Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling

Gerty Schreibelt,*[,1] Gijs Kooij,*[,1] Arie Reijerkerk,* Ruben van Doorn,* Sonja I. Gringhuis,* Susanne van der Pol,* Babette B. Weksler,[†] Ignacio A. Romero,[‡] Pierre-Olivier Couraud,[§] Jörg Piontek,[‖] Ingolf E. Blasig,[‖] Christine D. Dijkstra,* Eric Ronken,[¶] and Helga E. de Vries*[,2]

*Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands; [†]Weill Medical College, Cornell University, New York, USA; [‡]Department of Biological Sciences, The Open University, Walton Hall, Milton Keynes, UK; [§]Institut Cochin, Departement de Biologie Cellulaire, Paris, France; [‖]Institute of Molecular Pharmacology, Berlin, Germany; and [¶]Solvay Pharmaceuticals Research Laboratories, Weesp, The Netherlands

ABSTRACT **The blood-brain barrier (BBB) prevents the entrance of circulating molecules and immune cells into the central nervous system. The barrier is formed by specialized brain endothelial cells that are interconnected by tight junctions (TJ). A defective function of the BBB has been described for a variety of neuroinflammatory diseases, indicating that proper regulation is essential for maintaining brain homeostasis. Under pathological conditions, reactive oxygen species (ROS) significantly contribute to BBB dysfunction and inflammation in the brain by enhancing cellular migration. However, a detailed study about the molecular mechanism by which ROS alter BBB integrity has been lacking. Here we demonstrate that ROS alter BBB integrity, which is paralleled by cytoskeleton rearrangements and redistribution and disappearance of TJ proteins claudin-5 and occludin. Specific signaling pathways, including RhoA and PI3 kinase, mediated observed processes and specific inhibitors of these pathways prevented ROS-induced monocyte migration across an *in vitro* model of the BBB. Interestingly, these processes were also mediated by protein kinase B (PKB/Akt), a previously unknown player in cytoskeleton and TJ dynamics that acted downstream of RhoA and PI3 kinase. Our study reveals new insights into molecular mechanisms underlying BBB regulation and provides novel opportunities for the treatment of neuroinflammatory diseases.—Schreibelt, G., Kooij. G., Reijerkerk, A., van Doorn, R., Gringhuis, S. I., van der Pol, S., Weksler, B. B., Romero, I. A., Couraud, P.-O., Piontek, J., Blasig, I. E., Dijkstra, C. D., Ronken, E., de Vries, H. E. Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase and PKB signaling. *FASEB J.* 21, 3666–3676 (2007)**

Key Words: blood-brain barrier · claudin-5 · occludin · protein kinase B

The blood-brain barrier (BBB) is a tight barrier between the central nervous system (CNS) and the systemic circulation and is essential for maintenance and regulation of the neuroparenchymal environment and optimal neuronal functioning. The BBB is primarily formed by specialized brain endothelial cells (ECs), which form a tight seal due to the presence of well-developed tight junctions (TJ) that impede the entrance of circulating molecules and immune cells into the CNS (1).

TJs are continuous membrane strands located at the apical site between brain ECs, which consist of transmembrane and cytoplasmic proteins that are associated with the actin cytoskeleton. The transmembrane proteins occludin and the claudins mediate cellular interaction between brain ECs and play a major role in TJ functioning. Occludin is a phosphoprotein that spans the plasma membrane four times with intracellular location of both the amino and the carboxyl termini (2, 3). Occludin expression is associated with increased electrical resistance and decreased paracellular transport (4). Claudins comprise a multigene family consisting of more than 20 members and contain 2 extracellular loops and 4 transmembrane domains and interact in both a homophilic and heterophilic way with claudins of adjacent cells (5, 6). Claudin-5 is a critical component of the BBB as it closes the BBB for small molecules up to 800 Da (7). The carboxyterminal parts of both occludin and claudins interact with membrane-associated recruiting proteins of the zona occludens (ZO) protein family (8, 9). ZO proteins are reported to link transmembrane proteins to the actin cytoskeleton and have signaling potential (10, 11). Through its interaction with TJ molecules, the actin cytoskeleton

[1] These authors contributed equally to this work.

[2] Correspondence: Department of Molecular Cell Biology and Immunology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands, E-mail: he.devries@vumc.nl

doi: 10.1096/fj.07-8329com

0892-6638/07/0021-3666 © FASEB

plays an active role in maintaining TJ integrity and BBB function (12). Several cytoplasmic signaling molecules, such as Rho, PI3 kinase, protein kinase C (PKC), Ca2+, heterotrimeric G proteins, cyclic adenosine monophosphate (cAMP), and phospholipase C have been localized to TJ complexes and may regulate their assembly and disassembly [for review see (13, 14)].

A defective function of the BBB is reported in neuroinflammatory diseases like multiple sclerosis (MS) (15, 16), HIV-associated dementia (17) and encephalitis (18, 19), stroke, and brain trauma (20). Pathological events that may occur at the BBB include structural and spatial alterations of the TJ, enhanced permeability for blood-derived components, and infiltration of inflammatory cells into the CNS. In these processes, proinflammatory mediators like chemokines (21) and cytokines (22, 23) play an important role. Previously we showed that reactive oxygen species (ROS), which are highly reactive molecules, are produced during monocyte migration and contribute to BBB injury and subsequent inflammation in the brain (24, 25). Indeed, scavenging extracellular ROS by lipoic acid and luteolin prevented the development of clinical signs in animal models for MS, acute and chronic experimental allergic encephalomyelitis (24, 26). Currently, specific ROS signaling pathways within brain ECs that target the TJ to disengage are unclear, although previous studies from our group revealed that ROS induced enhanced Ca^{2+} mobilization and inositol (1, 4, 5)-trisphosphate (IP_3) formation in brain ECs via phospholipase C (25). Furthermore, ROS may activate protein tyrosine kinases in brain endothelial cells, which in turn may lead to tyrosine phosphorylation of TJ proteins (27).

Aim of current study was to elucidate the ROS-dependent signaling pathways regulating TJ dynamics in brain ECs. We provide evidence that ROS selectively activate signaling cascades involving RhoA, PI3 kinase, and protein kinase B (PKB/Akt) leading to rearrangements of the actin cytoskeleton and spatial redistribution and disappearance of occludin and claudin-5, inducing altered BBB integrity. Selective inhibitors of identified signaling pathways and antioxidants reversed observed alterations and prevented ROS-induced monocyte migration across an *in vitro* model of the BBB. Molecular understanding of the regulation of the function and integrity of the BBB is essential to identify agents that can prevent BBB dysfunction in neuroinflammatory diseases thus limiting neurological deficits.

MATERIALS AND METHODS

Chemicals and antibodies

The following agents were purchased: wortmannin and triciribine (Tocris Bioscience, Ellisville, MO, USA); toxin B (Calbiochem, San Diego, CA, USA); FITC-dextran, xanthine oxidase, hypoxanthine, superoxide dismutase, catalase and lipoic acid (Sigma-Aldrich, St. Louis, MO, USA); mannitol (BDH Chemicals, Poole, UK); luteoline (Kaden Biochemicals, Hamburg, Germany); monoclonal anti-occludin, polyclonal anti-claudin-5, and polyclonal anti-ZO-1 (Zymed, San Francisco, CA, USA); monoclonal anti-PKB and polyclonal anti-phosphoserine-PKB (Cell Signaling Technology Inc., Danvers, MA, USA); polyclonal anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Alexa 594-conjugated goat anti-rabbit F(ab′)2 (Molecular Probes, Leiden, The Netherlands); HRP-conjugated antibodies (Vector Laboratories, Burlingame, CA, USA); Ham's F12 medium and endothelial-serum free medium (Life Technologies, Inc., Carlsbad, CA, USA). C3 transferase was a kind gift from Prof. Dr. J. Greenwood, Institute of Ophthalmology, University College London, UK; the retroviral vector pLZRS-IRES-zeocin was kindly provided by Dr. P. L. Hordijk, Sanquin Research, Amsterdam, The Netherlands.

Construction of brain cell line overexpressing claudin-5-YFP

The DNA sequence of mouse claudin-5 fused to the N terminus of YFP was subcloned from the pEYFP-N1 vector (28) into the modified retroviral vector pLZRS-IRES-zeocin. The resulting construct, pLZRS-Claudin-5-YFP-IRES-zeocin, was transfected into amphotropic Phoenix retrovirus producer cells. Subsequently, virus containing supernatant was used to transduce immortalized rat GP8/3.9 brain endothelial cells (ECs) as described (29). Expression and localization of claudin-5-YFP were determined by confocal laser scanning microscopy (CLSM) at room temperature (Leica TCS SP2 AOBS microscope, HCX PL APO 63x/1.30 lens; Leica Microsystems B.V., Rijswijk, The Netherlands). The cells were grown on collagen-coated glass coverslips and fixed with 4% paraformaldehyde. Claudin-5-YFP was labeled with polyclonal rabbit anticlaudin-5 followed by Alexa 594-conjugated secondary antibodies. Mounted coverslips were analyzed by sequential excitation at 514 /594 nm.

