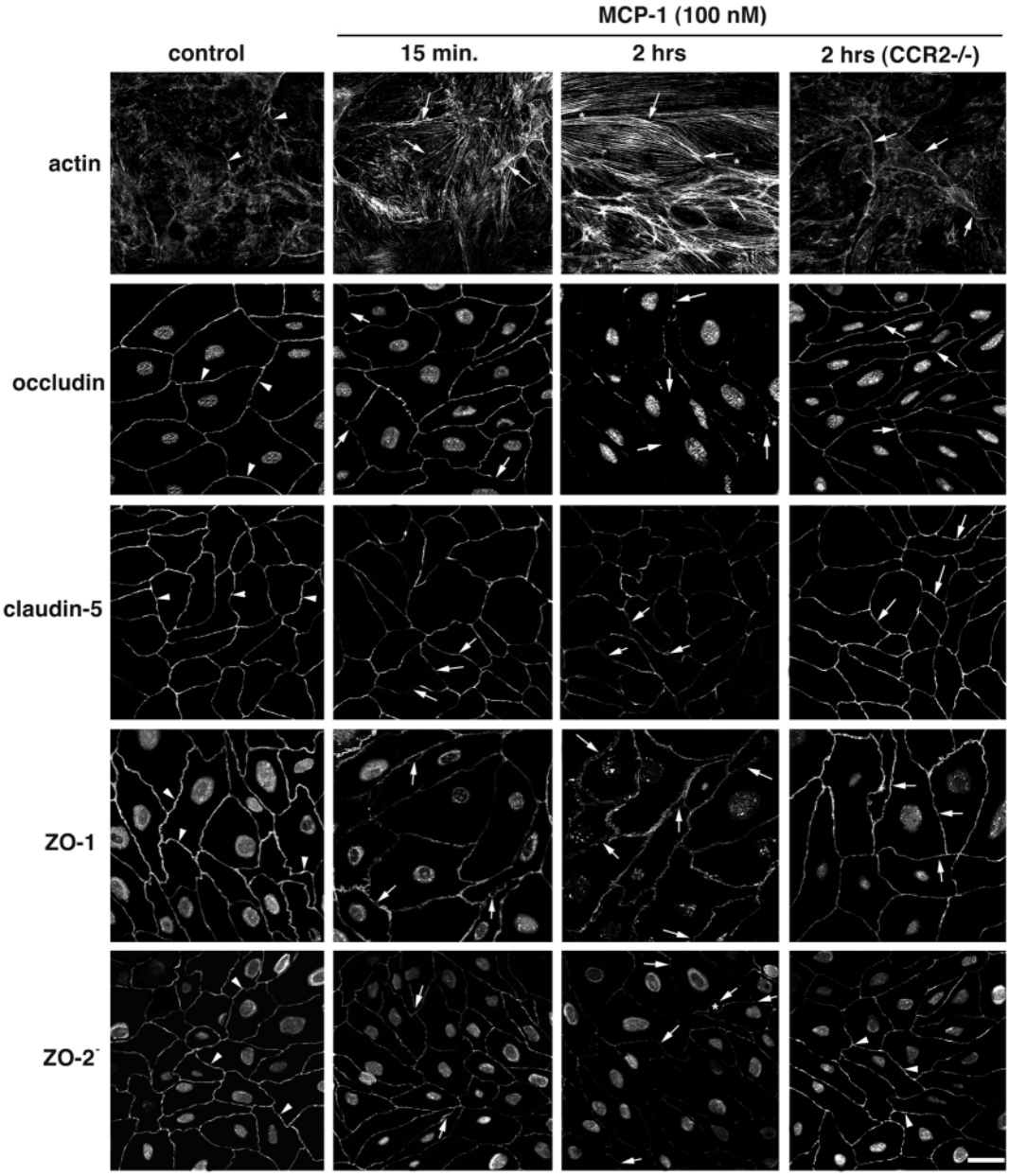

Fig. 3. Effect of MCP-1 on actin cytoskeleton and intercellular TJs. Confluent *CCR2*$^{+/+}$ and *CCR2*$^{-/-}$ mBMEC, were treated with recombinant mouse MCP-1 (100 nM) for the indicated time period (15 minutes and 2 hours) or served as normal controls (control). The cells were then fixed and stained with anti-occludin, ZO-1, ZO-2, claudin-5 antibodies or phalloidin Alexa 488 for F-actin. Untreated quiescent mBMEC (control, 7 days after initial plating) showed a typical polygonal shape, with actin filament distributed primarily in the cortical ring with a few stress fiber spanning the cells. They also had very specific continuous staining for occludin, ZO-1, ZO-2 and claudin-5 localized along the cell margins, possibly at the sites of cell-cell contact. In *CCR2*$^{+/+}$ cells, treatment with MCP-1 induced marked structural alterations in the distribution of actin filaments and TJ proteins in a time-dependent manner. In the absence of CCR2 receptors, the effect of MCP-1 on the actin cytoskeleton and TJ proteins were abrogated. Scale bar: 200 μm.

disrupted in mBMEC treated with MCP-1. The chemokine induced actin cytoskeleton rearrangement in a time-dependent manner. Analysis of the early MCP-1-induced cytoskeletal events (15 minutes) demonstrated an increase in stress fiber formation, while the majority of F-actin remained in the peripheral cortical actin ring. At 2 hours, changes in the actin cytoskeleton revealed loss of the cortical actin rim and intense stress fiber formation with a random orientation. In contrast, MCP-1 did not induce any changes in F-actin organization when the CCR2 receptor was absent; that is, MCP-1-induced morphological changes are receptor mediated (Fig. 3).

