Doctoral Thesis

Influence of tyrosine phosphorylation and ischemia on paracellular permeability in endothelial cells

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Influence of tyrosine phosphorylation and ischemia on paracellular permeability in endothelial cells

A dissertation submitted to the
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presented by

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Zusammenfassung


Der Transport von Molekülen durch die endotheliale Barriere kann prinzipiell auf zwei Arten erfolgen: (1) Durch die Zelle, den transzellulären Weg, und (2) zwischen den Zellen, den parazellulären Weg. In Endothelien ist der parazelluläre Weg durch sog. Schlussleisten (tight junctions), Zell-Zellverbindungen, die in der apikalen Region die Zelle vollständig umschließen, abgedichtet. Die Barriereeigenschaften der Schlussleisten kann durch verschiedene pathologische Signale signifikant verändert werden. Zu diesen Signalen gehören u.a. Sauerstoffmangel (Hypoxie) rsp. Mangel an Sauerstoff und Nährstoffen (ischaemische Bedingungen). Unter hypoxischen rsp. ischaemischen Bedingungen nimmt die normalerweise sehr geringe und selektive Durchlässigkeit der parazellulären Schranke zu, was zu Ödemen führen kann.


zeigen, dass die durch PTP-Hemmung selektive induzierte Proteolyse von Occludin zu einer Erhöhung der parazellulären Durchlässigkeit in Endothelzellen führt.

3. Summary

Compartmentalisation of the organism necessitates cellular linings, which function as building barriers between compartments. Endothelial cells between blood and lymph vessels and tissue constitute such a lining, who’s failure is conditioned by numerous pathological processes such as inflammatory and allergic reactions, often as a consequence of infections, leading to edemas and haemorrhages. This is apt to compromise organ function.

The movement of molecules across the endothelial lining can principally occur by two routes: (1) through the cell, the transcellular pathway, and (2) between adjacent cells, the paracellular pathway. In endothelia, the paracellular route is obstructed by tight junctions (TJs), which are cell-cell junctional complexes arranged in a belt-like structure in the apical region of plasma membranes. In response to pathological signals the usually effective paracellular barrier can undergo significant alterations of its barrier characteristics. Such signals are e.g. the lack of oxygen (hypoxia), or the lack of oxygen and nutrients (ischemia). Under hypoxic/ischemic conditions, the endothelial paracellular permeability increases, resulting in tissue edema.

Signal transduction pathways responsible for ischemia-induced endothelial permeability changes include the mitogen-activated protein kinase (MAPK), especially the extracellular, signal-regulated, kinase (ERK). In addition, there is evidence that tyrosine phosphorylation participates in the control of TJ function. However, a general understanding of endothelial barrier control is still elusive. Therefore, we developed a suitable endothelial in vitro barrier model and directly examined the influence of tyrosine phosphorylation on the integrity and functional characteristics of the TJs with the help of protein tyrosine phosphatase (PTP) inhibitors and analyzed the role of ERK in endothelial barrier dysfunction during ischemia.

We found that inhibition of PTPs leads to progressive disappearance of the transmembrane TJ protein occludin from the cell periphery concomitant to a rise in paracellular permeability. Other junctional marker proteins remained at their junctional localisation. Further characterisation of this process demonstrated that PTP inhibition induces occludin proteolysis by a metalloproteinase. PTP-inhibitor-induced occludin proteolysis and the accompanying rise in permeability and occludin redistribution was abolished by the metalloproteinase inhibitor 1,10-phenanthroline. These results indicate that selective proteolysis of occludin by inhibition of PTP activity results in paracellular permeability increase.

Ischemic conditions also elevated paracellular permeability in endothelial monolayers without, however, significantly disrupting TJ. Instead, a major redistribution of the actin cytoskeleton was found, characterised by nearly complete loss of stress fibres and
Summary

increased circumcellular localisation of the F-actin. All these effects were reversed after reoxygenation. During ischemia ERK first was rapidly activated, followed by nearly complete inactivation under ischemic conditions. After reoxygenation ERK was reactivated. Inhibition of the ERK-pathway by the selective inhibitor U0126 during reoxygenation prevented reestablishment of the actin cytoskeleton and also the decrease of permeability. These results show that ERK is necessary for proper (re)organisation of the actin cytoskeleton and suggests that reactivation of ERK might protect against ischemia-induced barrier breakdown.
4. Introduction

4.1. Barrier function of cell layers

4.1.1. General considerations

Compartmentalisation of the organism is the evolutionary solution for increasing the efficiency of the metabolism. At the border between different compartments specialised cells build a barrier. These cells are epithelial and endothelial in origin. Epithelial cells cover the outer surface of the body many inner cavities. From the more than 200 different cell types in vertebrates, the majority is organised into epithelial tissues. Endothelial cells form the barrier in the cardiovascular system between blood and tissue. The two cell types have the same functions for the organs and the whole organism as the cell membrane for the cell. They keep some molecules in the inner compartment, others in the outer compartment; they import nutrients and export waste; they transduce signals across the barrier and they protect the inner compartment against microorganisms and loss of liquid.

To fulfil all these functions, permeability across the barriers must be precisely and dynamically regulated. The failure of this function is associated with a variety of pathological conditions such as inflammatory and allergic reactions and haemorrhages, often compromising organ function. An example is the breakdown of the so called blood brain barrier, e.g. after reperfusion following stroke, which can lead to irreversible neuronal damage.

4.1.2. Barrier function of endothelia

Endothelial cells line all the vessels in the cardiovascular and lymphatic system. Their presence in all tissues makes the endothelial cell one of the most abundant cell types in vertebrates. The endothelial surface area in the cardiovascular system of a 70 kg adult male has been estimated to reach 7100 m². Functionally, the endothelium is the site of exchange of material between blood and tissue and thus behaves as transporting system. With regard to barrier function there is a great heterogeneity of endothelial cells. There are vascular structures permeable for large proteins, whereas others even restrict passage of ions. A very general classification scheme makes use of morphologically detectable features of barrier function to distinguish between 3 different types of vessels (Bennet et al., 1959):
The first type is characterised by the presence of interendothelial gaps or discontinuities and solutes pass relatively freely. This type is present in e.g. liver, spleen, choroid plexus, intestinal villi, hematopoetic tissues and lymph nodes.

The second type of vessels contains so called fenestrae, intracellular perforations of approximately 100 nm width sealed by a very thin nonmembranous diaphragm which allow increased exchange of solutes up to a mass of about 1000 Da. Therefore fenestrae do not increase the permeability to plasma proteins but to water, ions and small solute molecules. Fenestrated vessels are present e.g. in endocrine glands.

The third type, representing the great majority of vessels, lacks interendothelial discontinuities and fenestrae and has therefore the strongest barrier properties.

In another simple classification one uses functional features of endothelial barriers to characterise different types of endothelial cells. One distinguishes between “leaky” endothelial cells, which are relatively more permeable to certain substances, and “tight” endothelial cells, which are less permeable. Leaky endothelial cells are e.g. found in spleen and endocrine glands, whereas tight endothelial cells e.g. are found in muscles. The “tightest” endothelial cells are those in the mature brain, retina and thymus cortex. With the help of these tightest endothelial cells it is easy to illustrate that there is considerable tissue-specific specialisation of endothelial cells with regard to barrier function and that a tight barrier is often a necessity for proper function of the organs. The endothelial barrier in the brain, the blood brain barrier, prevents diffusion of solutes from the blood into the brain, which would, as already mentioned, severely impair brain function. Examples for such solutes are the amino-acids glycine and glutamate, which serve as neurotransmitters in the brain and which are present in the blood in concentrations that would lead to uncontrolled synaptic activities and neuronal damage.

A similar barrier also exists in the retina. The endothelial barrier in the cortex of the thymus prevents transfer of antigenic substances from the blood to the site where maturation of lymphoid cells takes place. The thymus cortex is the site where immature lymphocytes that recognise molecules originating from the organism itself (self antigens) are eliminated in order to gain self tolerance. Contact of immature lymphocytes in the thymus cortex with their specific antigens leads to apoptosis of the cells. Maturing T-lymphocytes specific for nonself antigens must be protected from their specific antigens in order to be able to survive. A barrier which prevents entering of nonself molecules into this area is therefore a prerequisite for the proper development of antigen specific T-lymphocytes.

Endothelial barrier function not only varies between different organs but also from region to region within any given vascular bed. The postcapillary venules (except in the brain and the retina) appear to be more permeable than any other area of the vasculature. This heterogeneity between arteries and veins is called “segmental differentiation” (Simionescu et al., 1975).
4.1.3. Electrophysiological considerations

There are two routes of movement for solutes across a cellular monolayer, each with its own barriers: (1) The route through the cell cytoplasm is called transcellular route and consists of two barriers, an apical and a basolateral cell membrane. (2) The route between the cells, the paracellular pathway can be considered to have either a single barrier, the intercellular contact site tight junction (TJ), or two barriers, the tight junction and the intercellular space (Fig. 1).

Each of the barriers has two measurable properties: the general permeability of the barrier, and the permselectivity of the barrier, which is the qualitative measure of its preference for certain cations or anions.

Fig. 1. The permeation routes across a cell layer. Two routes, the transcellular with the transcellular resistance ($R_c$) and the paracellular with the paracellular resistance ($R_s$), operate in parallel. $R_c$ and $R_s$ each contain two resistances in series: $R_a$ and $R_b$ are the resistances of apical and basolateral cell membranes, respectively, while $R_{TJ}$ and $R_{ICS}$ are the resistances of tight junction and intercellular space, respectively. M, C, and S stand for mucosal solution, cell, and serosal solution, respectively. (From Powell, 1981)

The general permeability of a cellular monolayer is simply quantified by its electrical resistance. As an approximation, the cell layer can be seen as an electrical circuit with resistances (Fig. 1). $R_a$ and $R_b$ are the resistances of apical and basolateral membranes, respectively, while $R_{TJ}$ and $R_{ICS}$ are the resistances of the tight junctions and the intercellular space. Total resistances for the transcellular and the paracellular pathway can be calculated by simple addition of the two resistances in series. The transcellular resistance $R_c$ is equal to the sum of $R_a$ and $R_b$, while the paracellular resistance $R_s$ is...
equal to the sum of $R_{T2}$ and $R_{ICS}$. The tissue resistance $R_T$ of the cell layer is composed of the parallel resistances $R_C$ and $R_s$ and is $R_T = \frac{R_C \times R_s}{R_C + R_s}$.

Generally, the resistance $R$ is proportional to the reciprocal value of the cross-section-area $A$ of a conductor ($R \approx 1/A$). To be able to compare the values from different cell layers, the resistances are normalised with the area of the cell layer and given in $\Omega \cdot \text{cm}^2$. There exists a wide range of values of cellular resistances in different barrier building tissues of an organism. Table 1 shows a selection of such values. The variety can be explained largely by differences in paracellular resistance. The few exceptions from this rule come from tissues with cell membranes that have unusually high ionic permeabilities (Augustus et al., 1977). To reflect the variability in paracellular permeability, tissues are classified as leaky, tight or impermeable according to the relation of $R_C$ to $R_s (R_C / R_s)$. Leaky tissues are those with a $R_C / R_s > 1$, tight tissues are those with a $R_C / R_s$ between 1 and 1/100 and impermeable tissues are those with a $R_C / R_s < 1/100$ (Schmidt et al., 2000).

**TABLE 1. Electrical resistances of various tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Species</th>
<th>Resistances [$\Omega \cdot \text{cm}^2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallbladder</td>
<td>Rabbit (Hénin et al., 1977)</td>
<td>$R_a$ 156  $R_b$ 143  $R_C$ 299  $R_s$ 21  $R_T$ 20</td>
</tr>
<tr>
<td>Colon</td>
<td>Rabbit (Schultz et al., 1977)</td>
<td>$R_a$ 1570  $R_b$ 100  $R_C$ 1670  $R_s$ 345  $R_T$ 286</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>Necturus (Spenney et al., 1974)</td>
<td>$R_a$ 1779  $R_b$ 1047  $R_C$ 2826  $R_s$ 10573  $R_T$ 2230</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Rabbit (Lewis et al., 1976)</td>
<td>$R_a$ 4000-150000  $R_b$ 5000-10000  $R_C$ 9000-160000  $R_s$ 6000-300000  $R_T$ 5000-10000</td>
</tr>
<tr>
<td>Brain endothelia</td>
<td>Human (Crome et al., 1982)</td>
<td>$R_a$ up to 3000  $R_b$ up to 3000</td>
</tr>
</tbody>
</table>

$R_a$ apical membrane resistance; $R_b$ basolateral membrane resistance; $R_C$ total transcellular resistance; $R_s$ paracellular resistance; $R_T$ total resistance

The permselectivity also combines contributions from both cell membrane and paracellular pathway. The permselectivities of the apical and basolateral cell membranes are a reflection of the carriers, pumps and passive permeation channels in these individual membranes. The permselectivity of the paracellular pathway is determined by the charge of the molecular groups lining this pathway.
4.1.4. Methods to analyse cell layer permeability in vitro

4.1.4.1. Measurement of resistance

Electrophysiological measurements provide the most sensitive assays of cellular permeability. Of the variety of techniques that are available, the simplest method involves electrodes placed on opposite sides of the cellular monolayer. To measure the resistance, a pulse of known amplitude is passed across the cell layer, the voltage deflection is measured and Ohm's law is used to calculate the resistance. This method is often used under the simplified view that the TJ is rate limiting to solute movement across the cellular monolayer and total resistance therefore reflects TJ permeability. However, some cellular systems show a variable transcellular resistance, which makes this interpretation complex. Conditions have been identified in both high and low resistance cellular monolayers where total resistance does not equate directly with tight junction ionic permeability (Reuss, 1992).

4.1.4.2. Measurement of flux

The analysis of the passive movement or flux of inert solutes over time through a cell layer is widely used to determine the paracellular permeability characteristics of the cellular barrier. The method can be used to determine the permeability in in vitro cell assays as well as in in vivo assays. In a widely used in vitro cell assay, cells are grown on a permeable support, which separates an apical from a basal compartment. The tracer can be placed into one of the compartments and its transfer to the other compartment is recorded.

An example for an in vivo assay is the use of marker substances to determine the leakiness of the blood brain barrier. The tracers are injected directly into the bloodstream and their permeation into the brain is then followed by different methods in situ or after preparation of the brain by histochemical methods.

Tracer molecules are selected in part for their lack of affinity for any transcellular transport systems (Madara et al., 1992). Often used tracer-substances are carbohydrates such as mannitol (Mw 182 Da), inulin (Mw ~5000 Da) and dextrans of different sizes (4-40 kDa) or proteins such as horse radish peroxidase (44 kDa) and albumin (66 kDa). In general, the tracers are labelled with an easily detectable substance such as radioactive isotopes or fluorescent dyes.

Flux studies of this kind measure barrier properties different than electrical measurements, since even relatively small tracers, such as mannitol, differ from Na⁺ and Cl⁻ ions in size, shape, charge and hydration sphere. Furthermore flux measurements represent the sum of all fluxes across barrier pathways of all the cells. The influence of the barrier around one cell in such a system is low. Measurements of the resistance, in contrast, consider the barriers of all cells as parallel resistances. Electrophysiological
and flux assays consequently provide complementary approaches to assessing paracellular permeability.

4.2. Molecular architecture leading to barrier function

4.2.1. General

To limit diffusion across a cellular monolayer, cells have acquired different structural and functional specialisations. This can best be explained with the use of the brain micro-capillary endothelial cells forming the blood brain barrier. To supply the brain with essential nutrients and to exchange metabolites across the barrier, these endothelial cells have a number of highly selective transport systems. To restrict non-selective uptake along the transcellular route the cells have minimised pinocytosis, the endocytotic uptake of solutes from the environment and the cells are not fenestrated. Diffusion along the paracellular pathway is prevented by the presence of continuos rows of junctions between the cells, which obstruct the intercellular space. Beside these cellular specialisations limiting diffusion of water-soluble substances, also the transfer of lipid-soluble substances across the barrier is partially prevented. Lipid-soluble substances generally are able to diffuse passively through the cell membranes of any cell. That’s the reason why substances such as ethanol, nicotine and heroin are able to pass the endothelial barrier. However, there is an active system transporting toxic substances out of the inner compartment, which is called multi-drug-resistance protein or P-glycoprotein. The P-glycoprotein is present in the apical membrane of endothelial cells in the brain (but also in many other types of cells in the organism) and it is optimally placed to extrude lipid-soluble substances from the cells into the blood. The protein binds to a wide range of natural and synthetic drugs. Efficiency of outward transport depends on the affinity of the P-glycoprotein for the substance. Additionally, the passage of a substance across the endothelial barrier can also be inhibited by its selective enzymatic inactivation within the endothelial cells (enzymatic barrier). The best example of this involves the action of monoamine oxidase (MAO) on catecholamine neurotransmitters synthesised in the brain. Dopamine, transported into the brain endothelial cells via abluminal amino acid transporters, is intracellularly converted into its pharmacologically inactive metabolite dihydroxyphenyl acetic acid (DOPAC) by the action of MAO. DOPAC then effluxes into the blood circulation where it produces minimal systemic effects and is mostly excreted.
4.2.2. Junctional complex
4.2.2.1. General

As mentioned in the above section, intercellular junctions obstruct the paracellular pathway in endothelia and also epithelia (and also a few other cell types such as odontoblasts (Bishop, 1985), certain fibroblasts in the eye (Hageman and Kelly, 1984), myoid cells of the testes (Dym and Fawcett, 1970) and oligodendrocytes (Dermietzel and Kroczek, 1980)). These junctions were originally described in epithelial systems. Over a century ago, the barrier between polarised epithelial cells was attributed to an intercellular thickening, termed the terminal bar, which was visualised with vital dyes at the apical-lateral boundary of adjacent cells. In the early parts of the 20th century, physiological studies demonstrated that the barrier was not complete and varied among tissues. With the availability of the electron microscope in the early 1960s, the terminal bar could be recognised as several morphologically distinct intercellular junctions.

These junctions are: gap junctions (epithelial and some endothelial cells), desmosomes (epithelial cells) or complexus adherentes (endothelial cells), adherens junctions (AJs) and tight junctions (TJs). Each junctional unit is formed by specific transmembrane proteins that interact through their cytoplasmic tails with signalling proteins and the cytoskeleton. Among these units the TJs and the AJs are the molecular structures responsible for the paracellular permeability barrier. The two junctions are positioned next to each other and both together are two aspects of a single functional unit referred as the apical junction complex (AJC). Both junction types form continuous circumferential zones of contact between adjacent cells.

The TJs usually occupy the most apical position in the junctional complex. This type of junction seals the paracellular cleft to form a semipermeable diffusion barrier (Mitic and Anderson, 1998; Stevenson and Keon, 1998). This blockade is referred to as gate function of TJs. Furthermore, TJs also separate the apical from the basolateral part of the plasma membrane, thereby restricting the movement of plasma membrane components (lipids, proteins) between these two domains. This capacity is referred to as fence function of TJs (van Meer and Simons, 1986). It allows different molecular composition of the membrane compartments leading to polarisation of the cells, a prerequisite for vectorial transport of substances across the cellular barriers. Recent evidence suggests that TJs also play a role in signal transduction. At least one transcription factor can interact with the TJs and its localisation in the nucleus or at the TJs is dependent upon cell density (Balda and Matter, 2000a) (see below).

