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

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FASEB Journal, accepted for publication
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 the 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.
Introduction

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.

TJs are continuous membrane strands located at the apical site between brain ECs, consisting 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 carboxy termini. Occludin expression is associated with increased electrical resistance and decreased paracellular transport. Claudins comprise a multigene family consisting of more than 20 members that contain two extracellular loops and four transmembrane domains and interact in both a homophilic and heterophilic way with claudins of adjacent cells. Claudin-5 is a critical component of the BBB as it closes the BBB for small molecules up to 800 Da. The carboxyterminal parts of both occludin and claudins interact with membrane-associated recruiting proteins of the zona occludens (ZO) protein family. ZO proteins are reported to link transmembrane proteins to the actin cytoskeleton and have signaling potential. Through its interaction with TJ molecules, the actin cytoskeleton plays an active role in maintaining TJ integrity and BBB function. 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).

A defective function of the BBB is reported in neuroinflammatory diseases like multiple sclerosis (MS), HIV-associated dementia and encephalitis, stroke and brain trauma. 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, pro-inflammatory mediators like chemokines and cytokines 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. 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. Currently, specific ROS signaling pathways within brain ECs that target the TJ to disengage are unclear, although previous studies form our group revealed that ROS induced enhanced Ca2+ mobilization and inositol (1,4,5)-trisphosphate (IP3) formation in brain ECs via phospholipase C. Furthermore, ROS may activate protein tyrosine kinases in brain ECs, which in turn may lead to tyrosine phosphorylation of TJ proteins.
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, Tocris Bioscience, Ellisville, 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); 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 (Gibco, Carlsbad, CA). 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 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 GP8/3.9 rat brain endothelial cells as described. 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 anti-claudin-5 followed by Alexa 594-conjugated secondary antibodies. Mounted coverslips were analyzed by sequential excitation at 514 /594 nm.

Cell culture
The Lewis rat brain endothelial cell line GP8/3.9, the GFP-occludin and the claudin-5-YFP expressing GP8/3.9 cell lines were cultured as described. The human brain endothelial cell line hCMEC/D3 was cultured as described.

ROS generating system
A mixture of 0.02 U/ml xanthine oxidase and 100 µM hypoxanthine was used to generate ROS as previously described. This mixture is known to produce constant levels of predominantly superoxide and to a lesser extent hydrogen peroxide and hydroxyl radicals.
**Live cell analysis of TJ rearrangements**

The GFP-occludin- and claudin-5-YFP expressing 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, NY, USA) was used to measure TEER in confluent monolayers of hCMEC/D3 cells. In short, 250 µl of cell suspension (8x10^5 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.²⁴

**Permeability of the BBB in vitro**

hCMEC/D3 cells were cultured onto collagen (upper side, Sigma, St. Louis, USA) coated Costar Transwell filters (pore-size 0.4 µm; Corning Incorporated, Corning, NY, USA) in endothelial cell (EC) growth medium 1:1 mixed with astrocyte-conditioned medium.³⁵ Permeability for FITC-dextran (150 kDa) was assayed as described before³⁶ 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 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. GP8/3.9 cells were grown to confluence and maintained for 48 h in serum-free cell culture medium. GP8/3.9 cells were 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% low fat milk powder and incubated with primary antibodies. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies. Supersignal West Dura Extended Duration Substrate (Pierce) was used for detection of immunoreactive proteins. Protein band intensity was quantified using ImageQuant TL software (Amersham Biosciences, NJ) and presented as percentage of control.

**Rho-activation assay**

Rho-activation was assayed using a RhoA G-LISA kit (Cytoskeleton, Denver, CO, USA). 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 lysis buffer. The RhoA G-LISA assay was performed according to manufacturer's prescription.

**AMAXA transfection of GP8 cells**

1.0x10^6 GP8/3.9 cells were transfected with 3 µg of DNA constructs (empty vector, dominant negative Rho A N19, or constitutively active RhoA V14^37, a 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 hCMEC/D3 cells was studied with time-lapse video microscopy as previously described. 7.5x10^5/ml freshly isolated monocytes 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.