Furthermore, MCP-1 also induced reorganization of TJ complexes (Fig. 3). In the basal condition denoted as control, confluent mBMEC show a characteristic polygonal shape and linear pattern of immunostaining for occludin, claudin-5, ZO-1 and ZO-2 at cell-cell borders. In MCP-1-treated cells, continuous lines of occludin and ZO-2 staining became slightly segmented and discontinuous by 15 minutes and became more discontinuous and punctate at 2 hours. ZO-1 was localized in a serrated pattern at cell-cell borders after 15 minutes treatment with MCP-1 and in a discontinuous pattern by 2 hours. However, there was no marked alteration in claudin-5 distribution after 15 minutes of exposure to MCP-1, but fragmentation and a discontinuous pattern of distribution were seen at 2 hours. Visible gaps between endothelial cells was found after treatment with MCP-1, particularly at 2 hours. MCP-1 did not induce alterations in TJ protein organization in endothelial cells that did not express the CCR2 receptor (Fig. 3).

Based on the premise that increased brain endothelial permeability and qualitative changes in TJ protein staining patterns are likely to represent important alterations in TJ assembly, we next sought to address this question biochemically. TJ disassembly is accompanied by the

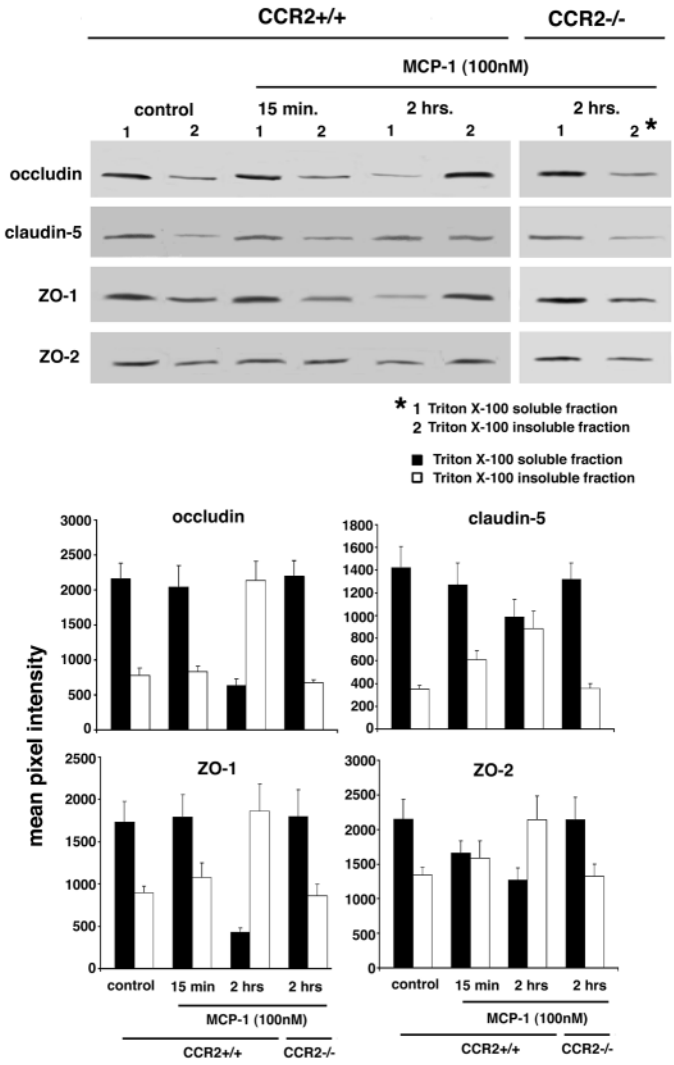

Fig. 4. Shift of TJ proteins from soluble to insoluble phase. Confluent *CCR2*$^{+/+}$ and *CCR2*$^{-/-}$ mBMEC were subjected to 100 nM MCP-1 for 15 minutes or 2 hours. Triton X-100-soluble and Triton X-100-insoluble fractions were collected. Immunoblots of those fractions were then probed with anti-occludin, anti-claudin-5, anti-ZO-1 and anti-ZO-2 antibodies. (B) Immunoblots were analyzed and quantified with NIH Image software. Data represent means±s.e.m. of three independent experiments.

