OXIDATIVE AND OSMOTIC STRESS INDUCED CHANGES IN CEREBRAL ENDOTHELIAL CELLS

Ph.D. Thesis

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Publications related to the thesis:

I. Krizbai IA, Bauer H, Bresgen N, Eckl PM, **Farkas A**, Szatmári E, Traweger A, Wejksza K, Bauer HC: Effect of oxidative stress on the junctional proteins of cultured cerebral endothelial cells. *Cell Mol Neurobiol*. 2005 Feb;25(1):129-39. IF_{2005}: 2,022

II. **Farkas A**, Szatmári E, Orbók A, Wilhelm I, Wejksza K, Nagyőszi P, Hutamekalin P, Bauer H, Bauer HC, Traweger A, Krizbai IA: Hyperosmotic mannitol induces Src kinase-dependent phosphorylation of beta-catenin in cerebral endothelial cells. *J Neurosci Res*. 2005 Jun 15;80(6):855-61. IF_{2005}: 3,239

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i. Wilhelm I, Nagyőszi P, **Farkas EA**, Couraud PO, Romero IA, Weksler B, Fazakas C, Dung NKT, Bauer H, Bauer HC, and Krizbai IA. Hyperosmotic stress induces Axl activation and cleavage in cerebral endothelial cells. J Neurochem. Oct;107(1):116-26. IF_{2007}: 4,451

ii. Hutamekalin P, Orbók A, Wilhelm I, **Farkas AE**, Nagyőszi P, Veszelka S, Deli MA, Buzás K, Hunyadi-Gulyás E, Medzihradszky KF, Meksuriyen D, Krizbai IA: Effect of nicotine and polyaromatic hydrocarbons on cerebral endothelial cells. *Cell Biol Int* 2008 Feb;32(2):198-209. IF_{2007}: 1,547

iii. Traweger A, Lehner C **Farkas A**, Krizbai IA, Tempfer H, Klement E, Guenther B, Bauer HC, Bauer H: Nuclear ZO-2 alters gene expression and junctional stability in epithelial and endothelial cells. *Differentiation* 2008 Jan;76(1):99-106. IF_{2007}: 2,894

iv. Wilhelm I, **Farkas AE**, Nagyőszi P, Váró G, Bálint Z, Végh GA, Couraud PO, Romero IA, Weksler B, and Krizbai IA: Regulation of Cerebral Endothelial Cell Morphology by Extracellular Calcium. *Phys Med Biol* 2007 Oct 21;52(20):6261-74. IF_{2007}: 2,528

v. Bálint Z, Krizbai IA, Wilhelm I, **Farkas AE**, Párducz A, Szegletes Z, Váro G: Changes induced by hyperosmotic mannitol in cerebral endothelial cells: an atomic force microscopic study. *Eur Biophys J*. 2007 Feb;36(2):113-20. IF_{2007}: 2,238

vi. Veszelka S, Pásztói M, **Farkas AE**, Krizbai I, Dung NT, Niwa M, Ábraham CS, Deli MA: Pentosan polysulfate protects brain endothelial cells against bacterial lipopolysaccharide-induced damages. *Neurochem Int.* 2007 Jan;50(1):219-28. IF_{2007}: 2,975

vii. Krizbai IA, Lenzser G, Szatmári E, **Farkas AE**, Wilhelm I, Fekete Z, Erdős B, Bauer H, Bauer HC, Sándor P, Komjáti K: Blood-brain barrier changes during compensated and decompensated hemorrhagic shock. *Shock.* 2005 Nov;24(5):428-33. IF_{2005}: 3,122

viii. Szabó H, Novák Z, Bauer H, Szatmári E, **Farkas A**, Wejksza K, Orbók A, Wilhelm I, Krizbai IA: Regulation of proteolytic activity induced by inflammatory stimuli in lung epithelial cells. *Cell Mol Biol* (Noisy-le-grand). 2005 Sep 2;51 Suppl:OL729-35. IF_{2005}: 1,018

Abbreviations

AJ: adherens junction
BBB: blood-brain barrier
BCA: bicinchoninic acid
bFGF: basic fibroblast growth factor
Caco-2 cell line: heterogeneous human epithelial colorectal adenocarcinoma cells
CEC: cerebral endothelial cell
CNS: central nervous system
CSK: c-Src tyrosine kinase
DMNQ: 2,3-dimethoxy-1,4-naphthoquinone
ECGF: endothelial cell growth factor
EDTA: ethylenediaminetetraacetic acid
ERK: extracellular signal-regulated kinase
FCS: fetal calf serum
GUK: guanylate kinase
HUVEC: human umbilical vein endothelial cell
ICAM: intercellular adhesion molecule
INT: 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride
JAM: junctional adhesion molecule
LDH: lactate dehydrogenase
MAGI: MAGUKs with inverted domain structure
MAGUK: membrane-associated guanylate kinase homologue
MAP kinase: mitogen-activated protein kinase
MDCK cells: Madin-Darby canine kidney cells
PAGE: polyacrylamide gel electrophoresis
PKC: protein kinase C
PMA: phorbol 12-myristate 13-acetate
PVDF: polyvinylidene fluoride
PY: phospho-tyrosine
ROK: Rho-kinase
SDS: sodium dodecyl sulphate
TJ: tight junction
Tris: tris(hydroxymethyl)aminomethane
VE-cadherin: vascular endothelial cadherin

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1. Introduction

The blood-brain barrier (BBB) plays an essential role in maintaining the extracellular milieu necessary for neuronal function. Unraveling the molecular mechanisms of BBB function is paramount in understanding the pathomechanisms of - and designing treatments for - several neurological disorders.

1.1. Anatomy and physiology of the blood-brain barrier

The blood-brain barrier is formed by several cell types, the most important being the brain endothelial cells that line the cerebral blood vessels. The capillaries are surrounded by astrocytic endfeet. The cerebral endothelial cells (CECs) are ensheathed by the basement membrane which is shared with pericytes (Figure 1). In contrast to peripheral capillaries which are fenestrated permitting the free exchange of solutes between the blood and the interstitial space, brain endothelial cells are tightly interconnected through tight junctions (TJs).

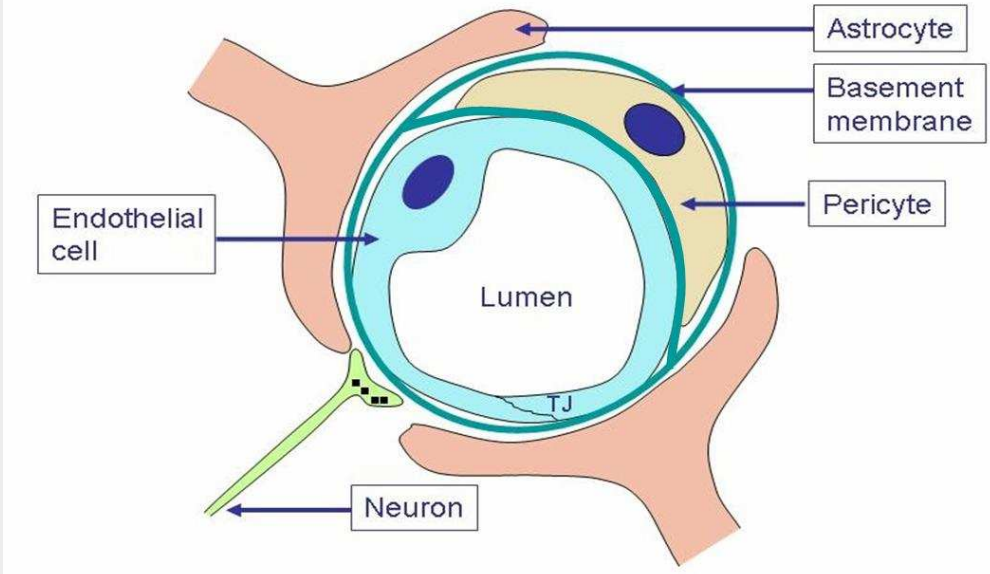

Figure 1: Schematic drawing of the blood-brain barrier

Tight junctions severely restrict paracellular transport of molecules through the BBB. This confines the passage of solutes to the transcellular path. However, that pathway is also restricted by the low pinocytic, high enzymatic and specific transporter activity of the CECs.

Thus the brain endothelial cells and the BBB control the transport of substances between blood and brain, effectively regulating the influx of nutrients and the removal of metabolites, protecting the central nervous system (CNS) from harmful substances and fluctuations in blood composition (e.g.: caused by digestion/absorption, exertion etc.).

1.2. Interendothelial junctions

One of the unique features of CECs is that paracellular transport is limited by the close and strong connection of the cells. The structure responsible for this is the interendothelial junctional complex, which consists of several proteins (Figure 2). Transmembrane proteins interconnect the endothelial cells, while cytoplasmic plaque proteins anchor the transmembrane proteins to cytoskeletal structures. Proteins involved in signal transduction also interact with building blocks of the interendothelial complex. Moreover, certain structural proteins like ZO-2 have signaling roles as well (Traweger et al., 2008).

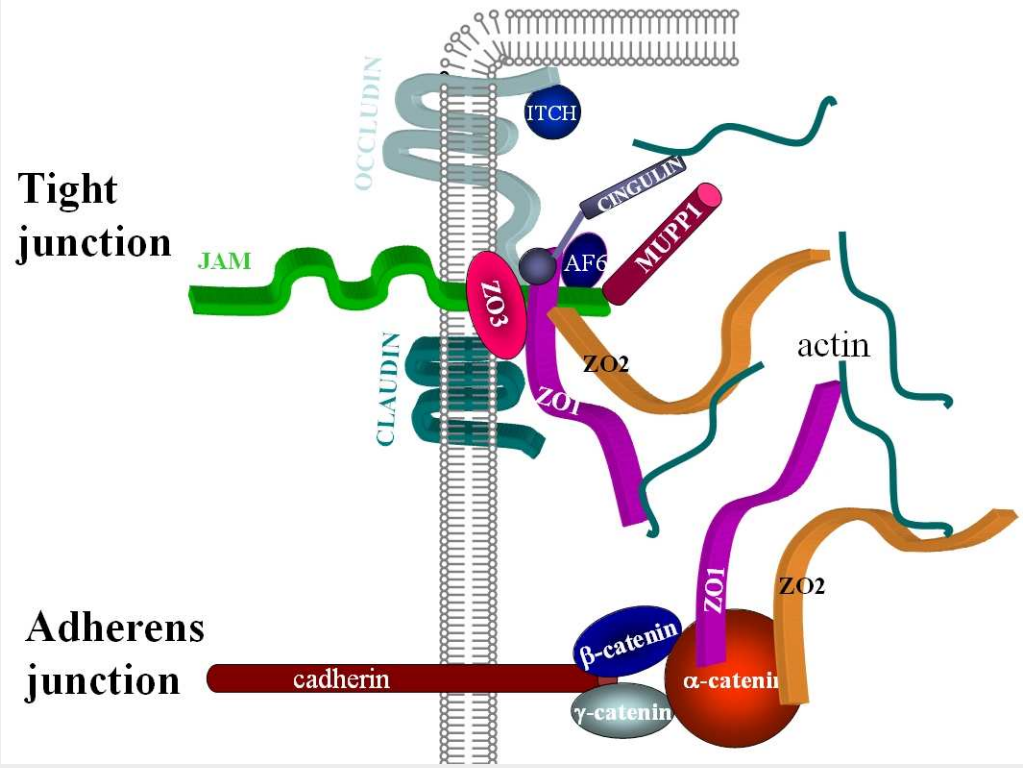

Figure 2: Proteins of the interendothelial junctions
The picture shows some of the most important constituents of the interendothelial complex and their interactions.

Tight junctions (TJs) are specialized contact zones of epi-, meso- and endothelial cells. They are responsible for the polarization of the cell leading to the separation of an apical from a basolateral membrane domain and the restriction of the paracellular pathway: these two

roles are the fence and the gate function respectively. Freeze-fracture electronmicrographs show complex networks of continuous multistranded tight junctions in capillaries and postcapillary venules (Nagy et al., 1984). The transmembrane proteins of TJs are occludin, claudins and junctional adhesion molecules (JAMs); these proteins are anchored to the cytoskeleton through the junctional plaque proteins, such as zonula occludens (ZO-1, -2 and -3) proteins.

Adherens junctions (AJs) are located basal to TJs and participate in the organization and maintenance of tight junctions. In epithelial cells the appearance of TJs during development is preceded by the E-cadherin mediated formation of AJs. Furthermore, the disruption of AJ increases TJ permeability (Gumbiner and Simons, 1986). The main transmembrane proteins of AJs are cadherins which are connected through catenins to the actin cytoskeleton.

1.2.1 Transmembrane proteins of the tight junction

The first transmembrane protein discovered to be localized to tight junctions was occludin. Occludin is an approximately 60 kDa tetraspan membrane protein with two extracellular loops, a short intracellular turn, a short N- and a longer C-terminal cytoplasmic domain (Furuse et al., 1993).

Overexpression experiments using full-length and mutated occludin in Madin–Darby canine kidney (MDCK) epithelial cells or *Xenopus* cells (Balda et al., 1996 and Chen et al., 1997), as well as a study using a synthetic peptide corresponding to the second extracellular loop of occludin (Wong and Gumbiner, 1997) suggested a role for occludin in the barrier and fence functions of tight junctions. However, overexpressing occludin in cell types which do not possess tight junctions (L1 fibroblast, Furuse et al., 1998b) leads to the formation of short TJ like strands only, indicating that occludin is not the only TJ protein participating in the composition of the paracellular seal. This is supported by the finding that occludin-deficient embryonic stem cells differentiated into polarized epithelial cells bearing well-developed tight junctions that exhibited no barrier dysfunction (Saitou et al., 1998).

The mystery of how TJs can be present without occludin was resolved when claudin-1 and -2 were discovered in 1998 (Furuse et al., 1998). Of the twenty-four claudins known to date, claudin-5 is characteristic to endothelial cells. Additionally, claudin-1, -3 and -12 have also been observed in CECs, while the other members of the claudin family are expressed in a tissue specific combination resulting in tissue specific barrier characteristics (Krause et al.,

2008). Similarly to occludin, claudins have four transmembrane domains; however, there is no sequence homology. Claudins overexpressed in endothelial cells are recruited to the existing TJ strands. Moreover, when expressed in fibroblasts lacking tight junctions, claudin-1 and 2 produce very long strands that have a morphology resembling very closely endogenous TJs (Furuse et al., 1998b).