The AJs confer mechanical connection between adjacent cells by members of the cadherin family of adhesion molecules. These transmembrane proteins mediate intercellular adhesiveness in a Ca\(^{2+}\)-dependent, homophilic manner via their extracellular domains. The cadherins interact with their C-termini with several cytoplasmic proteins of the catenin family. In contrast to epithelial cells, where AJs are
Introduction

localised just below TJs, AJs of endothelial cells are not clearly separated from the TJs but rather intermingled with them (Schulze and Firth, 1993). Like TJs, AJs are also involved in signal transduction (Behrens et al., 1996). The formation of TJs both in developing embryonic tissues (Collins and Fleming, 1995) and cultured cell model systems (Gumbiner et al., 1988) is dependent on the prior formation of AJs, suggesting that established AJs are a prerequisite for the building of the TJs. Whether AJs initiate intracellular signals leading to the assembly of TJs or whether TJs use AJs as a physical scaffold for assembly, or both, however, is a matter of debate.

4.2.2.2. Tight junctions
4.2.2.2.1. Morphology

Consistent with their role in maintaining barriers, TJs form continuous gasket-like contacts between adjacent cells. Electron microscopic (EM) observation of thin sections across the regions of intercellular contact reveals TJs as a series of contacts or "kisses" between plasma membranes of adjacent cells (Fig.2A, arrows) (Farquhar and Palade, 1963). These sites of intimate membrane contact are thought to represent the physical areas at which paracellular diffusion of molecules is embedded. In freeze-fracture EM, TJs appear as a series of branching and anastomosing fibrils that partition either to the cytoplasmic (=protoplasmic) leaflet (P-face) or to the extracytoplasmic leaflet (E-face) of the plasma membranes, with complementary grooves in the opposite leaflet (Fig.2B).

![Fig.2](image.png)

**Fig.2.** A, Thin section electron microscopic (EM) picture showing tight junctions (arrows) between two cells. (From Stevenson et al., 1998) B, Freeze fracture EM picture showing the junctional region of a cell with the characteristic network of TJ strands. (From Troxell et al., 2000)

The extent of TJ particles association to the P-face was demonstrated to correlate with the tightness of TJs (Zampighi et al., 1991; Wolburg et al., 1994).
Introduction

When different tissues are compared, the number of fibrils correlates with the paracellular electrical resistance and impedance to solute flux (Claude, 1978; Reuss, 1992). Therefore, each contact is likely to contribute a resistive element in the paracellular pathway. The complexity of the fibrillar network (number of anastomoses between individual strands) may be also related to transjunctional permeability (Claude and Goodenough, 1973) (Claude, 1978).

The expression “tight” junction is somewhat misleading by suggesting an absolute blockade of paracellular permeation for all solutes. However this is not the case. Detailed electrophysiological analyses suggested the existence of aqueous pores within the TJs (Diamond, 1977) (Claude, 1978) (Reuss, 1992) (Gumbiner, 1993). Permeability studies with epithelia revealed that these pores have radii of 30 to 40 Å (Lindemann and Solomon, 1962). The pores principally allow permeation of solutes with the right physiochemical properties. It has been proposed that the pores fluctuate between open and closed states (Claude, 1978). Hence, differences in paracellular permeability between tight and leaky cells are thought to be a consequence of the number of serially arranged TJs, their opening state and the specificity of their pores (Reuss, 1992).

Two molecular models have been proposed to explain the ultrastructural and functional observations of TJ strands. The first model postulated integral membrane proteins as the backbone of the TJ strands (the „protein“ model) (Fig.3).

![Fig.3. Protein model of TJ structure. TJ strands are composed of proteins. Proteins from strands of adjacent cells interact extracellularly. (From Cereijido et al., 1992)](image-url)
Introduction

Proteins from TJ strands of adjacent cells are thought to interact extracellularly thereby bringing membranes of neighboured cells into close contact and closing the intercellular space.

In the second model, TJ strands were thought to be composed of cylindrical inverted lipid micelles, sandwiched between linear fusions of the external membrane leaflets of adjacent cells (the "lipid" model) (Fig.4) (Kachar and Reese, 1982). The latter model had its origin in the appearance of TJs in thin section micrographs, where TJs often look like apparent hemifusions between the two neighbouring cell membranes. In these pictures, only the exoplasmic leaflets of adjacent membranes look like they were continuous.

Fig.4. Lipid model of TJ structure. a, Diagram of a cross section of a tight junction strand illustrating the intramembrane cylinders. b, Diagram illustrating the paired cylinders at a tight junction strand and the different possible planes of fracture through such a cylinder. c, Proposed organisation of the phospholipids at a tight junction strand. d, Diagram of phospholipids combined with freeze fracture micrograph to show how fractures through lipid micelles could produce images characteristic of tight junctions. E, extracytoplasmic leaflet, P, protoplasmic leaflet of the plasma membrane. (From Kachar et al., 1982)

There are several arguments against the lipid model. First, examination of the movement of lipid probes between the surface of adjacent cells argues that the membranes are not fused at the contact sites (van Meer and Simons, 1986). Second, the fibrils are structurally resistant to treatment with acetone and neutral and anionic detergents, which suggests that they are composed, at least in part, of proteins (Stevenson and Goodenough, 1984). Third, the identification of the TJ-specific integral membrane proteins (see below) supports the protein model. However, all these arguments do not exclude a possible involvement of lipids in the formation of TJ strands.
4.2.2. Tight junctions in endothelial cells

TJs are present in all endothelial cells with the exception of the endothelial cells of the bone marrow (Tavassoli and Shaklai, 1979). In general, the morphology of endothelial TJs appears similar to epithelial TJs. One special characteristic of most endothelial TJs is their tendency to associate with the E-face in freeze fracture experiments (Cereijido, 1992), in contrast to TJ in epithelia, which tend to associate with the P-face. One exception from this rule are the impermeable TJ from brain endothelial cells, which are P-face-associated. TJs from different areas of the vasculature differ in both morphology and barrier characteristics (Fig.5).

![Fig.5. Comparison of tight junctions from different areas of the vasculature.Insets show diagrammatic representations of tight junctional complexes and intermingled gap junctions from corresponding vessel types. Beads represent tight junction particles, shaded areas are gap junctions. (From Cereijido et al., 1992)](image)

In high-pressure large vessels such as the aorta and muscular arteries, TJs are relatively simple and do not restrict movement of macromolecules along the paracellular route. In arterioles, TJs are more complex and are less likely permeated by tracers. Capillaries possess the most complex TJs. Freeze fracture EM from capillaries shows a network of anastomosing TJ strands like the network found in tight epithelia. This complexity is not always directly correlated with low overall permeability. Microvessels in kidney and bronchii e.g. have complex TJs but are also fenestrated and exhibit high permeability to protein. Post capillary venules have simple TJs that appear to be the most leaky to protein (except the ones in the brain and the retina, which are as tight as the capillaries.)
in these organs). Considering the large surface area they comprise (12% of the whole vasculature), post capillary venules represent the greatest avenue for the movement of proteins via the paracellular route. In veins TJs are also relatively simple and resemble those of arteries.

4.2.2.2.3 Proteins

The determination of the protein composition of the tight junction strands proved to be a difficult task. The transmembrane proteins were difficult to isolate and their antigenicity in whole cell immunisations was low. Therefore, the discovery of the first transmembrane TJ protein was an important step forward. Subsequent investigations led to the identification and characterisation of more than 10 TJ-specific integral as well as peripheral membrane proteins with a series of different functions (see Tab.2).

**TABLE 2. Proteins of the tight junctions**

<table>
<thead>
<tr>
<th>Name of protein</th>
<th>membrane-association</th>
<th>Known functions within TJ complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occludin</td>
<td>integral</td>
<td>gate and fence function</td>
</tr>
<tr>
<td>Claudins</td>
<td>integral</td>
<td>gate and fence function</td>
</tr>
<tr>
<td>JAM</td>
<td>integral</td>
<td>monocyte transmigration ?</td>
</tr>
<tr>
<td>ZO-1</td>
<td>peripheral</td>
<td>Providing scaffold in junctional complex; signal transduction</td>
</tr>
<tr>
<td>ZO-2</td>
<td>peripheral</td>
<td>Providing scaffold in junctional complex</td>
</tr>
<tr>
<td>ZO-3</td>
<td>peripheral</td>
<td>Providing scaffold in junctional complex</td>
</tr>
<tr>
<td>Cingulin</td>
<td>peripheral</td>
<td>Providing link to actomyosin cytoskeleton?</td>
</tr>
<tr>
<td>Symplekin</td>
<td>peripheral</td>
<td>?</td>
</tr>
<tr>
<td>7H6</td>
<td>peripheral</td>
<td>?</td>
</tr>
<tr>
<td>Rab3B, Rab13</td>
<td>peripheral</td>
<td>?</td>
</tr>
<tr>
<td>ASIP</td>
<td>peripheral</td>
<td>?</td>
</tr>
<tr>
<td>VAP-33</td>
<td>peripheral</td>
<td>Vesicle targeting</td>
</tr>
<tr>
<td>ZONAB</td>
<td>peripheral</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>AF-6</td>
<td>peripheral</td>
<td>?</td>
</tr>
</tbody>
</table>
4.2.2.3.1. Integral membrane proteins

Occludin

Occludin is the first and best characterised transmembrane protein of TJs (Furuse et al., 1993). It is expressed by essentially all epithelial and endothelial tissues in vivo. Neurons, astrocytes and activated T-lymphocytes can express occludin at least in vitro (Alexander et al., 1998; Bauer et al., 1999).

![Diagram of occludin membrane topology](image)

**Fig.6.** The membrane topology of occludin. Occludin consists of a N- and C-terminal part in the cytoplasm, four transmembrane domains and two extracellular loops. (From Tsukita et al., 1999b)

Sequence analysis predicts that the 60 kDa protein spans the membrane four times with both the amino and the carboxy terminus located intracellularly (Fig.6). The two extracellular loops of occludin comprise about 45 amino acids each. Both extracellular domains consist solely of uncharged residues, with the exception of one or two charged residues adjacent to the transmembrane regions (Ando-Akatsuka et al., 1996). Remarkably, the first loop has an extremely high content of alternating Gly and Tyr residues of 36 and 24%, respectively.

Claudins

Besides occludin a whole family of transmembrane proteins specifically localising at TJs, the claudins (named from the Latin claudere, “to close”), has been found recently (Furuse et al., 1998a). To date the claudin family consists of about 20 members (Mitic et al., 2000).

These 22-24 kDa proteins have a membrane topology similar to the one of occludin, but when compared with occludin, the N-terminal and C-terminal cytoplasmic domains and
the second extracellular loop are fairly short (Fig.7). They range in sequence identity from 12.5% to 69.7% and can be grouped in subfamilies. All claudins end in V-Y- at the C-terminus, a sequence which has been shown to interact with PDZ domains (Hata, 1996).

\[\text{extracellular}\]

\[\text{intracellular}\]

**Fig.7.** Membrane topology of claudins. All claudins have two extracellular loops, four transmembrane domains and a N- and C-terminal part in the cytoplasm. (From Tsukita et al, 1999b)

**JAM (Junction-associated membrane protein)**

JAM is a ~ 40 kDa type I single-span membrane protein belonging to the Ig superfamily (Martin-Padura et al., 1998). The protein consists of an extracellular part with two immunoglobulin-like domains of the V-type and a short intracellular part.

**4.2.2.3.2. Peripheral membrane proteins\n\nZO-1,-2,-3**

ZO-1, ZO-2 and ZO-3 belong to the MAGUK (membrane-associated guanylate kinase homologues) (Anderson, 1996) family of proteins. MAGUK proteins are cytoplasmic molecules sharing a multidomain organisation including one or three PDZ domains (in the case of the ZO-proteins three), an SH3 domain and a region with homology to the enzyme guanylate kinase (Fig.8).
PDZ domains are named for their appearance in the MAGUK proteins post-synaptic density protein-95 (PSD-95), the Drosophila tumor suppressor Discs-large and ZO-1. Meanwhile they have been detected in more than 50 proteins. PDZ domains are protein-binding modules that recognise the last three to five residues at the C-terminus of their target proteins. Some PDZ domains bind ligands containing aromatic or hydrophobic residues at the three terminal positions, others bind to the consensus motif E-(S/T)-X-(V/I).

The SH3 (src homology domain 3) domain is also a protein-binding structure that recognises target-sequences with several prolines. SH3 domains have been described in different cytoskeletal and signalling proteins, where they mediate protein-protein binding and subcellular localisation. Binding partners for SH3 domains of MAGUKs have thus far not been described.

The guanylate kinase (GUK) domain in MAGUKs lacks the catalytic activity for the conversion of GMP to GDP. In ZO-1, ZO-2 and ZO-3 the GUK domains have specific deletions at both sites required for binding of ATP and GMP. This suggests that these functions are not required in TJs and thus are lost in evolution. The GUK domain may be involved in protein-protein interaction.

Non-MAGUKs

Several other cytoplasmic proteins are specifically located permanently or transiently at TJs. Many of them were found by light microscopy of cells specifically labelled with antibodies and their localisation often remained the only junctional characteristic known for a long time. To this group belong the proteins cingulin (Citi et al., 1988), 7H6 antigen (Zhong et al., 1993), the small G-proteins rab3B (Weber et al., 1994) and rab13 (Zahraoui et al., 1994), ASIP (Izumi et al., 1998), AF-6 (Yamamoto et al., 1997),
symplekin (only in epithelial cells) (Keon et al., 1996), the transcription factor ZONAB (Balda and Matter, 2000a), and VAP-33 (Lapierre et al., 1999).

4.2.2.2.4. Molecular model of TJ

Several studies demonstrated a direct involvement of occludin in TJ functionality and in paracellular barrier properties:

- Immuno electronmicroscopy demonstrated that occludin is directly associated with the intramembrane strands (Fujimoto, 1995).
- Overexpression of occludin in cultured MDCK (an epithelial cell line from canine kidney) cells increased the number of TJ strands, with concomitant elevation of their transepithelial electrical resistance (TER) (McCarthy et al., 1996).
- Introduction of C-terminally truncated occludin into MDCK cells or Xenopus embryo cells results in increased paracellular leakage of tracers of low molecular mass (Balda et al., 1996b; Chen et al., 1997).
- The TER of cultured Xenopus epithelial cells is reduced by addition into the culture medium of a synthetic peptide corresponding to the second extracellular domain of occludin (Wong and Gumbiner, 1997).
- The TJ fence function is affected by introduction of C-terminally truncated occludin into MDCK cells (Balda et al., 1996b).
- In transfected fibroblasts, occludin shows some cell-adhesion activity (Van Itallie and Anderson, 1997).

The primary structure of the extracellular loops of occludin with their high content of Gly and Tyr residues suggests that the loops on adjacent cells may interact simply through energetically favourable hydrophobic contacts. Gly residues content could create a flexible structure, and the Tyr side chains may stack. This could lead to an interaction of loops from adjacent cells in a zipper-like fashion to create a tight cell-cell ligation.

However, the expression of occludin in fibroblasts, which are deficient of occludin and TJs, did not result in the formation of a large network of TJ strands. Instead, only short TJ-like structures were observed (Furuse et al., 1998b). Additionally, the redistribution of endogenous occludin in MDCK cells in a punctuate pattern along the cell-cell border after introduction of C-terminally truncated occludin had no influence on the continuos network of the TJ strands (Balda et al., 1996b). Furthermore, endothelial cells in non-neuronal tissues and Sertoli cells in some species bear TJs but express only low amounts of occludin (Hirase et al., 1997) (Moroi et al., 1998). And finally, occludin-deficient embryonic stem cells are able to differentiate into epithelial cells forming well-developed TJ structures between adjacent cells (Saitou et al., 1998). These results suggest that apart from occludin further molecules are required to constitute the TJ strands. These molecules appeared to be the claudins.
Introduction

As occludin, claudins possess several functional characteristics consistent with a role in barrier formation.

- The extracellular loops of the claudins mediate Ca\(^{2+}\)-independent cell-adhesion activity (Kubota et al., 1999).
- Claudins have an intrinsic ability to polymerise into linear fibrils. The ectopic expression of claudins in fibroblasts, which neither produce occludin and claudin nor build TJs, allows to induce the formation of an extensive network of TJ-like structures (Furuse et al., 1998b).
- Claudin-11 (oligodendrocyte-specific protein OSP) constitutes TJ strands between lamellae of myelin sheaths of oligodendrocytes and between adjacent Sertoli cells in the testes. Claudin-11 null mice fail to assemble intramembrane strands in Sertoli cells and CNS myelin TJs (Gow et al., 1999).
- When occludin is expressed in claudin-producing fibroblasts, it is recruited into the claudin fibrils, suggesting that the claudins are the major element driving fibril formation (Furuse et al., 1998b).

Given the experimental evidence concerning the behaviour of occludin and claudins, one can propose a TJ model in which the basic framework of the intramembrane strands is formed by polymers of one or several types of claudins. As an additive, occludin is introduced into these polymers, which changes their functional properties. The exact molecular architecture of these claudin-occludin polymers is still obscure. Interestingly, the thickness of the TJ strands of 10 nm is similar to the diameter of gap junctions, which consist of six connexin molecules, each of which bears four transmembrane domains like claudins and occludin (Tsukita and Furuse, 1999a). Thus it may be possible that occludin and claudins constitute a heterohexamer 10 nm in diameter and that such hexamers are aligned in a linear fashion (Tsukita and Furuse, 1999a).

In various tissues more than 1 claudin species are coexpressed in a single cell type. In contrast to occludin however, which is ubiquitously expressed in TJ forming cells, individual claudins are generally expressed in only a restricted number of specific cell types, suggesting that they are associated with tissue-specific functions of TJs. An illustrative example is the claudin family member paracellin-1, which is specifically expressed in the kidney in the epithelial cell layer of the thick ascending layer of Henle. In this tubule segment the divalent cations Mg\(^{2+}\) and Ca\(^{2+}\) are resorbed from the tubule. Both ions are transported largely through the paracellular pathway, driven by an electrochemical gradient across the tubule epithelium (Di Stefano et al., 1993). Mutational disruption of paracellin-1 function causes hypomagnesemia, a wasting syndrome characterised by excessive loss of Mg\(^{2+}\) in the urine (Simon et al., 1999). The clinical phenotype is recessive, so both copies of the paracellin-1 gene must be affected. An obvious interpretation of this finding is that paracellin-1 regulates the resorption of Mg\(^{2+}\) through paracellular channels in the kidney tubule. Those data led to the hypothesis, that each member of the claudin family contributes to a distinct paracellular
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channel which possesses selective permeability (Wong and Goodenough, 1999). Another example for the cell specific localisation of claudins is claudin-5, which is specifically expressed in endothelial cells (Morita et al., 1999). The expression of different claudins would allow a flexible system for regulating the paracellular pathway in different cellular contexts by building different selective pores.

Recent detailed analysis of the manner of interaction of heterogeneous claudin species within and between TJ strands provided important information to further uncover this point (Furuse et al., 1999). (1) Coexpression of claudin-1, -2, and -3 in fibroblasts led to TJ strands in which the three claudins were copolymerized in any combination. (2) When different fibroblasts expressing only one of the three claudin species were cocultured, claudin-3-based strands laterally associated with claudin-1 or claudin-2-based strands in a heterotypic manner, but such heterotypically paired strands were not formed between claudin-1 and claudin-2 based strands. (3) When transfected fibroblasts only expressing claudin-1 were cocultured with those expressing both claudin-1 and claudin-2, claudin-1 homopolymeric strands laterally associated with claudin1/2 heteropolymers to form paired strands. These observations provide a possible explanation how claudins constitute the pores within the TJs. In cells expressing e.g. claudin-1 and claudin-2, individual TJ strands are heteropolymers of these two claudin types. If such two strands from neighbouring cells interact to form a functional TJ, interaction is only possible between claudin1/1 and claudin2/2 in a homotypical way, but not by heterotypic interaction between claudin-1 and -2.