![Figure 1. ROS decrease BBB integrity.](image)

(A) TEER was measured in real-time in confluent monolayers of hCMEC/D3 cells in the absence or presence of ROS. Data are expressed as percentage of the mean resistance ± SEM of two independent experiments performed in duplicate. * p<0.05 vs control. (B), Confluent monolayers of hCMEC/D3 cells 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 4 individual Transwell filters. * p<0.05 vs control.
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. ROS time-dependently reduced TEER with a maximum effect after 20 min (35 ± 3.9% decrease, p<0.05, Figure 1A). We next determined the paracellular permeability towards 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, Figure 1B). 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) upon exposure to ROS as described previously. ROS induced a significant increase of stress fiber formation at 60 min upon the addition of ROS (31 ± 6.1% increase, p<0.001, Figure 2A). In addition, hydrogen peroxide (100 µM) solely similarly increased the number of F-actin fibers (26 ± 8.6%, p<0.05, Figure 2B). Hydrogen peroxide did not affect brain EC viability as was assayed by MTT and 7AAD (data not shown).

Figure 2. ROS induce cytoskeleton rearrangements in brain ECs. (A), Quantification of F-actin stress fiber formation in phalloidin-rhodamin stained 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 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.
In addition, 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 rat 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 rat brain EC line overexpressing fluorescently labeled claudin-5, in which claudin-5-YFP was localized at the plasma membrane, recognized by anti-claudin-5 antibodies and colocalized with endogenous ZO-1 (supplementary Figure 1A,B). Live cell microscopy demonstrated that ROS, within 30 min, induced ruffling of cellular junctions, which coincided with redistribution of occludin (Figure 3A) and claudin-5 (Figure 3C) 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 (Figure 3B). In addition, ROS-induced claudin-5 disappearance was detected after 60 min (Figure 3D). Cell viability was assayed by MTT and 7AAD and remained unaffected (data not shown).

**Figure S1. Localization of Claudin-5-YFP in rat brain ECs.** (A), Immunostaining with anti-claudin-5 on GP8/3.9 cells expressing claudin-5-YFP showing that claudin-5-YFP (in yellow) is recognized by polyclonal antibodies directed against claudin-5 (in red) and is localized at the plasma membrane. (B), Immunostaining with anti-ZO-1 polyclonal antibody (in red) showing that claudin-5-YFP exactly coincides with endogenous ZO-1 at cell-cell junctions.
Figure 3. ROS induce redistribution and degradation of GFP-occludin and claudin-5-YFP. (A), Occludin dynamics were followed in GP8/3.9 cells expressing GFP-occludin using live cell confocal microscopy. Thirty minutes after addition of ROS occludin started to ruffle 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 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.
ROS specifically and transiently activate PKB in brain endothelial cells
A number of signal transduction pathways, including the PI3 kinase pathway\textsuperscript{38,39}, 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 rat brain ECs, we determined the phosphorylation state of PKB by Western blotting. Addition of ROS significantly induced transient PKB phosphorylation at Ser473, which is essential for PKB activation\textsuperscript{40}, without affecting total PKB levels. PKB phosphorylation was maximal at 30 min after the addition of ROS (52 ± 7.1% increase, \( p<0.001 \), Figure 4A), which was more potent than hydrogen peroxide alone (32 ± 7.7% increase, \( p<0.01 \), Figure 4B). 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, \( p<0.01 \), Figure 4A). 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 dismutated by SOD to hydrogen peroxide. In turn, hydrogen peroxide can be decomposed by 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 (Figure 4C).

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 (Figure 5A). In addition, our results showed that ROS induced RhoA activation (Figure 5B). 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)\textsuperscript{37} in rat 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 (Figure 5C). These results indicate that ROS induce PKB phosphorylation through RhoA-activation.
Figure 4. ROS induce PKB phosphorylation in brain endothelial cells. (A), Western blot analysis and quantification of PKB phosphorylation in 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 GP8.3 cells. Cells were exposed to ROS and hydrogen peroxide for 60 minutes. 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 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 three independent experiments and are presented as percentage of control. *** p<0.001 compared to ROS treated cells.
Figure 5. RhoA is an upstream mediator of ROS-induced PKB phosphorylation. (A), Quantification of PKB-phosphorylation. GP8/3.9 cells were treated with the Rho-inhibitor toxin B (5 ng/ml) 2 hours 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 three independent experiments and are presented as percentage of control. *** p<0.001. (B), ROS induced Rho-activation in GP8/3.9 cells. Data are expressed as mean ± SEM of 3 independent experiments and are presented as percentage of control. (C), Western blot analysis and quantification of PKB phosphorylation relative to total PKB in 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.