In the same year another membrane spanning protein of the TJ has been found: the junctional adhesion molecule or JAM (Martin-Padura et al., 1998). Today six JAMs are known, divided into two groups based on sequence homology. JAM-A (also called JAM-1) is involved in the barrier function of tight junctions in both endothelial and epithelial cells and in the development of apicobasal cell polarity in epithelial cells.

1.2.2 PDZ proteins of the junctional complex

The PDZ domain is a common structural domain of 80-90 amino-acids found in the signaling proteins of bacteria, yeast, plants, and animals (Ponting, 1997). PDZ is an acronym combining the first letters of three proteins — post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (ZO-1) — which were first discovered to share the domain. These domains help anchor transmembrane proteins to the cytoskeleton and hold together signaling complexes (Ranganathan and Ross, 1997).

The transmembrane proteins of tight junctions are anchored to the cytoskeleton through zonula occludens proteins ZO-1, ZO-2 and ZO-3. ZO proteins are members of the MAGUK family (membrane-associated guanylate kinase homologues) with binding domains to both TJ and AJ proteins, as well as to actin. ZO-1 (220 kDa) was the first TJ-associated protein identified in 1986 (Stevenson et al., 1986). ZO-2 and ZO-3 were identified later (Gumbiner et al., 1991; Balda et al., 1993 and Haskins et al., 1998). The zonula occludens proteins share a similar structural organization with the N-terminal region containing three PDZ domains, followed by a Src homology 3 domain and a GUK (guanylate kinase) domain, which is enzymatically inactive. ZO proteins are at the center of a network of protein interactions. Experiments with transfected fibroblasts and epithelial cells show that the first PDZ domain of ZO-1, ZO-2 and ZO-3 binds directly to the C-termini of claudins (Itoh et al., 1999). The second PDZ domain of ZO-1 is responsible for a homo- and heterodimerization through interaction with another ZO protein (Utepbergenov et al., 2006; Umeda 2006); and for binding to connexins in epithelial cells (Giepmans et al., 1998; Kausalya et al., 2001) and

also in brain capillary endothelium (Nagasawa et al., 2006). The third PDZ domain of ZO-1 interacts in vitro with JAM-1. The GUK region is responsible for occludin (Fanning et al., 2007 and Schmidt et al., 2004) and α-catenin (Rajasekaran et al., 1996 and Itoh et al., 1997) interaction. The C-terminal, proline-rich domain of ZO-1 interacts with actin and cortactin (Itoh et al., 1997; Fanning et al., 1998 and Katsube et al., 1998), functionally linking TJ membrane proteins to the actin cytoskeleton, by binding to membrane proteins through its N-terminal half, and to actin through its C-terminal half. ZO-2 differs from ZO-1 in that its C-terminal region is shorter; otherwise the domain structure of the two proteins is very similar. The interactions of ZO-2 are very similar to that of ZO-1 (Itoh et al., 1999; Itoh et al., 1999b and Wittchen et al., 1999). ZO-1 and ZO-2 are expressed in both epithelia and endothelia, whereas ZO-3 is exclusively expressed in epithelia (Inoko et al., 2003). Also, ZO-3 knockout mice did not show any phenotypic effects, which implies that the physiological function of ZO-3 might be redundant (Adachi et al., 2006).

Other PDZ domain containing proteins of the junctional plaque are AF-6/afadin (Ikeda et al., 1999 and Zhadanov et al., 1999), MUPP1 (Latorre et al., 2005 and Sugihara-Mizuno et al., 2007) and the MAGI (MAGUKs with inverted domain structure) proteins. Among several differentially spliced members of the MAGI family, only MAGI-1 and MAGI-3 are associated with TJ (Ide et al., 1999 and Laura et al., 2002). These PDZ proteins interact both with other junctional proteins, and proteins involved in signal transduction, beside their scaffolding function they are important in the organization of the junctional complex.

1.2.3 Non-PDZ proteins of the junctional plaque

Cingulin and JACOP/paracingulin are two vertebrate-specific, junction-associated proteins characterized by a globular head domain, a coiled-coil rod domain, and a small globular tail. Cingulin was identified as a TJ-specific protein which co-purified with myosin, and was specifically expressed in tissues containing epithelial TJ (Citi et al., 1998). The head domain interacts with several TJ proteins (Cordenonsi et al., 1999; Cordenonsi et al. 1999b and Bazzoni et al. 2000), and with actin (D'Atri and Citi, 2001). However, the key functional interaction is with ZO-1, through the conserved “ZIM” (ZO-1 Interaction Motif) which is required both for ZO-1 binding, and for junctional recruitment of cingulin in transfected cells (D'Atri et al., 2002). Paracingulin has sequence and domain organization similar to cingulin; however, unlike cingulin, it is present at both TJs and adherens-type junctions, and is absent from junctions of the small intestine, whereas it is detected in endothelial cells (Ohnishi et al.,

2004). Since JACOP/paracingulin contains the ZIM domain, it is likely that ZO-1 is involved in its junctional recruitment.

Angiomotin (Amot), JEAP (Angiomotin-like-protein1) and MASCOT (Angiomotin-like-protein2) are three members of a family of proteins characterized by the presence of a central coiled-coil domain, and a C-terminal PDZ interaction sequence motif. The first member of this family: Amot, plays a role in the growth factor stimulated migration of endothelial cells (Ernkvist et al., 2008). MASCOT and JEAP interact with MAGI-1, thus localizing to the TJ (Patrie, 2005).

1.2.4 Proteins of the adherens junction

The transmembrane proteins of the adherens junctions are the cadherins; in the case of vascular endothelial cells mainly VE-cadherin, which is linked through the catenins (α-, β- and γ-catenin) to the cytoskeleton. A proper function of the adherens junction is needed for tight junction formation and blockade of cadherin with peptides directed against the extracellular region of cadherin causes an increase in BBB permeability (Pal et al., 1997).

Catenins are members of the armadillo protein family; β-catenin is the mammalian homologue of the Drosophila armadillo protein. The first catenin discovered was the 94 kDa β-catenin (Nagafuchi et al., 1989), which is 65% homologous to the 85 kDa γ-catenin (also called plakoglobin). Cadherin is bound by the middle region of β- and γ-catenin, while the 102 kDa α-catenin is bound by their N-terminal region. β- and γ-catenin anchor cadherin to the cytoskeleton through α-catenin. An intimate connection between TJ and AJ is suggested by the finding that the TJ protein ZO-1 and the AJ protein α-catenin are able to interact (Imamura et al., 1999). However, recently it has been shown that α-catenin does not bind to β-catenin and actin at the same time. Further investigation of the interactions of α-catenin suggests that its main role is in restricting the formation of lamellipodia present in migrating cells after the formation of cadherin mediated cell contacts (Drees et al., 2005).

1.2.5 Regulation of the junctional complex by phosphorylation

Phosphorylation is one of the key mechanisms by which the cell regulates protein function. The relationship of protein phosphorylation and TJ function was first analyzed in 1989 using MDCK I and II kidney epithelial cell cultures (Stevenson et al., 1989). These cultures show a pronounced difference in the tightness of their junctions characterized by transepithelial electrical resistance measurement. It was found that in the low resistance MDCK II cultures phosphorylation of ZO-1 was twice as strong as in high resistance MDCK I

cultures while the total amount and localization did not differ. Ever since, there is intensive research focused on the regulation of the junctional complex. There is considerable amount of data indicating that various kinase systems can affect the permeability properties of the tight junction.

Tyrosine phosphorylation plays a crucial role in the assembly and function of TJs. However, the exact role of tyrosine phosphorylation is not yet understood completely and may depend on the developmental status of the tight junction. In the case of mature tight junction an increased phosphorylation has been associated with decreased TEER (Staddon et al., 1995). In both MDCK and brain endothelial cells an increased tyrosine phosphorylation of occludin was observed. Similar results have been obtained by Wachtel et al. (1999) showing that increased tyrosine phosphorylation resulted in occludin degradation and increased permeability. Furthermore, tyrosine phosphorylation of VE-cadherin is also accompanied by an increase in vascular permeability and leukocyte diapedesis (Turowski et al., 2008).

Src kinases could also be major players in the regulation of TJ function. The effect of Src kinase seems also to be dependent on the developmental status of the TJ. In mature epithelia activation of Src is involved in the disruption of interendothelial junctions induced by LPS (Sheth et al., 2007), progastrin (Hollande et al., 2003) or plasmodium falciparum (Gillrie et al., 2007).

Mitogen activated protein kinases (MAPKs) form a family of protein kinases, with ERK1/2, JNK and p38 as the best characterized members. MAPKs may be involved in the regulation of junctional permeability, especially under pathological conditions. Oxidative stress-induced activation of MAPK pathways is accompanied by an increase in paracellular permeability in endothelial cells. By using a specific ERK1/2 inhibitor PD98059, Kevil et al. (2000) were able to inhibit the increase in permeability and occludin redistribution elicited by hydrogen peroxide. A similar result has been obtained with the p38 inhibitor SB202190 (Kevil et al. 2001), indicating that ERK1/2 and p38 may be key players in the junctional damage elicited by oxidative stress. On the other hand in an ischemia/reoxygenation model, inhibition of ERK activity during the reoxygenation phase prevented barrier restoration, demonstrating that ERK activity could be essential to rebuild a disrupted endothelial barrier (Wachtel et al., 2002).

A crosstalk between PKC and the MAPK signaling pathway also regulates TJ opening: in corneal epithelial cell lines activation of PKC with PMA (phorbol 12-myristate 13-acetate) triggers a decrease in TEER through the activation of MAPK (Wang et al., 2004).

The interaction between TJ proteins and MAP kinases is bidirectional. Not only MAP kinases can regulate TJ proteins but TJ proteins themselves can play a role in activating the ERK pathway. Silencing JAM-1 hinders the migration of HUVEC cells on vitronectin induced by basic fibroblast growth factor (bFGF), due to the inability of the growth factor to activate ERK (Naik et al., 2003). Occludin also appears to participate in the activation of the MAPK signaling pathway. Thus in hepatic cell lines derived from occludin-deficient mice, MAPK activation is downregulated triggering apoptosis and increased claudin-2 expression. When occludin is transfected into these cultures MAPK becomes activated, reversing the increase in claudin-2 expression and apoptosis (Murata et al., 2005).

1.3. Oxidative stress and the blood brain barrier

Cerebral hypoxia and ischemia have severe consequences regarding the central nervous system (CNS). The most vulnerable cells in the CNS are neurons; however, increasing number of experimental data suggests that cerebral endothelial cells are also affected by hypoxia and may play an important role in the pathogenesis of hypoxic or ischemic damage. Oxygen free radicals play an important role in the damage caused by hypoxia and ischemia which are released mainly during reperfusion (McCord and Roy, 1982 and Granger, 1988), when blood supply returns to the tissue after a period of ischemia.

It has been shown that exposure of endothelial cells to reactive oxygen species is one of the main causes of endothelial dysfunction (Lagrange et al., 1999 and Wu et al., 1998). In endothelial cells, hypoxic environment regulates the expression of a number of genes with products that are vasoactive or mitogenic for vascular tissue such as cyclooxygenase (North et al., 1994), nitric oxide synthase (Xu et al., 2000) or endothelin (Tsang et al., 2001). Moreover, oxidative stress can induce chromosomal aberrations, micronuclei and apoptosis in these cells (Bresgen et al., 2003). Several proteins which have been shown to be regulated by hypoxia play significant roles in cell adhesion or intercellular interactions such as hypoxia inducible factor (HIF-1, Wang and Semenza, 1993), integrin (Suzuma et al., 1998) or ICAM (intercellular adhesion molecule) (Hess et al., 1994).

Hypoxia and ischemia may affect the integrity of the BBB (Olesen, 1986). The increased cerebrovascular permeability leads to vasogenic brain edema which contributes significantly to the development of cerebral edema as well. The combination of hypoxia and aglycemia may result in an even more rapid increase in permeability as shown by Abbruscato and Davis (1999).

The permeability of the endothelial barrier is largely determined by the integrity of the tight junctions and adherens junctions. In this respect the transmembrane proteins of the junctional complexes may have a special importance.

The mechanism by which oxidative stress leads to an increased endothelial permeability is not completely understood. The experimental data obtained from epithelial cells and endothelial cells of non cerebral origin suggest that proteins of the tight junction may be directly involved (Park et al., 1999). Results indicate that junctional proteins of CECs are regulated by oxidative stress (Mark and Davis, 2002; Brillaut et al., 2002 and Fischer et al., 2002); however, the results are not consistent.

1.4. Hyperosmosis and the blood-brain barrier

One of the most important functions of the mammalian BBB is to restrict the free movement of different substances between blood and neural tissue. Despite its undisputed importance in the normal function of the central nervous system, BBB can be a major impediment for the treatment of diseases of the CNS as well: due to the relative impermeability of the barrier many drugs are unable to reach the brain at therapeutically relevant concentrations. Different attempts have been made to overcome the limited access of drugs to the brain, such as chemical modifications of the drug, development of more hydrophobic analogs or linking the active compound to a specific carrier (Pardridge et al., 2002). An alternative possibility would be a controlled and reversible opening of the BBB. Disruption of the BBB by rapid intracarotid infusion of hyperosmolar solutions such as mannitol has been used both experimentally and clinically to increase the transport of different substances to the brain parenchyma (Neuwelt et al., 1991; Kroll and Neuwelt, 1998; Rapoport et al., 2000 and Doolittle et al., 2000). Mannitol has been successfully used in the concentration of 1.4-1.6 M in perfusion. This causes a rapid opening (within minutes) of the BBB which is reversible. The barrier function starts to recover approximately 1 hour after treatment, but complete recovery is achieved only after 6-8 hours (Rapoport et al., 1980 and Siegal et al., 2000).

Despite clinical and experimental interest, to date the mechanism of osmotic disruption of the BBB is poorly understood.

Since the permeability of the BBB is largely determined by the intercellular junctions (TJ and AJ) these structures may play a significant role in the permeability increase elicited by hyperosmolar solutions. This is supported by earlier studies which have demonstrated

hypertonic solution induced opening of tight junction (Brightman et al., 1973; Dorovini-zis et al., 1983 and Lossinsky et al., 1995).