![Diagram](image)

**Fig.9.** Schematic representation of a putative manner of interaction of different claudin species. Homotypic interaction and heterotypic interaction between claudin-1 and -3 is possible, whereas heterotypic interaction between claudin-1 and -2 is not possible and could result in the formation of a pore. Asterisks represent putative pores. (From Tsukita et al., 2000)

This results in the formation of pores at the sites where a claudin-1 and a claudin-2 are neighbouring in the opposite strands (see Fig.9).
Introduction

As TJ strands are composed of various combinations of different claudin species, this model provides an explanation how the tightness of paired TJ strands is determined by the number and type of claudin-species and their mixing ratio in strands. The model doesn't explain the function of occludin in the formation of the TJ strands. In recent experiments in MDCK cells with dominant-negative occludin mutants having deletions in the extracellular loops or with chimeric proteins in which the extracellular part of the Fc receptor was coupled to the C-terminal part of occludin the paracellular diffusion for mannitol and HRP was inhibited (Balda et al., 2000). This suggests that occludin is also involved in the formation of the pathway for selective diffusion. As the expression of occludin is quite ubiquitous it seems reasonable to speculate that occludin has a more general function in TJs.

Another characteristic of the junctions seems to be that the paracellular pores are not permanently in an open state, but are opened and closed in a regulated manner. Experimental evidence comes from expression studies in MDCK cells. Overexpression of occludin resulted in increased TER but also in increased paracellular permeability for tracer molecules (Balda et al., 1996b; McCarthy et al., 1996). This apparently contradictory result was explained with a model in which several TJ pores are in series, each of them on a different TJ strand. The opening and closing of the pores is synchronised in such a way that within a segment only the pores in one strand are open at a given time. When the pores of the first strand open, solutes with the right physiochemical properties can diffuse through the pores into the space between first and second TJ strand. The solutes remain there until the pores in the second strand open. Then the solutes can pass the second strand. By this process a solute is able to pass successively all the strands in series. In this model regulation of the rate of opening and closing of the pores can influence the paracellular permeability for solutes. This does not influence the TER, which is a parameter measured at a certain time, and therefore represents the sum of opened and closed pores at this time.

The function of the third transmembrane tight junctional protein, JAM, is not completely understood. Initial experiments suggested that this molecule is not involved in the gate or fence functions of TJs. JAM lacks the ability to reconstitute TJ strands in fibroblasts (Tsukita and Furuse, 2000). However, antibody-blocking of the extracellular domain inhibits transmigration of monocytes through endothelial monolayers (Martin-Padura et al., 1998). Thus, JAM may plays a role in monocyte transmigration through cellular barriers.

Occludin and claudin strands do not work for their own, but intracellularly interact with a variety of cytoplasmic proteins, which build a submembrane plaque to enable TJ functionality. One function of the plaque is to link the junctional strands to the actin cytoskeleton. This link is mediated by the three MAGUKs ZO-1, -2, and -3. All three ZO proteins bind to the cytoplasmic tail of both occludin and claudin and to actin (Furuse et al., 1994) (Itoh et al., 1999b) (Haskins et al., 1998) (Itoh et al., 1999a).
link to actin stabilises the junctional complex and may provide the possibility of regulation of the junctional permeability via mechanical force from the cytoskeleton (see chapter 3.4.1). Also interactions among the different ZO proteins have been reported, namely between ZO-1 and ZO-2 (Itoh et al., 1999b) and ZO-1 and ZO-3 (Haskins et al., 1998). These interactions may lead to clustering of the transmembrane proteins of the junctional strands thereby stabilising the whole structure. Thus, the protein binding domains of ZO-1, ZO-2 and ZO-3 are thought to provide a scaffold to organise the protein-components within the junctional complex. However, the ligands for many of their different protein binding domains are still uncharacterised. A function similar to the one of the ZO-proteins is supposed for cingulin, which interacts with myosin and all three ZO-proteins (Cordenonsi et al., 1999a).

Recently, a second function of the junctional plaque has been described, which is signal transduction from the junctions at the cell periphery to the nucleus to alter gene expression. The transcription factor ZONAB (ZO-1-associated nucleic acid binding protein) is assumed to be involved (Balda and Matter, 2000b). In subconfluent cells the amount of ZO-1 is not sufficient to bind all ZONAB, which therefore is present both in ZO-1-bound and free form. The free form is localised in the nucleus and is involved in regulation of gene expression. In confluent cells the amount of expressed ZO-1 is much higher and sufficient to bind all the ZONAB, which therefore no longer is partially localised in the nucleus but exclusively in the junctional region.

Fig. 10. Schematic model of the currently known protein interactions at the tight junctions. (From Mitic et al., 2000)
Introduction

The functions of many of the numerous non-MAGUK proteins of the submembrane plaque are currently not fully understood and further studies are necessary to define their role. A summary of the interactions of all the currently known proteins localising to the TJ is shown in figure 10. Some of the molecules of the junctional plaque are cell specifically expressed. For example from the two known splice forms of ZO-1 (see figure 8), one is expressed only in epithelial cells, while the other is expressed in both endothelial and epithelial cells (Balda and Anderson, 1993). Symplekin is expressed only in epithelial cells (Keon et al., 1996). So there is a high molecular diversity among TJs of different cells types, a condition one would expect given the many different tissues where TJs are required.

4.3. Regulation of barrier function

4.3.1. General

As already indicated above, cellular barrier properties are not static. Both changes in transcellular and paracellular pathways can contribute to barrier modulation. For example, stimulation of transcellular vesicular transport across the endothelium of the human brain has been reported in acute and chronic hypertension (Nag, 1984) or seizures (Westergaard et al., 1978).

Regulation of the paracellular pathway was first reported in a study investigating the influence of histamine on endothelial cell properties (Majno and Palade, 1961). The increase in permeability in response to this pro-inflammatory substance occurs via retraction of the cells from each other resulting in the formation of interendothelial gaps. This result was the basis for the early concept for paracellular permeability regulation, which was thought to result from the formation of intercellular gaps. However, this model may be too simple and recent progress in the understanding of permeability regulation indeed revealed that there may also exist more subtle mechanisms changing endothelial paracellular permeability. Cells are able to rapidly change permeability of TJs to allow passage of fluids or even cells. In most cases such effects are reversible and the cells are able to open and close their intercellular junctions within minutes. Up to date, the literature suggests that TJ permeability may vary widely according to:

1. the number of junctional strands and anastomoses between the strands (as indicated in chapter 4.2.2.1.)
2. the state of the cytoskeleton (see chapter 4.3.4.1.)
3. the phosphate content of the junctional components (see chapter 4.3.4.2.)
4. the degree of maturation of the cellular monolayer
5. physiological requirements
(6) environmental factors, such as osmolarity, pH, ionic strength and temperature
(7) the passage of leukocytes
Because of the tight interplay between AJs and TJs, effects on both types of junctions are of importance for permeability regulation. For example, regulatory events can be directed to components of the AJs, which subsequently can influence the integrity of TJs and paracellular permeability. A classical experiment demonstrating this interaction is the removal of the extracellular Ca\(^{2+}\), which disrupts the AJs because the cadherins need Ca\(^{2+}\) for adhesion (Martinez-Palomo et al., 1980). At the same time also TJs are disrupted. In accordance, treatment of endothelial cells with anti-cadherin antibodies results in increased permeation of horseradish peroxidase (Lampugnani et al., 1992). Regulatory events can also take place directly at the TJs. The following section describes such regulations.

4.3.2. Regulation of paracellular barrier function in endothelial cells

Regulation of the paracellular permeability in endothelial cells occurs in response to environmental stimuli leading to alterations of the microenvironment of the tissue. There are examples for experimental, physiological and pathological stimuli changing barrier properties. An example for an experimental stimulus is the intravascular infusion of hyperosmotic solutions containing e.g. mannitol (Nagy et al., 1979). Injection of such a solution directly into a brain vessel, such as the carotis, leads to increased permeability of the blood brain barrier through opened TJs. Although no physical separation of the TJs has been observed, opening of the TJ was proven by permeability studies with different tracers. It is thought that the hyperosmotic conditions lead to shrinkage of the vascular endothelial cells with subsequent rapid, transient opening of the TJ (Cosolo et al., 1989; Nagy et al., 1979). This method has clinical relevance and is widely used to open the blood brain barrier to increase drug delivery into the brain. There is also an increasing list of physiological and pathological stimuli changing endothelial paracellular permeability (table 3). Most of the substances are produced in acute or chronic inflammatory situations.

The time course of the action of the various stimuli is quite different. Some substances like thrombin or histamine induce a rapid and short-lived (minutes) increase in vascular permeability in vitro. In contrast, cytokines or hypoxia induce a sustained response after a delay of hours or days. Multiple stimuli may be present in vivo leading to complex responses. An important point is that, as the endothelium is functionally different in various regions of the vascular tree, the stimuli may alter barrier properties in different endothelia with different strength or even show the opposite effect. For instance, histamine or adenosine, when tested on endothelial cells of different origin, can increase or decrease vascular permeability (Hoek, 1992).
TABLE 3. Biological modulators of vascular permeability

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>in vitro</th>
<th>mechanism via</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>+</td>
<td>+</td>
<td>[Ca$^{2+}$], /PKC</td>
</tr>
<tr>
<td>Histamine</td>
<td>+</td>
<td>+/-</td>
<td>cAMP</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>+</td>
<td>+</td>
<td>PLD / PKC</td>
</tr>
<tr>
<td>Oxidative products</td>
<td>+</td>
<td>+</td>
<td>[Ca$^{2+}$], /PKC</td>
</tr>
<tr>
<td>(H$_2$O$_2$/O$_2$/OH$^-$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>LTB-4</td>
<td>+°</td>
<td>+°</td>
<td>[Ca$^{2+}$],</td>
</tr>
<tr>
<td>PAF</td>
<td>+</td>
<td>+</td>
<td>cAMP</td>
</tr>
<tr>
<td>Cytokines (TNF-α, IL-1, INF-γ)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>+</td>
<td>+</td>
<td>[Ca$^{2+}$]/NO/cGMP</td>
</tr>
<tr>
<td>Hypoxia/Ischemia</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>ANF</td>
<td>+</td>
<td>+/-</td>
<td>cAMP</td>
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<td>NT</td>
<td>+</td>
<td>?</td>
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<td>Adenosine</td>
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<td>+/-</td>
<td>cAMP</td>
</tr>
<tr>
<td>β-Adrenoreceptor agonists</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>PGE$_1$, PGE$_2$</td>
<td>-</td>
<td>-</td>
<td>cAMP</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>-</td>
<td>-</td>
<td>cAMP</td>
</tr>
</tbody>
</table>

Enhanced permeability (+); decreased permeability (-); different effects in different endothelial cells measured (+/-); not tested (NT); neutrophil-dependent effect (°); LPS, lipopolisaccharide; LTB-4, leukotriene B; PAF, platelet activating factor; TNF-α tumor necrosis factor α; IL-1, interleukin 1; INF-γ, interferon γ; ANF, atrial natriuretic factor; VEGF, vascular endothelial growth factor; AGE-proteins, advanced glycosylation end products; PGE$_1$, prostaglandin E$_1$; PGE$_2$, prostaglandin E$_2$; PGI$_2$, prostacyclin; PLD, phospholipase D; PKC, protein kinase C; NO, nitric oxide (From Dejana et al., 1995)

Intracellularly, many stimuli increase the intracellular calcium concentration ([Ca$^{2+}$]). Studies in cultured endothelial cells demonstrated that the increase in [Ca$^{2+}$] in response to inflammatory mediators was associated with an increase in nitric oxide (NO) and cGMP (Gosink and Forsberg, 1993). Further extending this, the [Ca$^{2+}$]-transduced increase in permeability could be attenuated by blocking NO-synthase or guanylate cyclase (He et al., 1997) (He et al., 1998). Other studies linked the [Ca$^{2+}$]-induced increase in permeability to the activation of protein kinase C (PKC). Different agonists activate PKC in parallel to increased endothelial permeability (Lynch et al., 1990) (Siflinger-Birnboim et al., 1992). Furthermore, inhibitors of PKC prevented the increase in transendothelial permeability in response to pro-inflammatory agents such as α-thrombin or H$_2$O$_2$ (Lynch et al., 1990) (Siflinger-Birnboim et al., 1992). Taken together these results demonstrate that [Ca$^{2+}$] activates different pathways leading to barrier
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dysfunction and once more stress the diversity of mechanisms regulating barrier properties.
The effect of cAMP on the permeability barrier in endothelial cells is more complicated. Depending on the type of endothelial cell, cAMP increases or decreases barrier properties. For instance, cAMP prevents or reverses permeability-induced pulmonary edema in every animal species and model studied, suggesting that cAMP induces resistance to the increase in permeability in pulmonary endothelial cells (Moore et al., 1998). In response to inflammatory agents in different endothelial cell types elevation of endothelial cAMP levels lowers basal permeability and attenuates the increase in permeability (He and Curry, 1998) (Michel and Curry, 1999). In addition, TNF-α induced permeability increase was shown to involve a reduction in intracellular cAMP (Koga et al., 1995). The opposite effect of cAMP has been reported e.g. in rat coronary endothelial cells, where an increase in cAMP accompanies enhanced permeability (Dejana et al., 1995).
Taken together, although many agonists and some second messenger pathways have been described that lead to permeability changes, their molecular effector targets and the precise mechanism are currently only poorly understood.

4.3.3. Mechanisms of barrier regulation by hypoxia/ischemia: implication of MAP kinases

4.3.3.1. Hypoxia/ischemia and permeability

Hypoxia and ischemia are two of the stimuli affecting vascular barrier function mentioned in table 3. Hypoxia is defined as the condition in which the oxygen supply is insufficient to answer metabolic demands. This can be the case for the whole organism under conditions of low oxygen pressure (pO₂) in the air e.g. in high altitude or for a single organ or even part of an organ after occlusion of a blood vessel. In the latter case there is additionally to the lack of oxygen also a lack of nutrients, conditions, which are defined as ischemia. The first tissue that is confronted with insufficient supply is the endothelium. The vascular endothelium is located at the crucial interface between blood as oxygen transporter and tissue as oxygen consumer. The oxygen-pressure in the blood decreases during passage through the body from 95 mm Hg (12.5% v/v) in arterial blood to 40 mm Hg (5.3% v/v) in venous blood (Schmidt et al., 2000). Arterial, capillary and venous endothelia are exposed to quite different pO₂ in the blood. It is therefore not surprising that there is a difference in the sensitivity of endothelia from diverse vascular beds to hypoxia-induced perturbation of barrier function (Ogawa et al., 1990a). Nevertheless a prerequisite for a normal oxidative metabolism in every cell is a minimal pO₂ of 0.1-1 mm Hg in the area of the mitochondria, the so-called critical pO₂ of the mitochondria (Schmidt et al., 2000). Below this value the electron transport in the
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respiratory chain stops. This limits the energy metabolism. Different alterations in the vasculature can be detected under hypoxic/ischemic conditions.

It is well established that hypoxic or ischemic conditions increase the vascular permeability resulting in several forms of organ and tissue edema. The clinical consequences of this perturbation are evident in the pulmonary edema, which results from the hypoxia of high altitude, and the cerebral edema, which can occur also in high altitude or after occlusion of a cerebral artery (stroke) (Stelzner et al., 1988) (Oleson, 1986). However, it is not always clear whether the increased permeability of the vasculature is directly linked to low oxygen supply or whether it is a secondary effect produced e.g. by inflammatory reactions.

A lot of studies tried to simulate these in vivo phenomenons in in vitro cell culture systems. In endothelial monolayers obtained from various segments of the vascular tree, hypoxia increases permeability to macromolecules (Ogawa et al., 1990a) (Partridge, 1995) (Park et al., 1999). The sequence of molecular events that leads to this increase in monolayer permeability during hypoxia is not completely known. The first step in a potential signal transduction cascade is the detection of low pO₂ by the cell. In contrast to bacteria and yeast, in which the principles of oxygen sensing have been elaborated (for a review see Bunn and Poyton, 1996), the oxygen sensor in mammalian cells is still unknown. In a second step, the detection of low pO₂ then must activate signal transduction cascades which leads to permeability changes of the cell layer. As to such transduction cascades again cAMP appears to participate. Exposure of bovine endothelial cells to hypoxia led to a progressive decrease of cAMP in parallel to an increase in permeability and addition of the cAMP analogue dibutyryl-cAMP abolished the hypoxia-induced increase in permeability (Ogawa et al., 1992).

4.3.3.2. MAP kinases

Likely candidate signalling molecules further downstream in a signalling cascade initiated by hypoxia/ischemia are the mitogen-activated protein kinases (MAPKs), a family of Ser/Thr-specific protein kinases. MAPKs are found in all eukaryotes and are common participants in signal transduction pathways from the membrane to the nucleus (Cobb, 1999). Based on the number of MAPK in S.cerevisiae, about 40 different members of this family are predicted to exist in mammals (English et al., 1999). It is speculated that nearly all cell surface receptors utilise one or more of the MAPK cascades in their repertoire of signal transduction mechanisms (Robinson and Cobb, 1997). A cascade of phosphorylation events strictly regulates the activity of the MAPKs. This MAPK cascade is composed of three protein kinases that work in series and form a module. The last enzyme of such a module is the MAPK itself. It is activated by phosphorylation of a Thr and a Tyr residue in a Thr-X-Tyr motif by a MAPK activator (MAPK kinase (MAPKK) or MEK (mitogen- or extracellular-regulated...
kinase)). The motif is located in the so-called activation loop, which emerges from the active site (Zhang et al., 1995). Upon phosphorylation the loop undergoes a conformational rearrangement exposing the active site and making it accessible for the substrate. Phosphorylation of both residues leads to an increase of activity of up to several thousand-fold, whereas phosphorylation of only one residue activates no more than 5-10-fold (Cobb, 1999). Thus both sites must be phosphorylated for high activity. The activity of the MAPKs is “proline-directed”, requiring a proline at position +1 relative to the phosphorylated residue in potential substrates.

MAPKKs are highly selective for their MAPK targets. Their activity again is regulated by Ser/Thr-phosphorylation by a MAPKK activator (MAPK kinase kinase (MAPKKK) or MEK kinase (MEKK)). While the MAPKs and MAPKKs are found in equimolar amounts in the cells and are very abundant proteins comprising of about 0.05% of the total soluble cellular protein (Huang and Ferrel, 1996), the MAPKKK are less abundant. Therefore the first phosphorylation step in the MAPKs module results in amplification of the signal. The second step with its dual phosphorylation mechanism and the high relative concentration of MAPKK builds a mechanism for cooperative activation of the MAPKs over a narrow range of input signal and creates a threshold to filter noise from subthreshold signals (English et al., 1999). Inactivation of the MAPKs is under the control of different sets of phosphatases. There are both examples for phosphatases specific for MAPKs such as the MAPK phosphatases (MAKP) and more broad-spectrum phosphatases such as the phosphoprotein phosphatases 1 and 2A (PP1 and PP2A).

Activated MAPKs exert downstream effects by translocation into the nucleus to activate transcription factors controlling genes important for cell proliferation and differentiation, and by activating cytosolic factors involved in cytoskeletal organisation and cell matrix interactions (Reszka et al., 1995) (Fincham et al., 2000). Based on sequence similarity, mechanisms of upstream regulation and sensitivity to activation by different MAPKKs, MAPKs are grouped into different subfamilies. In mammals six distinguishable subgroups of MAPK modules have been identified up to date (Fig.11) (Robinson and Cobb, 1997).