Cell culture

The Lewis immortalized rat brain endothelial cell line GP8/3.9 (30), the GFP-occludin- (29), and the claudin-5-YFP expressing GP8/3.9 cell lines were cultured as described (31). The human immortalized brain endothelial cell line hCMEC/D3 was cultured as described (32). Primary rat brain ECs were isolated and cultured as described (33)

ROS generating system

A mixture of 0.02 U/ml xanthine oxidase and 100 μM hypoxanthine was used to generate ROS as described previously (25). This mixture is known to produce constant levels of predominantly superoxide and to a lesser extent hydrogen peroxide and hydroxyl radicals (34).

Live cell analysis of TJ rearrangements

The GFP-occludin and claudin-5-YFP expressing immortalized rat GP8/3.9 cells were cultured to confluence on collagen-coated 42 mm diameter glass cover slips in Ham's F12 medium supplemented with 2.5% fetal calf serum. ROS was generated as described above. CLSM was performed at 37°C

Electrical cell substrate impedance sensing (ECIS) assay

ECIS™ Model 1600R (Applied BioPhysics, Troy, NY, USA) was used to measure TEER in confluent monolayers of immortalized human hCMEC/D3 cells. In short, 250 μl of cell suspension (8×10^5 cells per ml) was seeded to each well

of an 8W10 ECIS array equilibrated with EC growth medium without serum and coated with collagen. When monolayers reached maximum resistance (~850 Ω) xanthine oxidase (0.08 U/ml) and 100 μM hypoxanthine were added to generate ROS and the endothelial integrity was measured in real-time as described (35).

Permeability of the BBB *in vitro*

Immortalized human hCMEC/D3 cells were cultured onto collagen (upper side, Sigma) coated Costar Transwell filters (pore-size 0.4 μm; Corning Incorporated, Corning, NY, USA), and rat primary ECs were cultured onto collagen en fibronectin coated Costar Transwell filters in endothelial cell growth medium 1:1 mixed with astrocyte-conditioned medium (36). Permeability for FITC-dextran (150 kDa) was assayed as described before (37), and the influence of ROS on the permeability was tested. At various time-points after addition of ROS samples were collected from the acceptor chambers for measurement of fluorescence intensity using a FLUOstar Galaxy microplate reader (BMG Labtechnologies, Offenburg, Germany), excitation 485 nm, emission 520 nm.

Western blot of tight junction proteins and PKB phosphorylation

GFP-occludin- or claudin-5-YFP expressing immortalized rat GP8/3.9 cells were grown to confluence and incubated with inhibitors for Rho GTPases (toxin B, 5 ng/ml), PI3 kinase (wortmannin, 2 μM) or PKB (triciribine, 12.5 μM) 2 h prior to ROS treatment. Immortalized rat GP8/3.9 cells were grown to confluence and maintained for 48 h in serum-free cell culture medium and incubated with wortmannin (2 μM), toxin B (5 ng/ml), superoxide dismutase (5000 U/ml), catalase (5000 U/ml), mannitol (50 mM), lipoic acid (300 μM), or luteolin (50 μM) 2 h prior to ROS treatment. After ROS treatment cells were lysed and proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Membranes were blocked with 5% lowfat milk powder and incubated with primary antibodies. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies. Supersignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) was used for detection of immunoreactive proteins. Protein band intensity was quantified using ImageQuant TL software (Amersham Biosciences, Piscataway, NJ, USA) and presented as percentage of control.

Rho-activation assay

Rho-activation was assayed using a RhoA G-LISA kit (Cytoskeleton, Denver, CO, USA). Immortalized rat GP8/3.9 cells were grown to 60% confluence and maintained for 48 h in serum-free cell culture medium. Cells were treated with ROS for 30 min, washed with ice-cold PBS, and scraped in ice-cold lysisbuffer. The RhoA G-LISA assay was performed according to manufacturer's prescription.

AMAXA transfection of GP8/3.9 cells

Immortalized rat GP8/3.9 cells (1.0×10^6) were transfected with 3 μg of DNA constructs (empty vector, dominant-negative Rho A N19, or constitutively active RhoA V14 (38), kind gift from Alan Hall (MRC Laboratory for Molecular Cell Biology, University College London, London, UK) using the Basic Nucleofector Kit for Primary Mammalian Endothelial Cells™ (Amaxa Biosystems, Gaithersburg, MD, USA).

Monocyte migration

Monocyte migration of primary human monocytes across confluent monolayers of immortalized human hCMEC/D3 cells was studied with time-lapse video microscopy as described previously (25). Freshly isolated monocytes (7.5×10^5/ml) were added to confluent monolayers of hCMEC/D3 cells, and the number of migrated monocytes was assessed after 4 h. The migration assay was conducted in the absence and presence of ROS and inhibitors for Rho GTPases (toxin B, 5 ng/ml), PI3 kinase (wortmannin, 2 μM), or PKB (triciribine, 12.5 μM).

Statistical analysis

Data were analyzed statistically by means of analysis of variance (ANOVA) and Student-Newman-Keuls test. Statistical significance was defined as $P < 0.05$.

RESULTS

ROS affect BBB integrity

To investigate the influence of ROS on the integrity of the brain endothelial barrier *in vitro*, transendothelial electrical resistance (TEER) of confluent human brain endothelial monolayers was measured in time. To mimic ROS production *in vitro*, we used a mixture of xanthine oxidase and hypoxanthine, which is known to produce constant levels of predominantly superoxide and to a lesser extent hydrogen peroxide and hydroxyl radicals (34). ROS time-dependently reduced TEER across a monolayer of immortalized human hCMEC/D3 brain ECs (**Fig. 1*A***; maximal effect after 20 min (35±3.9% decrease, $P<0.05$) or a monolayer of primary rat brain ECs (Fig. 1*B*; maximal effect after 45 min (28±2.5% decrease, $P<0.05$). We next determined the paracellular permeability toward large hydrophilic molecules. Addition of ROS to a human brain endothelial monolayer on Transwell filters enhanced leakage of FITC-dextran (150 kDa; 34±12.5% increase after 45 min, rising to 100±32.6% increase after 180 min, $P<0.05$, Fig. 1*C*). Similarly, ROS enhanced leakage of FITC-dextran across a monolayer of primary rat brain ECs (maximal effect after 60 min, 27±4.4% increase, $P<0.05$, Fig. 1*D*). ROS did not affect brain EC viability, which was routinely checked by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay and 7-amino-actinomycin D (7AAD) exclusion by flow cytometry within this time frame (data not shown).

BBB integrity is dynamically regulated by the actin cytoskeleton and dependent on the presence of tight junctions. Therefore, cytoskeletal alterations induced by ROS may be responsible for increased BBB permeability. To gain insight into the effect of ROS on actin cytoskeleton dynamics in brain endothelium, we determined the formation of F-actin bundles (stress-fibers) on exposure to ROS as described previously (24). ROS induced a significant increase of stress fiber formation at 60 min on the addition of ROS (31±6.1% increase, $P<0.001$, **Fig. 2*A***). In addition, hydrogen peroxide (100