association of TJ proteins into large macromolecule complexes, movement of TJ proteins into an insoluble pool and an increased association between TJ proteins and the actin based cytoskeleton. Since detergent extractability is an established biochemical means for analyzing protein-cytoskeleton interactions (Stuart and Nigam, 1995; Stuart et al., 1996; Tsukamato and Nigam, 1997) we examined the Triton X-100 solubility properties of TJ proteins after MCP-1 treatment. As shown in Fig. 4, in steady state monolayers of mBMEC, occludin, ZO-1, ZO-2 and claudin-5 were mostly in the Triton X-1000-soluble pool, although a small amount of these proteins could be found in the Triton X-100-insoluble pool. ZO-1, ZO-2 and occludin become more Triton X-100 insoluble after 2 hours of MCP-1 treatment (Fig. 4A), suggesting a close association of these proteins with the actin cytoskeleton. There was a less obvious shift in claudin-5. Densitometric analysis of the blots revealed that TJ proteins move independently into insoluble fractions after MCP-1 treatment (Fig. 4B). MCP-1 did not induce a shift in TJ proteins from the Triton X-100-soluble to Triton X-100-insoluble fraction in *CCR2*$^{-/-}$ mBMEC.

Role of small GTPase Rho in MCP-1- induced alteration of brain endothelial permeability

To test which signal pathways could be activated upon MCP-1 stimulation, we performed cDNA microarray analysis. As shown in Fig. 5A, MCP-1 induced activation of several signal pathways: PI 3-kinase, MAP kinase, protein kinase C (PKC), small GTPase, etc (Fig. 5A). These signal pathways have been described as being involved in stress fiber formation. Therefore, to detect which of these pathways might be involved in altering brain endothelial permeability, mBMEC was pretreated with either a Rho kinase inhibitor Y27632, a PKC inhibitor Ro-31-7549, a PI-3K inhibitor LY294002, a MEK/ERK kinase inhibitor PD98059, a p38 inhibitor SB203580, a calmodulin inhibitor W7 hydrochloride, or a PLC inhibitor U7322. Following this, cells were stimulated with 100 nM MCP-1 for 2 hours. Fig. 5B shows the alteration in actin cytoskeleton and ZO-1 in mBMEC treated with MCP-1 in the presence of these inhibitors. It was notable that inhibitors of ERK1/2 and p38 had no effect on actin stress fiber formation and redistribution of TJ proteins under stimulation with MCP-1 while U7322, W7 and LY294002 partially inhibited the MCP-1 effect. In contrast, Y27632 and Ro-31-7549 inhibited the effects of MCP-1 on the actin cytoskeleton and ZO-1. These data were supported by measurements of TEER. Y27632 completely and Ro-31-7549, LY294002, W7 and U7322 partially abolished the effect of MCP-1 on the brain endothelial permeability, whereas SB203580 and PD98059 did not affect TEER (Fig. 5C). These data indicate that Rho kinase and PKC could be key players in MCP-1-induced cytoskeleton and TJ reorganization.

The activation of Rho kinase is mostly regulated by the small GTPase RhoA, suggesting that RhoA could be a potential regulator of endothelial permeability. To evaluate this possibility, we investigated the activity of RhoA protein, in mBMEC during exposure to MCP-1, by affinity precipitation of active RhoA-GTP and the role of RhoA in MCP-1-induced actin cytoskeleton and TJ reorganization by using specific inhibitors of RhoA activity, C3 exoenzyme, and transient transfection of T17 Rho dominant negative mutant. Our results showed that MCP-1 induced activation of RhoA, with the peak occurring after 20 minutes (Fig. 6A,B). Treatment of mBMEC with C3 exoenzyme, or a transient transfection of T17 Rho dominant negative mutant into mBMEC prevented MCP-1-induced activation of RhoA protein (Fig. 6C).

RhoA inhibition or Rho kinase inhibition completely abolished MCP-1-induced alterations in mBMEC permeability. Western blot analysis showed that pretreatment with RhoA inhibitors or Rho kinase inhibitor prevented the MCP-1-induced redistribution of TJ proteins between the Triton X-100-soluble and Triton X-100-insoluble fraction, emphasizing the critical role of RhoA and Rho kinase in MCP-1-induced actin cytoskeleton and TJ reorganization (Fig. 7A). Furthermore, inhibition of RhoA and Rho kinase significantly