Two well described modules are the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and the p38 MAPK cascades. These cascades are often activated by cellular stressors such as UV light, heat shock, cytokines, protein synthesis inhibitors, antioxidants, antibiotics and DNA-damaging agents. Because JNK and p38 are generally activated by the same stress signals, they have been collectively referred to as the stress kinases (Kyriakis and Avruch, 1996).
A further module is built by the extracellular signal-regulated kinases 1 and 2 (ERK1/2) cascades, which are among the cascades most commonly activated in signal transduction pathways. The MAPKKs in the ERK pathways are called MEK1/2 and the MAPKKKs are members of the Raf-family. Raf is often activated via the small G-protein Ras. The ERK-cascades have particularly been linked to cell proliferation, but have also important roles in many other events (Cobb, 1999; Lewis et al., 1998). The ERK pathways are activated by variety of growth factors (Robinson and Cobb, 1997), but also by oxidative stress (Aikawa et al., 1997). The other three cascades (p38-like, ERK5 and ERK3; see Fig.11) are only poorly understood at the moment.

The different mammalian cascades act in concert and cross talk is crucial to the coordinated responses of the cells. They can act antagonistically or can cooperate. The physiological responses associated with a certain MAPK module can be cell type-specific. For example, although ERKs generally regulate cell growth and cell differentiation and JNKs participate in stress response, in certain cell types activation of JNKs can induce proliferation (Smith et al., 1997). This suggests that signal specificity is also determined by other mechanisms than just the activation of a certain MAPK module.
4.3.3. MAP kinase and hypoxia/ischemia-induced permeability change

There is growing evidence that the activity of multiple MAPKs is regulated during hypoxia/ischemia and that MAPKs contribute to the structural and functional changes following these events. Considering the diversity of functional modulations following hypoxia in a cell, it is not surprising that different MAPK pathways participate in hypoxia-induced signal transduction. There are examples for regulation of all three major MAPK cascades under hypoxic conditions both in vivo and in vitro. ERK1/2, p38 and SAPK all were activated in whole hippocampus after transient forebrain ischemia in gerbils (Sugiono et al., 2000). Essentially a similar activation was detected in cardiac myocytes in culture under hypoxic conditions (Yue et al., 2000). Different from this broad activation, in the pheochromocytoma cell line PC12 (catecholaminergic excitable cell line widely used as in vitro model for neural cells) short-time hypoxia induced activation of the p38 subtypes p38α and p38γ but not of the subtypes p38β and p38δ or SAPK. ERK1/2 was slightly activated after prolonged hypoxia (Conrad et al., 1999). These examples demonstrate that there exist cell type-specific differences in hypoxia-induced MAPK-activation. Furthermore, also the severity of the oxygen lack seems to influence the MAPK-regulation. In the rat myocyte cell line H9c2 hypoxia (in this case conditions during which the cells consumed all O₂ from initial 140 µM down to 0 µM) had no influence on the activity of ERK1/2 or p38. However under anoxia (0 µM O₂) ERK1/2 activity progressively decreased while p38 activity even increased (Abas et al., 2000). Taken together the impact of hypoxia on MAPK activity seems to depend on the cell type and the severity of the hypoxic conditions.

This leads to the question whether the above-described regulation of MAPK cascades under hypoxic conditions are involved in permeability modulation of endothelia. An interesting candidate module for such an involvement is the ERK-pathway, as other barrier modulating stimuli have been shown to transduce their effect via ERK. There is evidence for both activation of ERK but also inhibition of the basal ERK-activity affecting barrier function.

Activation of ERK is responsible for barrier dysfunction following exposure to different stimuli. In many of the studies, (some of which are described below), inhibition of MEK1/2 by the specific inhibitor PD98059 (Alessi et al., 1995) attenuated the barrier opening effect of the stimulus. Using phorbol 12-myristate 13-acetat (PMA), a direct activator of protein kinase C (PKC), to modulate the permeability of human umbilical vein endothelial cell (HUVEC) monolayers, a reduction in TER was measured, which was paralleled by PKC-mediated activation of the ERK-pathway (Verin et al., 2000). Inhibition of different proteins of the ERK module attenuated PMA-induced permeability increase, suggesting that the ERK cascade is involved in some mechanisms that influence barrier properties in this cellular system. In another study vascular endothelial growth factor (VEGF) was used as stimulus to decrease barrier properties.
(Kevil et al., 1998). VEGF, also known as vascular permeability factor, increased permeability of HUVECs monolayers several-fold. PD98059 partially blocked this effect. In a third study the human immunodeficiency virus (HIV) protein Tat, a protein secreted from virally infected cells, was used to modulate barrier properties (Oshima et al., 2000). Tat increased permeability of HUVEC monolayers in vitro and in parallel activated the ERK pathway. Inhibition of the ERK-pathway with PD 98059 attenuated the permeability increase. Similar results were obtained using ischemia as stimulus in vivo and in vitro. Inhibition of MEK1/2 by PD98059 resulted in decreased edema formation in mouse brain following transient focal cerebral ischemia (Alessandrini et al., 1999). The same inhibitor also partially inhibited the increase of permeability of endothelial monolayers in culture. In this case ischemia was simulated with the O2 chelator thioglycollate in combination with glucose deprivation (Park et al., 1999).

As mentioned, also inhibition of ERK may affect barrier function. A basal level of ERK-activity has been detected in different cell types (Lu et al., 1998) (Pombo et al., 1994). This basal ERK activity is mainly localised in the cytoplasm, in some cells associated with microtubules (Morishima and Kosik, 1996). This localisation suggests that this basal MAPK activity is rather involved in some regulatory events in the cytoplasm than in the regulation of gene transcription for which MAPKs normally translocate into the nucleus. The signals that maintain the basal ERK activity are currently not known. It was speculated that integrins could play a role, because in different cells MAPKs are activated when cells adhere to integrin ligands (Chen et al., 1994). In PC12 cells the basal ERK activity seemed to be necessary for proper organisation of the actin cytoskeleton and adherens junctions (Lu et al., 1998). Competition of the basal ERK activity by overexpression of a dominant negative ERK2 protein led to redistribution of actin filaments and cadherin (Lu et al., 1998). During hypoxic conditions basal ERK activity could, therefore, be abolished by simple depletion of cellular ATP. Indeed, chemical ATP depletion by inhibition of both glycolysis with 2-deoxyglucose and the citric acid cycle with cyanide suppressed ERK1/2 activity in MDCK cells (Pombo et al., 1994).

The activity of MAPKs is ATP-dependent in two ways: (1) kinase activity of ERK1/2 requires ATP, which serves as donor of phosphoryl groups; (2) ERK activity depends on phosphorylation by another kinase. Dephosphorylation by phosphatases is ATP-independent. Therefore, under longterm hypoxic conditions ERK is likely to be completely inactivated.

Only limited information is available on the mechanisms leading to barrier modulation downstream of the ERK-pathway. The time course of some of the above described permeability changes via the ERK-pathway lies in the range of less than 1 hour and suggests peripherally located ERK-induced effects rather than regulation of gene transcription. In this case it is reasonable to assume that the events downstream of ERK directly influence the properties of proteins building or regulating the barrier such as
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junctional or cytoskeletal proteins. In other cases such as that of the Tat protein, the time course of permeability changes is in the range of up to one day. In these cases ERK-regulated transcriptional modulations, possibly of genes coding for structural or regulatory proteins, are more likely.

A permeability-relevant ERK target belonging to the group of potential cytoskeletal components could be the myosin light chain kinase (MLCK). This Ca\(^2+\)/calmodulin-dependent enzyme phosphorylates myosin light chains (MLCs). Actin and myosin together build a molecular machinery that is able to initiate and sustain cytoskeletal contraction leading to inwardly directed tension, which is thought to increase barrier permeability (see chapter 4.3.4.1.). Phosphorylation of MLCs by MLCK is one of several regulatory mechanisms initiating this actomyosin-contraction. MLCK is phosphorylated by both ERK1 and ERK2 in vitro and in vivo. Phosphorylation enhances its kinase activity and leads to increased phosphorylation of MLCs (Klemke et al., 1997) (Fincham et al., 2000). Therefore, activation of the ERK pathway could induce MLC phosphorylation via MLCK activation and this could lead to actomyosin contraction and increased permeability.

Another possible ERK-target is the protein caldesmon, another actin-cytoskeleton component found in different cell types (Sobue and Sellers, 1991). Like MLCK, caldesmon also influences actomyosin contraction. Caldesmon modulates actin-myosin interactions by binding to both actin and myosin in the “resting” or non-contractile state, thus forming an actin-caldesmon-myosin complex, which effectively inhibits direct actin and myosin interaction and myosin ATPase (Walsh, 1987) (Stasek et al., 1992). Caldesmon binding thus inhibits actomyosin-driven contraction. Ca\(^2+\)/calmodulin-binding to or phosphorylation of caldesmon abolishes its inhibitory effects. Phosphorylation of caldesmon was shown to be accompanied with profound changes in the cytoskeleton of fibroblasts (Mak et al., 1991) (Lamb et al., 1990) (Yamashiro et al., 1991) (Yamashiro et al., 1995) and was linked with endothelial barrier dysfunction (Stasek et al., 1992). Since ERK phosphorylates caldesmon (Adam and Hathaway, 1993), caldesmon is a possible linker between ERK activation and permeability increase.

Along with cytoskeletal modifications there is also initial evidence for ERK-induced direct modifications of TJs leading to decreased barrier properties. Occludin and VE-cadherin were both redistributed by ERK-induced mechanisms in endothelial cells incubated with VEGF, and this incubation led to barrier dysfunction (Kevil et al., 1998). Occludin rearrangement from TJs with concomitant disruption of the paracellular barrier was also induced via the ERK-pathway in cells incubated with H\(_2\)O\(_2\) (Kevil et al., 2000). In this case, occludin rearrangement involved a loss of occludin at junctional localisation and dissociation of occludin from ZO-1. Occludin also was heavily Ser-phosphorylated. These results support the idea that the ERK pathway promotes occludin phosphorylation leading to its redistribution from the TJs. However, in in vitro
phosphorylation assays MAPK failed to phosphorylate occludin directly (Cordenonsi et al., 1999b). Thus, ERK rather activates an unknown Ser/Thr kinase pathway which then phosphorylates occludin. In another study removal of claudin-2 from TJ complexes and redistribution into intracellular granules was demonstrated in epithelial cells treated with PD98059 (Kinugasa et al., 2000). Also claudin-1 expression was less focused in the tight junctional region in this experiment, indicating that TJ assembly is dependent on a functional ERK-pathway. Another indication that the ERK pathway influences TJ integrity was provided by experiments in which epidermal growth factor (EGF) was used to activate the ERK pathway in an epidermal carcinoma cell line (Van Itallie et al., 1995). In these experiments ZO-1 became transiently phosphorylated and colocalised with a transiently redistributed actin band in the perijunctional region.

There is also evidence for ERK-mediated regulatory mechanisms of permeability via regulation of gene transcription. Interleukin-17-induced expression of claudin-1 and -2 as well as basal expression of claudin-2 were downregulated following incubation of cells of the intestinal epithelial cell line T84 with PD98059 (Kinugasa et al., 2000). In a salivary gland epithelial cell line activation of the ERK pathway down-regulated occludin and claudin-1 mRNAs, resulting in disruption of TJs (Li and Mrsny, 2000b). In A549 cells (an airway epithelial cell line), which have high Ras-Raf signalling activity due to an oncogenic K-ras mutation and therefore an activated ERK-pathway, upregulation of occludin by overexpression of a dominant negative Raf-1 construct or by treating the cells with PD98059 was observed (Li and Mrsny, 2000a). These results suggest that the transcription of some tight junctional proteins is under the control of the ERK-pathway. A transcription factor that could link hypoxia-induced ERK activation and gene expression is the hypoxia-inducible factor-1 (HIF-1). It was shown that HIF-1 is phosphorylated by ERK-1 during hypoxia thereby increasing its transcriptional activity (Minet et al., 2000).

Transcriptional regulation via ERK not only affects genes of proteins directly involved in formation of the barrier, but also influences barrier properties indirectly. An example for such a mechanism is the interplay between VEGF and ERK. The ERK-pathway not only is activated by VEGF, leading to barrier dysfunction in some cellular systems as mentioned above, but ERK seems to play a key role in the transcriptional regulation of the VEGF gene in endothelial cells and in fibroblasts. Activation of the ERK pathway in these cells by overexpression of constitutively active Raf-1 leads to an increase of the VEGF transcription (Milanini et al., 1998) (Milanini et al., 2000), demonstrating that activated ERK is able to upregulate VEGF transcription. This leads to the question whether stimuli activating the ERK pathway initiate an upregulation of VEGF-expression. When hypoxia acts as stimulus, this seems to be the case.

It is well established that hypoxic conditions lead to upregulation of VEGF expression in many cells in vitro and also in various organs in vivo (Ikeda et al., 1995) (Gleadle et al., 1995) (Marti and Risau, 1998). This upregulation has been linked to increased
permeability properties of cells in culture (Fischer et al., 1999), blood vessels in tumours (hypoxic conditions are often found in tumours) (Berse et al., 1992) or brain microvessels after stroke (Zhang et al., 2000). Thus VEGF, which can be upregulated during hypoxia via ERK, may mediate hyperpermeability in a circuit involving its own transcriptional activation by activating ERK.

Taken together all the examples describing the potential mechanisms for ERK to influence permeability characteristics show that there are many different pathways within a cell that influence a simple measurable characteristic, the paracellular permeability. A selective usage of pathways could allow each cell type to respond in an appropriate manner to environmental disturbances such as hypoxia.

4.3.4. Modes of action at the TJ itself contributing to barrier regulation
4.3.4.1. Actin-cytoskeletal contributions to TJ barrier regulation

Several investigations showed that regulation of junctional permeability is at least in part under cytoskeletal control. The cytoskeleton in eukaryotic cells is composed of different elements, such as microtubules, intermediate filaments and actin filaments. Microtubules do not exert a great influence on TJ permeability, since even dramatic alterations in these structures, such as depolymerisation, had no effect on transepithelial resistance in MDCK cells (Cereijido et al., 1992). Only limited information is available on a possible involvement of the intermediate filaments in permeability regulation. Some physiological agents which exert alterations in TJ structure and function also exert parallel effects on intermediate filament distribution (Madara and Carlson, 1991). But it remains unclear whether intermediate filament redistribution was a cause or a consequence of perturbation.

Much more research has been focused on the actin cytoskeleton. In most eukaryotic cells actin is concentrated in a layer underneath the plasma membrane, in the so-called cell cortex, where it mediates mechanical stability to the cell surface. The cellular junctions responsible for permeability control, AJs and TJs, interact both with the actin-cytoskeleton. Actin was the first protein shown by morphological techniques to be associated with the membrane in the junctional region (Hirokawa and Tilney, 1982). At the level of AJs, a dense band of bidirectional actin filaments and myosin is visible. The protein α-catenin from the submembrane plaque of AJs mediates the connection between AJs and the actin cytoskeleton, either directly, or indirectly via other linkage proteins such as α-actinin and vinculin (Knudsen et al., 1995) (Weiss et al., 1998).

A smaller number of actin filaments enters TJs and terminates at sites of cell-cell contact, presumably near occludin/claudins. As described, the three MAGUK proteins ZO-1, ZO-2 and ZO-3 are thought to link TJs to the actin cytoskeleton via an actin-ZO-occludin/claudin complex (Fanning et al., 1998; Itoh et al., 1997), although the existing evidence for this linkage does not rule out the participation of other unidentified
proteins. In vitro occludin, ZO-1, ZO-2 and ZO-3 directly interact with F-actin (Wittchen et al., 1999). The in vitro data are further substantiated by the finding that ZO-2, ZO-3 and occludin colocalize with cytochalasin D (an agent which has many effects on F-actin microfilaments including depolymerisation, a capping like effect at the elongating end and severing if microfilaments are under tension)-disrupted actin aggregates at cell borders in vivo. Furthermore, the TJ protein cingulin binds to myosin (Cordenonsi et al., 1999a). These results indicate that the actomyosin cytoskeleton has multiple potential binding partners both at AJs and TJs. Multiple linkage mechanisms may provide the versatility required for differential regulation.

Several lines of evidence suggest that paracellular permeability is influenced by the state of perijunctional actin. Different molecules involved in virtually all intracellular signalling pathways affect permeability and their effects often correlate with changes in actin organisation (Balda et al., 1991; Madara et al., 1992). Among them are tyrosine kinases, Ca²⁺, calmodulin, protein kinase C, heterotrimeric G-proteins, cAMP, lipid second messengers and phospholipase C.

Two models for the role of microfilaments in junctional regulation have been proposed:

(1) The microfilaments which are directly linked to TJs and AJs have the ability to influence TJ properties leading to permeability changes in an unknown manner (Bentzel et al., 1980).

(2) Lateral tension induced by cytoskeletal contraction of the actin belt connecting AJs leads to mechanical opening of the TJs (Madara et al., 1986).

Experimental support for the first model is the finding that if preexisting actin filaments are directly depolymerised, the junctions are dissociated. This has been shown in cultured endothelial cells by application of clostridium botulinum C2-toxin (Suttorp et al., 1991). Clostridium botulinum C2-toxin ADP-ribosylates actin monomers and, therefore, causes actin filament net depolymerisation. This increases endothelial permeability by intercellular gap formation.

The second model is also experimentally supported. The mentioned contractile process is thought to result from actin-myosin interactions that occur within the circumferential ring of microfilaments lying adjacent to the membrane at the site of the junctional complex. The existence of such a myosin-dependent mechanism was found in isolated enterocyte membranes, where the actomyosin ring was capable of ATP-dependent contraction (Mooseker, 1985). Cytochalasin D altered the permeability of epithelial cell layers and these functional changes were accompanied by both alterations in TJ structure (Madara et al., 1986) and condensation of perijunctional actomyosin (Dharmsathaphorn et al., 1984). Furthermore, manoeuvres that prevented microfilament condensation also prevented cytochalasin D-elicited decrease of junctional resistance (Madara et al., 1987).

In non-muscle cells, myosin ATPase and contraction are activated by phosphorylation of its regulatory light chain. The phosphorylation state of MLC is under the control
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MLCK and the myosin light chain phosphatase (MLCP). Several lines of evidence suggest that the actomyosin contraction is regulated through MLCK and MLCP leading to changes in permeability. Elevating the levels of MLCK by transfection of MDCK cells increased light chain phosphorylation and decreased TER (Hecht et al., 1996). Enhancement of permeability was also achieved by direct microinjection of a fragment of MLCK into hepatocytes (Hecht et al., 1996).

Evidence for the influence of MLCP is more indirect. MLCP is thought to be inactivated by the small G-protein rho via a rho-dependent kinase. Rho activates the rho-dependent kinase which, in its activated form, phosphorylates and inactivates MLCP. The net effect is enhanced myosin light chain phosphorylation and contractile tone. Evidence for such a mechanism comes from rho overexpression experiments. Transfection of MDCK cells with constitutively active rho mutants induced a synchronous change in junctional actin, dissociation of ZO-1 from the junction and a decrease in TER (Ridley, 1996).

These results support a significant role of the cytoskeleton in permeability control, although the molecular knowledge of all mechanisms is still rudimentary. Consequently this topic represents an area of intense investigation.