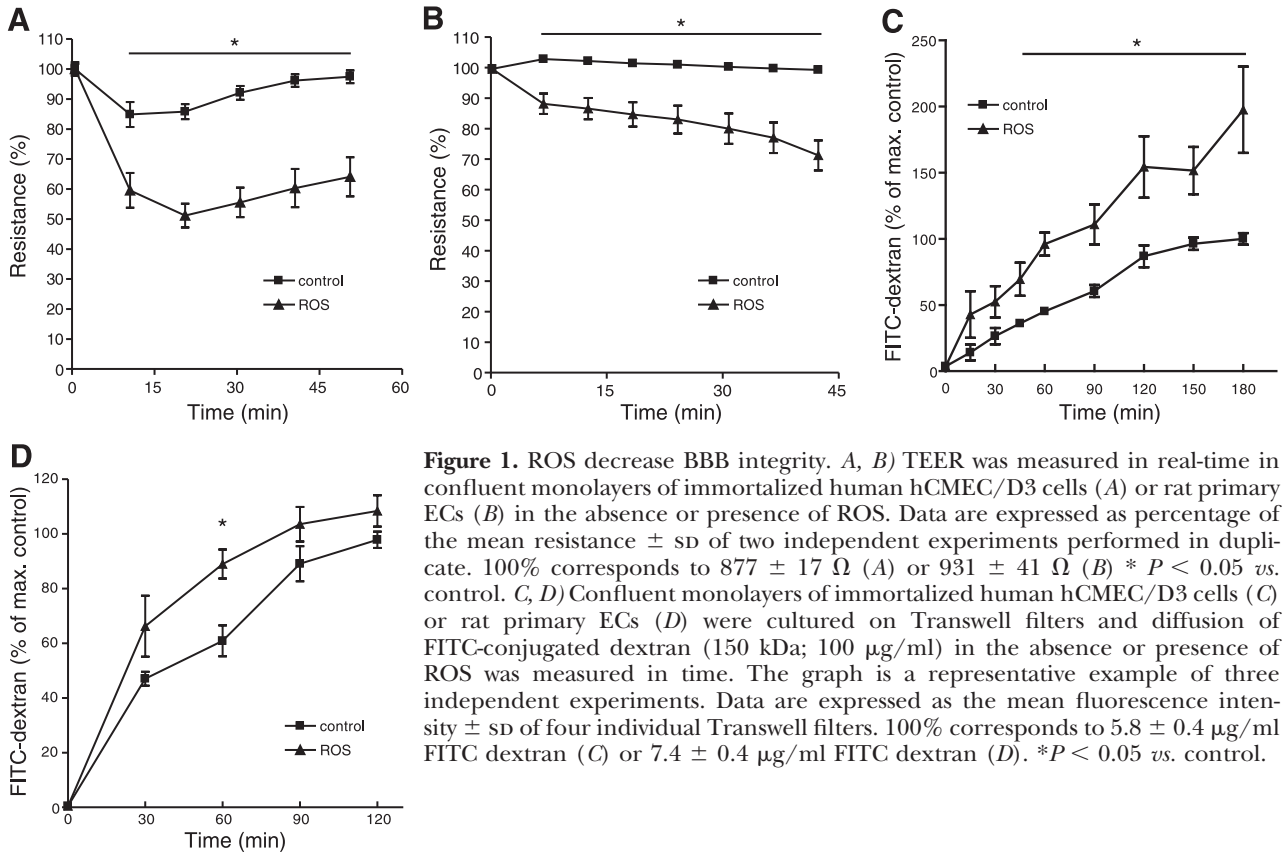


Figure 1. ROS decrease BBB integrity. *A, B)* TEER was measured in real-time in confluent monolayers of immortalized human hCMEC/D3 cells (*A*) or rat primary ECs (*B*) in the absence or presence of ROS. Data are expressed as percentage of the mean resistance ± SD of two independent experiments performed in duplicate. 100% corresponds to 877 ± 17 Ω (*A*) or 931 ± 41 Ω (*B*) * $P < 0.05$ *vs.* control. *C, D)* Confluent monolayers of immortalized human hCMEC/D3 cells (*C*) or rat primary ECs (*D*) were cultured on Transwell filters and diffusion of FITC-conjugated dextran (150 kDa; 100 μg/ml) in the absence or presence of ROS was measured in time. The graph is a representative example of three independent experiments. Data are expressed as the mean fluorescence intensity ± SD of four individual Transwell filters. 100% corresponds to 5.8 ± 0.4 μg/ml FITC dextran (*C*) or 7.4 ± 0.4 μg/ml FITC dextran (*D*). *$P < 0.05$ *vs.* control.

μM) solely similarly increased the number of F-actin fibers (26±8.6%, $P<0.05$, Fig. 2*B*). Hydrogen peroxide did not affect brain EC viability as was assayed by MTT and 7AAD (data not shown).

Furthermore, we have investigated the effect of ROS on the behavior of the TJ proteins claudin-5 and occludin. To study real-time dynamics of TJ proteins, we used a immortalized rat GP8/3.9 brain EC line overexpressing GFP-occludin, which was previously used in our group to study occludin dynamics under inflammatory conditions (29). In addition, we generated a immortalized rat brain EC line overexpressing fluorescently labeled claudin-5, in which claudin-5-YFP was localized at the plasma membrane, recognized by anticlaudin-5 antibodies and colocalized with endogenous ZO-1 (supplementary Fig. 1*A–F*). Further characterization of this cell line is shown in supplementary Fig. 1*G–I*. Live cell microscopy demonstrated that ROS, within 30 min, induced ruffling of cellular junctions (indicated by white arrows), which coincided with redistribution of occludin (**Fig. 3*A***) and claudin-5 (Fig. 3*C*) from the cellular junctions. Western blot analysis demonstrated that occludin expression decreased within a similar time frame, indicating that loss of membrane-associated occludin is directly followed by loss of protein expression (Fig. 3*B*). In addition, ROS-induced claudin-5 disappearance was detected after 60 min (Fig. 3*D*). Cell viability was assayed by MTT and 7AAD and remained unaffected (data not shown).

ROS specifically and transiently activates PKB in brain endothelial cells

A number of signal transduction pathways, including the PI3 kinase pathway (39, 40), have been suggested to be involved in the regulation of TJs and the cytoskeleton and may regulate ROS-induced signals. Therefore, we hypothesize that its downstream target PKB could also be implicated in these processes. To study the effect of ROS on PKB activation in immortalized rat GP8/3.9 brain ECs, we determined the phosphorylation state of PKB by Western blotting. Addition of ROS significantly induced transient PKB phosphorylation at Ser-473, which is essential for PKB activation (41), without affecting total PKB levels. PKB phosphorylation was maximal at 30 min after the addition of ROS (52±7.1% increase, $P<0.001$, **Fig. 4*A***), which was more potent than hydrogen peroxide alone (32±7.7% increase, $P<0.01$, Fig. 4*B*). Incubation with wortmannin, a selective inhibitor of PI3 kinase, revealed that ROS-induced PKB phosphorylation was dependent on PI3 kinase (69±11.3% decrease compared to ROS alone, $P<0.01$, Fig. 4*A*). Another selective inhibitor of PI3 kinase, LY294002, equally inhibited ROS-induced PKB phosphorylation (data not shown). ROS can be scavenged by broad-spectrum antioxidants like lipoic acid and luteolin, or by more specific scavengers like catalase, superoxide dismutase (SOD) and mannitol. Superoxide can be converted by SOD to hydrogen peroxide. In turn, hydrogen peroxide can be decomposed by

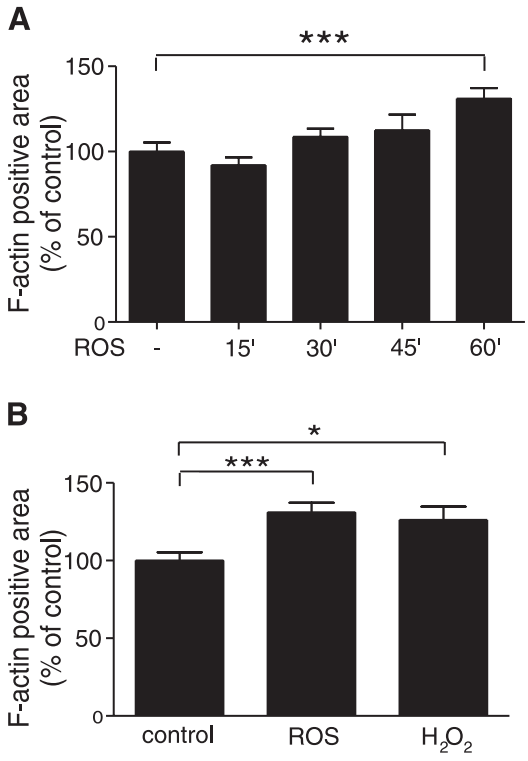


Figure 2. ROS induce cytoskeleton rearrangements in brain ECs. *A)* Quantification of F-actin stress fiber formation in phalloidin-rhodamin stained immortalized rat GP8/3.9 cells at various time-points after exposure to ROS. ROS significantly induced stress fiber formation after 60 min. Data are expressed as mean ± SEM of at least 75 cells per condition and are presented as percentage of control. ***$P < 0.001$. *B)* Quantification of stress fiber formation in phalloidin-rhodamin stained immortalized rat GP8/3.9 cells 60 min after addition of ROS or 100 μM hydrogen peroxide. Data are expressed as mean ± SEM of at least 75 cells per condition and are presented as percentage of control. *$P < 0.05$; ***$P < 0.001$.

catalase into water and oxygen. Alternatively, hydrogen peroxide can be used to generate hydroxyl radicals, which can be scavenged by mannitol. All these antioxidants completely inhibited ROS-induced PKB phosphorylation, demonstrating that superoxide, and to a certain extent hydrogen peroxide and hydroxyl radical, contribute to the ROS-induced PKB phosphorylation (Fig. 4*C*).