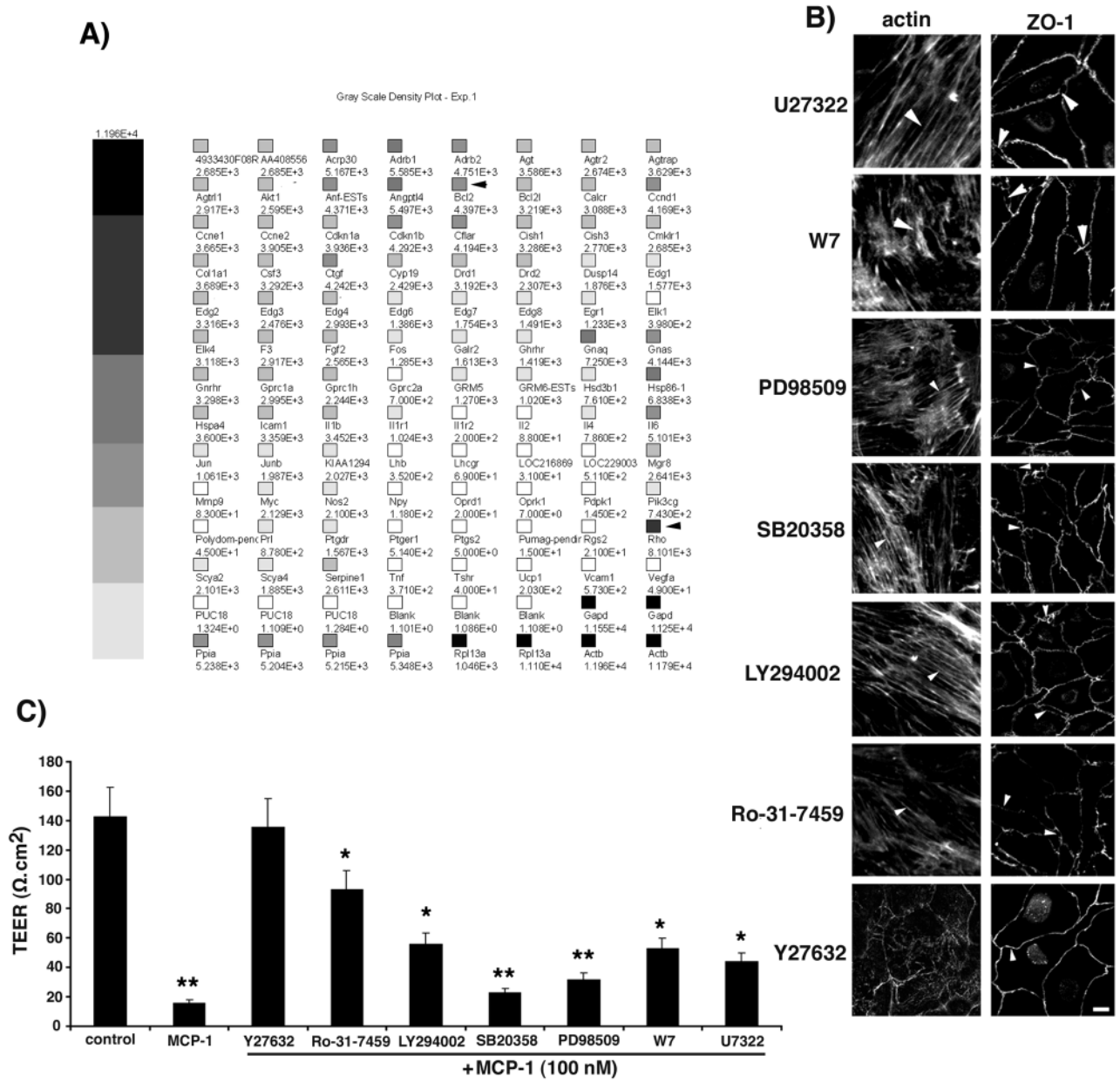

Fig. 5. Analysis of signal pathways in response to MCP-1. **(**A) cDNA microarray analysis of signal pathways induced by MCP-1. Data represent relative expression levels of specific genes normalized using a housekeeping gene control and compared with the group of untreated cells. Arrowheads indicate several representative genes whose levels increased in response to MCP-1. (B) Confluent mBMEC were pretreated with the following inhibitors: PD98059, SB 203580, LY294002, Ro-37840, Y27632, W7 and U7322 for 1 hour at 37°C. Recombinant mouse MCP-1 (100 nM) was then added for 2 hours. The cells were then fixed and processed for immunocytochemistry using anti-ZO-1 and Alexa 488 Phalloidin. The samples were viewed on a laser scanning Zeiss confocal microscope. Scale bar: 200 μm. (C) Changes in TEER during treatment with specific inhibitors of different signal pathways. Confluent mBMEC were grown on the filters in a Transwells chamber system and pretreated with the following inhibitors: PD98059, SB 203580, LY294002, Ro-37840, Y27632, W7 and U7322 for 1 hour at 37°C. After that the cells were exposed to MCP-1 (100 nM) in the presence of inhibitors. TEER was measured every 15 minutes over a time period of 2 hours. Results are presented as means±s.e.m., *P<0.01; **P<0.001.

diminished the effect of MCP-1 on mBMEC electrical resistance (Fig. 7B) and [^{14}C]inulin permeability (Fig. 7C). These results were supported by immunocytochemistry. Immunofluorescence microscopy revealed that when RhoA or Rho kinase were inhibited, MCP-1 had no effect on the actin cytoskeleton and TJ complex of mBMEC (Fig. 8). Taken together, these data imply that MCP-1 alters actin and TJ structure reorganization and, thus, permeability through activation of Rho small GTPase RhoA and Rho kinase.