Tyrosine phosphorylation of junctional components, and in particular phosphorylation of catenins, has been proposed as a critical step in modulating cell-cell adhesion and contact. Several lines of evidence suggest that tyrosine phosphorylation of the cadherin-catenin complex regulates the association of cadherin-catenin complexes with the cytoskeleton (Roura et al., 1999 and Gaudry et al., 2001) which could influence paracellular permeability as well.

Furthermore, the junctional complex is under the regulation of several signaling pathways among which MAPKs have been shown to be activated by osmotic stress (Koh et al., 2001 and Duzgun et al., 2000).

1.5. Aims

Understanding the finely tuned regulation of BBB permeability is of critical importance for the treatment of CNS diseases. In this respect both controlled opening for therapeutic purposes and counteracting increased permeability in stroke, trauma or neurodegenerative disorders has been the long term goal of BBB research.

The main aims of our studies were to elucidate the molecular changes appearing in BBB endothelial cells in response to stress triggered by hypoxia/reoxygenation and hypoglycemia or hyperosmotic mannitol treatment.

Specifically:

- Do the aforementioned stress factors affect:
  - the expression,
  - localization and
  - interaction of junctional proteins?
- What signal transduction pathways are activated by oxidative or hyperosmotic stress?
- How do these changes affect the integrity of the BBB?

2. Materials and Methods

2.1. Chemicals and antibodies

All chemicals if not otherwise stated were purchased from Sigma. PP-1, Y27632 were from Tocris, Genistein from Gibco and U0126 was from Cell Signaling Technology. Protein G sepharose was purchased from GE Healthcare. We have used the following polyclonal antibodies: anti-pan-cadherin, anti-α-catenin, anti-β-catenin (Sigma), anti-VE-cadherin (Santa Cruz), anti-occludin (Zymed), anti-ERK1/2, phosphorylated anti- ERK1/2 (phosphorylated at Thr183/Tyr185) and p38 (phosphorylated at Thr180/Tyr182) (Cell Signaling Technology). Anti-β-actin (Sigma) and anti-phosphotyrosine (clone PY20, Beckton Dickinson) are monoclonal antibodies. Goat anti-mouse and anti-rabbit HRP conjugated secondary antibodies were purchased from GE Healthcare. DMEM (Dulbecco's modified Eagle's medium) was from Life Technologies and ECGF (endothelial cell growth factor) from Collaborative Research. DMNQ was purchased from Alexis. DMEM/F12 (1:1 mixture of DMEM and Ham's F-12 Nutrient Mixture) was purchased from Sigma; plasma-derived bovine serum (PDS) was from First Link.

2.2. Cell cultures

Cloned capillary endothelial cells from murine brain cortex microvessels were isolated and cultured according to Tontsch and Bauer (1989) with slight modifications. Briefly, after removing meninges under sterile conditions, brains were cut into small pieces and digested in two steps with collagenase type II (Sigma) and collagenase/dispase (Roche), followed by centrifugation on Percoll gradient. The microvessel fragments were seeded on plastic culture dishes. Cultures were maintained in DMEM supplemented with 10% FCS (fetal calf serum), ECGF alpha (4 ng/ml) and heparin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

The GP8 rat brain endothelial cell line was obtained from Dr. John Greenwood (London, UK). The cells were cultured in 6 cm Petri dishes (Greiner) coated with rat tail collagen in DMEM/F12 supplemented with 20% PDS, 100 μg/ml heparin and 50 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5% CO_2. GP8 cells cultured under these conditions have been shown to maintain good BBB characteristics (Greenwood et al., 1996) and have been successfully used in a great number of BBB studies (Adamson et al., 1999; Etienne et al., 1998 and Reijerkerk et al., 2006).

2.3. Induction of oxidative stress

For the production of hypoxia, confluent cultures of cloned murine brain endothelial cells were maintained in a special air tight chamber (Billups-Rothenburg) at 37^0C in an atmosphere containing 5% CO_2 and 95% N_2 for 16 hours followed by reoxygenation performed in an atmosphere containing 5% CO_2, 70% N_2 and 25% O_2 for 4 hours. In addition, the effects of oxidative stress have been investigated by treating the cultures with 10 μM DMNQ (2,3-Dimethoxy-1,4-naphthoquinone) for 1-2 hours as well. DMNQ is a redox-cycling quinone widely used in the research of oxidative stress, because it induces intracellular superoxide anion formation (Powel et al., 2004 and Namgaladze et al. 2005). During the experiments the culture medium contained either 1g/dm^3 glucose or was glucose free (hypoglycemia) depending on the experiment. The MAP kinase inhibitor U0126 was used in 10 μM concentration concurrently with either DMNQ treatment or induction of hypoxia/reoxygenation.

2.4. Hyperosmotic mannitol treatment

Confluent monolayers of GP8 cells were treated in serum free culture medium with 10 or 20 % mannitol for 10 or 30 minutes. 10 μM U0126 (MAP kinase inhibitor), 10 μM Y27632 (Rho kinase inhibitor), 50 μM Genistein (tyrosine kinase inhibitor), 10 μM verapamil (L-type Ca^{++} channel inhibitor), 5 μM EDTA (ethylenediaminetetraacetic acid) or 10 μM PP-1 (Src kinase inhibitor) was applied simultaneously with mannitol treatment. For testing the reversibility of the mannitol induced changes, the hyperosmotic medium was replaced after treatment by standard culture medium. The cultures were allowed 10-60 minutes of recovery before the cells were harvested.

2.5. Cell viability test

The viability of the endothelial cells in response to oxidative stress was assessed by measuring the LDH (lactate dehydrogenase) activity in the cell culture medium after each experiment. For this purpose a commercially available cytotoxicity detection kit (Roche) was used. For further analysis we used only cultures with no increased LDH activity.

LDH is a stable cytoplasmic enzyme that is present in all cells. It is rapidly released into the cell-culture supernatant when the plasma membrane is damaged. LDH activity is determined in a coupled enzymatic reaction; during this reaction, the tetrazolium salt INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) is reduced to formazan. The increase in supernatant LDH activity directly correlates to the amount of formazan

formed over time. This formazan dye is easy to assay, since it is water-soluble and has a broad absorption maximum at approximately 500 nm.

2.6. Western-blot

After our experiments cells from a 6 cm diameter dish were washed twice with PBS (phosphate buffered saline) and scraped into 300 µl ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton-X-100 0.5% NP-40, 2 mM $CaCl_2$, 5 mM NaF, 1 mM Na orthovanadate and 1 mM Pefabloc (Roche)) and incubated on ice for 30 minutes. Lysates were clarified by centrifugation at 10000 g at 4 ºC for 10 minutes and protein concentration was determined with the BCA (bicinchoninic acid) method (Pierce). The supernatant was mixed with 5x Laemmli sample buffer (60mM Tris-HCl (pH 6,8), 25% glycerol, 2% SDS (sodium dodecyl sulfate), 14,4 mM 2-mercaptoethanol, 0,1% bromophenol blue) and was used as the Triton X-100 soluble fraction. The pellet was resuspended in Laemmli sample buffer and used as the Triton X-100 insoluble fraction. Samples in Laemli sample buffer were boiled for 3 minutes. Proteins were electrophoresed using standard, denaturing, SDS-PAGE (SDS polyacrylamide gel electrophoresis) procedures (electrophoresis buffer: 25 mM Tris, 192 mM glycine, 0,1% SDS) and blotted (transfer buffer: 15,6 mM Tris, 120 mM glycine, 20% methanol) onto PVDF (polyvinylidene fluoride) membranes (Pall). Blocking the nonspecific binding capacity of the membranes was carried out at room temperature for 30 minutes in TBS-T (Tris buffered saline (10 mM Tris-HCl (pH 7,5), 150 mM NaCl) with 0.1% Tween 20) containing either 5% casein (nonfat milk powder), or 3% bovine serum albumin in the case of phospho-specific antibodies. After blocking the blots were incubated with the primary antibodies using the following dilutions in TBS-T: anti-pan-cadherin: 1:2000, anti-α-catenin: 1:1000, anti-β-catenin: 1:2000, anti-β-actin: 1:5000, anti-VE-cadherin: 1:1000, anti-phosphotyrosine (clone PY20): 1:1500, anti-occludin: 1:1000, anti-ERK: 1:1000, anti-phospho-ERK: 1:1000 and anti-phospho-p38: 1:1000. After washing the membranes three times for 10 minutes in TBS-T the blots were incubated with the secondary antibodies diluted 1:5000 in TBS-T, then washed again three times for 10 minutes in TBS-T. The immunoreaction was visualized by an ECL chemiluminescence detection kit (GE healthcare) on x-ray film (AGFA).

2.7. Immunoprecipitation

For Immunoprecipitation and co-immunoprecipitation experiments, cells from a 6 cm diameter dish were homogenized in 500 µl lysis buffer (20 mM TrisHCl pH 7.5, 150 mM

NaCl, and 0.5% Triton-X 100, 0.5% NP-40, 2 mM $CaCl_2$, 5 mM NaF, 1 mM Na orthovanadate and 1 mM Pefabloc). The lysates were centrifuged at 10000 g in a microfuge and the supernatant was subjected to immunoprecipitation. After preclearing with protein G-Sepharose supernatants were incubated with 5 μg primary antibody (phosphotyrosine or β-catenin) at 4 ºC for 4 hours. The formed immunocomplexes were precipitated by incubating the samples overnight with protein G-Sepharose beads (GE Healthcare). The precipitates were washed 4 times with lysis buffer, boiled in Laemmli sample buffer, and subjected to SDS-PAGE and immunoblotting.

2.8. Immunofluorescence

For immunofluorescent studies cells were cultured on collagen/fibronectin coated coverslips. Cells were fixed using a mixture of ice cold ethanol/acetic acid (95/5) for 10 minutes and then washed three times for 5 minutes in PBS. After blocking with 3% bovine serum albumin for 30 minutes coverslips were incubated with primary antibodies against β-catenin (1:100) for 90 minutes. Excess primary antibodies were removed by three washes in PBS lasting 5 minutes each. The coverslips were incubated with Cy3 conjugated anti-rabbit secondary antibody diluted 1:500 in PBS, and then washed three times for 5 minutes in PBS. Coverslips were mounted in anti-fading embedding medium (Biomeda) and the distribution of the signal was studied using a Nikon Eclipse TE2000 fluorescent microscope (Nikon, Japan) and photographed with a Spot RT digital camera (Diagnostic Instruments, USA).

2.9. Measurement of transendothelial electrical resistance

For the measurement of transendothelial electrical resistance (TEER) cells were grown on Millicell-CM filter inserts (filter area: 1.1 cm^2, pore size: 0.4 μm, Millipore) coated with fibronectin. The TEER was measured using a chamber electrode and an EVOM epithelial voltohmmeter (World Precision Instruments). The resistance of the cell free filter was measured in each experiment and was subtracted from the resistance obtained with cells on the filter to find the resistance of the endothelial monolayer itself. With this method resistance values above 100 $\Omega \times cm^2$ could be obtained.

3. Results

3.1. Oxidative stress

3.1.1 Cell viability

To exclude that the changes observed after hypoxia/reoxygenation arises from nonspecific cell injury, LDH (lactate dehydrogenase) release was determined in order to asses cell viability and membrane integrity. Hypoxia followed by 4 hour reoxygenation caused no significant increase in the LDH activity of the culture medium (166+/-28 and 184+/-32 U/ml) indicating that the endothelial cells maintained their membrane integrity during the experiment.

3.1.2 Expression and interaction of junctional proteins

Exposure of cerebral endothelial cells to 16 hours hypoxia and 4 hours reoxygenation led to a significant decrease in the expression of occludin as shown by western-blot analysis (Figure 3). Although maintenance of the cells in glucose free medium alone caused no significant change in occludin levels, the decrease of its expression after hypoxia/reoxygenation was more pronounced in glucose free medium than in glucose containing medium. Similar results were obtained using DMNQ to produce oxidative stress. Exposure to 10 μM DMNQ for 2 hours caused a decline in occludin levels whereas β-actin levels remained unchanged (data not shown).

The changes observed in proteins associated with the adherens junctional complex were less pronounced. Expression of cadherin was slightly reduced after hypoxia/reoxygenation in the presence and absence of glucose as well, whereas actin levels remained unchanged during the treatment (Figure 3). Immunoprecipitation experiments have shown that the interaction between cadherin and β-catenin is disrupted after oxidative stress (Figure 4). Since β-catenin levels did not change significantly and the decrease of cadherin levels is moderate, the low level of cadherin in the β-catenin precipitate can be only partly explained by the downregulation of cadherin. Similarly the association of β-catenin with α-catenin is also reduced (Figure 4).

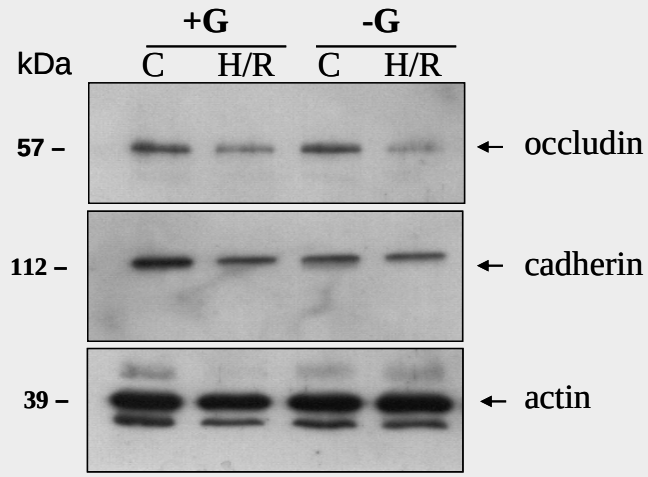

Figure 3: Effect of hypoxia/reoxygenation on the expression of occludin and cadherin in cultured cerebral endothelial cells.

Confluent monolayers were exposed to 16 h hypoxia followed by 4 h reoxygenation or kept under normoxic condition in the presence (lanes 1, 3) and absence (lanes 2 and 4) of glucose. As control the expression of actin is shown under the same conditions. One representative of six independent experiments is shown.