4.3.4.2. Phosphorylation of junctional proteins

From the different possible phosphorylations of proteins, tyrosine phosphorylation seems to be most important in the regulation of paracellular permeability. Basic evidence showing the influence of tyrosine phosphorylation came from studies using different mechanisms to increase the intracellular content of tyrosine phosphate. With inhibitors of protein tyrosine phosphatases (PTPs) a correlation between PTP inhibition and enhanced permeability was found both in epithelial and endothelial cells (Staddon et al., 1995). Increasing the phosphotyrosine content by transfection of cells with the protein tyrosine kinase (PTK) src, led to disassembly of junctions and rounding-up of the cells (Volberg et al., 1992). After treatment with PTK-inhibitors, the src-induced effect could be antagonised. These results suggest that tyrosine phosphorylation decreases adhesiveness and cell-cell interaction, while tyrosine dephosphorylation has the opposite effect.

Many molecular alterations leading to the results mentioned above seem to take place at AJs. In fact, phosphorylation events regulating barrier properties are typical examples for the concept that both changes at AJs and TJs can ultimately lead to altered permeability characteristics of TJs. Immunofluorescent labelling of phosphotyrosine in epithelial and endothelial cells treated with PTP inhibitors revealed most staining at the cell border in the junctional region, suggesting localisation and enzymatic activity of different PTKs and PTPs in this area of the cell (Staddon et al., 1995). Several members from both enzyme families are localised at AJs. AJs isolated from rat liver were
immunopositive for the PTKs src and lyn (Tsukita et al., 1991) and at least four 
PTPs were associated with the cadherins in AJs. These were on the one hand the 
membrane-bound PTPs PTPγ, PTPκ and PTPLAR, and on the other hand the cytosolic 
PTP1B [Brady-Kalnay, 1995 #191 (Fuchs et al., 1996) (Kypta et al., 1996) (Balsamo et 
al., 1996). The major tyrosine phosphorylation targets within the cadherin/catenin 
complex are at least three of the different catenins of the complex, namely β-catenin, γ-
catenin and p120-catenin. These three molecules are heavily tyrosine phosphorylated in 
src-transformed cells and also in response to different growth factors (Downing and 
Reynolds, 1991; Hamaguchi et al., 1993). Direct in vitro tyrosine phosphorylation of 
cadherins has also been reported, but the extent of cadherin phosphorylation in vivo is 
generally low relative to the three catenins and its significance is unknown. Increased 
tyrosine phosphorylation of the catenins is associated with a decrease in adhesive 
strength of AJs (Daniel and Reynolds, 1997). Two different mechanisms could explain 
this effect. In the first, the interaction of the proximal membrane domain of cadherins 
with p120-catenin is of importance. The intracellular membrane proximal part of the 
cadherin molecule is involved in lateral dimerisation of the cadherins, which is 
important to stabilise the adhesion made by the extracellular part of the molecule and 
leads to cadherin clustering critical for stabilisation of adhesion (Yap et al., 1997). 
Interaction of p120 catenin with the membrane proximal part of cadherins modulates 
cadherin clustering and thus stabilisation of adhesion (Thoreson et al., 2000). Selective 
uncoupling of p120 catenin from cadherin disrupts strong adhesion. Tyr-
phosphorylation of p120 catenin increases its binding to cadherin (Roura et al., 1999), 
providing a regulatory influence of Tyr-phosphorylation on cell-cell adhesion. The 
second mechanism is the uncoupling of the cytoskeleton from AJs, which is mediated 
under normal conditions by the linkage cadherin-β-catenin-α-catenin-actin. 
Phosphorylation of β-catenin disrupts the chain, either by dissociation of β-catenin 
from cadherin or from α-catenin. There is evidence for both of these possibilities 

Phosphorylation was also detected at TJs. Several of the proteins of the TJ complex are 
known to be phosphorylated. In detail these are occludin, ZO-1, ZO-2, cingulin and the 
7H6 antigen. Unlike at AJs the phosphorylation of both Tyr and Ser/Thr residues seems 
to be important at TJs. Phosphorylation of some of these proteins appears important in 
TJ assembly. The localisation of occludin to TJ correlated with an increase in Ser/Thr 
phosphorylation of its C-terminal domain (Sakakibara et al., 1997; Wong, 1997). In 
parallel to phosphorylation, the resistance of occludin to non-ionic detergent extraction 
increased, which is seen as a measure for enhanced cytoskeletal association. Whether 
this influences TJ permeability is not known. Another study showed that ZO-1 
phosphorylation levels correlated with the localisation of this protein in epithelial cells 
(Howarth et al., 1994), with the most phosphorylated ZO-1 localised at TJs and the less 
phosphorylated in inner compartments of the cells.
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Several kinases were detected to be localised at TJs, e.g. two atypical PKC isotypes PKC\textsubscript{\textgamma} and PKC\textalpha, as well as their specific binding protein (ASIP; atypical PKC isotype-specific interacting protein) (Stuart and Nigam, 1995) (Izumi et al., 1998). Another kinase localising at TJs is the Ser/Thr-specific kinase ZAK (Balda et al., 1996a). However compared to AJFs much less information is available concerning a direct relation between phosphorylation of TJ proteins and tight junction function. Only a temporal correlation of the two events was demonstrated so far. Incubation of cells with PTP inhibitors led to increased tyrosine phosphorylation of ZO-1 and ZO-2 in parallel to increasing permeability (Staddon et al., 1995). However, under these conditions AJFs proteins such as \textbeta-catenin are phosphorylated too. An inverse relationship between phosphorylation, in this case total phosphorylation and TER exists in the two generally used MDCK cell lines (strain I and strain II): strain I cells have a much higher phosphate content in ZO-1 than MDCK strain II cells, which have a much lower TER (Stevenson et al., 1989).

4.3.4.3. Proteolysis of junctional proteins

An increasing amount of evidence suggests that proteolytic events specifically taking place at TJ can regulate barrier function. Especially under pathological conditions permeability changes of cellular barriers often arise because of proteolysis of junctional proteins. There are both examples for regulation of the junctional permeability by factors of the host or by the pathogen itself. An example for the former is the mode of action of mucosal mast cells in the intestine as initial immune defence against invading pathogens. These cells undergo hyperplasia within and immediately below the intestinal epithelium in response to invading pathogens such as nematodes. This mast cell response leads to increased epithelial permeability of the intestinal epithelium (Scudamore et al., 1995). It is suggested that this allows increased translocation of antibodies into the gut lumen to opsonise the pathogens and to play an important role in the defense mechanism. Data from in vitro studies suggest that this permeability change in the epithelium of the gastrointestinal tract is due to the action of the protease mucosal mast cell protease II (RMCP-II) leading to elimination of occludin and ZO-1 at TJs and formation of gaps separating epithelial cells (Scudamore et al., 1998). Inhibition of the protease abrogated the response, demonstrating that proteolysis was required.

Other studies suggest an influence of proteolytic activity on the integrity of the blood brain barrier. An increase in blood brain barrier permeability and subsequent brain edema formation occur as a result of inflammation of the nervous system during many diseases such as multiple sclerosis, brain trauma, stroke and infection. As one possible cause, increased production of matrix metalloproteinases (MMPs) has been linked to this disruption of the blood brain barrier. Experimentally opening of the blood brain barrier can occur after intracerebral injection of activated gelatinase A, and inhibition of
the gelatinase A activity by TIMP-2, a natural inhibitor of MMPs, blocked the opening (Rosenberg et al., 1992). Also endogenously upregulated gelatinase expression in the brain, induced by intracerebral injection of the inflammatory cytokine TNF-α, correlated with increased permeability of brain capillaries. In this case, the TNF-α-induced barrier failure could be inhibited by the synthetic MMP inhibitor Batimastat, suggesting that MMP activity was indeed responsible for the permeability increase (Rosenberg et al., 1995).

Evidence for a direct influence of pathogens on TJ integrity came from studies investigating the route of invasion of different microorganisms through epithelial into subepithelial tissues. One of these microorganisms is the gram-negative bacterium *Porphyromonas gingivalis*, which is recognised as one of the primary agents of adult periodontal disease. This bacterium is able to invade the deeper structures of connective tissue via the paracellular pathway by degrading epithelial cell-cell junctional complexes (Katz et al., 2000). Occludin as well as E-cadherin are lost in MDCK cells during incubation with these bacteria, probably by proteolytic degradation. Proteolysis of a junctional protein was also demonstrated by another important microorganism, the bacterium *Bacteroides fragilis*. This bacterium is a member of the normal colonic microflora of most mammals. Some strains produce a toxin, which causes diarrhoeal disease in farm animals and humans. The toxin is a zinc metalloproteinase (fragilysin) able to disrupt the paracellular pathway of epithelial cells by proteolysis of junctional proteins such as cadherin (Obiso et al., 1997) (Wu et al., 1998).

Another study demonstrated the influence of the faecal pellets of dust mites, *Dermatophagoides pteronyssinus*, on TJs. These pellets are responsible for allergic reactions leading to the development of asthma when inhaled. However, the lung epithelium forms a barrier that allergens must cross before they can cause sensitisation. When inhaled, pellets become hydrated and are deposited on the mucosal barrier. The pellets then cause a disruption of TJs. This is possible because the pellets contain a cysteine proteinase which cleaves the first extracellular loop of occludin (Wan et al., 1999). Putative cleavage sites for this protease were also identified in the first extracellular loop of claudin-1. This proteolysis of occludin and possibly of claudin-1 leads to an increase of TJ permeability, allowing the allergens to cross the epithelial boundary.

### 4.4. Aims of this study

In summary it can be said that there may exist many different pathways that influence the permeability of TJs. An overview over the general mechanisms leading to increased TJ permeability is given in figure 12.
Aim of this study was to gain insight into paracellular permeability regulation in endothelial cells. Based on the current knowledge we hypothesised that phosphorylation could play a critical role in permeability control. Thus, in a first experiment the influence of Tyr-phosphorylation on junctional integrity was to be investigated with the help of inhibitors of PTPs, such as pervanadate and phenylarsine oxide. Then the role of the MAP kinase ERK under hypoxic/ischemic conditions in permeability regulation was to be examined. Effects on the permeability should be monitored with the help of a permeability assay, measuring the flux of tracer molecules through endothelial monolayers grown on filters. We also planned to analyse structural changes at the TJ complex, such as the distribution of the different junctional molecules and also cytoskeletal modulations by immunofluorescence and biochemical assays. Assessment of the role of ERK by using antibodies recognising selectively the phosphorylated
activated form of ERK and by using specific inhibitors to suppress the ERK pathway, was to complete this investigation.
5. Research papers

5.1. OCCLUDIN PROTEOLYSIS AND INCREASED PERMEABILITY IN ENDOTHELIAL CELLS THROUGH TYROSINE PHOSPHATASE INHIBITION

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Occludin proteolysis by metalloproteinase

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SUMMARY

Regulation of epithelial and endothelial permeability is essential for proper function of compartmentalised organisms and tyrosine phosphorylation plays an important role in this process. We analysed the impact of protein tyrosine phosphatase (PTP) inhibition on the structure of endothelial junctional proteins. In human umbilical vein endothelial cells (HUVECs) the PTP inhibitors phenylarsine oxide (PAO) and pervanadate induced proteolysis of the tight junction protein occludin. Occludin proteolysis was inhibited by the metalloproteinase inhibitor 1,10-phenanthroline (PHEN), but not by inhibitors against other types of proteases. The junctional proteins ZO-1, cadherin and β-catenin were not cleaved. Under conditions of occludin proteolysis, PAO treatment elevated permeability for FITC-dextran. Simultaneous incubation of HUVECs with PAO and PHEN inhibited the rise of permeability by more than 60%. PAO treatment lead to progressive disappearance of occludin from the cell periphery. In contrast, ZO-1, cadherin and β-catenin retained their positions at the sites of intercellular contact. Simultaneous administration of PAO and PHEN greatly prevented the redistribution of occludin. These results demonstrate a selective cleavage of occludin by a metalloproteinase and suggest that this process can contribute to the control of paracellular permeability in endothelial cells.
INTRODUCTION

Epithelia and endothelia form a dynamic interface between body compartments allowing the selective vectorial passage of molecules through this barrier. This gatekeeper function is essential for homeostasis and the proper function of the entire organism. The movement of molecules between two compartments physically separated by a cellular layer can principally occur on two routes: through the cell, the transcellular pathway, and between adjacent cells, the paracellular pathway (Madara, 1998). Whereas transcellular passage involves various mechanisms like passive diffusion of lipophilic compounds, receptor-mediated shuttling and transcytosis, traffic on the paracellular pathway is not assisted by such mechanisms. Instead, ions and solutes diffuse between adjacent cells down their concentration gradient. In epithelia and endothelia that form a tight barrier this paracellular space is obstructed by tight junctions (TJs), a cell-cell junctional complex arranged in a belt-like structure in the apical region of lateral plasma membranes (Mitic and Anderson, 1998; Stevenson and Keon, 1998). TJs also separate the apical from the basolateral fraction of the plasma membrane thereby restricting the movement of plasma membrane constituents within these two domains. This ability is usually referred to as fence function (van Meer and Simons, 1986).

The molecular structure of TJs becomes increasingly understood, whereas much less is known about their regulation. The first and best characterized transmembrane protein of TJs is occludin (Furuse et al., 1993). Sequence analysis predicts that the protein spans the membrane four times with both the amino and the carboxy terminus located intracellularly. The two extracellular loops of occludin comprise about 45 amino acids each. The first loop has a high proportion of glycine and tyrosine residues, suggesting that it could play a role in tight cell-cell ligation (Ando-Akatsuka et al., 1996). Several studies demonstrated a direct involvement of occludin in the formation of TJs and the generation of transepithelial barrier properties (Balda et al., 1996; McCarthy et al., 1996; Hirase et al., 1997; Wong and Gumbiner, 1997). The presence of TJs in occludin-deficient cells, however, was the first indication for the existence of further transmembrane molecules (Saitou et al., 1998). Indeed, two novel transmembrane molecules with no similarity to occludin, claudin-1 and -2, were recently discovered in epithelial junctions (Furuse et al., 1998a). Although the ectopic expression of claudins in fibroblasts induced the formation of TJ-like structures (Furuse et al., 1998b), their precise role in the formation of TJs and in the control of transcellular permeability remains to be uncovered in greater detail.

The carboxy-terminal cytosolic domain of occludin directly or indirectly interacts with a series of cytoplasmic proteins such as ZO-1 (Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994) and ZO-3 (Haskins et al., 1998). These proteins belong to the MAGUK family (membrane-associated guanylate kinase
homologues) (Anderson, 1996), a family of multidomain cytoplasmic molecules involved in the coupling of transmembrane proteins to the cytoskeleton. Additional proteins found in epithelial TJs are 7H6 (Zhong et al., 1993), cingulin (Citi et al., 1988), and symplekin (Keon et al., 1996). The precise role of these molecules in the formation/regulation of TJs remains to be assigned.

Endothelial TJs, although basically implicated in the same tasks, appear to be very similar, but not identical to epithelial junctions. For example, 7H6 and symplekin are absent from endothelial TJs. The junctions are likely to be regulated in a different manner, since, e.g., isolated epithelial cells retain much of their barrier properties also in culture like the formation of high transepithelial electrical resistance of several thousand $\Omega \text{ cm}^2$ (see e.g. (Wong and Gumbiner, 1997)), whereas in particular endothelial cells from brain microvessels loose much of their high electrical resistance barrier capacities when cultured in vitro (Heller et al., 1997), suggesting different regulatory mechanisms to be at work in either epithelium or endothelium. Grafting experiments demonstrated that brain tissue strongly influenced the highly specific barrier properties of cerebral microvessels (Janzer and Raff, 1987). Compatible with this view in vitro reconstitution experiments identified astrocytes as possible source to induce blood-brain barrier properties (for review see (Reinhardt and Gloor, 1997)). Epithelial cells do not obviously depend on such tissue interactions; the different structural organisation of epithelial and endothelial TJs might thus be a consequence of the existence of tissue- and cell type-specific mechanisms controlling permeability.

Both physiological and pathophysiological conditions influence cellular permeability and the molecular mechanisms of these controls are manifold: in IL-1$\beta$-injected rats adhesion of neutrophils to cerebral endothelium leads to loss of occludin and ZO-1 and the cytokine-induced breakdown of the blood-brain barrier is accompanied by elevated phosphotyrosine on blood vessels in vivo (Bolton et al., 1998); proinflammatory cytokines which increase permeability cause also rearrangements of the cytoskeleton in endothelial cells (Blum et al., 1997); dexamethasone, which decreases permeability, downregulates the expression of fascin and directs occludin to the cell periphery (Wong et al., 1999). Tyrosine phosphorylation is implicated in elevation of transcellular permeability in epithelial and also endothelial cells (Staddon et al., 1995; Gloor et al., 1997; Esser et al., 1998) and occludin, ZO-1 and ZO-2 can be phosphorylated at either serine, threonine or tyrosine residues (Singer et al., 1994; Staddon et al., 1995; Van Itallie et al., 1995; Sakakibara et al., 1997), suggesting that these molecules could be targets during permeability changes. However, precise functional linkages between tyrosine phosphorylation and regulation of permeability are not yet understood.

Given the well established properties of human umbilical vein endothelial cell lines (HUVECs) (Soker et al., 1996; Abedi and Zachary, 1997), we have used these cells in
the present study to examine the consequences of protein tyrosine phosphatase (PTP) inhibition on permeability and the structural organisation of TJ components. We show that inhibition of PTP activity leads to cleavage of occludin through a metalloproteinase-dependent step. The cleavage of occludin was found to be associated with enhanced endothelial permeability and redistribution of the molecule from sites of cell contact.
MATERIALS AND METHODS

Materials and reagents

Tissue culture treated, collagen-coated polycarbonate transwell® filters (0.4 μm pore size) were from Costar. All other tissue culture materials were ordered from Life Technologies. Phenylarsine oxide (PAO), BSA and FITC-labeled dextran (average Mr 4400 and 38260, respectively) were from Sigma. PAO was prepared as a 100 mM stock solution in DMSO and, when needed, sequentially diluted into the culture medium at the desired final concentration. Sodium orthovanadate was from Sigma. A 5 mM solution in PBS was prepared by heating to 100°C ten minutes before use, pervanadate (PV) was prepared by adding 30% H2O2 to a final concentration of 50 mM. This solution was then diluted into the medium used for the experiments. The protease inhibitors aprotinin, pepstatin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Boehringer, dissolved in PBS or methanol (PMSF) and stored in aliquots at -20°C. 1,10-phenanthroline (PHEN) was from Aldrich and a stock solution (1.5 M) was prepared in methanol and stored at -20°C. Gel electrophoresis reagents and all standard laboratory chemicals were of the highest grade commercially available and purchased from Fluka (Buchs, Switzerland).

Antibodies

Mouse monoclonal (Oc-3F10) and polyclonal antibodies against occludin and the polyclonal antibody against ZO-1 were from Zymed (Gebr. Mächler AG, Basel, Switzerland). Monoclonal anti-phosphotyrosine antibody PY20 and the polyclonal antibody against focal adhesion kinase were from Santa Cruz (Glaser AG, Basel, Switzerland). The polyclonal antibodies against β-catenin and pan-cadherin were both from Sigma (Buchs, Switzerland). Secondary antibodies for immunofluorescence (FITC- and Cy3-labeled) and for immunoblots (HRP-labeled) were from Sigma. Phalloidin-Cy5 was a gift of Prof. H. Faulstich (University Heidelberg).