Discussion

MCP-1, like other chemokines, was initially recognized as playing a role in migration and activation of specific leukocyte

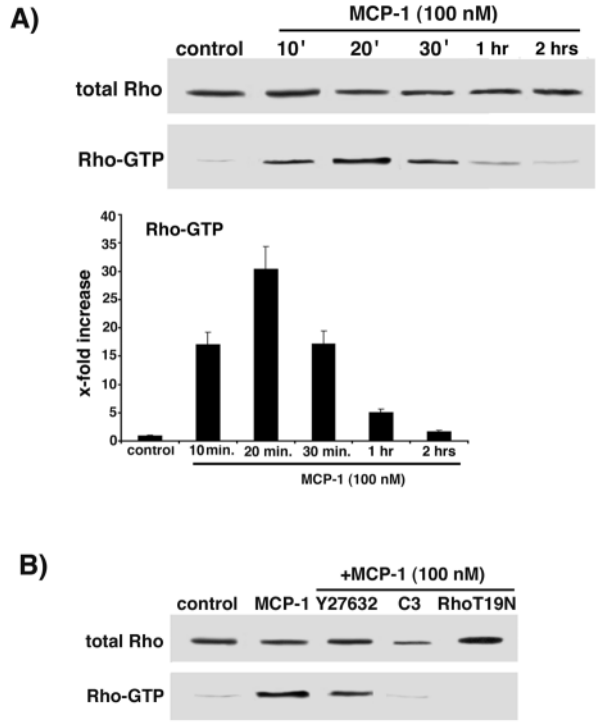

Fig. 6. MCP-1 transiently activates Rho in mouse BMEC. (A) Confluent mBMEC were treated with 100 nM murine MCP-1 for 10, 20, 30 minutes, 1 or 2 hours. Cell lysates were subject to affinity precipitation using Rhotek in recombinant protein agarose conjugated which specifically precipitates active RhoA (Rho-GTP). Total Rho indicates total amount of active and inactive Rho in the mBMEC. The immunoblot represents one of three independent experiments. (B) Densitometric analysis of MCP-1-induced activation of RhoA. Data are means±s.e.m., n=3 independent experiments *P<0.001; (C) Confluent mBMEC were subject to treatment with a Rho kinase inhibitor Y27632 10 μM for 30 minutes) or with specific inhibitor of RhoA, C3 exoenzyme (5 μg/ml for 18 hours), or transiently transfected with dominant negative mutant T19NRho. Western blot was performed using an antibody specific for RhoA.

subpopulations in both physiological and pathological contexts. (Rollins, 1997; Mantovani, 1999a; Mantovani, 1999b). In addition to chemotactic activity for leukocytes, several recent studies have indicated that MCP-1 also plays a role in tumor metastasis and angiogenesis, in development of CNS, immune and vascular systems, as well as in modulation of cell proliferation, apoptosis, protein synthesis, etc (Gu et al., 1999; Salcedo et al., 2000; Sasayama et al., 2000; Liss et al., 2001; Luther and Cyster, 2001; Rezaie et al., 2002). In line with this new evidence, the present study highlights a possible role for MCP-1 in the regulation of brain endothelial permeability.

Brain endothelial cells form a very tight and highly impermeable barrier serving to regulate and protect the brain microenvironment. The endothelial barrier function is highly dependent on specific adhesion molecules in interendothelial junctions and contractile forces within the endothelial cells which have the capacity to retract cells and subsequently form interendothelial gaps (Lum and Malik, 1994; Malik and Lo, 1996; Van Hinsbergh, 1997). Under many pathological conditions, particularly those associated with inflammation and angiogenesis, this barrier becomes high permeable (Liebsch et al., 1996; Vestweber, 2000; Huber et al., 2001b; Brown and Davis, 2002). The increase in brain endothelial permeability is attributed to rearrangement of the actin microfilament system and redistribution of TJ proteins. These changes induce endothelial contractile forces directed at interendothelial junctions, decrease in intercellular adhesive forces and gap formation (Moy et al., 1996; van Nieuw Amerongen et al., 1998). At the functional level, endothelial barrier dysfunction is manifested as increase permeability for paracellular pathway tracers, associated with changes in distribution of tight junction proteins and their shifting into an insoluble pool (Tsukamoto and Nigam, 1997; Tsukamoto and Nigam, 1999). Our results also affirm that MCP-1 affects brain endothelial permeability. MCP-1 induces prolonged increase of endothelial permeability lasting for 1-2 hours. Direct application of MCP-1 to mBMEC resulted in a time- and dose-dependent decrease in TEER. MCP-1 treatment also increased the permeability of cultured mBMEC to [^{14}C]inulin, a large molecular mass paracellular pathway tracer. These functional permeability changes were associated with morphologic changes including disruption of the linear distribution of TJ proteins at intercellular junctions, stress fiber formation and the appearance of gaps between cells. MCP-1 treatment also caused shifting of TJ