Rho is an upstream mediator of PI3 kinase signaling

To identify upstream mediators of ROS-induced PKB phosphorylation, we examined the effects of specific inhibitors of candidate signal transduction molecules in this pathway. Inhibition of Rho GTPase-mediated signaling by toxin B (5 ng/ml) completely blocked ROS-induced PKB phosphorylation ($P<0.001$), indicating that activation of Rho family GTPases is a prerequisite of ROS-induced PKB activation in brain ECs (**Fig. 5*A***). In addition, our results showed that ROS induced RhoA activation (Fig. 5*B*). To test whether RhoA activity was required for ROS-induced activation of PKB, we overexpressed a dominant-negative mutant of RhoA (RhoA N19) or a constitutively active mutant of RhoA (RhoA V14) (38) in immortalized rat GP8/3.9 brain EC. The expression of constitutively active RhoA coincided with increased baseline PKB phosphorylation levels, which slightly increased after ROS addition, whereas the presence of dominant-negative RhoA resulted into decreased baseline PKB phosphorylation levels and blocked ROS-induced PKB phosphorylation (Fig. 5*C*). These results indicate that ROS induces PKB phosphorylation through RhoA-activation.

PKB phosphorylation and Rho-activation mediate the effects of ROS on brain EC integrity

Next, we examined whether Rho, PI3 kinase, and PKB are involved in cytoskeleton and TJ alterations induced by ROS. Western blot analysis revealed that the Rho-inhibitor toxin B, the PI3 kinase inhibitor wortmannin, and the PKB inhibitor triciribine prevented ROS-induced disappearance of GFP-occludin (**Fig. 6*A***) and claudin-5-YFP (Fig. 6*B*). Moreover, these inhibitors also reduced ROS-induced formation of actin stress fibers (Fig. 6*C*). Together, these results suggest that signaling pathways involving Rho, PI3 kinases, and PKB mediate ROS-induced cytoskeleton and TJ reorganization.

Specific inhibitors for Rho, PI3 kinase, and PKB can prevent ROS-induced transendothelial monocyte migration

Under pathological conditions, ROS significantly contribute to BBB dysfunction and inflammation in the brain by enhancing cellular migration. To study the role of Rho, PI3 kinase, and PKB in ROS-induced monocyte migration, we used an *in vitro* model of the BBB as described previously (25). ROS induced the migration of primary human monocytes across immortalized human hCMEC/D3 brain ECs (38±3.8% increase, **Fig. 7**). Specific inhibitors of Rho (42±3.5% decrease), PI3 kinase (40±3.5% decrease), and PKB (41±3.3% decrease) prevented this ROS-induced migration (Fig. 7, ***$P<0.001$). In addition, the same inhibitors also prevented ROS-induced migration of primary rat monocytes across immortalized rat GP8/3.9 brain ECs (data not shown) and a specific RhoA inhibitor (C3 transferase) prevented ROS-induced migration of primary human monocytes across immortalized human hCMEC/D3 brain ECs (Supplementary Fig. 2). Furthermore, basal monocyte migration across untreated human brain ECs ($^{\#\#}P<0.01$) was also inhibited by the same specific inhibitors for Rho (16±2.4% decrease), PI3 kinase (17±2.3% decrease), and PKB (14±1.6% decrease). Together, these results indicate that these signaling pathways mediate ROS-induced monocyte migration across an *in vitro* model of the BBB.

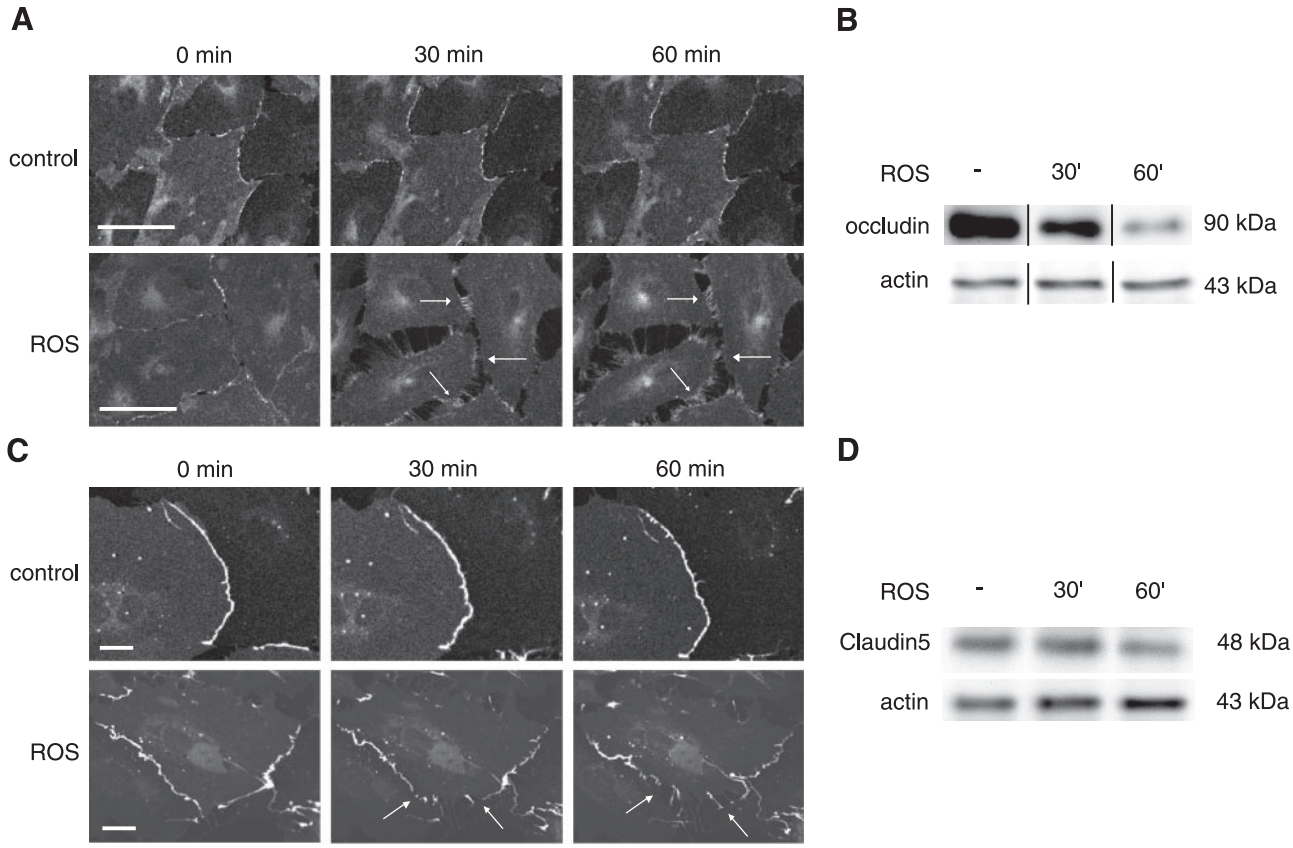


Figure 3. ROS induce redistribution and degradation of GFP-occludin and claudin-5-YFP. *A)* Occludin dynamics were followed in immortalized rat GP8/3.9 cells expressing GFP-occludin using live cell confocal microscopy. Thirty minutes after addition of ROS occludin started to ruffle (indicated by arrows) and brain ECs detached, which further increased after 60 min. Per condition one representative area out of 10 is shown. Bars: 25 μm. *B)* Representative Western blot of GFP-occludin expressing GP8/3.9 cells showing that ROS induced loss of occludin expression already after 30 min, which further decreased after 60 min. GFP-occludin was detected with monoclonal antibodies directed against occludin. Actin was used as a control for protein loading. Dividing lines represent the grouping of different parts of the same Western blot. *C)* Claudin-5-YFP dynamics were followed using live cell confocal microscopy. Thirty minutes after addition of ROS, claudin-5 started to ruffle (indicated by arrows) and brain ECs detached, which further increased after 60 min. Per condition one representative area out of 10 is shown. Bars: 10 μm. *D)* Representative Western blot of claudin-5-YFP expressing GP8/3.9 cells showing that ROS induced claudin-5 disappearance after 60 min. claudin-5-YFP was detected with monoclonal antibodies directed against claudin-5. Actin was used as a control for protein loading.