Cell culture

All cells were maintained at 37°C under 5% CO2 in humidified air. HUVECs (ATCC clone CRL-1730 and ATCC clone CRL-1998) were maintained in DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 50μg/ml gentamycin. MDCK cells were kindly provided by Dr. Patrick Keller (EMBL Heidelberg). For experimental use 8x10^4 HUVECs per cm² were plated on 12 mm collagen-coated transwell filters or on gelatine-coated 12 mm glass coverslips. The medium was changed every third or fourth day and the cells were usually used seven days after plating. Both endothelial cell lines were routinely checked for mycoplasma
contamination and found to be negative. For the experiments including PAO and PHEN the cells were washed twice with Ca- and Mg-supplemented PBS (DPBS) and then incubated with medium supplemented with 1.5 mM PHEN or methanol alone for ten minutes. Then the medium was replaced by medium containing 30 μM PAO and 1.5 mM PHEN or methanol alone and incubated for three hours. For the experiments including 2,3-dimercapto-1-propanol the cells were washed twice with DPBS and incubated with 30 μM PAO in medium for 60 minutes. Then the cells were washed twice with DPBS and incubated with 150 μM 2,3-dimercapto-1-propanol in medium for five minutes. After washing several times with DPBS, cells were supplemented with fresh medium and incubated for another two hours in the absence of the additives.

Paracellular flux

After washing the cells cultured on transwell inserts once with DPBS, the culture medium was replaced in the apical (0.5 ml) and basolateral chamber (1.5 ml) by fresh medium with PHEN or methanol alone. The transwell filters were incubated for ten minutes at 37°C under 5% CO₂ in humidified air and PAO or DMSO alone was then added to the apical chamber. At the same time the medium in the apical chamber was supplemented with 100 μM FITC-dextran (Mr 4400) or 25 μM FITC-dextran (Mr 38260). The transwell filters were then shaken at 60 rpm on a Bioblock Scientific rotomix shaker at room temperature in the dark and 50 μl aliquots were taken from the basolateral chamber at the indicated time points. Fluorescence was measured with a TD-700 fluorometer (Witec AG, Switzerland) and the amount of diffused tracer was calculated from a standard curve containing known amounts of FITC-dextran. Basal-to-apical flux studies revealed similar results.

Immunocytochemistry

Cells grown on collagen- or gelatine-coated glass coverslips were washed once with DPBS, fixed and permeabilized with acetone/methanol (1:1) for ten minutes at -20°C and air dried for ten minutes at room temperature, with the exception of the cells which were processed for actin staining. These cells were fixed with 3% paraformaldehyde in PBS for 15 minutes, washed and incubated with 0.1 M glycine in PBS for five minutes. After washing, the cells were permeabilized with 0.2% Triton X-100 in PBS for 15 minutes and then washed. For all preparations incubation with the primary antibody in PBS containing 3% BSA was for one hour at room temperature. The cells were then washed 3 times for five minutes with DPBS and incubated with a 1:200 dilution of FITC- or Cy3-conjugated secondary antibody in PBS containing 3% BSA for one hour at room temperature. When required, a 1:200 dilution of Cy5-labeled phalloidin was included with the secondary antibody. After washing 3 times for five minutes with DPBS, the coverslips were drained and mounted in 70% glycerol, 5% n-propyl-gallate.
in 30 mM Tris-HCl pH 9.5. The coverslips were sealed with nail polish and examined by confocal microscopy on a Leica inverted microscope DM IRB/E.

**Image processing**

The imaging system consisted of a Leica true confocal scanner TCS NT and a Silicon Graphics workstation. The images were recorded using a Leica PL APO 63x/1.4 oil immersion objective. The system was equipped with an argon/krypton mixed gas laser. Image processing was done on a Silicon Graphics workstation using Imaris® (Bitplane AG, Zurich, Switzerland), a 3D multi-channel image processing software specialized for confocal microscopy images (Messerli et al., 1993). The images were resized, grouped and labeled using Adobe Photoshop® 3.0 and finally printed on a laser printer equipped with photographic paper. The images are representative of the original data.

**Electrophoresis and immunoblotting**

Whole cell extracts were prepared by lysing the cells in extraction buffer containing: 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.2 mM EGTA, 1 mM Na vanadate, 50 mM Na molybdic acid, 1% Triton X-100, and protease inhibitors (0.01 volumes of a mixture consisting of 0.25 mg/ml pepstatin, 0.06 mg/ml aprotinin, 1.1 mg/ml leupeptin, 4.7 mg/ml benzamidin, 0.24 mg/ml bestatin and 38.3 mg/ml PHEN). Protein concentration in the extracts was measured by a Bradford assay (Bio Rad). Equal amounts of protein were mixed with SDS sample buffer (Lämmli, 1970) and incubated for 3 minutes at 100°C before loading. Proteins were resolved by SDS-PAGE (Lämmli, 1970) and transferred to nitrocellulose membranes (Schleicher & Schuell). The quality of the transfer was controlled by Ponceau S staining of the membrane. All blots were blocked in 5% non-fat dried milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) containing 0.05% Tween-20 for 45 minutes at room temperature except the anti-phosphotyrosine immunoblots, which were blocked in 1% non-fat dried milk, 1% BSA, 0.05% Tween-20 in TBS for 45 minutes at room temperature. The filters were then incubated with the primary antibodies diluted in blocking buffer for one hour. After washing 3 times for five minutes in TBS containing 0.05% Tween-20, the primary antibody was reacted with a horseradish peroxidase-conjugated secondary antibody in blocking buffer for 30 minutes. After washing 3 times with TBS plus 0.05% Tween-20 and once with TBS, immunoreactive bands were detected by enhanced chemiluminescence (Amersham) following the manufacturer’s instructions. The chemiluminescent signals were scanned from autoradiographic films (Fuji RX), imported into Adobe Photoshop and processed as described above. For reprobing the blots, the filters were stripped in a solution containing 0.2 M glycine pH 2.2, 0.1% SDS and 1% Tween-20 for 90 minutes. After washing three times with PBS containing 1% Tween-20, the filters were reprobed beginning with the blocking step.
Immunoprecipitation

For Immunoprecipitation extracts were prepared with ice-cold lysis buffer (50 mM Tris-Cl pH 7.2, 150 mM NaCl, 10 mM EDTA, 40 mM NaF, 1 mM Na vanadate, 1% NP-40, 0.1% Na deoxycholate and protease inhibitors). Extracts were incubated for 30 minutes on ice and homogenized with a dounce homogenizer. After centrifugation at 10000xg for 20 minutes, the protein concentration in the supernatant was determined and adjusted to 0.5 mg/ml. 0.5 ml extracts were precleared with 20 μl pansorbin cells (10% cell suspension (w/v), Calbiochem) for 1 hour at 4°C on a rotating wheel. After centrifugation the lysates were incubated for 1 hour at 4°C with 20 μl pansorbin conjugated to anti-occludin antibodies (previously prepared by incubating 20 μl pansorbin with 2 μg anti-occludin antibodies for one hour at 4°C in 1 ml 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA). Immunoprecipitates were washed three times for five minutes with lysis buffer and then resuspended in 40 μl SDS sample buffer.
RESULTS

Inhibition of tyrosine phosphatase activity leads to proteolysis of occludin in HUVECs

We first asked, whether inhibition of PTP activity triggered structural alterations of junctional molecules. HUVEC cultures were incubated with 30 μM PAO for increasing time periods. Cell extracts were first probed with antibodies against occludin, since this molecule is known to be directly involved in barrier formation. Occludin appeared as a broad band (apparent Mr about 61000) in control cells (Fig. 1A). In PAO-treated cells this band progressively disappeared with incubation time and a second band of apparent Mr of about 50000, presumably a product of specific proteolysis of occludin, accumulated (Fig. 1A). PAO-induced proteolysis occurred also in MDCK type I and type II cells (Fig. 1A), demonstrating that the proteolysis of occludin is not restricted to one cell type. Immunoprecipitation of occludin from PAO-treated and control cells with polyclonal and subsequent detection with monoclonal anti-occludin antibodies revealed the same pattern (Fig. 1B). Densitometric analysis of the bands pointed to a clear precursor-product relationship, suggesting that the Mr 50000 band is an authentic occludin fragment (Fig. 1C). Minimal amounts of the Mr 50000 fragment were discovered also in untreated MDCK cells (Fig. 1A, lanes 6, 8). To exclude proteolysis that could occur during the preparation of the detergent extracts we directly lysed the cells with hot SDS sample buffer. Western blot analysis gave virtually identical results. Like PAO, also the PTP inhibitor PV induced occludin proteolysis (data not shown).

Effect of protease inhibitors on PAO-induced proteolysis of occludin

Cleavage of plasma membrane proteins has been observed for various classes of molecules like cell adhesion molecules, growth factor receptors, receptor ligands and receptor PTPs (for review see (Hooper et al., 1997)), resulting in the release of extracellular domains from the plasma membrane. Metalloproteinases appear to play a major role in this process (Campan et al., 1996; Reiland et al., 1996; Black et al., 1997; Moss et al., 1997; Herren et al., 1998; Vecchi et al., 1998), but also members of the prohormone convertase family are involved (Campan et al., 1996). As occludin most likely spans the membrane four times and both the N- and C-terminus are located intracellularly, proteolysis of occludin at a single site would not lead to shedding of any part of the molecule into the extracellular compartment. To obtain first insights into the identity of the protease(s) implicated in the proteolysis of occludin, HUVECs were incubated with PAO in the presence of different protease inhibitors. Among the tested inhibitors, only the metalloproteinase inhibitor PHEN could inhibit PAO-induced proteolysis, whereas PMSF, aprotinin and pepstatin, inhibitors of serine, cysteine or
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aspartate proteases were ineffective (Fig. 2A). The more selective metalloendopeptidase inhibitor phosphoramidon was also ineffective (Fig. 2A). Inhibition of occludin proteolysis by PHEN occurred in a concentration-dependent manner (Fig. 2B, lanes 1-4). To provide additional evidence for a metalloproteinase-mediated cleavage of occludin we studied the impact of Zn\(^{2+}\) ions on enzyme activity. HUVECs, grown in medium at pH 6.8 to minimize zinc precipitation, were treated with PAO in the presence of either PHEN, ZnCl\(_2\), or both Fig. 2B). 1 mM free Zn\(^{2+}\) partially recovered proteolytic activity, whereas 2.5 mM Zn\(^{2+}\) in the absence of PHEN inhibited proteolysis. 2.5 mM Zn\(^{2+}\) in the absence of PAO did not induce proteolysis. These results are indicative for a metalloproteinase-mediated process leading to the proteolysis of occludin. The inhibitory effect observed with higher Zn\(^{2+}\) concentrations is likely to be due to a covalent modification by Zn\(^{2+}\) ions in the active site (Larsen and Auld, 1991).

**Inhibition of PTP activity does not induce general proteolysis of junction-associated proteins**

Treatment of HUVECs with the specific, but not very selective, PTP inhibitor PAO is likely to inhibit tyrosine phosphatases implicated in various cellular signalling pathways. We therefore assessed, whether PTP inhibition induced proteolysis of other junctional proteins in HUVECs. As demonstrated in Fig. 3A, conditions which resulted in cleavage of occludin had no influence on the occludin binding protein ZO-1, nor on the adherens junctions proteins β-catenin and cadherin, nor on the focal adhesion kinase. Furthermore, apart from occludin (see Fig. 1), in none of these cases the amount of protein detected by the respective antibody significantly changed during treatment with PAO (Fig. 3B). These data support that the PAO-induced proteolysis did not lead to general non selective proteolysis of junction-associated proteins.

**Inhibition of PTP activity elevates endothelial permeability**

To investigate whether conditions inducing occludin proteolysis also influenced permeability, HUVECs were seeded on collagen-coated transwell filters and maintained for seven days. At this stage the cells covered the entire surface of the insert and cell-free regions were virtually absent. Permeability was assessed with FITC-dextran (Mr 44000), which was added to the upper compartment. Cells were incubated with 30µM PAO for up to three hours before fluorescence in the medium of the lower compartment was recorded. Permeability was barely different from the control after one hour, but clearly detectable after two and three hours (Fig. 4A). The PAO concentration-dependent effect is shown in Fig. 4B. Dissociation analysis with cells incubated with 30 µM PAO for 3 hours demonstrated a slightly more rapid detachment of the PAO-treated cell monolayer from the substrate in the presence of trypsin. Cell-cell dissociation,
however, was not affected and cell aggregation was comparable between control and PAO assays with the control cells forming slightly larger aggregates after one hour (data not shown), suggesting that enhanced permeability was not primarily due to a PAO-induced reduced cell-cell adhesion. Dye exclusion analysis with trypan blue in the presence of the same concentrations of PAO confirmed the virtual absence of dead cells. Even concentrations from 100 μM up to 300 μM PAO did not result in more than about 5% of cells which had taken up the dye. Higher concentrations, however, led to progressive disintegration of the homogeneity of the cell layer with rounding up and ultimately detaching of the cells from the substrate. Therefore, we used low non-toxic PAO concentrations up to 30 μM for permeability studies to ensure the complete integrity of the cells.

To verify that PAO significantly inhibited PTP activity under these conditions, total cell extracts from HUVECs treated with increasing PAO concentrations for one hour were prepared. Probing with the anti-phosphotyrosine antibody PY20 revealed multiple protein bands (Fig. 4C). The most intense bands had apparent M_r of about 180000, 100000-120000, 70000 and 55000. Treatment of the cells with 1 mM PV for one hour lead to a much stronger result with tyrosine-phosphorylated proteins of a broad molecular mass range (Fig. 4D). The less restricted pattern of tyrosine-phosphorylated proteins in cells incubated with PV as compared to incubation with PAO is consistent with our observation that PV leads to rapid rounding up and detachment of HUVECs from the substrate (data not shown).

**Inhibition of occludin proteolysis reverses the PAO-induced elevated permeability**

Although PAO-induced occludin proteolysis and permeability increase occurred under the same experimental conditions, no functional relation can be inferred from these findings. To provide evidence for a linkage between occludin proteolysis and elevated permeability, HUVECs, grown on transwell inserts, were incubated with PAO and PHEN under conditions which prevented proteolysis of occludin. Co-administration of PAO and PHEN prevented the elevation of FITC-dextran (M_r 4400) permeability by more than 60% (Fig. 5A), whereas the metalloproteinase inhibitor alone had no effect on HUVEC permeability. To further assure that the rise in permeability was due to alterations in the paracellular pathway, we used M_r 38260 FITC-dextran. Essentially identical permeability profiles were obtained with this tracer (Fig. 5B). The apparently lower permeability of M_r 38260 FITC-dextran is unlikely to reflect any size selectivity, as this tracer had to be used in lower concentrations due to its higher fluorescence per mole of dextran. These results indicate a possible correlation between occludin proteolysis and the elevation of paracellular permeability.
PAO treatment leads to redistribution of occludin from sites of tight cell-cell contact in HUVECs

To investigate the consequences of PAO treatment on occludin distribution we used indirect immunofluorescence analysis. In HUVECs grown for seven days to reach tight confluence, occludin was predominantly found at cell-cell contact sites (Fig. 6A). Treating the cells with 30 μM PAO and incubating them for one or three hours revealed that occludin immunoreactivity progressively disappeared from the sites of cell contacts and, instead, accumulated in a diffuse distribution throughout the cells (Fig. 6C, E). We next assayed the distribution of some of the other tight and adherens junctions proteins. The same incubation conditions did not lead to the rearrangement of ZO-1 (Fig. 6B,D,F); the protein retained its circumcellular distribution. Double immunofluorescence analysis of occludin with either cadherin or β-catenin showed both proteins to retain their distribution around the cell periphery both in the absence (Fig. 7A,C) and presence (Fig. 7B,D) of PAO. These results provide evidence that the principal organisation of tight and adherens junctions was not strongly perturbed by the PAO treatment. The immunofluorescence images also demonstrated that the cells maintained their close apposition to each other, since intercellular openings were virtually absent. Furthermore, also actin fibres did not visibly alter their belt-like distribution (Fig. 7E,F), emphasising that PAO-induced elevation of endothelial permeability at low concentrations does not lead to gross changes in the architecture of HUVECs.

Inhibition of occludin proteolysis inhibits its redistribution from sites of cell-cell contact

The data presented so far did not allow to distinguish whether proteolysis of occludin was a cause or a consequence of its redistribution. To look into this question we explored the influence of PHEN on the cellular distribution of occludin in cells treated with PAO. HUVECs grown under conditions identical to those used for the preceding analyses were preincubated for ten minutes with 1.5 mM PHEN prior to the addition of PAO and then incubated for three hours in the presence of both inhibitors. Immunofluorescence with the monoclonal antibody revealed occludin distribution in cells treated with both inhibitors that was only weakly different from the distribution observed with PAO alone (data not shown). We then repeated the experiment with the polyclonal anti-occludin antibody, which was shown to require either mild preextraction or acetone precipitation for optimal reactivity (Balda et al., 1996). In the absence of PAO, occludin was detected only at the cell periphery (Fig. 8A). Incubation with PAO led to the redistribution of the molecule (Fig. 8B). In the presence of PAO and PHEN a circumcellular distribution of occludin was detected and only a few cells showed patterns similar to those seen in the absence of the metalloproteinase inhibitor (Fig 8C, 58.
arrowhead). Virtually identical results were obtained with mild preextraction instead of acetone precipitation (not shown). Since simultaneous incubation with PAO and PHEN prevented occludin proteolysis (see Fig. 2), this experiment indicates that proteolysis precedes the redistribution detected by indirect immunofluorescence. Finally, cells were incubated with PAO for one hour and then washed. 2,3-dimercapto-1-propanol was briefly added to reverse the inhibitory activity of PAO (Staddon et al., 1995) and incubation without additives was continued for another two hours before analysis of occludin distribution. Under these conditions, most occludin staining was localized at the cell periphery (Fig. 8D), demonstrating that reactivation of PTP activity allowed a significant restoration of the distribution of occludin at the cell periphery.
DISCUSSION

In the present study the inhibition of PTP activity in endothelial cells through PAO resulted in the proteolysis of occludin leading to the formation of an approximately Mr 50000 cleavage product and to cellular redistribution of occludin proteins. The proteolysis was sensitive to the metalloprotease inhibitor PHEN. Under the same conditions PAO raised endothelial permeability which could also be suppressed by PHEN, suggesting that the elevation of endothelial permeability was related to PAO-induced proteolytic cleavage and redistribution of occludin.

The role of tyrosine phosphorylation in the control of proteolysis

A linkage between tyrosine phosphatase inhibition and proteolysis is only poorly understood. PTP inhibition was shown to induce proteolysis of the ErbB-4 receptor (Vecchi et al., 1998), cleavage of the amyloid precursor protein (Slack et al., 1995) and shedding of syndecan I (Reiland et al., 1996). In the case of the amyloid precursor and syndecan I the class of protease was not identified. Inhibitory studies using batimastat demonstrated that proteolysis of ErbB-4 was dependent on a metalloprotease activity (Vecchi et al., 1998), but, although the cytoplasmic domain of ErbB-4 was tyrosine phosphorylated after PV treatment, the authors did not emphasize that the phosphorylation rendered the receptor susceptible to proteolysis, since other stimuli which also induced ErbB-4 phosphorylation did not result in proteolysis. This observation leads to the question of the mechanism of occludin proteolysis. Changes in the state of tyrosine phosphorylation of occludin could make the protein susceptible to proteolysis. This mechanism does not appear to occur under our conditions, since probing immunoprecipitated occludin from HUVECs for phosphotyrosine revealed no signal under our experimental conditions (data not shown). Inhibition of PTP activity could, indirectly, result in activity alterations of serine/threonine kinases ultimately leading to changes in the phosphoserine and/or phosphothreonine level of occludin. We have no evidence for such a mechanism either, since treatment of immunoprecipitated occludin from HUVECs with alkaline phosphatase did not alter the apparent molecular mass of both the intact and cleaved protein (data not shown). Reported restrictions and selective reactivities of available occludin antibodies (Balda et al., 1996; Sakakibara et al., 1997), however, make a conclusive interpretation of these observations difficult.