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

Next, we examined whether Rho and PI3 kinase are involved in cytoskeleton and TJ alterations induced by ROS. Western blot analysis revealed that the PI3 kinase inhibitor wortmannin and the Rho-inhibitor toxin B both prevent ROS-induced disappearance of GFP-occludin (Figure 6A). Moreover, inhibitors of Rho, PI3 kinase, and PKB (triciribine) prevented claudin-5-YFP disappearance (Figure 6B) and reduced ROS-induced formation of actin stress fibers (Figure 6C). 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 previously described. ROS induced the migration of primary monocytes across
human brain ECs (38 ± 3.8% increase, p<0.001, Figure 7). Specific inhibitors of Rho (42 ± 3.5% decrease, p<0.001), PI3 kinase (40 ± 3.5% decrease, p<0.001), and PKB (41 ± 3.3% decrease, p<0.001) prevented this ROS-induced migration (figure 7). In addition, the same inhibitors also prevented ROS-induced migration of primary rat monocytes across rat GP8/3.9 ECs (data not shown). Together, these results indicate that these signaling pathways mediate ROS-induced monocyte migration across an *in vitro* model of the BBB.

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 disappearance is prevented by the Rho-inhibitor toxin-B (5 ng/ml) and the PI3-kinase inhibitor wortmannin (2 µM; A) and that ROS-induced claudin-5 disappearance is prevented by toxin-B, wortmannin and a specific PKB inhibitor triciribine (12.5 µM; B). Cells were treated with inhibitors for 2 h prior to ROS exposure and cells were exposed to ROS for 60 min (A) or 120 (B) min. Actin was used as a control for protein loading. (C), Quantification of stress fiber formation in phalloidin-rhodamin stained 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.
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 upon adhesion of monocytes to brain ECs and contribute to BBB disruption\(^\text{24}\). 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 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\(^\text{7}\), 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\(^\text{27,41,42}\) as well as facilitates the formation of actin stress fibers in bovine brain endothelium\(^\text{42}\). Our data confirm and extend these results as superoxide, which is the 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\(^\text{43-45}\) and brain ECs\(^\text{42,46}\). 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, 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

![Figure 7 ROS-induced monocyte migration is mediated through Rho, PI3 kinase and PKB.](image-url)
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. Furthermore, evidence suggests that the phosphorylation state of occludin is important in the regulation of TJ assembly and disassembly. Occludin can be phosphorylated on Ser, Thr and Tyr residues and in epithelial cells extracellular ROS induced tyrosine phosphorylation and dissociation of occludin from intercellular junctions. 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. 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. 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 RhoA resulted in decreased baseline PKB phosphorylation levels and blocked ROS-induced PKB phosphorylation. We therefore suggest that RhoA is an upstream activator of the PI3 kinase pathway, which is in line with previous studies. 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. In our brain EC model however, Rho acts upstream of PI3 kinase and ensuing activation of PKB allows direct interaction with the cytoskeleton.

Specific inhibitors for Rho, PI3 kinase and PKB reduce ROS-induced monocye 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. Furthermore, inhibition of RhoA in brain endothelium resulted in decreased cellular migration in vitro and in vivo. 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 (Figure 8). Furthermore, inhibition of 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, thus supporting an inflammatory response. Furthermore, PKB phosphorylation and RhoA activation may be early markers of BBB dysfunction. Agents that selectively inhibit the effect of ROS on TJ integrity, like antioxidants or signaling inhibitors for RhoA, PI3 kinase or PKB, may be used therapeutically to modulate neuroinflammatory diseases complicated by BBB dysfunction.

Figure 8. Schematic representation of the effect of ROS on blood-brain barrier integrity.
Acknowledgements

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 (phosphorylated) 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), the Dutch Asthma Foundation (grant 3.2.03.39, S. Gringhuis).
References


Chapter 3