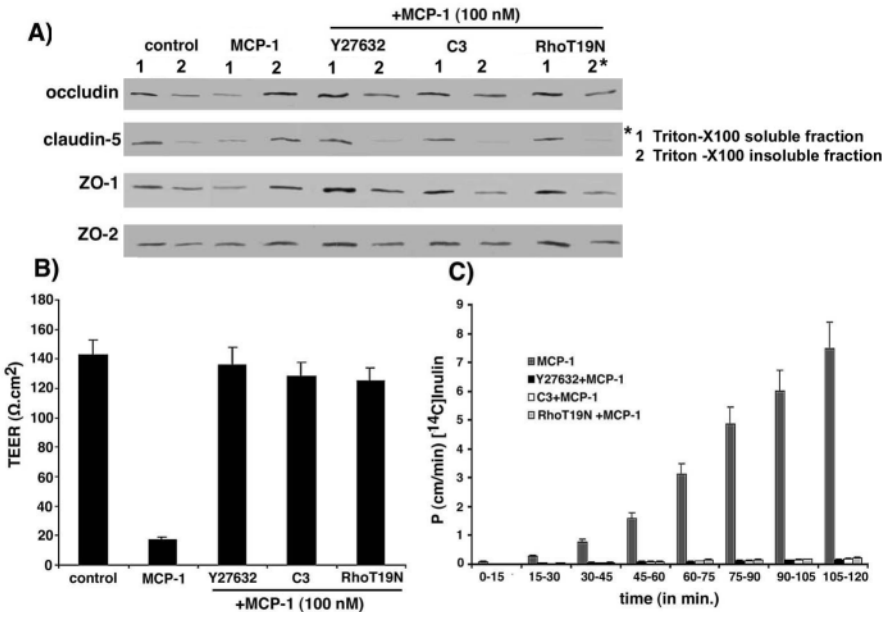

Fig. 7. Effect of inhibition of RhoA and Rho kinase on MCP-1-induced changes in brain endothelial permeability. (A) Western blot analysis of TJ proteins (occludin, claudin-5, ZO-1 and ZO-2) after treatment of cell with Rho kinase and RhoA inhibitors, Y27632 and C3 or transiently transfected with dominant negative mutant T19NRho. Effect of Rho and Rho kinase inhibitors on changes (B) TEER and (C) [^{14}C]inulin permeability (P) induced by MCP-1. Results are presented as means±s.e.m., n=3 independent experiments.