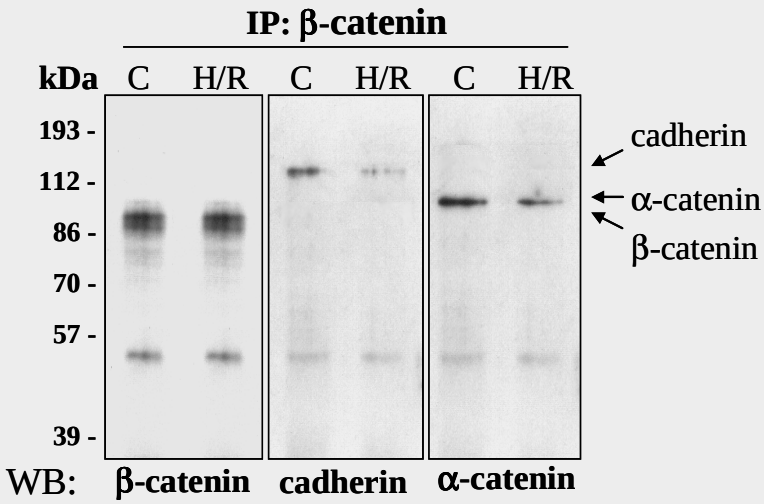

Figure 4: Interaction of cadherin, β-catenin and α-catenin in response to hypoxia/reoxygenation in cerebral endothelial cells.

β-catenin was immunoprecipitated from control cells and cells exposed to hypoxia/reoxygenation. The associated cadherin and α-catenin was detected by immunoblotting. One representative of two independent experiments is shown.

3.1.3 Changes of transendothelial resistance

Due to changes observed in the expression and interaction of junctional proteins we decided to study the effect of oxidative stress on the transendothelial resistance which reflects junctional integrity. Exposure of cells to 10μM DMNQ caused a rapid drop of TEER to 50% of the initial value after 20 minutes. Continuous exposure of the cells to DMNQ caused a further drop in TEER which was reduced almost to zero after 1 hour treatment (Figure 5).

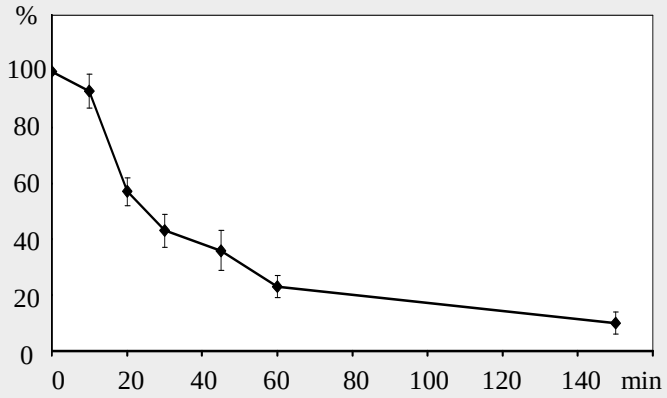

Figure 5: Effect of oxidative stress induced by DMNQ on the transendothelial electrical resistance of cerebral endothelial cells.

Cultures of brain endothelial cells were grown on filter inserts and treated with 10μM DMNQ. The integrity of the barrier was assessed by TEER measurement (average±SD, n=8).

3.1.4 Activity of MAPK

In looking for signal transduction pathways whose activation could lead to the dysfunction of the junctional complex we decided to study the activation of the MAPK pathways. By using an antibody which recognizes only the phosphorylated and thus the active form of the ERK1/2 we have shown that oxidative stress produced by the treatment of cerebral endothelial cells with 10μM DMNQ for 2 hours leads to an increased phosphorylation of ERK1/2 (Figure 6, lane 3). The presence of glucose can strongly influence the activity of ERK1/2. Glucose free medium has no effect on the activation of the ERK1/2 under control conditions (Figure 6, lane 2); however, absence of glucose strongly potentiates the activation of ERK1/2 in response to oxidative stress (Figure 6, lane 4). The activation of ERK1/2 could be almost completely blocked by its inhibitor U0126 (Figure 6, lanes 5 and 6).

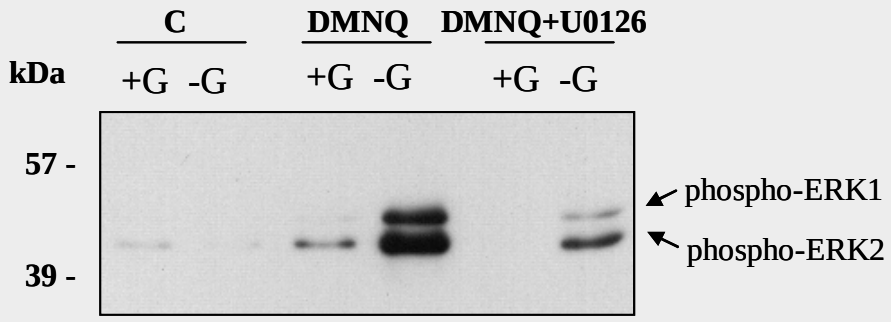

Figure 6: Effect of glucose on the activation of ERK1/2 by oxidative stress.
Cells were treated with 10 µM DMNQ for 2 h in the presence (lanes 3 and 5) and absence (lanes 4 and 6) of glucose. Activation of ERK can be almost completely blocked by its inhibitor U0126. One representative of four independent experiments is shown.

3.2. Hyperosmotic stress

3.2.1 Tyrosine phosphorylation

To determine endothelial signaling mechanisms activated by hyperosmotic environment we treated CECs with 20% mannitol (1.1 M) a concentration which is used to reversibly open the BBB in clinical trials. Under control conditions two phosphorylation bands can be detected at 50-60 kDa and 110 kDa (Figure 7A). Mannitol induces a rapid and strong phosphorylation on tyrosine residues of proteins in the range of 50-190 kDa. A 30 minute hyperosmolar treatment followed by the replacement of the hyperosmotic medium with standard medium lead to a remarkable reduction of tyrosine phosphorylation after only 10 minute recovery time which returned to normal levels after 60 minutes (Figure 7A). We have used inhibitors of different signaling pathways to determine the mechanisms through which mannitol induces tyrosine phosphorylation. Addition of the ERK inhibitor U0126 (10 µM) and the Rho inhibitor Y27632 (10 µM) did not affect the induction of tyrosine phosphorylation by mannitol. However, the Src kinase inhibitor PP-1 (10 µM) and the tyrosine kinase inhibitor genistein (50 µM) blocked the activation of tyrosine kinases (Figure 7A). To get an insight into the role of extracellular Ca^{2+} we used verapamil and EDTA. Neither the L-type calcium channel blocker verapamil nor EDTA was able to inhibit tyrosine phosphorylation induced by mannitol (Figure 7B). Furthermore we have shown that tyrosine phosphorylation is more intense in the Triton X-100 soluble cellular fraction compared to the Triton X-100 insoluble fraction (Figure 9A).

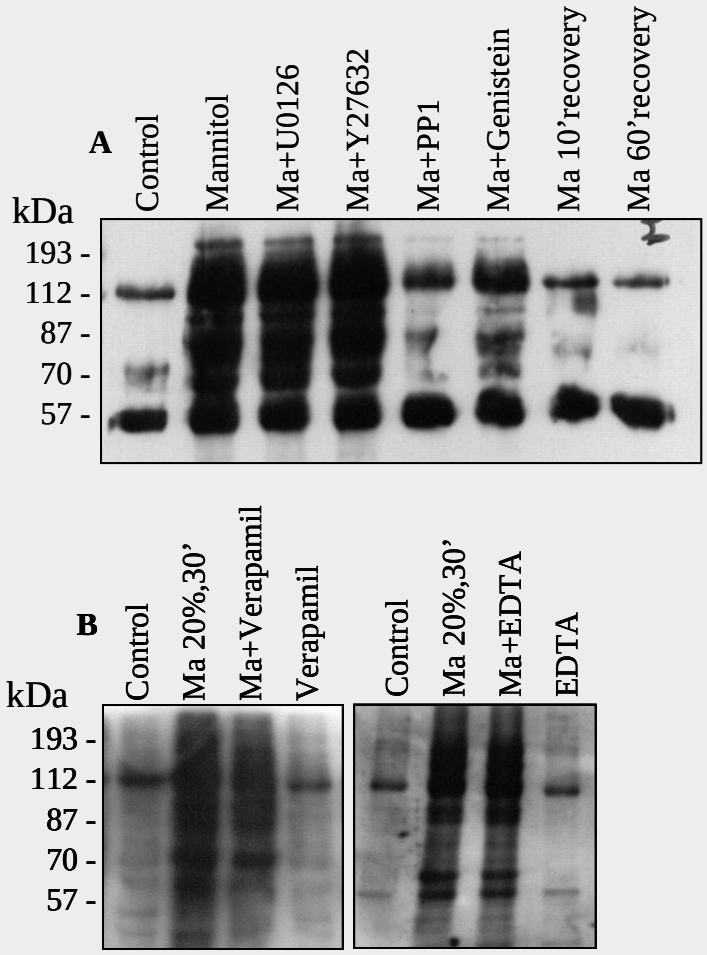

Figure 7: Tyrosine phosphorylation in CECs in response to mannitol treatment.

(A) Cells were treated with 20% (1.1 M) mannitol for 30 min. Recovery time and the effect of the Erk inhibitor U0126 (10 μM), Rho-kinase inhibitor Y27632 (10 μM), Src inhibitor PP1 (10 μM), tyrosine kinase inhibitor genistein (50 μM) was tested. (B) Effect of the L-type Ca^{++} channel blocker verapamil (10 μM) and EDTA (5 mM) on the mannitol induced tyrosine phosphorylation. One representative of four independent experiments is shown. (Ma=mannitol)

3.2.2 Activation of MAP kinases

To detect possible activation of different MAP kinases by hyperosmosis we used antibodies which detect the phosphorylated and thus activated form of MAP kinases. Addition of 10% mannitol caused phosphorylation of ERK1/2 which was more pronounced when we used 20% mannitol (Figure 8A). The phosphorylation was considerably weaker after 60 minutes recovery time and could be inhibited by the ERK inhibitor U0126 but not the Src inhibitor PP1 (Figure 8A). Under basal conditions we could not detect phosphorylation and

thus activation of ERK1/2 (Figure 8A). The total amount of ERK1/2 was not changed by mannitol treatment. Furthermore, we found no activation of p38 (Figure 8B).

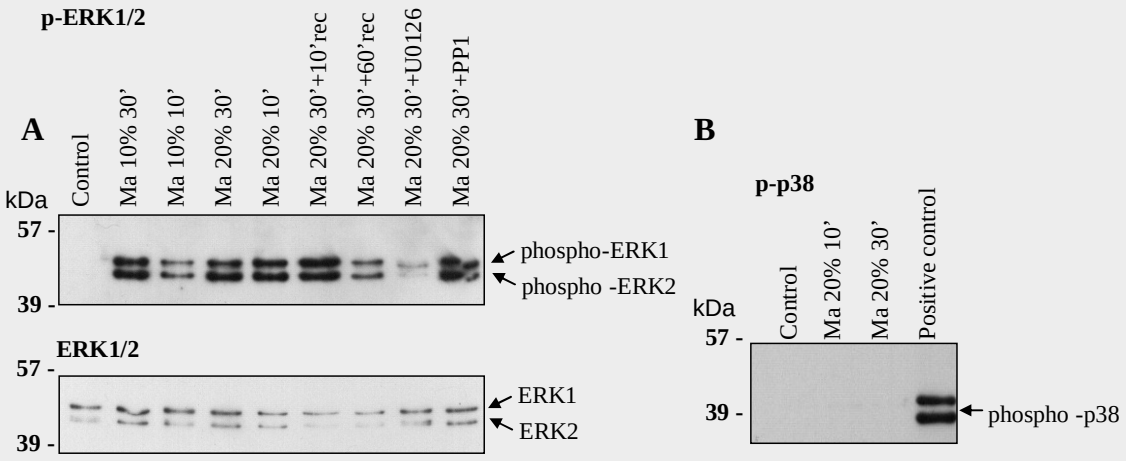

Figure 8: Induction of MAP kinases by mannitol in CECs.
The effect of 10% (0.55 M) or 20% (1.1 M) mannitol treatment for 10 or 30 min was tested on the activation (phosphorylation) of ERK1/2 (A) and p38 (B). The total amount of ERK1/2 is shown on (A), lower panel. Positive control (B): endothelial cells treated with phenylarsine oxide. One representative of three independent experiments is shown. (Ma=mannitol)

3.2.3 Phosphorylation of β-catenin

AJ proteins are often phosphorylated on tyrosine residues. By immunoprecipitation using phosphotyrosine antibodies we have shown that a target of the hyperosmosis induced tyrosine phosphorylation is the adherens junction protein β-catenin and the tyrosine phosphorylation of the Triton X-100 soluble β-catenin is stronger compared to the phosphorylation of the Triton X-100 insoluble β-catenin (Figure 9B). Under control conditions there were no signs of tyrosine phosphorylation of β-catenin (Figure 10A). Mannitol induced a strong phosphorylation on tyrosine residues of β-catenin which almost disappeared after only 10 minutes recovery time (Figure 10A). Tyrosine phosphorylation of β-catenin could be prevented by the Src-kinase inhibitor PP-1 and the tyrosine kinase inhibitor genistein but not with the ERK1/2 inhibitor U0126 or the Rho inhibitor Y27632 (Figure 10A). The L-type calcium channel blocker verapamil was also ineffective in inhibiting tyrosine phosphorylation of β-catenin induced by mannitol (Figure 10B).

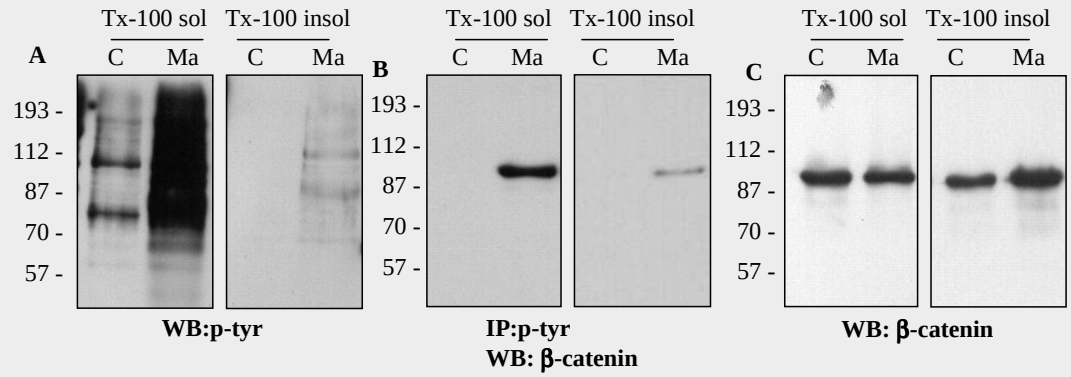

Figure 9: Triton X-100 solubility of the proteins phosphorylated in response to mannitol.