Activation of a metalloprotease through steps involving inhibition of PTP activity could be a possible mechanism. Although direct regulation of proteolytic activity of metalloproteinases has not been observed to date, such a mechanism seems possible, as some members of the disintegrin-like family of metalloproteinases carry SH3 domain binding motifs in their cytoplasmic domain (Wolfsberg and White, 1996) and Src can
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interact with disintegrin-like metalloproteinase MDC9 through its SH3 domain (Weskamp et al., 1996). The disturbance of this interaction by PAO/PV could then interfere with the activity of the metalloproteinases and/or influence their localization. That the subcellular localization of metalloproteinases influences biological functions has been found in the case of the membrane-bound matrix metalloproteinase MT1-MMP (Nakahara et al., 1997).

Possible relation between proteolysis/redistribution of occludin and elevated permeability

Our finding that proteolysis of occludin and enhanced permeability tightly coincided and the facts that the metalloproteinase inhibitor PHEN almost completely prevented proteolysis, strongly suppressed the enhanced permeability and greatly arrested redistribution suggests that proteolysis of occludin is involved in the elevated paracellular flux. Deduced from the apparent molecular mass of the proteolytic fragment cleavage appears to occur in the first loop, although we cannot strictly exclude proteolysis to occur elsewhere, as the antibodies against occludin used in this study were made against a peptide comprising the C-terminal 150 amino acids. N-terminal amino acid sequence analysis is ultimately required to address this point precisely. Studies using peptides derived from either the first (Van Itallie and Anderson, 1997) or second (Wong and Gumbiner, 1997) extracellular loop of occludin provided evidence for a direct contribution of these sequences in adhesion and barrier formation, respectively. It is therefore likely that cleavage in either of these two domains would interfere with these probably interdependent functions of occludin, resulting in increased paracellular permeability. A selective disappearance of occludin, but not ZO-1, from its location at the cell periphery, was induced by peptides against the second, but not the first, extracellular loop of occludin (Wong and Gumbiner, 1997). Different from our observation, the disappearance was not accompanied by a specific proteolysis of the molecule, but more reminiscent of increased turnover. The complete absence of occludin protein (or an immunologically detectable cleavage fragment) under those conditions, therefore, suggests a different mechanism to be involved.

Redistribution of occludin, but not ZO-1, was found in MDCK cells transfected with constitutively active mutants of RhoA and Rac1 (Jou et al., 1998). Since Rho-induced activation of protein tyrosine kinase activity is required for the formation of stress fibres (Ridley and Hall, 1994), PAO-induced effects may possibly interfere with signals in Rho-dependent pathways. Independent regulation of occludin and ZO-1 was furthermore observed during the formation of the chick neural tube: whereas ZO-1 expression increased, occludin levels decreased with the formation of the tube (Aaku-Sarraste et al., 1996). Finally, dexamethasone was able to induce concomitant reversible translocation of occludin and β-catenin to the apex of mammary epithelial cells (Wong
et al., 1999) and in parallel the cellular resistance increased. These different observations strongly suggest highly specialized regulation of occludin expression and distribution in various tissues under different conditions.

Since occludin was not tyrosine phosphorylated in our system, inhibition of as yet unknown PTPs could lead to light microscopically not detectable rearrangements at TJs, due to increased tyrosine phosphorylation of other junction-associated molecules. For example, ZO-1 and ZO-2 are tyrosine-phosphorylated under conditions of elevated permeability in MDCK cells (Staddon et al., 1995) and the extent of ZO-1 tyrosine phosphorylation relates to the transcellular resistance of epithelial cells (Stevenson et al., 1989). The altered phosphorylation of these proteins could then influence permeability functions of occludin, with or without occludin proteolysis, or elevate permeability through direct or indirect modulation of other transmembrane junctional proteins.

Other mechanisms contributing to elevated permeability in HUVECs

The enhanced permeability was significantly (more than 60%), but not completely, inhibited by the metalloproteinase inhibitor PHEN. Similarly, the metalloproteinase inhibitor strongly, but not completely, abolished occludin redistribution, whereas, under the same conditions, the inhibitor was able to prevent the proteolysis of occludin almost completely. Therefore, this small fraction of occludin cleavage product could correspond to those molecules that remained in a scattered cellular distribution (Fig. 8D) and accounted for the incomplete reversibility. Alternatively, the residual permeability might be caused by the still existing inhibition of PTP activity in the presence of PHEN, leading to junctional changes independent of occludin proteolysis and/or occludin. The discontinuous distribution of occludin found in cells treated with 2,3-dimercapto-1-propanol subsequent to the one hour PAO treatment would be in agreement with the first possibility. Independent of occludin, PTP inhibition could e.g. induce proteolysis and concomitant increase of permeability of junctional proteins not investigated in this study. Candidates are the recently discovered JAM (Martin-Padura et al., 1998) and the claudins (Furuse et al., 1998a). The observation that occludin-deficient stem cells could differentiate into epithelial cells with TJ structures (Saitou et al., 1998) in fact indicates that the formation of TJs does not solely depend on the presence of occludin. However, claudins might also act as (partial) surrogates under these conditions. A detailed investigation of the barrier properties of such junctions will be essential to provide insight into this possibility. The complex expression pattern of claudin proteins could furthermore be an indication that the composition of TJs exhibits subtle tissue-specific differences in order to precisely respond to the demands of a particular tissue.
ACKNOWLEDGEMENT

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REFERENCES


FIGURE LEGENDS

**Figure 1:** Proteolysis of occludin. (A) HUVECs and MDCK cells were incubated with 30 μM PAO for the indicated time periods. Total cell detergent extracts were prepared, separated on an 8% acrylamide gel (30 μg protein per lane) and subsequently transferred to nitrocellulose. Occludin was detected with monoclonal anti-occludin antibodies (identical results were obtained with the polyclonal anti-occludin antibodies). Bound antibodies were visualized with peroxidase-conjugated secondary antibody and chemiluminescence. Lanes 1-5: HUVECs; lanes 6, 7: MDCK type I cells; lanes 8, 9: MDCK type II cells. Molecular sizes are given in kDa. (B) Immunoprecipitation of occludin with polyclonal and subsequent detection with monoclonal anti-occludin antibodies. Lane 1: PAO-treated cells; lane 2: control cells. (C) Relative expression level of occludin proteins. The blot shown in (A) was used for densitometric quantification of signal intensity. Intensity values are given as arbitrary units.

**Figure 2:** Inhibition of occludin proteolysis. (A) HUVECs were incubated with solvent (lane 1) or with 30 μM PAO (lane 2-7) for three hours in the presence of different protease inhibitors which were used in the following concentrations: PHEN: 2 mM (lane 3); PMSF: 0.4 mM (lane 4); aprotinin (Apro): 80 U/ml (lane 5); peptatin (Peps): 10 μM (lane 6); phosphoramidon (Phos): 130 μM (lane 7). Total cell detergent extracts were prepared, separated on an 8% acrylamide gel (30 μg protein per lane), subsequently transferred to nitrocellulose and examined by Western blot analysis with polyclonal anti-occludin antibodies. Bound antibodies were visualized with peroxidase-conjugated secondary antibody and chemiluminescence. (B) HUVECs were treated with 30 μM PAO for three hours in the presence of different concentrations of PHEN. Lane 1: control; lane 2: 1.5 mM; lane 3: 0.3 mM; lane 4: 0.06 mM. The impact of Zn^{2+} ions was examined with 2.5 mM ZnCl\textsubscript{2} (lanes 8-10). Lane 5: control; lane 6: 30 μM PAO; lane 7: PAO and 1.5 mM PHEN.

**Figure 3:** Influence of PAO on the integrity of junction-associated proteins. (A) Cells were treated as indicated in figure 1. After occludin detection the blot was stripped for 90 minutes in 0.2 M glycine pH 2.2, containing 0.1% SDS and 1% Tween-20, washed three times with PBS containing 1% Tween-20, and reprobed with antibodies against ZO-1, β-catenin, pan-cadherin and focal adhesion kinase (FAK). Bound antibodies were detected with peroxidase-conjugated secondary antibodies and chemiluminescence. (B) Relative expression level of junction-associated proteins. The blots shown in (A) were exposed for shorter time and used for densitometric quantification of signal intensity. Intensity values are given as arbitrary units.
Figure 4: Time- and dose-dependent effect of PAO on endothelial FITC-dextran permeability. (A) HUVECs were grown for seven days on collagen-coated transwell filters (0.4 μm pores) and incubated with 30 μM PAO for the time indicated. 100 μM FITC-dextran (Mr 4400) was present in the upper compartment during the entire incubation period. Control cultures received solvent only. Data are means± s.d. of triplicate experiments. (B) HUVECs were incubated with increasing concentrations of PAO from 1 to 30 μM for three hours. Flux was determined as in A. Data are means± s.d. of triplicate experiments. (C) HUVECs were incubated with 30 μM PAO for one hour. Total cell detergent extracts were prepared, separated on an 8% acrylamide gel (30 μg protein per lane) and subsequently transferred to nitrocellulose. To determine the tyrosine phosphorylation pattern of the proteins the monoclonal anti-phosphotyrosine antibody PY20 was used. Bound antibodies were visualized with peroxidase-conjugated secondary antibody and chemiluminescence. (D) Tyrosine phosphorylation pattern after treatment of HUVECs with 1 mM PV for one hour. Molecular sizes are given in kDa.

Figure 5: Influence of the metalloproteinase inhibitor PHEN on the PAO-induced proteolysis of occludin. HUVECs were grown for seven days on collagen-coated transwell filters (0.4 μm pores). The cells were then incubated with solvent alone (control), with 30 μM PAO, with 1.5 mM PHEN, or with 30 μM PAO and 1.5 mM PHEN (added to the cells ten minutes before the addition of PAO) for two hours. (A) 100 μM FITC-dextran (Mr 4400), present in the upper compartment during the entire incubation period, was used as tracer. (B) 25 μM FITC-dextran (Mr 38260), present in the upper compartment during the entire incubation period, was used as tracer. Permeability was measured by recording the fluorescence of 50 μl aliquots removed from the lower compartment after three hours of incubation and expressed as pmoles FITC-dextran/(cm² minutes). Data shown in (A) are the means± SD of triplicate transwell cultures. Data shown in (B) are the means± s.d. of triplicate transwell cultures of two experiments.

Figure 6: Cellular distribution of occludin and ZO-1. HUVECs were grown on gelatine-coated coverslips for 7 days. The cells were then treated with solvent alone (A, B), or incubated with 30 μM PAO for one hour (C, D) or three hours (E, F). After the incubation the cells were fixed with acetone/methanol (1:1) and processed for double immunofluorescence with a monoclonal anti-occludin antibody (A, C, E) and with an anti-ZO-1 antibody (B, D, F). The slides were analyzed by confocal microscopy. Images represent the summarized Z-axis. The scale bar in (F) is 10 μm.

Figure 7: Cellular distribution of proteins of adherens junctions and focal adhesion points. HUVECs were grown on gelatine-coated coverslips for 7 days. The cells were then treated with solvent alone (A, C, E), or incubated with 30 μM PAO for three hours (B, D, F). After incubation the cells were fixed with methanol/acetone (1:1) and
processed for double immunofluorescence with a monoclonal anti-occludin antibody (image inserts) and either the anti-cadherin (A, B) or anti-β-catenin antibody (C, D), respectively. For actin detection the cells were fixed with paraformaldehyde and stained with Cy5-phalloidin (E, F). The slides were analyzed by confocal microscopy. Images represent the summarized Z-axis. The scale bar in (F) is 10 μm.

**Figure 8:** Cellular distribution of occludin in the presence of PHEN. HUVECs were grown on gelatine-coated coverslips for 7 days. The cells were then treated with solvent alone (A), or incubated with 30 μM PAO for three hours either alone (B), or together with and 1.5 mM PHEN (added to the cells ten minutes before the addition of PAO) (C). In (D) the cells were incubated with 30 μM PAO for one hour, then washed twice with DPBS and incubated with 150 μM 2,3-dimercapto-1-propanol in medium for five minutes. After washing several times with DPBS, cells were supplemented with fresh medium and incubated for another two hours in the absence of the additives. Cells in (A, B, C) were fixed and permeabilized with methanol/acetone (1:1) prior to immunofluorescence analysis with polyclonal anti-occludin antibodies. Cells in (D) were processed for immunofluorescence with monoclonal anti-occludin antibodies without acetone or detergent treatment. The slides were analyzed by confocal microscopy. Images represent the summarized Z-axis. The scale bar in (D) is 10 μm.
Figure 1

A

<table>
<thead>
<tr>
<th>HUVEC</th>
<th>MDCK</th>
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<tbody>
<tr>
<td>1 2 3 4 5</td>
<td>6 7 8 9</td>
</tr>
<tr>
<td>116- 97-</td>
<td>66-</td>
</tr>
<tr>
<td>66-</td>
<td>45-</td>
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</table>

hrs in 0 0.5 1 2 3 0 1.5 0 1.5 PAO

B

| 1 2 |
| 116- 97- |
| 66- |
| 45- |

C

*Chloroquine levels over time (30 μM PAO)*

hours 0 1 2 3 4

0 50 100 150
Figure 2

A

PAO
Inhibitor

PHEN
PMSF
AprO
Peps
Phos

B

PAO
PHEN (mM)
ZnCl₂ (mM)
Figure 3

A

occludin
ZO-1
β-catenin
cadherin
FAK

0 0.5 1 2 3
hours 30 μM PAO

B

cadherin
ZO-1
β-catenin
FAK

0 1 2 3 4
hours 30 μM PAO
Figure 4

A

B

C

D

Figure 4
Figure 5

A

B

control  PAO  PHEN  PAO+PHEN

flux (pmol/cm² min)

0  5  10  15  20  25  30  35  40  45  50

control  PAO  PHEN  PAO+PHEN
5.2. Inhibition of ERK1/2 during reperfusion prevents restoration of ischemia-induced endothelial barrier failure

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Running title: ERK1/2 restores barrier function after ischemic injury

Key words: ischemia, ERK, cytoskeleton, U0126, FAK
Summary

The MAP kinase ERK1/2 has been implicated in various aspects of ischemic injury. We investigated the role of ERK1/2 activity in the modulation of endothelial permeability during ischemia and reoxygenation. Ischemia led to a 2.5-fold increased phosphorylation of ERK1/2 within 30 min and to a subsequent drastic decrease below control levels after 5 hours. 2 hours after reoxygenation ERK1/2 phosphorylation had returned to basal levels. During ischemia paracellular permeability increased, but returned to control levels during reoxygenation. Suppression of ERK1/2 phosphorylation with the MEK1/2 inhibitor U0126 prevented full recovery of paracellular permeability during reoxygenation, but did not induce barrier failure under normoxic conditions. During ischemia a redistribution of the actin cytoskeleton, including a complete loss of actin stress fibres occurred, which was reversed 2 hours after reoxygenation. U0126 also prevented complete reformation of the stress fibres, and mimicked actin reorganisation in cells under normoxic conditions. Parallel to stress fibre disappearance FAK was redistributed and dephosphorylated. Endothelial leakage was accompanied by a drop of the cellular ATP level, which was fully restored during reoxygenation. These data support that ERK1/2 activation is required to restore endothelial barrier function after ischemic injury and suggest that loss of endothelial ERK1/2 activity contributes to vascular leakage during ischemia.
Introduction

Tissue ischemia is a consequence of various disorders such as thrombosis, artheriosclerosis, stroke and tumor growth. Ischemic conditions are associated with increased permeability of the vascular endothelium leading to the development of edema, evident e.g. in the high altitude pulmonary and cerebral edema (Olesen, 1986; Stelzner et al., 1988). Also in endothelial monolayers obtained from various segments of the vascular tree, ischemic conditions increase permeability to macromolecules (Ogawa et al., 1992) (Park et al., 1999). This disturbance of the endothelial barrier function is reversible upon reoxygenation. (Ogawa et al., 1990b). Vascular permeability is dependent on structural integrity of tight junctions (TJs), and the function of TJs is dependent on the presence of adherens junctions and the linkage to the actin-cytoskeleton, respectively. Because of the mutual structural and functional interrelation of these three structural units, vascular permeability can be influenced by alterations of each of these structures. However little is known regarding the signalling cascades modulating vascular permeability under conditions of ischemia and reperfusion.

Different stimuli including phorbol 12-myristate 13-acetate (PMA) (Verin et al., 2000), vascular endothelial growth factor (VEGF) (Kevil et al., 2000) and the human immunodeficiency virus (HIV) protein Tat (Oshima et al., 2000) affect endothelial barrier properties via the extracellular signal-regulated kinase 1/2 (ERK1/2). ERK1/2 are serine/threonine protein kinases and belong to the mitogen-activated protein kinase (MAPK) family. ERK1/2 is activated by the MAPK kinases MEK1/2 leading to dual phosphorylation of tyrosine and a threonine residue in a TEY motif (Schaeffer and Weber, 1999). ERK1/2 can affect cytosolic and cell surface proteins and also to translocate into the nucleus to regulate transcription factors (Alvarez et al., 1991; Childs et al., 1992; Drechsel et al., 1992; Nakajima et al., 1993; Northwood et al., 1991). ERK1/2 activity influences paracellular permeability by both phosphorylation of cytoplasmic proteins and by regulation of gene expression. Basal ERK1/2 activity was reported to be necessary for proper organisation of the actin cytoskeleton and adherens junctions (Lu et al., 1998). In different cell types ERK has been shown to influence the expression of various TJ proteins (Kinugasa et al., 2000; Li and Mrsny, 2000a). The MAPK pathways are also known to play a critical role in the cellular adaptation to different environmental stresses such as UV light, osmotic shock, heat shock or cytokines. There is growing evidence that the activity of ERK1/2 is also modulated during ischemia and reoxygenation. (Yue et al., 2000) (Abas et al., 2000; Sugiono et al., 2000). Furthermore, using adult mice, inhibition of MEK1/2 resulted in reduced edema formation in the brain after focal cerebral ischemia (Alessandrini et al., 1999). We therefore wondered whether ERK1/2 participate in the ischemia/reoxygenation-induced modulation of vascular permeability.
In the current study we have investigated the participation of ERK1/2 in ischemia and reoxygenation-induced modulation of endothelial permeability. We show that ischemia reversibly leads to opening of the barrier properties in endothelial cells. Furthermore ERK1/2 activity is influenced by ischemia/reoxygenation in a complex pattern. ERK1/2 reactivation is necessary for the reestablishment of the original permeability of the endothelial cells during reoxygenation.
Materials and methods

Antibodies and reagents-Rabbit anti-phospho-p42/44 MAP kinase (anti-ppERK1/2) and rabbit anti-p42/44 MAP kinase (anti-ERK1/2) antibodies were obtained from Cell Signalling Technology. Rabbit anti-occludin and mouse anti-ZO-1 antibodies were from Zymed Laboratories. Rabbit anti-β-catenin, rabbit anti-α-catenin and rabbit anti-pan-cadherin antibodies were from Sigma. Mouse anti-p120 antibody was from Transduction Laboratories. Rabbit anti-FAK and mouse anti-phosphotyrosine (PY-20) antibodies were from Santa Cruz. Fluorescent- and HRP-labelled secondary antibodies were obtained from Sigma. TRITC-phalloidin conjugate was from Sigma. MEK inhibitor U0126 was purchased from Promega, dissolved in DMSO, stored up to 7 days in portions at -20°C and when needed further diluted into the culture medium at the desired final concentration. Protein G-sepharose was obtained from Sigma. Tissue culture-treated, collagen-coated polycarbonate transwell® filters (0.4 μm pore size) were from Costar. All other tissue culture materials were from Life Technologies. Gel electrophoresis reagents and all standard laboratory chemicals were of the highest grade commercially available and purchased from Fluka (Buchs, Switzerland).