DISCUSSION

Under basal physiological conditions, the BBB acts as a barrier to the immune system limiting the entry of leukocytes. Nevertheless, in neuroinflammatory diseases, a disruption of BBB integrity occurs, which can be accompanied by the transmigration of activated leukocytes into the CNS. We have previously shown that ROS are produced on adhesion of monocytes to brain ECs and contribute to BBB disruption (24). Here, we demonstrate for the first time that extracellular ROS modulate BBB integrity by transient activation of the PI3 kinase and PKB pathway via RhoA, which acutely disrupts the integrity of the TJs, allowing paracellular transport to occur.

ROS altered brain EC integrity within minutes by decreasing TEER and increasing brain EC permeability. ROS also induced cytoskeletal rearrangements, redistribution, and disappearance of both occludin and claudin-5 in a time-dependent manner. As claudin-5 is thought to be a critical component of TJ in the BBB (7), ROS-induced redistribution and disappearance of claudin-5 may be an important mechanism underlying enhanced BBB permeability. Recently, it was shown that hydrogen peroxide exposure enhances BBB permeability and reduces TEER (27, 42, 43) as well as facilitate the formation of actin stress fibers in bovine brain endothelium (43). Our data confirm and extend these results as superoxide, which is the most predominant ROS produced in our ROS generating system, has the capacity to do this as well. The organization of TJ proteins occludin and ZO-1 is altered by exogenous ROS in both epithelial cells (44–46) and brain ECs (43, 47). However, we are the first to demonstrate that ROS affected claudin-5 expression at the cellular junction.

In this study we established that the PKB pathway is an important mediator of ROS-induced alterations in brain ECs. Exogenous superoxide and hydrogen peroxide activated PI3 kinase and its downstream target PKB in a transient manner in brain ECs. Moreover,

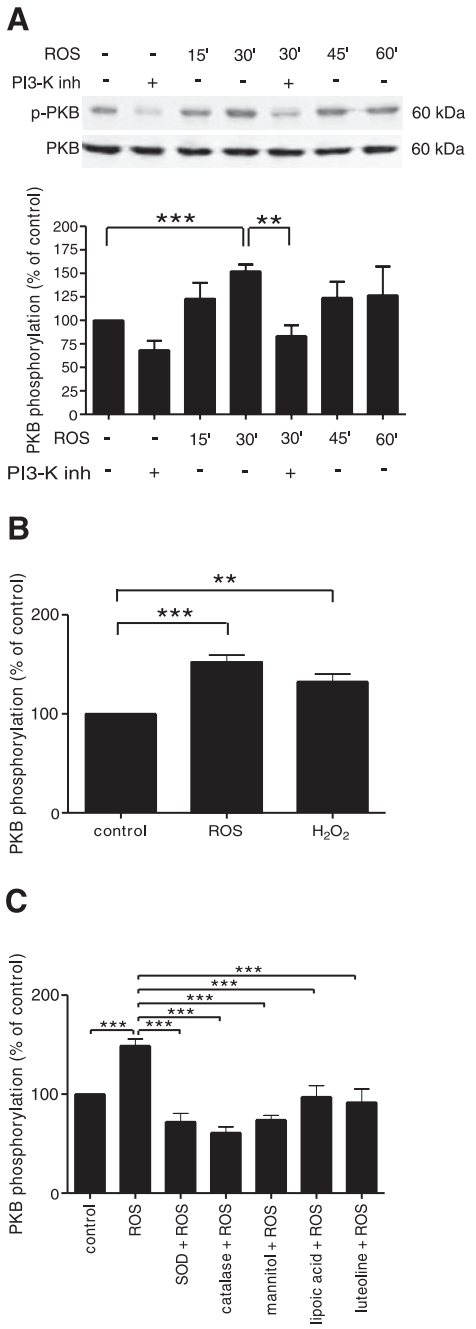


Figure 4. ROS induce PKB phosphorylation in brain endothelial cells. *A)* Western blot analysis and quantification of PKB phosphorylation in immortalized rat GP8/3.9 cells showing that ROS induced PKB phosphorylation time dependently. ROS-induced PKB-phosphorylation was prevented by the PI3-kinase inhibitor wortmannin (2 μM). One representative Western blot out of three experiments is shown. Data are expressed as mean ± SEM relative to total PKB of at least three independent experiments and are presented as % of control. $^{**}P < 0.01$; $^{***}P < 0.001$. *B)* Quantification of the influence of ROS or hydrogen peroxide (100 μM) on PKB-phosphorylation in immortalized rat GP8.3 cells. Cells were exposed to ROS and hydrogen peroxide for 60 min. Data are expressed as mean ± SEM of at least three independent experiments and are presented as % of control. $^{**}P < 0.01$; $^{***}P < 0.001$. *C)* Quantification of ROS-induced PKB phosphorylation in immortalized rat GP8/3.9 cells in the presence of the antioxidants SOD (5000 U/ml), catalase (5000 U/ml), mannitol (50 mM), lipoic acid (300 μM), and luteoline (50 μM). Cells were incubated with antioxidants 2 h prior to the addition of ROS. Cells were exposed to ROS for 60 min. All antioxidants used significantly prevented ROS-induced PKB-phosphorylation. Data are expressed as mean ± SEM of at least 3 independent experiments and are presented as percentage of control. $^{***}P < 0.001$ compared to ROS-treated cells.

inhibition of PI3-kinase or PKB activity prevented the formation of F-actin stress-fibers as well as the disappearance of occludin and claudin-5, indicating that the PI3 kinase and PKB signaling pathway have a pivotal role in ROS-induced loss of BBB integrity. Thus far, knowledge about the role of ROS-induced signal transfer in the regulation of claudin-5 in brain ECs is limited. It has been reported that claudin-5 can be phosphorylated after exposure of brain ECs to ROS (27, 48). Furthermore, evidence suggests that the phosphorylation state of occludin is important in the regulation of TJ assembly and disassembly (49). Occludin can be phosphorylated on Ser, Thr, and Tyr residues (50) and in epithelial cells extracellular ROS induced tyrosine phosphorylation and dissociation of occludin from intercellular junctions (44, 45). In addition, the ROS-induced claudin-5 and occludin disappearance may also be explained by protein degradation. However, no degradation products were detected using the current antibodies (data not shown). Previously (29), we postulated that the ubiquitin-proteasome pathway might affect tight junction stability, since *in vivo* evidence suggests that occludin is ubiquitinated and its turnover is retarded after treatment with a proteasome inhibitor (51). Whether such a pathway is involved in occludin and claudin-5 degradation remains to be determined. Together, our data provide conclusive evidence that the PI3 kinase and PKB pathway mediates ROS-induced dysregulation of occludin and claudin-5 and suggest that PKB is the putative kinase that may regulate occludin and claudin-5 dynamics.

Inhibition of Rho activation prevented ROS-induced cytoskeleton rearrangements and disappearance of occludin and claudin-5. These data indicate that ROS activate Rho, which subsequently may affect the actin cytoskeleton via PI3 kinase and PKB. It has been reported that cytoskeleton depolymerization causes redistribution of TJ molecules (52, 53). Hence, signaling molecules that control the organization of the actin cytoskeleton may indirectly be involved in the regulation of TJs. The family of Ras-related small GTP-binding proteins RhoA, Rac1, and Cdc42 are such regulators of the actin cytoskeleton, and it has been described that RhoA activation leads to phosphorylation of occludin and claudin-5 and TJ reorganization(18, 54, 55). The broad-spectrum Rho-GTPase inhibitor toxin B blocked ROS-induced PKB phosphorylation. However, a more specific inhibitor of RhoA (C3 transferase) prevented ROS-induced cytoskeleton rearrangements. Overexpression of constitutively active RhoA in our brain ECs coincided with increased baseline PKB phosphorylation levels, whereas the presence of dominant-negative