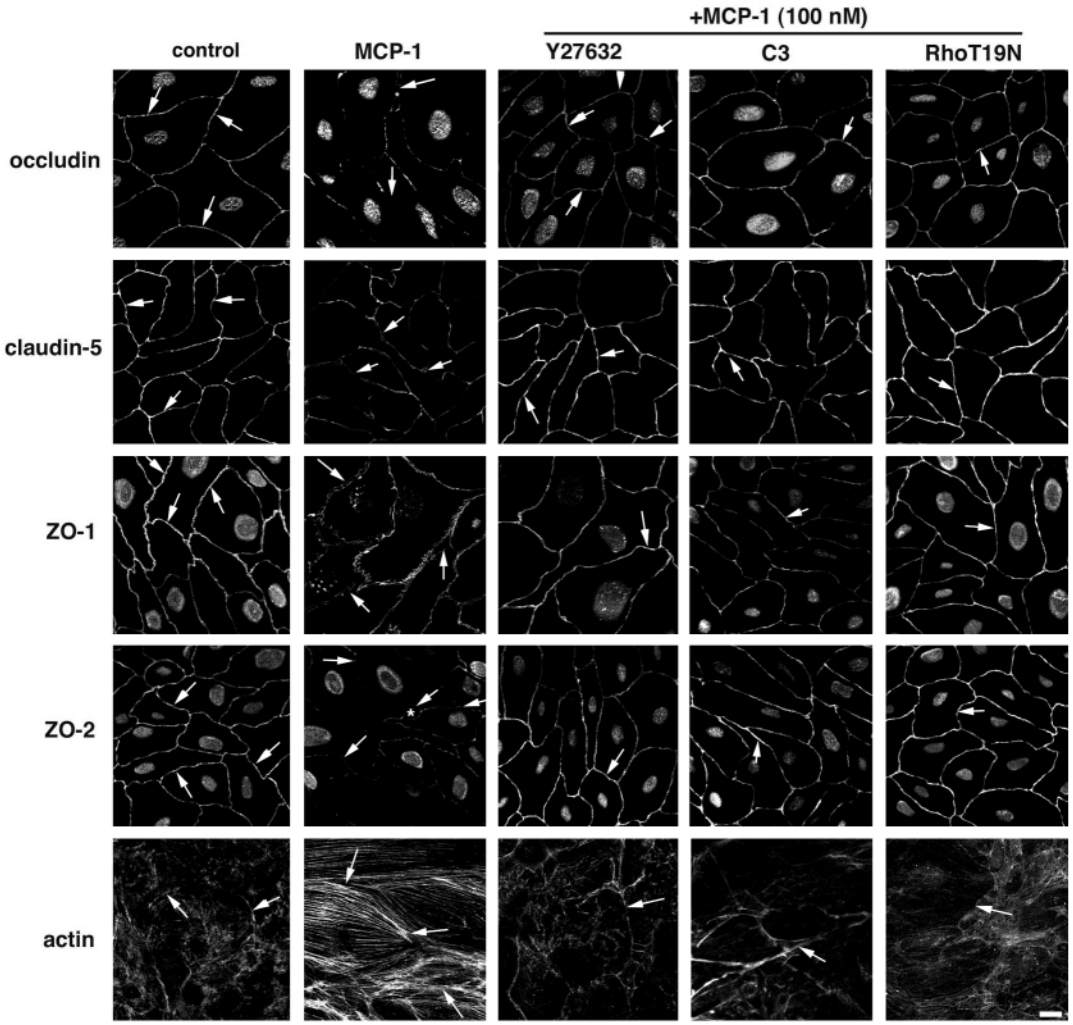

Fig. 8. Effect of inhibition of RhoA and Rho kinase on MCP-1-induced alterations in actin and TJ proteins. Confluent mBMEC were pretreated for 30 minutes with 10 μM Y27632, or 18 hours with 5 μg/ml C3 exoenzyme, or transiently transfected with T19NRho and then were exposed to 100 nM MCP-1 for 2 hours. The cells were then fixed and processed for immunocytochemistry using anti-ZO-1, -ZO-2, -occudin, and claudin-5 antibodies and Alexa 488 Phalloidin for F-actin. Arrows indicate organization of actin and TJ proteins in presented experimental groups. Scale bar: 200 μm.

proteins from Triton-soluble into Triton-insoluble fractions. Based on these results, we suggest that MCP-1 increases brain endothelial permeability by rearrangement of intracellular actin and alteration of TJ assembly. The previously published data have established that proinflammatory cytokines such as IL-1, IL-4, IL-10, IL-13, TNF-α and INF-γ alter tight junction organization and induce stress fiber formation in both epithelial and endothelial cells, and thus increase permeability (Ross and Joyner, 1997; Wojciak-Stothard et al., 1998; Yoakim and Ahdieh, 1999; Blamire et al., 2000; Ahdieh et al., 2001; Oshima et al., 2001; Coyne et al., 2002). Similar actions were described recently for IL-8, a member of CXC family of chemokines (Biffl et al., 1995). Some in vitro and in vivo data clearly suggest that IL-8, through a CXCR2 receptor on the endothelial cells, causes cell retraction and gap formation between adjacent cells leading to an increase in permeability of endothelial cells monolayers (Schraufstatter et al., 2001). In addition, IL-8 is defined as a factor that can cause brain edema formation (Matsumoto et al., 1997). Presumably, MCP-1 like other proinflammatory cytokines could contribute to increased permeability during an inflammatory process, making a junction between brain endothelial cells ‘porous’ and ready to allow leukocytes infiltration into brain tissue.

How does MCP-1 induce increased brain endothelial permeability? Because the current experiments utilized a monoculture system, MCP-1 must have exerted its activity on permeability directly. Our data clearly show that MCP-1-induced brain endothelial barrier dysfunction is receptor mediated. Depletion of CCR2, the sole receptor through which MCP-1 signals, prevented MCP-1-induced reorganization of the actin cytoskeleton and redistribution of TJ proteins. CCR2 is a G protein-coupled seven transmembrane receptor, and binding of MCP-1 to CCR2 could activate several different signal pathways. Some of these pathways could be actively involved in regulation of endothelial permeability by targeting the actin cytoskeleton and/or junctional complexes. Numerous signaling mechanisms have been reported to regulate vascular permeability. Evidence suggests that MAPK, ERK1/2 and p38 alone or in a coordinated fashion could be involved in modulating endothelial barrier function by altering the actin cytoskeleton and/or by phosphorylation and redistribution of occludin and ZO-1 (Hout et al., 1997; Tanaka et al., 1999; Kevil et al., 2000; Kevil et al., 2001; Niwa et al., 2001; Wachtel et al., 2002). It has also been shown that regulation of endothelial barrier function could be mediated by soluble agonist occupancy of PLC associated receptor (Rotrosen and Gallin, 1986; Yuan et al., 1993; Lum and Malik, 1994). Two typical intermediates that function downstream of activated

PLC are endothelial cytosolic Ca^{2+} and PKC (Lum and Malik, 1994; Huang and Yuan, 1997). Agonists, such as histamine, cause hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2) by PLC activation, subsequently leading to Ca^{2+} mobilization. This in turn results in Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase (MLCK) and actin cytoskeleton rearrangement and increased permeability (Rotrosen and Gallin, 1986; Lum and Malik, 1994; Stephan and Brock, 1996; van Nieuw Amerongen et al., 2000; Borbiev et al., 2001). Other groups of agonists, such as phorbol esters, bradykinin and platelet activating factor, alter endothelial barrier function via a PKC-dependent mechanism. They target TJ proteins (direct serine/threonine phosphorylation) and the actin cytoskeleton by increasing MLC phosphorylation, which occurs in conjunction with transient stress fiber formation or by acting directly on actin fibers by phosphorylation of caldesome (Lynch et al., 1990; Stasek et al., 1992; Kobayashi et al., 1994; Huang and Yuan, 1997; Ross and Joyner, 1997; Voung et al., 1998; Bogatcheva et al., 2003). Additionally, PI 3-kinase has been suggested as a central molecule in a putative common signal transduction pathway that is important for regulation of endothelial permeability (Pedram et al., 2002; Ericcson et al., 2003). Recently much attention has focused on members of the Rho family of GTPase (RhoA, Rac1, Cdc42) the activity of which have been linked to the regulation of both tight junction assembly and paracellular permeability (Ridley, 1997; Ridley, 2001; Carbajal and Schaffer, 1998; Fujita et al., 2000; Hirase et al., 2001; Adamson et al., 2002; Etienne-Manneville and Hall, 2002; Matter and Balda, 2003). RhoA has a prominent stimulatory effect on actin-myosin interaction by its ability to stabilize the phosphorylated state of MLC that occurs by activation of Rho associated kinase (ROCK) (Amano et al., 1996; Kimura et al., 1996; Kawano et al., 1999; Katoh et al., 2001; Wettschureck and Offermanns, 2002). To analyze the molecular mechanisms by which MCP-1 alters brain endothelial permeability, the involvement of all these listed pathways were tested. Our findings clearly indicated that only inhibition of Rho kinase completely prevented MCP-1-induced changes in permeability, actin cytoskeleton reorganization and TJ proteins redistribution, indicating that the Rho/Rho kinase pathway could have a critical role. This was confirmed by experiments where inhibitors for Rho associated kinase (Y27632), and RhoA (C3 transferase and T17 Rho dominant negative mutant) were used. We found that application of these inhibitors prevents functional, morphological and biochemical alterations in brain endothelial permeability under MCP-1 treatment. Taken together our findings highlight a Rho/Rho associated kinase as one of the major pathways in regulation of brain endothelial permeability.