CECs were treated with 20% (1.1 M) mannitol for 30 min. (A) Tyrosine phosphorylation of proteins in the Triton X-100 soluble fraction (lanes 1, 2) and insoluble fraction (lanes 3, 4) was tested. (B) Tyrosine phosphorylation induced by mannitol of Triton X-100 soluble (lanes 1, 2) and insoluble (lanes 3, 4) β-catenin. Immunoprecipitation was performed using anti-phosphotyrosine antibodies and the blot was stained with anti-β-catenin antibody. (C) Distribution of β-catenin between the Triton X-100 soluble (lanes 1, 2) and insoluble (lanes 3, 4) fractions. One representative of two independent experiments is shown. (Ma=mannitol)

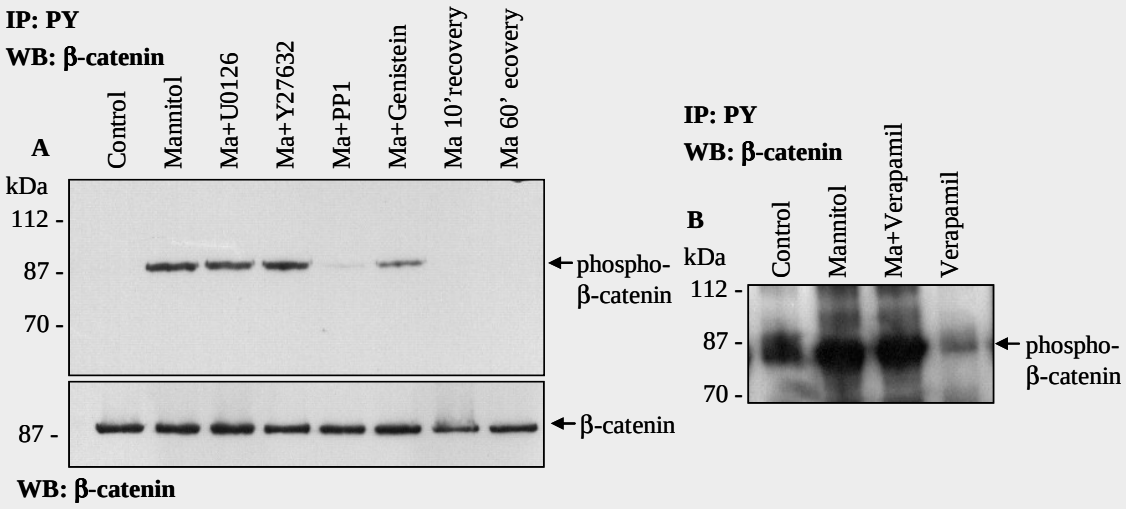

Figure 10: Phosphorylation of β-catenin.

CECs were treated with 20% (1.1 M) mannitol for 30 min. Recovery time and the effect of the ERK inhibitor U0126 (10 µM), Rho-kinase inhibitor Y27632 (10 µM), Src inhibitor PP1 (10 µM), tyrosine kinase inhibitor genistein (50 µM) was tested. A β-catenin control western-blot from the the same samples is also shown (A). (B) shows the effect of L-type calcium channel blocker verapamil (10 µM). One representative of three independent experiments is shown. (Ma=mannitol)

3.2.4 Localization of β-catenin

We have used immunofluorescent staining to study the localization of β-catenin. Under control conditions β-catenin is localized mainly to the cell membrane. In response to 20% mannitol treatment for 30 minutes the membrane staining became discontinuous without major change in staining intensity reflecting a redistribution rather than a change in expression of β-catenin (Figure 11).

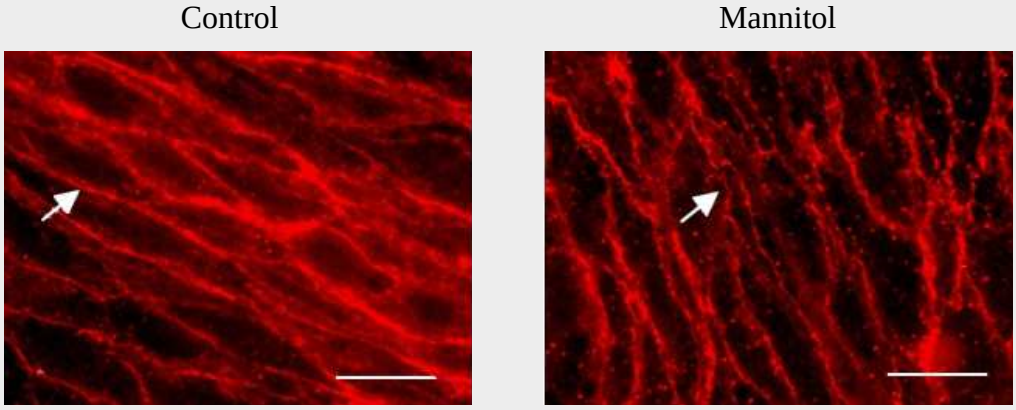

Figure 11: Redistribution of β-catenin in CECs treated with 20% mannitol. Arrows indicate β-catenin staining. Note the discontinuities of the membrane staining after 30 min of treatment with 20% (1.1 M) mannitol. Bar = 20 μm.

3.2.5 Interaction of the adherens junction proteins

Since tyrosine phosphorylation of AJ proteins may affect AJ function we tested the integrity of the adherens junctions following mannitol treatment by co-immunoprecipitation. Although total levels of cadherin remained unchanged in response to mannitol treatment considerably lower levels of cadherin were found in β-catenin precipitates from mannitol treated cels. Similarily α-catenin almost disappeared from β-catenin precipitates indicating a dissociation of the cadherin - β-catenin/α-catenin complex (Figure 12).

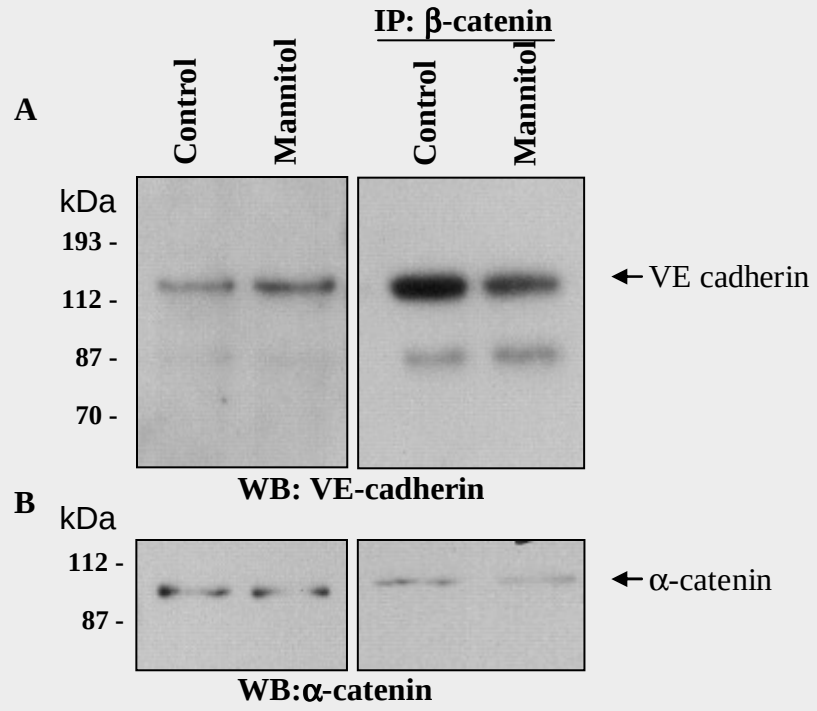

Figure 12: Effect of mannitol treatment on the interaction of β-catenin with α-catenin and cadherin.

CECs were treated with 20% (1.1 M) mannitol for 30 min. Immunoprecipitation was performed using anti β-catenin antibodies and the blots were stained with anti VE-cadherin (A) and α-catenin (B) antibodies. (Ma=mannitol)

4. Discussion

Being located at the forefront of the defense line of the CNS, the cerebral endothelium is exposed to a great variety of environmental stress factors. Any alteration in its function may have severe consequences for the CNS. In our study we have tried to address the question of how CECs respond to two different stress types: oxidative stress, that accompanies cerebral ischemia and osmotic stress, which has been successfully used to reversibly open the BBB for therapeutic purposes. Since a detailed in vivo biochemical and molecular study of CECs is not possible, we have used cultures of cloned murine brain endothelial cells and GP8 immortalized rat brain endothelial cells which according to literature data and our results retain in vivo characteristics, such as the expression of von Willebrand factor and tight junction proteins (Tontsch and Bauer, 1989 and Greenwood et al., 1996).

4.1. Oxidative stress and the blood brain barrier

Although there is a general agreement that CECs are sensitive to oxidative stress, its effect on endothelial viability is still controversial. Plateel et al. (1995) reported that LDH activity in the culture medium of CECs did not increase even after prolonged hypoxia. On the other hand Mertsch et al. (1995) demonstrated significant membrane damage even after 120 minutes hypoxia/30 minutes reoxygenation. This discrepancy may arise from the differences in the experimental conditions. To assess the membrane integrity of CECs after hypoxia/reoxygenation we have measured the LDH activity in the culture medium. Our results show that CECs do not exhibit a significant increase in LDH activity in response to hypoxia/reoxygenation indicating that the experimental conditions used do not affect cell viability.

The results presented here clearly indicate that CECs are sensitive to oxidative stress and changes in the endothelial function appear before the integrity and viability of the cells are seriously affected. Oxidative stress caused a rapid and intense decrease of the transendothelial resistance which is a sign of increased paracellular permeability. This led us to assume that junctional proteins which are responsible for the maintenance of the paracellular barrier may represent a potential target of reactive oxygen species. In our experiments oxidative stress led to a downregulation of occludin. The decrease of occludin was even more pronounced in the absence of glucose, a condition which better mimics the in vivo situation when the blood supply of the brain is impaired by different factors (e.g. stroke). Changes in the expression of junctional proteins in response to hypoxia/reoxygenation have

been demonstrated in several studies as well. Fischer et al. (2002) have shown that hypoxia causes a decrease in ZO-1 levels accompanied by an increased phosphorylation and redistribution of this protein. VEGF may play an important role in this process (Fischer et al., 2002). ZO-1 levels were also reduced by hypoxia in alveolar cells, while occludin was relocalized from the membrane to the cytoplasm (Bouvry et al., 2006). Results obtained from the study of endothelial cells of non-cerebral origin support the idea that glucose may be an important factor in the regulation of junctional proteins in oxidative stress. Consistent with our data presented here, Park et al. (1999) have shown that dermal microvascular endothelial cells respond to a combination of oxidative stress and absence of glucose with a significant downregulation of occludin and cadherin and concomitant increase in permeability. A proteolytic degradation of occludin associated with a reduction of TEER has been described in Caco-2 epithelial cells (Rao et al., 2002) as well. However, Mark and Davis (2002) found using bovine brain microvascular endothelial cells that hypoxia followed by reoxygenation causes an increase in the expression of junctional proteins like ZO-1 and occludin accompanied by an increased permeability to sucrose.

The mechanism by which occludin is downregulated is subject of extensive study. Most of our knowledge stems from experiments with epithelial cells or endothelial cells of non cerebral origin which makes it difficult to elucidate the mechanism of occludin downregulation in CECs. An important but not yet clarified issue in this respect is occludin phosphorylation. A decreased phosphorylation of occludin on threonine residues in response to PKC (protein kinase c) activation in LLC-PK1 cells led to a drop in the transendothelial resistance (Clarke et al., 2000). In accordance with these results it has been shown that increased serine phosphorylation of occludin is related to the localization of occludin to the tight junctions (Andreeva et al., 2001). An important role of occludin phosphorylation in the regulation of epithelial or endothelial permeability is supported by other experimental data as well (Antonetti et al., 1999 and Hirase et al., 2001). Moreover, it has been shown that inhibition of tyrosine phosphorylation leads to a degradation of occludin which is probably mediated by metalloproteases (Wachtel et al., 1999). An important pathway of occludin downregulation involves proteasomal degradation. It has been shown that occludin is able to bind the ubiquitin ligase Itch which mediates the ubquitination of occludin leading to its degradation (Traweger et al., 2002). However, no direct evidence of increased proteasomal degradation of occludin in response to oxidative stress is available so far.

MAP kinases are among the signaling pathways activated by oxidative stress. Our finding that ERK1/2 was phosphorylated and thus activated in response to oxidative stress is supported by results obtained from different types of endothelial and epithelial cells (Kevil et al., 2000 and Lee et al., 2006). An important element of the regulation of MAPK activity in conditions of oxidative stress is glucose deprivation. Although absence of glucose alone did not activate ERK1/2, absence of glucose combined with oxidative stress induced a drastic activation of ERK1/2. The precise role of ERK1/2 in the regulation of paracellular permeability is still controversial. It has been shown that activation of MAPK pathways lead to occludin downregulation and increased permeability (Chen et al., 2000) and inhibition of ERK1/2 during oxidative stress significantly reduces the downregulation of occludin (Park et al., 1999). The study performed by Kevil et al. (2000) on HUVECs led to a similar result. On the other hand, results by Wachtel et al. (2002) demonstrate that ERK activity is essential to rebuild a disrupted endothelial barrier after ischemia.

Besides tight junctions, adherens junctions play an important role in maintaining the paracellular barrier and the integrity of the BBB as well (Pal et al., 1997). Cadherin is also susceptible to ischemic downregulation (Abbruscato and Davis, 1999b and Bush et al., 2000) and a dissociation of the cadherin – catenin complex in response to ischemia or oxidative stress has been detected in MDCK (Madin-Darby canine kidney) cells (Bush. et al., 2000) caco-2 cells (Rao et al., 2002) and dermal microvascular endothelial cells (Park et al., 1999). Here we show that a dissociation of the cadherin - β-catenin and α-catenin - β-catenin complex occurs in cerebral endothelial cells in response to hypoxia/reoxygenation as well. Although cadherin levels were slightly reduced, β-catenin levels remained unaffected in response to oxidative stress.