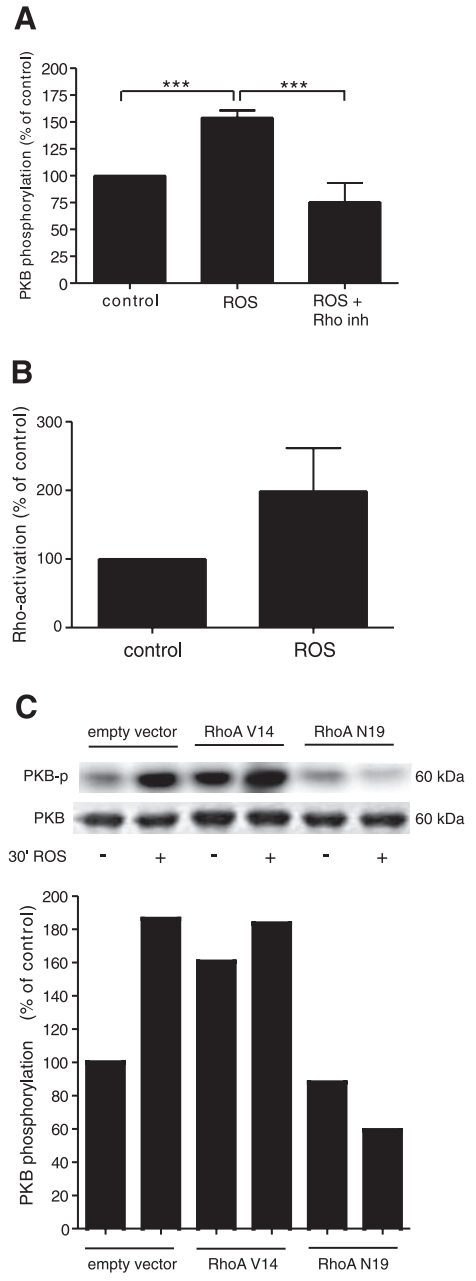


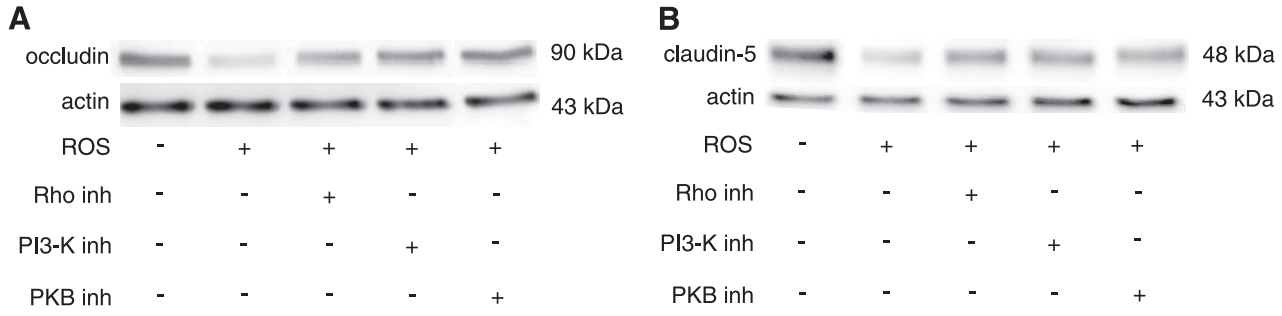
Figure 5. RhoA is an upstream mediator of ROS-induced PKB phosphorylation. *A)* Quantification of PKB-phosphorylation. Immortalized rat GP8/3.9 cells were treated with the Rho-inhibitor toxin B (5 ng/ml) 2 h prior to ROS treatment. Cells were treated for 30 min with ROS. Data are expressed as mean ± SEM relative to total PKB of at least 3 independent experiments and are presented as percentage of control. $***P < 0.001$. *B)* ROS induced Rho-activation in immortalized rat GP8/3.9 cells. Data are expressed as mean ± SEM of three independent experiments and are presented as percentage of control. *C)* Western blot analysis and quantification of PKB phosphorylation relative to total PKB in immortalized rat GP8/3.9 cells transfected with empty vector, constitutively active RhoA (RhoA V14), or dominant-negative RhoA (RhoA N19) in the absence or presence of ROS.

RhoA resulted into decreased baseline PKB phosphorylation levels and blocked ROS-induced PKB phosphorylation. We therefore conclude that RhoA is an upstream activator of the PI3 kinase pathway, which is in line with previous studies (56, 57). It should be noted that the relationship between PKB and the Rho family of GTPases is not unambiguous and may depend on stimuli and cell type. A number of reports suggest that Rho-activation is a downstream event of PI3 kinase signaling (58, 59). In our brain EC model, however, Rho acts upstream of PI3 kinase and ensuing activation of PKB allows direct interaction with the cytoskeleton (60).

Specific inhibitors for Rho, PI3 kinase, and PKB mediate ROS-induced monocyte migration across an *in vitro* model of the human BBB. Infiltration of leukocytes into the CNS is a crucial event during neuroinflammatory diseases. We have previously shown that PI3 kinase plays an important role during ROS-induced monocyte transmigration across rat brain ECs (25). Furthermore, inhibition of RhoA in brain endothelium resulted in decreased cellular migration *in vitro* and *in vivo* (61, 62). Our data confirm and extend these results as a specific inhibitor of PKB can prevent ROS-induced monocyte migration across an *in vitro* model of the BBB.

In summary, our data provide evidence that ROS specifically activate the PKB signaling pathway via RhoA in brain ECs. We have conclusively shown that activation of the PKB pathway, either directly or indirectly via the actin cytoskeleton, causes dissociation and subsequent disappearance of the TJ proteins claudin-5 and occludin. Furthermore, inhibiting PKB activity prevented ROS-induced monocyte migration across an *in vitro* model of the BBB. Our results may support the imagination of an immunomodulatory role of ROS in TJ dynamics. By down-regulating TJ assembly, ROS contribute to altered BBB integrity, thereby facilitating the influx of leukocytes and thus supporting an inflammatory response. Furthermore, PKB phosphorylation and RhoA activation may be early markers of BBB dysfunction. Agents that selectively inhibit this effect, like antioxidants or signaling inhibitors for RhoA, PI3 kinase, or PKB, may be used therapeutically to modulate neuroinflammatory diseases complicated by BBB dysfunction. FJ

We thank Dr. A. Hall (MRC Laboratory for Molecular Cell Biology, University College London, London, UK) for providing the pEXV/myc-RhoA V14 and pEXV/myc-RhoA N19 constructs; Dr. Peter L. Hordijk (Sanquin Research, Amsterdam, The Netherlands) for providing the retroviral construct LZRS-EGFP-actin-IRES-zeocin; L. Grijpink-Ongering (Solvay Pharmaceuticals Research Laboratories, Weesp, The Netherlands) for practical assistance with the (phosphorylation-) PKB Western blots; and Dr. R.J.P. Musters (Laboratory for Physiology, VU University Medical Center, The Netherlands) for technical assistance with the 3I MarianasTM digital microscopy workstation. This work was supported by grants from the Institute for Clinical and Experimental Neurosciences, VU University Medical Center, Amsterdam, The Netherlands (G. Schreibelt), The Netherlands Organization of Scientific Research (grant 016.046.314, G. Kooij, A. Reijerkerk), the Dutch foundation of MS Research, The Netherlands (grant MS 02–358, H.E. de Vries; grant 05–358c, R. van Doorn), and the Dutch Asthma Foundation (grant 3.2.03.39, S. Gringhuis).



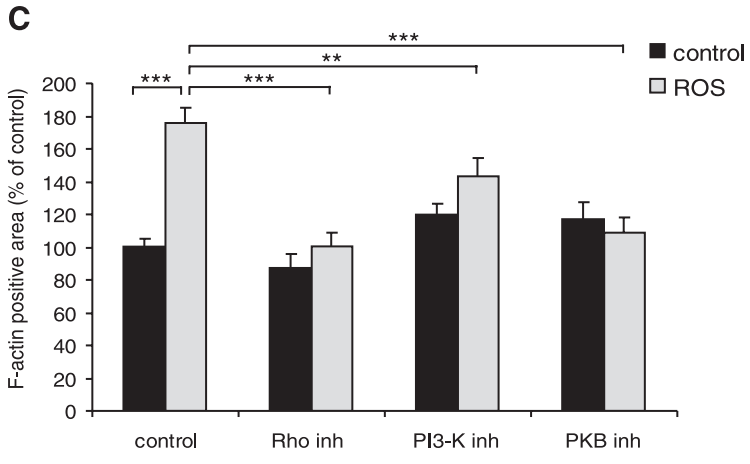


Figure 6. ROS-induced BBB alterations are mediated through Rho, PI3 kinase and PKB. *A, B)* Western blot analysis of total cell lysates of GFP-occludin and claudin-5-YFP expressing GP8/3.9 cells showing that ROS-induced occludin and claudin-5 disappearance is prevented by a Rho-inhibitor (toxin-B, 5 ng/ml), PI3-kinase inhibitor (wortmannin, 2 μM) and a specific PKB inhibitor (triciribine, 12.5 μM). Cells were treated with inhibitors for 2 h prior to ROS exposure, and cells were exposed to ROS for 120 min. Actin was used as a control for protein loading. *C)* Quantification of stress fiber formation in phalloidin-rhodamin stained immortalized rat GP8/3.9 cells 60 min after addition of ROS. Cells were incubated with a specific Rho-inhibitor C3-transferase (10 μg/ml) 24 h prior to ROS exposure or with the PI3 kinase inhibitor wortmannin (2 μM) and the PKB inhibitor triciribine (12.5 μM) 2 h prior to ROS exposure. All inhibitors significantly reduced ROS-induced stress fiber formation. Data are expressed as mean ± SEM of at least 75 cells per condition and are presented as % of control. $^{**}P < 0.01$; $^{***}P < 0.001$.