Based on our findings, we propose a possible scenario for the MCP-1-induced increase in brain endothelial permeability: the early step is interaction of MCP-1 with its receptor CCR2, expressed on endothelial cells. This interaction in turn activates Rho A which further, probably via coupling to Rho binding domain on Rho associated kinase, activates that kinase. Activation of Rho associated kinase causes phophorylation of regulator subunits of MLC phosphatase and inhibits myosin phosphatase activity. This results in stimulation of actin myosin interaction and a prolonged increase of cortical forces in the endothelial cells. Furthermore, the actin cytoskeleton, through interaction with certain TJ proteins (occludin, claudin-5, ZO proteins), could regulate TJs. The interaction of the actin cytokeleton with TJ proteins could also be possible through direct action of RhoA on the TJ proteins. Some studies have indicated that Rho directly stimulates phosphorylation of the carboxy domain of occludin and thereby regulates interaction of occludin and the submembrane cytoskeleton and, thus, paracellular permeability (Hirase et al., 2001; Benais Pont et al., 2003). This pattern may also apply for other TJ proteins.

The data in the current study strongly indicate that the RhoA/Rho kinase pathway has a major role in MCP-1-induced alterations in the actin cytoskeleton. However, in the case of regulation of tight junction proteins, we do not wish to exclude some other possible alternative pathways, such as activation of PKC or a direct affect of Rho on tight junction proteins. Our finding that PKC inhibition partially abolished MCP-1-induced alterations in mBMEC permeability favors a contribution by PKCs. An intriguing possibility is that activated Rho may interact with PKC isoforms, which in turn phosphorylate TJ proteins. Several recent studies indicate that this interaction can take place (Coghlan et al., 2000; Pal et al., 2001; Slater et al., 2001; Schmitz et al., 2002). Alternatively MCP-1 might also directly activate some of the PKC isoforms in order to induce phosphorylation of TJ proteins. This is an areas for our future investigations.

What are the implications of MCP-1 modulation of brain endothelial permeability? Recent findings indicate that MCP-1 is a potential angiogenic factor (Salcedo et al., 2000). Alteration of endothelial permeability is a critical early step during angiogenesis followed by endothelial cells migration toward a MCP-1 gradient and their possible proliferation and tube formation. However, MCP-1 is one of the major chemokines participating in CNS inflammatory processes. MCP-1 is secreted by astrocytes in order to make a chemotactic gradient. Endothelial cells alone or in interaction with invading monocytes could also be a source of MCP-1 (Zhang et al., 1999; Boven et al., 2000; Rimbach et al., 2000). Obviously MCP-1 is present in high concentration during the process of leukocyte transmigration and alteration of tight junction structure and brain endothelial permeability could be very important factor for brain endothelial ‘unzipping’. It is possible that MCP-1 could participate in vasogenic edema formation in the brain [as proposed in the lung (Sakao et al., 2001)] although this possibility awaits investigation.

In summary, this study provides clear evidence that MCP-1 induces prolonged brain endothelial hyperpermeability through a Rho A/Rho kinases pathway. Our data imply that MCP-1 secreted into the perivascular space of the BBB not only attracts leukocytes, but also has a role in ‘opening’ the BBB during leukocyte extravasation by acting directly on brain endothelial cells. In many CNS diseases, preventing BBB disruption and stopping leukocyte extravasation is an important goal for limiting inflammatory injury. MCP-1 could be an attractive therapeutic target, given that it is critical for leukocyte migration and, as shown here, is involved in regulating BBB permeability. This is an important area for further investigation.

This work was supported by grant 00050831T from the American Heart Association to A.V.A. The authors thank Chris Edwards, Bruce Donohoe and Dotty Sorenson from Microcopy and the Imaging Laboratory at the Department of Cell and Development Biology, University of Michigan Medical School, for their technical assistance.

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