4.2. Hyperosmosis and the blood-brain barrier

It is well known that one of the most important consequences of the hyperosmotic environment is cellular shrinkage which at the level of the cerebral endothelium may lead to the opening of the BBB (Greenwood et al., 1988). However, there is evidence that reversible osmotic disruption and reconstruction of the BBB is not due to simple mechanical shrinkage of the endothelial cells but is mediated by the activation of several intracellular signaling mechanisms. Nagashima et al. (1997) have shown that exposure of rat brain capillary endothelial cells to 1.4 M mannitol caused a rapid increase of intracellular Ca^{2+} concentration which reached its peak value within 10 seconds after the application of mannitol and returned

to the basal level within 200 seconds. Other investigators found similar results in aortic endothelial cells as well showing that hyperosmolar conditions leads to Ca^{2+} entry from the extracellular space (Marchenko et al., 2000).

We obserwed that 20% (1.1 M) mannitol induced a rapid and reversible phosphorylation of brain endothelial proteins. This finding is in line with the results of Ragette et al. (1997) showing that exposure of cultured lung microvascular endothelial cells to hyperosmotic conditions induced a genistein-inhibitable increase of protein tyrosine phosphorylation. However, tyrosine phosphorylation affected different protein bands in these cells and the kinetics of the phosphorylation was different as well. Hyperosmosis-induced protein phosphorylation has been reported by Szaszi et al. (1997) in Chinese hamster ovary cells which affected proteins at 42, 85 and 120 kDa. These and our data suggest that protein targets of hyperosmosis-induced tyrosine phosphorylation are cell specific. However, relatively few proteins have been identified so far to be phosphorylated on tyrosine residues in response to osmotic stress.

Another network of signaling pathways which could be activated by hyperosmosis are MAP kinases. Activation of ERK1/2, p38 and JNK has been observed in different cell types like inner medullary collecting duct cells (Berl et al., 1997) and bovine aortic endothelial cells (Gatsios et al., 1998 and Duzgun et al., 2000). These studies have demonstrated that different osmotic agents may induce MAPK activation differently. The downstream elements of MAPK pathways are less well characterized. p38, a member of the MAPK family has been shown to be involved in the activation of the transcription factor osmosis response element binding protein (OREBP/TonEBP) and thus may participate in the regulation of potentially osmoprotective genes. However, inhibition of p38 by SB203580 was only partially able to block the hypertonic induction of ORE reporter construct which suggests that other signaling pathways may play a role in this process (Ko et al., 2002). Beside regulation of osmosensitive genes MAPKs have been shown to mediate several other effects of hyperosmosis like increased leukocyte migration (Schäffler et al., 2000), L-selectin expression (Rizoli et al., 1999) and IL-8 production (Németh et al., 2002).

In our study mannitol was able to induce a rapid, concentration dependent activation of ERK1/2. No activation of p38 could be observed. Interestingly, activation of ERK1/2 was independent of tyrosine phosphorylation because U0126 was able to inhibit only the activation of ERK1/2 and had no influence on tyrosine phosphorylation.

Rho and the Rho/ROK (Rho-kinase) pathway could also be involved in mediating the effect of hyperosmosis. This activation has been shown to lead to the phosphorylation of myosin light chain (Ciano et al., 2003). Furthermore, Rho family-dependent cytoskeleton remodeling may be an important osmoprotective response that reinforces the cell cortex (Ciano et al., 2002). However, as demonstrated in our experiments, mannitol induced tyrosine phosphorylation in cerebral endothelial cells does not require activation of Rho and the Rho/ROK pathway.

In contrast, we have shown that PP1, a selective Src family inhibitor, strongly reduced tyrosine phosphorylation. It has been shown that members of the Src family, especially Fyn are volume sensitive enzymes and are able to phosphorylate protein components of the cell-cell contact apparatus in different cell types (Owens et al., 2000 and Ko et al., 2002). Recently Src has been found to localize to the AJ in HUVECs (Lambeng et al., 2005).

Furthermore, we found that one of the targets of tyrosine phosphorylation is β-catenin. β-catenin in the Triton X-100 soluble fraction is rapidly phosphorylated in response to mannitol treatment and its phosphorylation state returns to control level within 10 minutes after the removal of mannitol. No significant change in the Ser phosphorylation of β-catenin was observed (data not shown).

Alteration of cadherin-mediated cell-cell adhesion is frequently associated to tyrosine phosphorylation of β-catenin and the phosphorylation occurs mainly on Tyr-86 and Tyr-654 (Roura et al., 1999). Furthermore, it has been shown that tyrosine phosphorylation of β-catenin decreases the affinity of β-catenin for the cytosolic domain of E-cadherin (Roura et al., 1999). Tyrosine phosphorylation of β-catenin (Tyr-142) has been reported to be responsible for the disruption of α -catenin/β-catenin binding and altered cell adhesion as well (Ozawa and Kemler, 1998, Pai et al., 2004).

In our attempt to decipher the signaling pathways leading to phosphorylation of β-catenin we have found that the Src family kinase inhibitor PP-1 was able to inhibit β-catenin phosphorylation. Src family members have been shown to be important regulators of AJ protein phosphorylation and they can be activated by different extracellular stimuli including osmotic stress as demonstrated by Kapus et al. (1999) in CHO cells. The mechanism by which Src regulates BBB function is largely unknown. According to Kusaka et al. (2004) inhibition of Src activation is able to reduce the BBB permeability increase, edema formation and phosphorylation of VEGF and MAP kinases induced by subarachnoid hemorrhage.

Furthermore, in vitro studies have revealed that Src can directly phosphorylate junctional proteins (Kale et al., 2003) and Src induced tyrosine phosphorylation of β-catenin and ZO-1 in epithelial cells can lead to redistribution of junctional proteins and increase in transepithelial electric resistance (Basuroy et al., 2003). Similarly to general tyrosine phosphorylation, tyrosine phosphorylation of β-catenin could not be prevented by U0126 and Y27632 indicating that ERK1/2 and Rho dependent pathways do not play a significant role in this process.

An important regulator of tyrosine phosphorylation is calcium which may exert its effect through activation of different signaling pathways. Furthermore an increase in intracellular calcium in response to mannitol has already been demonstrated. Despite these findings our results show that extracellular calcium does not play a major role in mannitol induced tyrosine phosphorylation and β-catenin phosphorylation in CECs.

5. Summary

The blood-brain barrier plays an important role in maintaining the constant biochemical environment necessary for the physiological activity of neurons. Neurological disorders or different stress factors affecting the brain may affect the BBB as well which in turn could aggravate neuronal injury and negatively influence the outcome of the disease. In our experiments we have studied at molecular level two conditions which may occur in vivo as well: oxidative stress, which accompanies a large number of neurological disorders and hyperosmotic stress which has been used for therapeutical opening of the BBB.

Our results show, that oxidative stress induces a downregulation in the expression of the tight junction protein occludin. A significant factor in the regulation of occludin under these experimental conditions is glucose: its absence may significantly aggravate the damage caused by oxidative stress. Furthermore, oxidative stress leads to disruption of the cadherin-β-catenin complex and an activation of ERK1/2, which is more intense in the absence of glucose. We have shown that one of the causes of the BBB breakdown is probably the structural alteration of the junctional complex caused by oxidative stress, a process in which ERK1/2 may play an important role.

Regarding the effects of hyperosmotic mannitol we found that mannitol induced a rapid, concentration dependent and reversible tyrosine phosphorylation of a broad range of proteins between 50 and 190 kDa. One of the targets of tyrosine phosphorylation turned out to be the adherens junction protein β-catenin. Phosphorylation of β-catenin on tyrosine residues caused its subcellular redistribution and its dissociation from cadherin and α-catenin as shown by co-immunoprecipitation studies. All these effects could be inhibited by the Src-kinase inhibitor PP-1 but not by the Erk inhibitor U0126, the Rho-kinase inhibitor Y27632 or the calcium channel blocker verapamil. Since β-catenin is a key component of the junctional complex its Src-mediated phosphorylation may play an important role in the mannitol induced reversible opening of the BBB.

Our results clearly demonstrate, that CECs are active players in the studied processes, are able to respond quickly to environmental stimuli and we were able to unravel some molecular elements of the endothelial response.

6. References

Abbruscato, T. J. & Davis, T. P. (1999), 'Combination of hypoxia/aglycemia compromises in vitro blood-brain barrier integrity.', *J Pharmacol Exp Ther* 289(2), 668-675.

Abbruscato, T. J. & Davis, T. P. (1999b), 'Protein expression of brain endothelial cell E-cadherin after hypoxia/aglycemia: influence of astrocyte contact.', *Brain Res* 842(2), 277-286.

Adachi, M.; Inoko, A.; Hata, M.; Furuse, K.; Umeda, K.; Itoh, M. & Tsukita, S. (2006), 'Normal establishment of epithelial tight junctions in mice and cultured cells lacking expression of ZO-3, a tight-junction MAGUK protein.', *Mol Cell Biol* 26(23), 9003-9015.

Adamson, P.; Etienne, S.; Couraud, P. O.; Calder, V. & Greenwood, J. (1999), 'Lymphocyte migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a rho-dependent pathway.', *J Immunol* 162(5), 2964-2973.

Andreeva, A. Y.; Krause, E.; Müller, E. C.; Blasig, I. E. & Utepbergenov, D. I. (2001), 'Protein kinase C regulates the phosphorylation and cellular localization of occludin.', *J Biol Chem* 276(42), 38480-38486.

Antonetti, D. A.; Barber, A. J.; Hollinger, L. A.; Wolpert, E. B. & Gardner, T. W. (1999), 'Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors.', *J Biol Chem* 274(33), 23463-23467.

Balda, M. S.; Gonzalez-Mariscal, L.; Matter, K.; Cereijido, M. & Anderson, J. M. (1993), 'Assembly of the tight junction: the role of diacylglycerol.', *J Cell Biol* 123(2), 293-302.

Balda, M. S.; Whitney, J. A.; Flores, C.; González, S.; Cereijido, M. & Matter, K. (1996), 'Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein.', *J Cell Biol* 134(4), 1031-1049.

Basuroy, S.; Sheth, P.; Kuppuswamy, D.; Balasubramanian, S.; Ray, R. M. & 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(14), 11916-11924.

Bazzoni, G.; Martinez-Estrada, O. M.; Orsenigo, F.; Cordenonsi, M.; Citi, S. & Dejana, E. (2000), 'Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin.', *J Biol Chem* 275(27), 20520-20526.

Berl, T.; Siriwardana, G.; Ao, L.; Butterfield, L. M. & Heasley, L. E. (1997), 'Multiple

mitogen-activated protein kinases are regulated by hyperosmolality in mouse IMCD cells.', *Am J Physiol* 272(3 Pt 2), F305-F311.

Bouvry, D.; Planès, C.; Malbert-Colas, L.; Escabasse, V. & Clerici, C. (2006), 'Hypoxia-induced cytoskeleton disruption in alveolar epithelial cells.', *Am J Respir Cell Mol Biol* 35(5), 519-527.

Bresgen, N.; Karlhuber, G.; Krizbai, I.; Bauer, H.; Bauer, H. C. & Eckl, P. M. (2003), 'Oxidative stress in cultured cerebral endothelial cells induces chromosomal aberrations, micronuclei, and apoptosis.', *J Neurosci Res* 72(3), 327-333.

Brightman, M. W.; Hori, M.; Rapoport, S. I.; Reese, T. S. & Westergaard, E. (1973), 'Osmotic opening of tight junctions in cerebral endothelium.', *J Comp Neurol* 152(4), 317-325.

Brillault, J.; Berezowski, V.; Cecchelli, R. & Dehouck, M.-P. (2002), 'Intercommunications between brain capillary endothelial cells and glial cells increase the transcellular permeability of the blood-brain barrier during ischaemia.', *J Neurochem* 83(4), 807-817.

Bush, K. T.; Tsukamoto, T. & Nigam, S. K. (2000), 'Selective degradation of E-cadherin and dissolution of E-cadherin-catenin complexes in epithelial ischemia.', *Am J Physiol Renal Physiol* 278(5), F847-F852.

Chen, Y.; Lu, Q.; Schneeberger, E. E. & Goodenough, D. A. (2000), 'Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in ras-transformed Madin-Darby canine kidney cells.', *Mol Biol Cell* 11(3), 849-862.

Chen, Y.; Merzdorf, C.; Paul, D. L. & Goodenough, D. A. (1997), 'COOH terminus of occludin is required for tight junction barrier function in early Xenopus embryos.', *J Cell Biol* 138(4), 891-899.

Ciano, C. D.; Nie, Z.; Szászi, K.; Lewis, A.; Uruno, T.; Zhan, X.; Rotstein, O. D.; Mak, A. & Kapus, A. (2002), 'Osmotic stress-induced remodeling of the cortical cytoskeleton.', *Am J Physiol Cell Physiol* 283(3), C850-C865.

Ciano-Oliveira, C. D.; Sirokmány, G.; Szászi, K.; Arthur, W. T.; Masszi, A.; Peterson, M.; Rotstein, O. D. & Kapus, A. (2003), 'Hyperosmotic stress activates Rho: differential involvement in Rho kinase-dependent MLC phosphorylation and NKCC activation.', *Am J Physiol Cell Physiol* 285(3), C555-C566.

Citi, S.; Sabanay, H.; Jakes, R.; Geiger, B. & Kendrick-Jones, J. (1988), 'Cingulin, a new peripheral component of tight junctions.', *Nature* 333(6170), 272-276.

Clarke, H.; Soler, A. P. & Mullin, J. M. (2000), 'Protein kinase C activation leads to dephosphorylation of occludin and tight junction permeability increase in LLC-PK1 epithelial

cell sheets.', *J Cell Sci* 113 (Pt 18), 3187-3196.

Cordenonsi, M.; D'Atri, F.; Hammar, E.; Parry, D. A.; Kendrick-Jones, J.; Shore, D. & Citi, S. (1999), 'Cingulin contains globular and coiled-coil domains and interacts with ZO-1, ZO-2, ZO-3, and myosin.', *J Cell Biol* 147(7), 1569-1582.

Cordenonsi, M.; Turco, F.; D'atri, F.; Hammar, E.; Martinucci, G.; Meggio, F. & Citi, S. (1999b), 'Xenopus laevis occludin. Identification of in vitro phosphorylation sites by protein kinase CK2 and association with cingulin.', *Eur J Biochem* 264(2), 374-384.

D'Atri, F. & Citi, S. (2001), 'Cingulin interacts with F-actin in vitro.', *FEBS Lett* 507(1), 21-24.

D'Atri, F.; Nadalutti, F. & Citi, S. (2002), 'Evidence for a functional interaction between cingulin and ZO-1 in cultured cells.', *J Biol Chem* 277(31), 27757-27764.