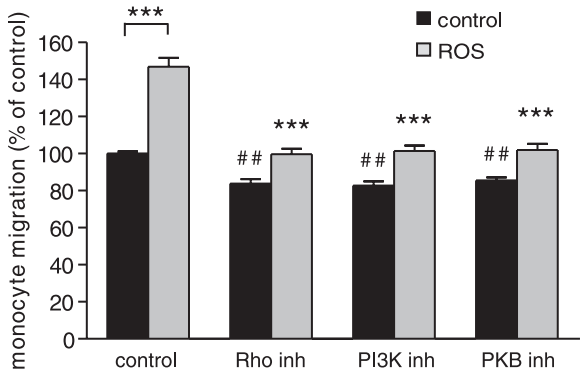


Figure 7. ROS-induced monocyte migration is mediated through Rho, PI3 kinase, and PKB. Monocyte migration of primary human monocytes across confluent monolayers of hCMEC/D3 cells was studied with time-lapse video microscopy. Before the migration experiment, hCMEC/D3 cells were incubated for 30 min with a Rho inhibitor (toxin-B, 5 ng/ml), PI-3 kinase inhibitor (wortmannin, 2 μM), or PKB inhibitor (triciribine, 12.5 μM), followed by 2 h incubation with these inhibitors (black bars) or by ROS exposure in the presence of these inhibitors (gray bars). Data are presented as the mean percentage of control migration of 8 wells ± SEM. Migration across untreated hCMEC/D3 cells served as a control and was regarded as 100%, which corresponds to 14.3 ± 0.3% of migrated cells of total cells. $^{\#\#}P < 0.01$: effect of inhibitors *vs.* control migration, $^{***}P < 0.001$: effect of inhibitors *vs.* ROS-induced migration.

REFERENCES

1. Pachter, J. S., de Vries, H. E., and Fabry, Z. (2003) The blood-brain barrier and its role in immune privilege in the central nervous system. *J. Neuropathol. Exp. Neurol.* **62,** 593–604
2. Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1993) Occludin: a novel integral membrane protein localizing at tight junctions. *J. Cell Biol.* **123,** 1777–1788
3. Tsukita, S., and Furuse, M. (1999) Occludin and claudins in tight-junction strands: leading or supporting players? *Trends. Cell Biol.* **9,** 268–273
4. Balda, M. S., Flores-Maldonado, C., Cereijido, M., and Matter, K. (2000) Multiple domains of occludin are involved in the regulation of paracellular permeability. *J. Cell. Biochem.* **78,** 85–96
5. Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., and Tsukita, S. (1998) Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J. Cell Biol.* **141,** 1539–1550
6. Furuse, M., Sasaki, H., and Tsukita, S. (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J. Cell Biol.* **147,** 891–903
7. Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., and Tsukita, S. (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J. Cell Biol.* **161,** 653–660
8. Fanning, A. S., Jameson, B. J., Jesaitis, L. A., and Anderson, J. M. (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J. Biol. Chem.* **273,** 29745–29753
9. Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1994) Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J. Cell Biol.* **127,** 1617–1626
10. Anderson, J. M. (1996) Cell signalling: MAGUK magic. *Curr. Biol.* **6,** 382–384

11. Matter, K., and Balda, M. S. (2003) Signalling to and from tight junctions. *Nat. Rev. Mol. Cell. Biol.* **4,** 225–236
12. Lai, C. H., Kuo, K. H., and Leo, J. M. (2005) Critical role of actin in modulating BBB permeability. *Brain. Res. Brain. Res. Rev.* **50,** 7–13
13. Kooij, G., van Horssen, J., and de Vries, H. E. (2005) Tight junctions of the blood-brain barrier. In *The Blood-Brain Barrier and Its Microenvironment* (de Vries, H. E., and Prat, A., eds) pp. 47–139, Taylor & Francis Group, New York, NY, USA
14. Hawkins, B. T., and Davis, T. P. (2005) The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol. Rev.* **57,** 173–185
15. Vos, C. M., Geurts, J. J., Montagne, L., van Haastert, E. S., Bo, L., van, d., V, Barkhof, F., and de Vries, H. E. (2005) Blood-brain barrier alterations in both focal and diffuse abnormalities on postmortem MRI in multiple sclerosis. *Neurobiol. Dis.* **20,** 953–960
16. Kirk, J., Plumb, J., Mirakhur, M., and McQuaid, S. (2003) Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood-brain barrier leakage and active demyelination. *J. Pathol.* **201,** 319–327
17. Boven, L. A., Middel, J., Verhoef, J., De Groot, C. J., and Nottet, H. S. (2000) Monocyte infiltration is highly associated with loss of the tight junction protein zonula occludens in HIV-1-associated dementia. *Neuropathol. Appl. Neurobiol.* **26,** 356–360
18. Persidsky, Y., Heilman, D., Haorah, J., Zelivyanskaya, M., Persidsky, R., Weber, G. A., Shimokawa, H., Kaibuchi, K., and Ikezu, T. (2006) Rho-mediated regulation of tight junctions during monocyte migration across blood-brain barrier in HIV-1 encephalitis (HIVE). *Blood* **107,** 4770–4780.
19. Dallasta, L. M., Pisarov, L. A., Esplen, J. E., Werley, J. V., Moses, A. V., Nelson, J. A., and Achim, C. L. (1999) Blood-brain barrier tight junction disruption in human immunodeficiency virus-1 encephalitis. *Am. J. Pathol.* **155,** 1915–1927
20. Mark, K. S., and Davis, T. P. (2002) Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation. *Am. J. Physiol. Heart. Circ. Physiol.* **282,** H1485–H1494
21. Song, L., and Pachter, J. S. (2004) Monocyte chemoattractant protein-1 alters expression of tight junction-associated proteins in brain microvascular endothelial cells. *Microvasc. Res.* **67,** 78–89
22. De Vries, H. E., Blom-Roosemalen, M. C., van Oosten, M., de Boer, A. G., van Berkel, T. J., Breimer, D. D., and Kuiper, J. (1996) The influence of cytokines on the integrity of the blood-brain barrier in vitro. *J. Neuroimmunol.* **64,** 37–43
23. Deli, M. A., Abraham, C. S., Kataoka, Y., and Niwa, M. (2005) Permeability studies on in vitro blood-brain barrier models: physiology, pathology, and pharmacology. *Cell. Mol. Neurobiol.* **25,** 59–127
24. Schreibelt, G., Musters, R. J., Reijerkerk, A., de Groot, L. R., Van Der Pol, S. M., Hendrikx, E. M., Dopp, E. D., Dijkstra, C. D., Drukarch, B., and de Vries, H. E. (2006) Lipoic acid affects cellular migration into the central nervous system and stabilizes blood-brain barrier integrity. *J. Immunol.* **177,** 2630–2637
25. Van der Goes, A., Wouters, D., Van Der Pol, S. M., Huizinga, R., Ronken, E., Adamson, P., Greenwood, J., Dijkstra, C. D., and de Vries, H. E. (2001) Reactive oxygen species enhance the migration of monocytes across the blood-brain barrier in vitro. *FASEB J.* **15,** 1852–1854
26. Hendriks, J. J., Alblas, J., Van Der Pol, S. M., van Tol, E. A., Dijkstra, C. D., and de Vries, H. E. (2004) Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. *J. Exp. Med.* **200,** 1667–1672
27. Haorah, J., Ramirez, S. H., Schall, K., Smith, D., Pandya, R., Persidsky, Y. (2007) Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J. Neurochem.* **101,** 566–576.
28. Blasig, I. E., Winkler, L., Lassowski, B., Mueller, S. L., Zuleger, N., Krause, E., Krause, G., Gast, K., Kolbe, M., and Piontek, J. (2006) On the self-association potential of transmembrane tight junction proteins. *Cell. Mol. Life. Sci.* **63,** 505–514
29. Reijerkerk, A., Kooij, G., Van Der Pol, S. M., Khazen, S., Dijkstra, C. D., and de Vries, H. E. (2006) Diapedesis of monocytes is associated with MMP-mediated occludin disappearance in brain endothelial cells. *FASEB J.* **20,** 2550–2552.
30. Greenwood, J., Pryce, G., Devine, L., Male, D. K., dos Santos, W. L., Calder, V. L., and Adamson, P. (1996) SV40 large T immortalised cell lines of the rat blood-brain and blood-retinal barriers retain their phenotypic and immunological characteristics. *J. Neuroimmunol.* **71,** 51–63
31. De Vries, H. E., Hendriks, J. J., Honing, H., De Lavalette, C. R., Van Der Pol, S. M., Hooijberg, E., Dijkstra, C. D., and van den Berg, T. K. (2002) Signal-regulatory protein alpha-CD47 interactions are required for the transmigration of monocytes across cerebral endothelium. *J. Immunol.* **168,** 5832–5839
32. Weksler, B. B., Subileau, E. A., Perriere, N., Charneau, P., Holloway, K., Leveque, M., Tricoire-Leignel, H., Nicotra, A., Bourdoulous, S., Turowski, P., Male, D. K., Roux, F., Greenwood, J., Romero, I. A., and Couraud, P. O. (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J.* **19,** 1872–1874
33. Perriere, N., Demeuse, P., Garcia, E., Regina, A., Debray, M., Andreux, J. P., Couvreur, P., Scherrmann, J. M., Temsamani, J., Couraud, P. O., Deli, M. A., and Roux, F. (2005) Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood-brain barrier-specific properties. *J. Neurochem.* **93,** 279–289
34. Shatos, M. A., Doherty, J. M., Orfeo, T., Hoak, J. C., Collen, D., and Stump, D. C. (1992) Modulation of the fibrinolytic response of cultured human vascular endothelium by extracellularly generated oxygen radicals. *J. Biol. Chem.* **267,** 597–601
35. Keese, C. R., Wegener, J., Walker, S. R., and Giaever, I. (2004) Electrical wound-healing assay for cells in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1554–1559
36. De Groot, C. J., Langeveld, C. H., Jongenelen, C. A., Montagne, L., van, d., V, and Dijkstra, C. D. (1997) Establishment of human adult astrocyte cultures derived from postmortem multiple sclerosis and control brain and spinal cord regions: immunophenotypical and functional characterization. *J. Neurosci. Res.* **49,** 342–354
37. De Vries, H. E., Blom-Roosemalen, M. C., de Boer, A. G., van Berkel, T. J., Breimer, D. D., and Kuiper, J. (1996) Effect of endotoxin on permeability of bovine cerebral endothelial cell layers in vitro. *J. Pharmacol. Exp. Ther.* **277,** 1418–1423
38. Nobes, C. D., and Hall, A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81,** 53–62
39. Massoumi, R., and Sjolander, A. (1998) The inflammatory mediator leukotriene D4 triggers a rapid reorganisation of the actin cytoskeleton in human intestinal epithelial cells. *Eur. J. Cell Biol.* **76,** 185–191
40. Nusrat, A., Chen, J. A., Foley, C. S., Liang, T. W., Tom, J., Cromwell, M., Quan, C., and Mrsny, R. J. (2000) The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. *J. Biol. Chem.* **275,** 29816–29822
41. Woodgett, J. R. (2005) Recent advances in the protein kinase B signaling pathway. *Curr. Opin. Cell Biol.* **17,** 150–157
42. Blasig, I. E., Mertsch, K., Haseloff, R. F. (2002) Nitronyl nitroxides, a novel group of protective agents against oxidative stress in endothelial cells forming the blood-brain barrier. *Neuropharmacology* **43,** 1006–1014
43. Lee, H. S., Namkoong, K., Kim, D. H., Kim, K. J., Cheong, Y. H., Kim, S. S., Lee, W. B., and Kim, K. Y. (2004) Hydrogen peroxide-induced alterations of tight junction proteins in bovine brain microvascular endothelial cells. *Microvasc. Res.* **68,** 231–238
44. Basuroy, S., Sheth, P., Kuppuswamy, D., Balasubramanian, S., Ray, R. M., and Rao, R. K. (2003) Expression of kinase-inactive c-Src delays oxidative stress-induced disassembly and accelerates calcium-mediated reassembly of tight junctions in the Caco-2 cell monolayer. *J. Biol. Chem.* **278,** 11916–11924
45. Rao, R. K., Basuroy, S., Rao, V. U., Karnaky Jr., K. J., and Gupta, A. (2002) Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. *Biochem. J.* **368,** 471–481
46. Sheth, P., Basuroy, S., Li, C., Naren, A. P., and Rao, R. K. (2003) Role of phosphatidylinositol 3-kinase in oxidative stress-induced disruption of tight junctions. *J. Biol. Chem.* **278,** 49239–49245

47. Fischer, S., Wiesnet, M., Renz, D., and Schaper, W. (2005) H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. *Eur. J. Cell Biol.* **84,** 687–697
48. Haorah, J., Knipe, B., Leibhart, J., Ghorpade, A., and Persidsky, Y. (2005) Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. *J. Leukoc. Biol.* **78,** 1223–1232
49. Wong, V. (1997) Phosphorylation of occludin correlates with occludin localization and function at the tight junction. *Am. J. Physiol.* **273,** C1859–C1867
50. Sakakibara, A., Furuse, M., Saitou, M., ndo-Akatsuka, Y., and Tsukita, S. (1997) Possible involvement of phosphorylation of occludin in tight junction formation. *J. Cell Biol.* **137,** 1393–1401
51. Traweger, A., Fang, D., Liu, Y. C., Stelzhammer, W., Krizbai, I. A., Fresser, F., Bauer, H. C., and Bauer, H. (2002) The tight junction-specific protein occludin is a functional target of the E3 ubiquitin-protein ligase itch. *J. Biol. Chem.* **277,** 10201–10208
52. Shen, L., and Turner, J. R. (2005) Actin depolymerization disrupts tight junctions via caveolae-mediated endocytosis. *Mol. Biol. Cell.* **16,** 3919–3936
53. Stevenson, B. R., and Begg, D. A. (1994) Concentration-dependent effects of cytochalasin D on tight junctions and actin filaments in MDCK epithelial cells. *J. Cell Sci.* **107 (Pt 3),** 367–375
54. Nusrat, A., Giry, M., Turner, J. R., Colgan, S. P., Parkos, C. A., Carnes, D., Lemichez, E., Boquet, P., and Madara, J. L. (1995) Rho protein regulates tight junctions and perijunctional actin organization in polarized epithelia. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10629–10633
55. Jou, T. S., Schneeberger, E. E., and Nelson, W. J. (1998) Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. *J. Cell Biol.* **142,** 101–115
56. Nguyen, K. T., Zong, C. S., Uttamsingh, S., Sachdev, P., Bhanot, M., Le, M. T., Chan, J. L., Wang, L. H. (2002) The role of phosphatidylinositol 3-kinase, rho family GTPases, and STAT3 in Ros-induced cell transformation. *J. Biol. Chem.* **277,** 11107–11115
57. Prasad, R., Giri, S., Nath, N., Singh, I., and Singh, A. K. (2005) Inhibition of phosphoinositide 3 kinase-Akt (protein kinase B)-nuclear factor-kappa B pathway by lovastatin limits endothelial-monocyte cell interaction. *J. Neurochem.* **94,** 204–214
58. Perez, V., Bouschet, T., Fernandez, C., Bockaert, J., and Journot, L. (2005) Dynamic reorganization of the astrocyte actin cytoskeleton elicited by cAMP and PACAP: a role for phosphatidylInositol 3-kinase inhibition. *Eur. J. Neurosci.* **21,** 26–32
59. Saci, A., and Carpenter, C. L. (2005) RhoA GTPase regulates B cell receptor signaling. *Mol. Cell.* **17,** 205–214
60. Cenni, V., Sirri, A., Riccio, M., Lattanzi, G., Santi, S., de, P. A., Maraldi, N. M., and Marmiroli, S. (2003) Targeting of the Akt/PKB kinase to the actin skeleton. *Cell Mol. Life Sci.* **60,** 2710–2720
61. Adamson, P., Etienne, S., Couraud, P. O., Calder, V., and Greenwood, J. (1999) Lymphocyte migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a rho-dependent pathway. *J. Immunol.* **162,** 2964–2973
62. Walters, C. E., Pryce, G., Hankey, D. J., Sebti, S. M., Hamilton, A. D., Baker, D., Greenwood, J., and Adamson, P. (2002) Inhibition of Rho GTPases with protein prenyltransferase inhibitors prevents leukocyte recruitment to the central nervous system and attenuates clinical signs of disease in an animal model of multiple sclerosis. *J. Immunol.* **168,** 4087–4094

Received for publication February 9, 2007.
Accepted for publication May 17, 2007.