Doolittle, N. D.; Miner, M. E.; Hall, W. A.; Siegal, T.; Jerome, E.; Osztie, E.; McAllister, L. D.; Bubalo, J. S.; Kraemer, D. F.; Fortin, D.; Nixon, R.; Muldoon, L. L. & Neuwelt, E. A. (2000), 'Safety and efficacy of a multicenter study using intraarterial chemotherapy in conjunction with osmotic opening of the blood-brain barrier for the treatment of patients with malignant brain tumors.', *Cancer* 88(3), 637-647.

Dorovini-Zis, K.; Sato, M.; Goping, G.; Rapoport, S. & Brightman, M. (1983), 'Ionic lanthanum passage across cerebral endothelium exposed to hyperosmotic arabinose.', *Acta Neuropathol* 60(1-2), 49-60.

Drees, F.; Pokutta, S.; Yamada, S.; Nelson, W. J. & Weis, W. I. (2005), 'Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly.', *Cell* 123(5), 903-915.

Duzgun, S. A.; Rasque, H.; Kito, H.; Azuma, N.; Li, W.; Basson, M. D.; Gahtan, V.; Dudrick, S. J. & Sumpio, B. E. (2000), 'Mitogen-activated protein phosphorylation in endothelial cells exposed to hyperosmolar conditions.', *J Cell Biochem* 76(4), 567-571.

Ernkvist, M.; Persson, N. L.; Audebert, S.; Lecine, P.; Sinha, I.; Liu, M.; Schlueter, M.; Horowitz, A.; Aase, K.; Weide, T.; Borg, J.-P.; Majumdar, A. & Holmgren, L. (2008), 'The Amot/Patj/Syx signalling complex spatially controls RhoA GTPase activity in migrating endothelial cells.', *Blood*.

Etienne, S.; Adamson, P.; Greenwood, J.; Strosberg, A. D.; Cazaubon, S. & Couraud, P. O. (1998), 'ICAM-1 signaling pathways associated with Rho activation in microvascular brain endothelial cells.', *J Immunol* 161(10), 5755-5761.

Fanning, A. S.; Jameson, B. J.; Jesaitis, L. A. & 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(45), 29745-29753.

Fanning, A. S.; Little, B. P.; Rahner, C.; Utepbergenov, D.; Walther, Z. & Anderson, J. M. (2007), 'The unique-5 and -6 motifs of ZO-1 regulate tight junction strand localization and scaffolding properties.', *Mol Biol Cell* 18(3), 721-731.

Fischer, S.; Wobben, M.; Marti, H. H.; Renz, D. & Schaper, W. (2002), 'Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1.', *Microvasc Res* 63(1), 70-80.

Furuse, M.; Fujita, K.; Hiiragi, T.; Fujimoto, K. & 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(7), 1539-1550.

Furuse, M.; Hirase, T.; Itoh, M.; Nagafuchi, A.; Yonemura, S.; Tsukita, S. & Tsukita, S. (1993), 'Occludin: a novel integral membrane protein localizing at tight junctions.', *J Cell Biol* 123(6 Pt 2), 1777-1788.

Furuse, M.; Sasaki, H.; Fujimoto, K. & Tsukita, S. (1998b), 'A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts.', *J Cell Biol* 143(2), 391-401.

Gatsios, P.; Terstegen, L.; Schliess, F.; Häussinger, D.; Kerr, I. M.; Heinrich, P. C. & Graeve, L. (1998), 'Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock.', *J Biol Chem* 273(36), 22962-22968.

Gaudry, C. A.; Palka, H. L.; Dusek, R. L.; Huen, A. C.; Khandekar, M. J.; Hudson, L. G. & Green, K. J. (2001), 'Tyrosine-phosphorylated plakoglobin is associated with desmogleins but not desmoplakin after epidermal growth factor receptor activation.', *J Biol Chem* 276(27), 24871-24880.

Giepmans, B. N. & Moolenaar, W. H. (1998), 'The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein.', *Curr Biol* 8(16), 931-934.

Gillrie, M. R.; Krishnegowda, G.; Lee, K.; Buret, A. G.; Robbins, S. M.; Looareesuwan, S.; Gowda, D. C. & Ho, M. (2007), 'Src-family kinase dependent disruption of endothelial barrier function by Plasmodium falciparum merozoite proteins.', *Blood* 110(9), 3426-3435.

Granger, D. N. (1988), 'Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury.', *Am J Physiol* 255(6 Pt 2), H1269-H1275.

Greenwood, J.; Luthert, P. J.; Pratt, O. E. & Lantos, P. L. (1988), 'Hyperosmolar opening of the blood-brain barrier in the energy-depleted rat brain. Part 1. Permeability studies.', *J Cereb Blood Flow Metab* 8(1), 9-15.

Greenwood, J.; Pryce, G.; Devine, L.; Male, D. K.; dos Santos, W. L.; Calder, V. L. & 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(1-2), 51-63.

Gumbiner, B.; Lowenkopf, T. & Apatira, D. (1991), 'Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1.', *Proc Natl Acad Sci U S A* 88(8), 3460-3464.

Gumbiner, B. & Simons, K. (1986), 'A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide.', *J Cell Biol* 102(2), 457-468.

Haskins, J.; Gu, L.; Wittchen, E. S.; Hibbard, J. & Stevenson, B. R. (1998), 'ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin.', *J Cell Biol* 141(1), 199-208.

Hess, D. C.; Zhao, W.; Carroll, J.; McEachin, M. & Buchanan, K. (1994), 'Increased expression of ICAM-1 during reoxygenation in brain endothelial cells.', *Stroke* 25(7), 1463-7; discussion 1468.

Hirase, T.; Kawashima, S.; Wong, E. Y.; Ueyama, T.; Rikitake, Y.; Tsukita, S.; Yokoyama, M. & Staddon, J. M. (2001), 'Regulation of tight junction permeability and occludin phosphorylation by Rhoa-p160ROCK-dependent and -independent mechanisms.', *J Biol Chem* 276(13), 10423-10431.

Hollande, F.; Lee, D. J.; Choquet, A.; Roche, S. & Baldwin, G. S. (2003), 'Adherens junctions and tight junctions are regulated via different pathways by progastrin in epithelial cells.', *J Cell Sci* 116(Pt 7), 1187-1197.

Ide, N.; Hata, Y.; Nishioka, H.; Hirao, K.; Yao, I.; Deguchi, M.; Mizoguchi, A.; Nishimori, H.; Tokino, T.; Nakamura, Y. & Takai, Y. (1999), 'Localization of membrane-associated guanylate kinase (MAGI)-1/BAI-associated protein (BAP) 1 at tight junctions of epithelial cells.', *Oncogene* 18(54), 7810-7815.

Ikeda, W.; Nakanishi, H.; Miyoshi, J.; Mandai, K.; Ishizaki, H.; Tanaka, M.; Togawa, A.; Takahashi, K.; Nishioka, H.; Yoshida, H.; Mizoguchi, A.; Nishikawa, S. & Takai, Y. (1999), 'Afadin: A key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis.', *J Cell Biol* 146(5), 1117-1132.

Imamura, Y.; Itoh, M.; Maeno, Y.; Tsukita, S. & Nagafuchi, A. (1999), 'Functional domains of alpha-catenin required for the strong state of cadherin-based cell adhesion.', *J Cell Biol* 144(6), 1311-1322.

Inoko, A.; Itoh, M.; Tamura, A.; Matsuda, M.; Furuse, M. & Tsukita, S. (2003), 'Expression and distribution of ZO-3, a tight junction MAGUK protein, in mouse tissues.', *Genes Cells* 8(11), 837-845.

Itoh, M.; Furuse, M.; Morita, K.; Kubota, K.; Saitou, M. & Tsukita, S. (1999), 'Direct

binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins.', *J Cell Biol* 147(6), 1351-1363.

Itoh, M.; Morita, K. & Tsukita, S. (1999b), 'Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin.', *J Biol Chem* 274(9), 5981-5986.

Itoh, M.; Nagafuchi, A.; Moroi, S. & Tsukita, S. (1997), 'Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments.', *J Cell Biol* 138(1), 181-192.

Kale, G.; Naren, A. P.; Sheth, P. & Rao, R. K. (2003), 'Tyrosine phosphorylation of occludin attenuates its interactions with ZO-1, ZO-2, and ZO-3.', *Biochem Biophys Res Commun* 302(2), 324-329.

Kapus, A.; Szászi, K.; Sun, J.; Rizoli, S. & Rotstein, O. D. (1999), 'Cell shrinkage regulates Src kinases and induces tyrosine phosphorylation of cortactin, independent of the osmotic regulation of Na+/H+ exchangers.', *J Biol Chem* 274(12), 8093-8102.

Katsube, T.; Takahisa, M.; Ueda, R.; Hashimoto, N.; Kobayashi, M. & Togashi, S. (1998), 'Cortactin associates with the cell-cell junction protein ZO-1 in both Drosophila and mouse.', *J Biol Chem* 273(45), 29672-29677.

Kausalya, P. J.; Reichert, M. & Hunziker, W. (2001), 'Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells.', *FEBS Lett* 505(1), 92-96.

Kevil, C. G.; Oshima, T.; Alexander, B.; Coe, L. L. & Alexander, J. S. (2000), 'H(2)O(2)-mediated permeability: role of MAPK and occludin.', *Am J Physiol Cell Physiol* 279(1), C21-C30.

Kevil, C. G.; Oshima, T. & Alexander, J. S. (2001), 'The role of p38 MAP kinase in hydrogen peroxide mediated endothelial solute permeability.', *Endothelium* 8(2), 107-116.

Ko, B. C. B.; Lam, A. K. M.; Kapus, A.; Fan, L.; Chung, S. K. & Chung, S. S. M. (2002), 'Fyn and p38 signaling are both required for maximal hypertonic activation of the osmotic response element-binding protein/tonicity-responsive enhancer-binding protein (OREBP/TonEBP).', *J Biol Chem* 277(48), 46085-46092.

Koh, Y. H.; Che, W.; Higashiyama, S.; Takahashi, M.; Miyamoto, Y.; Suzuki, K. & Taniguchi, N. (2001), 'Osmotic stress induces HB-EGF gene expression via Ca(2+)/Pyk2/JNK signal cascades in rat aortic smooth muscle cells.', *J Biochem* 130(3), 351-358.

Krause, G.; Winkler, L.; Mueller, S. L.; Haseloff, R. F.; Piontek, J. & Blasig, I. E. (2008), 'Structure and function of claudins.', *Biochim Biophys Acta* 1778(3), 631-645.

Kroll, R. A. & Neuwelt, E. A. (1998), 'Outwitting the blood-brain barrier for therapeutic purposes: osmotic opening and other means.', *Neurosurgery* 42(5), 1083-99; discussion 1099-100.

Kusaka, G.; Ishikawa, M.; Nanda, A.; Granger, D. N. & Zhang, J. H. (2004), 'Signaling pathways for early brain injury after subarachnoid hemorrhage.', *J Cereb Blood Flow Metab* 24(8), 916-925.

Lagrange, P.; Romero, I. A.; Minn, A. & Revest, P. A. (1999), 'Transendothelial permeability changes induced by free radicals in an in vitro model of the blood-brain barrier.', *Free Radic Biol Med* 27(5-6), 667-672.

Lambeng, N.; Wallez, Y.; Rampon, C.; Cand, F.; Christé, G.; Gulino-Debrac, D.; Vilgrain, I. & Huber, P. (2005), 'Vascular endothelial-cadherin tyrosine phosphorylation in angiogenic and quiescent adult tissues.', *Circ Res* 96(3), 384-391.

Latorre, I. J.; Roh, M. H.; Frese, K. K.; Weiss, R. S.; Margolis, B. & Javier, R. T. (2005), 'Viral oncoprotein-induced mislocalization of select PDZ proteins disrupts tight junctions and causes polarity defects in epithelial cells.', *J Cell Sci* 118(Pt 18), 4283-4293.

Laura, R. P.; Ross, S.; Koeppen, H. & Lasky, L. A. (2002), 'MAGI-1: a widely expressed, alternatively spliced tight junction protein.', *Exp Cell Res* 275(2), 155-170.

Lee, J. S.; Kim, S. Y.; Kwon, C. H. & Kim, Y. K. (2006), 'EGFR-dependent ERK activation triggers hydrogen peroxide-induced apoptosis in OK renal epithelial cells.', *Arch Toxicol* 80(6), 337-346.

Lossinsky, A. S.; Vorbrodt, A. W. & Wisniewski, H. M. (1995), 'Scanning and transmission electron microscopic studies of microvascular pathology in the osmotically impaired blood-brain barrier.', *J Neurocytol* 24(10), 795-806.

Marchenko, S. M. & Sage, S. O. (2000), 'Hyperosmotic but not hyposmotic stress evokes a rise in cytosolic Ca2+ concentration in endothelium of intact rat aorta.', *Exp Physiol* 85(2), 151-157.

Mark, K. S. & Davis, T. P. (2002), 'Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation.', *Am J Physiol Heart Circ Physiol* 282(4), H1485-H1494.

Martìn-Padura, I.; Lostaglio, S.; Schneemann, M.; Williams, L.; Romano, M.; Fruscella, P.; Panzeri, C.; Stoppacciaro, A.; Ruco, L.; Villa, A.; Simmons, D. & Dejana, E. (1998), 'Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration.', *J Cell Biol* 142(1), 117-127.

McCord, J. M. & Roy, R. S. (1982), 'The pathophysiology of superoxide: roles in inflammation and ischemia.', *Can J Physiol Pharmacol* 60(11), 1346-1352.

Mertsch, K.; Grune, T.; Siems, W. G.; Ladhoff, A.; Saupe, N. & Blasig, I. E. (1995), 'Hypoxia and reoxygenation of brain endothelial cells in vitro: a comparison of biochemical and morphological response.', *Cell Mol Biol (Noisy-le-grand)* 41(2), 243-253.

Murata, M.; Kojima, T.; Yamamoto, T.; Go, M.; Takano, K.-I.; Osanai, M.; Chiba, H. & Sawada, N. (2005), 'Down-regulation of survival signaling through MAPK and Akt in occludin-deficient mouse hepatocytes in vitro.', *Exp Cell Res* 310(1), 140-151.

Nagafuchi, A. & Takeichi, M. (1989), 'Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin.', *Cell Regul* 1(1), 37-44.

Nagasawa, K.; Chiba, H.; Fujita, H.; Kojima, T.; Saito, T.; Endo, T. & Sawada, N. (2006), 'Possible involvement of gap junctions in the barrier function of tight junctions of brain and lung endothelial cells.', *J Cell Physiol* 208(1), 123-132.

Nagashima, T.; Ikeda, K.; Wu, S.; Kondo, T.; Yamaguchi, M. & Tamaki, N. (1997), 'The mechanism of reversible osmotic opening of the blood-brain barrier: role of intracellular calcium ion in capillary endothelial cells.', *Acta Neurochir Suppl* 70, 231-233.

Nagy, Z.; Peters, H. & Hüttner, I. (1984), 'Fracture faces of cell junctions in cerebral endothelium during normal and hyperosmotic conditions.', *Lab Invest* 50(3), 313-322.

Naik, M. U.; Vuppalanchi, D. & Naik, U. P. (2003), 'Essential role of junctional adhesion molecule-1 in basic fibroblast growth factor-induced endothelial cell migration.', *Arterioscler Thromb Vasc Biol* 23(12), 2165-2171.

Namgaladze, D.; Shcherbyna, I.; Kienhöfer, J.; Hofer, H. W. & Ullrich, V. (2005), 'Superoxide targets calcineurin signaling in vascular endothelium.', *Biochem Biophys Res Commun* 334(4), 1061-1067.

Neuwelt, E. A.; Goldman, D. L.; Dahlborg, S. A.; Crossen, J.; Ramsey, F.; Roman-Goldstein, S.; Braziel, R. & Dana, B. (1991), 'Primary CNS lymphoma treated with osmotic blood-brain barrier disruption: prolonged survival and preservation of cognitive function.', *J Clin Oncol* 9(9), 1580-1590.

North, A. J.; Brannon, T. S.; Wells, L. B.; Campbell, W. B. & Shaul, P. W. (1994), 'Hypoxia stimulates prostacyclin synthesis in newborn pulmonary artery endothelium by increasing cyclooxygenase-1 protein.', *Circ Res* 75(1), 33-40.

Németh, Z. H.; Deitch, E. A.; Szabó, C. & Haskó, G. (2002), 'Hyperosmotic stress induces nuclear factor-kappaB activation and interleukin-8 production in human intestinal epithelial cells.', *Am J Pathol* 161(3), 987-996.

Ohnishi, H.; Nakahara, T.; Furuse, K.; Sasaki, H.; Tsukita, S. & Furuse, M. (2004), 'JACOP, a novel plaque protein localizing at the apical junctional complex with sequence similarity to cingulin.', *J Biol Chem* 279(44), 46014-46022.

Olesen, S. P. (1986), 'Rapid increase in blood-brain barrier permeability during severe hypoxia and metabolic inhibition.', *Brain Res* 368(1), 24-29.

Owens, D. W.; McLean, G. W.; Wyke, A. W.; Paraskeva, C.; Parkinson, E. K.; Frame, M. C. & Brunton, V. G. (2000), 'The catalytic activity of the Src family kinases is required to disrupt cadherin-dependent cell-cell contacts.', *Mol Biol Cell* 11(1), 51-64.

Ozawa, M. & Kemler, R. (1998), 'Altered cell adhesion activity by pervanadate due to the dissociation of alpha-catenin from the E-cadherin.catenin complex.', *J Biol Chem* 273(11), 6166-6170.

Pai, R.; Tarnawski, A. S. & Tran, T. (2004), 'Deoxycholic acid activates beta-catenin signaling pathway and increases colon cell cancer growth and invasiveness.', *Mol Biol Cell* 15(5), 2156-2163.

Pal, D.; Audus, K. L. & Siahaan, T. J. (1997), 'Modulation of cellular adhesion in bovine brain microvessel endothelial cells by a decapeptide.', *Brain Res* 747(1), 103-113.

Pardridge, W. M. (2002), 'Drug and gene delivery to the brain: the vascular route.', *Neuron* 36(4), 555-558.

Park, J. H.; Okayama, N.; Gute, D.; Krsmanovic, A.; Battarbee, H. & Alexander, J. S. (1999), 'Hypoxia/aglycemia increases endothelial permeability: role of second messengers and cytoskeleton.', *Am J Physiol* 277(6 Pt 1), C1066-C1074.

Patrie, K. M. (2005), 'Identification and characterization of a novel tight junction-associated family of proteins that interacts with a WW domain of MAGI-1.', *Biochim Biophys Acta* 1745(1), 131-144.

Plateel, M.; Dehouck, M. P.; Torpier, G.; Cecchelli, R. & Teissier, E. (1995), 'Hypoxia increases the susceptibility to oxidant stress and the permeability of the blood-brain barrier endothelial cell monolayer.', *J Neurochem* 65(5), 2138-2145.

Ponting, C. P. (1997), 'Evidence for PDZ domains in bacteria, yeast, and plants.', *Protein Sci* 6(2), 464-468.

Powell, C. S.; Wright, M. M. & Jackson, R. M. (2004), 'p38mapk and MEK1/2 inhibition contribute to cellular oxidant injury after hypoxia.', *Am J Physiol Lung Cell Mol Physiol* 286(4), L826-L833.

Ragette, R.; Fu, C. & Bhattacharya, J. (1997), 'Barrier effects of hyperosmolar signaling in microvascular endothelium of rat lung.', *J Clin Invest* 100(3), 685-692.

Rajasekaran, A. K.; Hojo, M.; Huima, T. & Rodriguez-Boulan, E. (1996), 'Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions.', *J Cell Biol* 132(3), 451-463.

Ranganathan, R. & Ross, E. M. (1997), 'PDZ domain proteins: scaffolds for signaling

complexes.', *Curr Biol* 7(12), R770-R773.

Rao, R. K.; Basuroy, S.; Rao, V. U.; Jr, K. J. K. & 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(Pt 2), 471-481.

Rapoport, S. I. (2000), 'Osmotic opening of the blood-brain barrier: principles, mechanism, and therapeutic applications.', *Cell Mol Neurobiol* 20(2), 217-230.

Rapoport, S. I.; Fredericks, W. R.; Ohno, K. & Pettigrew, K. D. (1980), 'Quantitative aspects of reversible osmotic opening of the blood-brain barrier.', *Am J Physiol* 238(5), R421-R431.

Reijerkerk, A.; Kooij, G.; van der Pol, S. M. A.; Khazen, S.; Dijkstra, C. D. & de Vries, H. E. (2006), 'Diapedesis of monocytes is associated with MMP-mediated occludin disappearance in brain endothelial cells.', *FASEB J* 20(14), 2550-2552.

Rizoli, S. B.; Rotstein, O. D. & Kapus, A. (1999), 'Cell volume-dependent regulation of L-selectin shedding in neutrophils. A role for p38 mitogen-activated protein kinase.', *J Biol Chem* 274(31), 22072-22080.

Roura, S.; Miravet, S.; Piedra, J.; de Herreros, A. G. & Duñach, M. (1999), 'Regulation of E-cadherin/Catenin association by tyrosine phosphorylation.', *J Biol Chem* 274(51), 36734-36740.

Saitou, M.; Fujimoto, K.; Doi, Y.; Itoh, M.; Fujimoto, T.; Furuse, M.; Takano, H.; Noda, T. & Tsukita, S. (1998), 'Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions.', *J Cell Biol* 141(2), 397-408.

Schmidt, A.; Utepbergenov, D. I.; Mueller, S. L.; Beyermann, M.; Schneider-Mergener, J.; Krause, G. & Blasig, I. E. (2004), 'Occludin binds to the SH3-hinge-GuK unit of zonula occludens protein 1: potential mechanism of tight junction regulation.', *Cell Mol Life Sci* 61(11), 1354-1365.

Schäffler, A.; Arndt, H.; Schölmerich, J. & Palitzsch, K. D. (2000), 'Amelioration of hyperglycemic and hyperosmotic induced vascular dysfunction by in vivo inhibition of protein kinase C and p38 MAP kinase pathway in the rat mesenteric microcirculation.', *Eur J Clin Invest* 30(7), 586-593.

Sheth, P.; Santos, N. D.; Seth, A.; LaRusso, N. F. & Rao, R. K. (2007), 'Lipopolysaccharide disrupts tight junctions in cholangiocyte monolayers by a c-Src-, TLR4-, and LBP-dependent mechanism.', *Am J Physiol Gastrointest Liver Physiol* 293(1), G308-G318.

Siegal, T.; Rubinstein, R.; Bokstein, F.; Schwartz, A.; Lossos, A.; Shalom, E.; Chisin, R. & Gomori, J. M. (2000), 'In vivo assessment of the window of barrier opening after osmotic blood-brain barrier disruption in humans.', *J Neurosurg* 92(4), 599-605.

Staddon, J. M.; Herrenknecht, K.; Smales, C. & Rubin, L. L. (1995), 'Evidence that tyrosine phosphorylation may increase tight junction permeability.', *J Cell Sci* 108 (Pt 2), 609-619.

Stevenson, B. R.; Anderson, J. M.; Braun, I. D. & Mooseker, M. S. (1989), 'Phosphorylation of the tight-junction protein ZO-1 in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance.', *Biochem J* 263(2), 597-599.

Stevenson, B. R.; Siliciano, J. D.; Mooseker, M. S. & Goodenough, D. A. (1986), 'Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia.', *J Cell Biol* 103(3), 755-766.

Sugihara-Mizuno, Y.; Adachi, M.; Kobayashi, Y.; Hamazaki, Y.; Nishimura, M.; Imai, T.; Furuse, M. & Tsukita, S. (2007), 'Molecular characterization of angiomotin/JEAP family proteins: interaction with MUPP1/Patj and their endogenous properties.', *Genes Cells* 12(4), 473-486.

Suzuma, K.; Takagi, H.; Otani, A. & Honda, Y. (1998), 'Hypoxia and vascular endothelial growth factor stimulate angiogenic integrin expression in bovine retinal microvascular endothelial cells.', *Invest Ophthalmol Vis Sci* 39(6), 1028-1035.

Szászi, K.; Buday, L. & Kapus, A. (1997), 'Shrinkage-induced protein tyrosine phosphorylation in Chinese hamster ovary cells.', *J Biol Chem* 272(26), 16670-16678.

Tontsch, U. & Bauer, H. C. (1989), 'Isolation, characterization, and long-term cultivation of porcine and murine cerebral capillary endothelial cells.', *Microvasc Res* 37(2), 148-161.

Traweger, A.; Fang, D.; Liu, Y.-C.; Stelzhammer, W.; Krizbai, I. A.; Fresser, F.; Bauer, H.-C. & Bauer, H. (2002), 'The tight junction-specific protein occludin is a functional target of the E3 ubiquitin-protein ligase itch.', *J Biol Chem* 277(12), 10201-10208.

Traweger, A.; Lehner, C.; Farkas, A.; Krizbai, I. A.; Tempfer, H.; Klement, E.; Guenther, B.; Bauer, H.-C. & Bauer, H. (2008), 'Nuclear Zonula occludens-2 alters gene expression and junctional stability in epithelial and endothelial cells.', *Differentiation* 76(1), 99-106.

Tsang, M. C.; Lo, A. C.; Cheung, P. T.; Chung, S. S. & Chung, S. K. (2001), 'Perinatal hypoxia-/ischemia-induced endothelin-1 mRNA in astrocyte-like and endothelial cells.', *Neuroreport* 12(10), 2265-2270.

Turowski, P.; Martinelli, R.; Crawford, R.; Wateridge, D.; Papageorgiou, A.-P.; Lampugnani, M. G.; Gamp, A. C.; Vestweber, D.; Adamson, P.; Dejana, E. & Greenwood, J. (2008), 'Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration.', *J Cell Sci* 121(Pt 1), 29-37.

Umeda, K.; Ikenouchi, J.; Katahira-Tayama, S.; Furuse, K.; Sasaki, H.; Nakayama,

M.; Matsui, T.; Tsukita, S.; Furuse, M. & Tsukita, S. (2006), 'ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation.', *Cell* 126(4), 741-754.

Utepbergenov, D. I.; Fanning, A. S. & Anderson, J. M. (2006), 'Dimerization of the scaffolding protein ZO-1 through the second PDZ domain.', *J Biol Chem* 281(34), 24671-24677.

Wachtel, M.; Frei, K.; Ehler, E.; Bauer, C.; Gassmann, M. & Gloor, S. M. (2002), 'Extracellular signal-regulated protein kinase activation during reoxygenation is required to restore ischaemia-induced endothelial barrier failure.', *Biochem J* 367(Pt 3), 873-879.

Wachtel, M.; Frei, K.; Ehler, E.; Fontana, A.; Winterhalter, K. & Gloor, S. M. (1999), 'Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition.', *J Cell Sci* 112 (Pt 23), 4347-4356.

Wang, G. L. & Semenza, G. L. (1993), 'General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia.', *Proc Natl Acad Sci U S A* 90(9), 4304-4308.

Wang, Y.; Zhang, J.; jin Yi, X. & Yu, F.-S. X. (2004), 'Activation of ERK1/2 MAP kinase pathway induces tight junction disruption in human corneal epithelial cells.', *Exp Eye Res* 78(1), 125-136.

Wittchen, E. S.; Haskins, J. & Stevenson, B. R. (1999), 'Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3.', *J Biol Chem* 274(49), 35179-35185.

Wong, V. & Gumbiner, B. M. (1997), 'A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier.', *J Cell Biol* 136(2), 399-409.

Wu, S.; Tamaki, N.; Nagashima, T. & Yamaguchi, M. (1998), 'Reactive oxygen species in reoxygenation injury of rat brain capillary endothelial cells.', *Neurosurgery* 43(3), 577-83; discussion 584.

Xu, J.; He, L.; Ahmed, S. H.; Chen, S. W.; Goldberg, M. P.; Beckman, J. S. & Hsu, C. Y. (2000), 'Oxygen-glucose deprivation induces inducible nitric oxide synthase and nitrotyrosine expression in cerebral endothelial cells.', *Stroke* 31(7), 1744-1751.

Zhadanov, A. B.; Provance, D. W.; Speer, C. A.; Coffin, J. D.; Goss, D.; Blixt, J. A.; Reichert, C. M. & Mercer, J. A. (1999), 'Absence of the tight junctional protein AF-6 disrupts epithelial cell-cell junctions and cell polarity during mouse development.', *Curr Biol* 9(16), 880-888.

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8. Appendix