Cell culture- HUVEC (ATCC clone CRL-1730) were cultured at 37°C under 5% CO₂ in DMEM supplemented with 10% foetal bovine serum (FCS), 2 mM L-glutamine and 50 μg/ml gentamycin. For experimental use 8×10⁴ HUVECs per cm² were plated on 6.5 mm collagen-coated transwell filters, gelatine-coated 12 mm glass coverslips or petridishes. The medium was changed every third or fourth day.

Exposition of HUVEC to ischemic conditions-HUVECs were washed once with PBS and then incubated with serum- and glucose-free medium in a humidified atmosphere of 5% CO₂ and 1% O₂ at 37°C for different periods (Oxygen level was adapted within 30 min from air levels to 1%). Control cells were held in the same medium under a normal atmosphere. For reoxygenation, medium was changed to DMEM/10%FCS and cells were cultured under normal atmosphere.

Permeability assay-The culture medium of the HUVECs grown on transwell filters and incubated under the desired experimental conditions was replaced by medium supplemented with 100 μM FITC-dextran (4.4 kDa) in the apical chamber (0.1 ml) and by normal medium in the basolateral chamber (0.6 ml). The cells were then incubated for 1 hour at 37°C under 5% CO₂ in humidified air. 50 μl aliquots were taken from the basolateral chamber and the amount of diffused tracer was measured with a TD-700 fluorometer (Witec AG, Switzerland).

Western Blot Analysis-Whole cell extracts were prepared by lysing the cells in extraction buffer containing 25 mM Hepes pH=7.4, 100 mM NaCl, 1 mM EGTA, 1 mM
sodium vanadate, 40 mM sodium fluoride, 1% Triton X-100, 25 μM phenylarsine oxide (PAO) and 1xcomplete® protease inhibitor mixture (Roche Molecular Biochemicals). Protein concentrations in the extracts were measured by a Bradford assay (Bio Rad). Equal amounts of protein were mixed with SDS-sample buffer (Laemmli, 1970) and incubated for 3 min at 100°C before loading. Proteins were resolved by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Schleicher & Schuell). The quality of the transfer was controlled by Ponceau S staining of the membrane. All the blots were blocked in 5% non-fat dried milk in 20 mM Tris-HCL, pH=8.0, 150 mM NaCl (TBS) containing 0.05% Tween-20 for 45 min at room temperature except the anti-phosphotyrosine immunoblots, which were blocked in 1% non-fat dried milk, 1% BSA, 0.05% Tween-20 in TBS for 45 min at room temperature. The filters were then incubated with the primary antibodies diluted in blocking buffer for 1 hour or over night (p42/44 MAP kinase and phospho-p24/44 MAP kinase). After washing three times with for 5 min in TBS containing 0.05% Tween-20, the primary antibody was reacted with a horseradish peroxidase-conjugated secondary antibody in blocking buffer for 30 min. After washing three times with TBS plus 0.05% Tween-20 and once with TBS, immunoreactive bands were detected by enhanced chemiluminescence (Amersham) following the manufacturer's instructions. The chemiluminescence signals were scanned from autoradiographic films (Fuji RX) and imported into Bio Rad Molecular Analyst for densitometric measurements or Adobe Photoshop for presentation. For reprobing the blots, the filters were stripped in a solution containing 0.2 M glycine, pH=2.2, 0.1% SDS and 1% Tween-20 for 90 min. After washing three times with PBS containing 1% Tween-20, the filters were reprobed, beginning with the blocking step.

**Immunofluorescence**-Cells grown on gelatine-coated glass coverslips were washed once with PBS and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature or 4°C. After incubation with 0.1 M glycine in PBS for 5 min, cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min. After washing three times with PBS, the cells were incubated with the primary antibody or TRITC-phalloidin in 3% BSA in PBS for 1 hour at room temperature. The cells were then washed again three times for 5 min with PBS and incubated with FITC- or Cy3-conjugated secondary antibody in 3% BSA in PBS for 1 hour at room temperature. After washing three times for 5 min with PBS, the coverslips were drained and mounted in 70% glycerol, 5% n-propyl-gallat in 30 mM Tris-HCl, pH=9.5. The coverslips were sealed with nail polish and examined by confocal microscopy on a Leica inverted microscope DM IRB/E.

For p42/44 MAP kinase (ERK1/2)- and phospho-p24/44 MAP kinase (ppERK1/2)-staining, the cells were fixed with 3% paraformaldehyde in 50 mM Tris-HCL, pH=7.4, 150 mM NaCl (TBS) for 20 min at 4°C. The cells were then washed three times in TBS plus 0.1% Triton X-100 (TBST) for 5 min at room temperature and blocked with 5.5%
normal goat serum in TBST for 1 hour. Incubation with primary antibody in TBS containing 3% BSA was done over night at 4°C. After washing once with TBS for 15 min and once with TBS plus 0.1% BSA for 15 min, cells were incubated with secondary antibody in TBS containing 3% BSA for 1 hour. Cells were then washed two times with TBST for 5 min and once with TBS for 5 min and mounted and examined as described above.

Immunoprecipitation-For immunoprecipitation cell extracts were prepared with ice-cold lysisbuffer (25 mM Hepes pH 7.4, 100 mM NaCl, 1 mM EGTA, 40 mM NaF, 1 mM Na vanadate, 25 µM phenylarsine oxide (PAO), 1% Triton X-100 and 1x complete® protease inhibitor mix). Extracts were incubated for 30 min on ice and homogenised by pressing several times through a 21g needle. After centrifugation at 10000xg for 20 min, the protein concentration in the supernatant was determined and adjusted to 0.5 mg/ml. 0.5 ml extracts were precleared with 25 µl of a 10% suspension of protein G sepharose (Sigma) for 1 hour at 4°C on a rotating wheel. After centrifugation the lysates were incubated for 4 hours or over night at 4°C with 25 µl protein G sepharose conjugated to the desired antibodies (previously prepared by incubating 25 µl 10% protein G sepharose suspension with 2 µg of the desired antibodies for one hour at 4°C in 1 ml 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA). Immunoprecipitates were washed five times for five minutes with lysisbuffer, resuspended and boiled for 8 min in 40 µl 2x SDS sample buffer and then used for SDS-PAGE analysis.

ATP-determination-The ATP concentration in the HUVEC extracts was determined using the ATP bioluminescence assay kit HS II (Roche molecular biochemicals) following the manufacturer’s instructions.
Results

Ischemia and reoxygenation influence ERK1/2 location differently

To investigate the impact of ischemia and reoxygenation on ERK1/2 and endothelial barrier functionality, HUVECs were exposed to ischemic conditions (1% O₂, 5% CO₂, 94% N₂, serum-free DMEM without glucose) for up to 5 hours with or without subsequent reoxygenation (readdition of glucose-containing complete medium and exposure to 21% O₂) for 2 h. Within 1 h after onset of ischemia ERK1/2 phosphorylation increased to about 2.5 fold of the control level of resting cells, and then continuously decreased to app. 10% of the initial level after 5 h (Fig. 1A). 2 h reoxygenation led to rephosphorylation of ERK1/2 above the control level (Fig. 1A). The amount of ERK1/2 protein did not change during the whole treatment (Fig.1A). To follow ERK1/2 in situ, cells exposed to ischemic and reperfusion conditions, respectively, were rapidly fixed and immunostained with the anti-ppERK1/2 antibody. In control cells ppERK1/2 was detected in the nucleus as well as in the cytoplasm (Fig. 1B). After 1 hour of ischemia, ppERK1/2 reactivity became more intense, with strongest staining at the cell periphery, whereas no signals were found after 5 h, consistent with the immunoblot analysis. After reoxygenation, ppERK1/2 immunoreactivity reappeared again with a predominantly nuclear distribution (Fig. 1B) These data indicate that phosphorylation of ERK1/2 during early ischemia or reperfusion, respectively, results in significant redistribution of ERK1/2.

Ischemia, but not hypoxia alone, leads to a reversible increase of endothelial permeability

To investigate the influence of ischemia and reoxygenation on the permeability of HUVEC monolayers, cells grown on transwell filters were exposed to ischemic conditions for 5 h with or without subsequent reoxygenation for 2 h. As shown in figure 2, ischemia led to a 50% increase of paracellular permeability. In the presence of glucose no barrier breakdown was observed. Reoxygenation led to reestablishment of the initial barrier characteristics. Exposure to ischemia for 16 hours resulted in a several-fold increased permeability, which, above about 10 h, however, was not restored during reoxygenation (data not shown). Under the conditions shown in figure 2 cell viability remained above 95%, as assessed by trypan blue exclusion (not shown). From these data we conclude that endothelial barrier maintenance requires metabolic activity and that short term ischemic conditions do not cause irreversible damage to endothelial cells.
Inhibition of ERK1/2 activation prevents barrier recovery during reoxygenation

To investigate the role of ERK1/2 on the barrier modulation induced by ischemia and reoxygenation, the selective MEK1/2 inhibitor U0126 was used. During reoxygenation, U0126 prevented barrier recovery in a dose-dependent manner (Fig. 3A). U0126 had no influence on the permeability of control monolayers. The effect of U0126 on the ERK1/2 phosphorylation was tested by immunoblot analysis. As shown in figure 3B, incubation of HUVECs for 2 hours with 60 μM U0126 led to complete disappearance of the ERK1/2 phosphorylation. These data suggest that restoration of barrier properties after ischemic insult requires ERK1/2 activation.

Ischemic treatment only subtly affects the distribution of occludin, ZO-1 and β-catenin

It is well established that paracellular permeability in endothelial cells is controlled by tight and adherens junctions together with the cytoskeleton. To understand ischemia-induced barrier failure, we immunostained proteins of the adherens and tight junctions in HUVECs after ischemia. As shown in figure 4, ischemia had only a moderate influence on the intensity and distribution of the tight junction proteins occludin and ZO-1 and the adherens junction protein β-catenin, as best visible by the more punctuated staining of β-catenin during ischemia. Since redistribution of adherens junction-associated proteins often results from a loss of interaction with other junctional proteins, we further investigated the structural integrity of adherens junctions. As depicted in figure 5, immunoprecipitation of β-catenin allowed to equally coprecipitate α-catentin and pan-cadherin both under normal and ischemic conditions. Likewise, immunoprecipitation of pan-cadherin resulted in coprecipitation of β-catenin under either condition (Fig. 5). Finally, immunoprecipitation of p120 revealed pan-cadherin in equal amounts under normal and ischemic conditions, respectively (Fig. 5) These results support that adherens junctions retain their structural integrity during ischemic treatment and suggest that redistribution of β-catenin may resulted from another influence, possibly involving the cytoskeleton.

Ischemia induces reversible cytoskeletal rearrangements

Anchoring of the junctional complexes to the cytoskeleton is essential for their function and depends on the interaction of the intracellular junctional plaque with the actin cytoskeleton. We thus investigated the effect of ischemia on the actin-made cytoskeleton. HUVECs were exposed to ischemia for 5 h with or without subsequent reoxygenation. Figure 6A shows that ischemic conditions led to a drastic redistribution of the F-actin. In basal regions of the cells (A,C,E) most of the stress fibres, clearly visible in control cells (A), disappeared in ischemic cells (C). Reoxygenation for 2 h
restored the initial distribution (E). In the apical part of the cells (B,D,F), the redistribution of the F-actin was more subtle but also clearly visible, with actin being more concentrated at the cell periphery after ischemia (D), as compared to control cells (B) or ischemic cells after reoxygenation (F). To test whether actin redistribution required ERK1/2, the same analysis was performed in the presence of U0126. Figure 6B shows that incubation of the cells with 60μM U0126 during the 2 h reoxygenation period prevented fully reformation of stress fibres (E), but had no influence on actin distribution in control cells identically treated under normoxic conditions (B). From these results we conclude that during reoxygenation ERK1/2-mediated stress fibre reformation is linked to endothelial barrier formation.

Focal adhesion kinase (FAK) is dephosphorylated and redistributed during ischemia

FAK has been proposed to play a role in stress fibre formation and/or maintenance (Defilippi et al., 1997). We therefore investigated the behaviour of FAK during ischemic conditions leading to stress fibre disappearance. Confocal images of immunostainings of FAK in cells exposed to ischemia for 5 h revealed a loss of the dash-like pattern reminiscent for well established focal adhesions (Fig.7). Instead, a dot-like pattern was visible. Immunoprecipitation of FAK and immunoblotting with anti-phosphotyrosine antibodies showed that in parallel to FAK redistribution the protein was also dephosphorylated to about 50% of the initial level after ischemic treatment (Fig.7). Analysis after suppression of ERK1/2 phosphorylation with U0126, however, did not induce FAK dephosphorylation (not shown), suggesting that although FAK seems to be a target of ischemia-induced structural rearrangements it is presumably not a downstream substrate of ERK1/2.

Cellular ATP is depleted in ischemic cells and fully replenished after reoxygenation

Protein kinase activity depends on the availability of ATP to phosphorylate the substrate. Since only ischemia but not hypoxia were sufficient to induce barrier failure, we speculated that reversible loss of ERK1/2 activity could be due to ATP depletion. Determination of the ATP content of the cells showed that after 5 h of ischemic treatment of the cells cytosolic ATP concentration decreased about 50% compared to control cells, but was completely restored after 2 h of reoxygenation (Fig. 8). These data are consistent with the assumption that ATP levels could be critical for the ERK1/2 phosphorylation pattern with a rapid, but transient increase, followed by a reversible hypophosphorylation period during an extended ischemic insult.
Discussion

Ischemic conditions are characterised by a lack of both oxygen and nutrients and occur in tissues as a consequence of impaired blood supply e.g. due to vessel occlusion. Endothelial cells forming the walls of the blood vessels and building the interface between blood and tissue are the first target to be affected by ischemia (and subsequent reoxygenation). A hallmark of ischemia is edema formation in the non-supplied tissue, induced by a failure of vascular permeability control (Ogawa et al., 1990a; Olesen, 1986; Park et al., 1999; Partridge, 1995).

The present study was undertaken to better characterise the role of ERK1/2 in endothelial cells during ischemia and reperfusion. We have demonstrated that in barrier-forming endothelial cells ischemia leads to a rapid, but transient increase of ERK1/2 phosphorylation to finally end in nearly complete inactivation of the enzyme. Reoxygenation restores ERK1/2 activity. During ischemia barrier dysfunction occurs, which is restored during reoxygenation. Inhibition of ERK1/2 phosphorylation prevents full recovery of barrier function during reoxygenation, but has no effect during ischemia. During ischemia a complete loss of actin stress fibres occurs, which is reversed 2 hours after reoxygenation in an ERK1/2-dependent manner.

Major structural alterations in the endothelial architecture during ischemia were found at the level of actin stress fibres. Modulation of endothelial stress fibers is involved in permeability control. A ring of actin microfilaments underlies the junctional complex comprised of AJ and TJ and its contraction has been proposed to regulate paracellular permeability. Actin has multiple binding partners both at the AJs and the TJs (Fanning et al., 1998; Knudsen et al., 1995; Wittchen et al., 1999). Coupling of the AJ to the actin-cytoskeleton is important for strong cell-cell adhesion (for a review see Provost and Rimm, 1999). Experimental disruption of F-actin with agents such as phalloidin or cytochalasin disrupt the permeability barrier and change the morphology of TJ (Madara et al., 1986). These data suggest that in the ischemia/reoxygenation-induced modulation of endothelial barrier function cytoskeletal rearrangements or rearrangements at the cytoskeletal-junctional link influencing junctional function may play an important role.

The permeability characteristics of endothelia (and epithelia) also depend on structural integrity of adherens and tight junctions and disruption of either junctional plaque leads to increased permeability, often detectable by redistribution of some of the junctional molecules from their initial circumcellular localisation (Wachtel et al., 1999; Wong and Gumbiner, 1997). However, in our system of simulated ischemic injury with a barrier-forming endothelial monolayer, we could observed only minimal differences in the subcellular distribution of several tight and adherens junctions proteins such as
occludin, ZO-1 and β-catenin and established coimmunoprecipitation studies confirmed structural preservation of adherens junctions. In contrast, when exposing endothelial cells which have not yet formed a fully developed permeability barrier to reversible ischemia, redistribution of junctional molecules and formation of intercellular gaps occurred (data not shown). This difference suggests that target molecules of ischemia may be preferentially associated with either junctional structures or the cytoskeleton, depending on the developmental stage of the system.

As a lot of environmental stresses such as UV light, osmotic shock, heat shock or cytokines transduce their effects on the cells via different MAP kinase pathways such as the ERK1/2 pathway, we investigated the role of this class of molecules in the permeability modulation induced by the environmental stress ischemia/reoxygenation. Ischemic treatment first led to 2.5 fold activation of ERK1/2 followed by a progressive decrease to very low activation levels after 5 hours of exposure. Reoxygenation led to reactivation of ERK1/2 above control levels after 2 hours. A possible explanation for this ERK1/2 activity pattern is that during ischemia ERK1/2 is first activated by signalling cascades that react upon the lower oxygen level or the continuously lowering ATP levels. The early increase in ERK1/2 activity can be inhibited by the protein tyrosine kinase (PTK)-inhibitor genistein, suggesting that the activating pathway may involve one or more PTK. This initial activation pattern also seems to be cell type-specific, as there are several reports which did (Punn et al., 2000; Yue et al., 2000), or did not find ERK1/2 activation during ischemia (Abas et al., 2000) (Pombo et al., 1994).

Downregulation of the ERK1/2-activity during prolonged ischemia may be explained with ATP depletion, leading to direct inactivation of ATP-dependent kinases and also indirect inactivation of ERK1/2 by dephosphorylation through ATP-independent phosphatases. Reoxygenation leads to reestablishment of the normal ATP level and in parallel the ERK1/2 activity is restored. Evidence for such a mechanism is given by studies of the influence of the mitochondrial ATP production on the ERK1/2 activity (Abas et al., 2000), showing necessity of ATP production for proper ERK1/2 activation.

Activation of ERK1/2 by exposition to ischemic conditions for 1 hour led to redistribution of active ERK1/2 mainly to sites at the periphery of the cells. Although many Map kinases have been shown to accumulate in the nucleus of cultured cells once they are activated (Chen et al., 1992a) (Lenormand et al., 1993) (Gonzalez et al., 1993) (Cheng et al., 1996), there is a growing number of reports demonstrating that activated ERK1/2 can also be translocated to different cytoplasmic localisations in a cell. ERK1/2 has been shown to be targeted to focal adhesion points where it is present in its active form (Fincham et al., 2000), and active ERK1/2 has also been found at the kinetochors, asters and midbody during certain stages of mitosis (Shapiro et al., 1998) and at the
centromer (Zecevic et al., 1998). In contrast to the situation after 1 hour of ischemic treatment, the activation of ERK1/2 during reoxygenation led to translocation of a significant part of the protein into the nucleus after 2 hours. Therefore although ischemic conditions and reoxygenation both activate ERK1/2, the two conditions influence ERK1/2-localisation in a different way.

Inhibition of ERK1/2 activation by the specific MEK1/2 inhibitor U0126 (Favata et al., 1998) during reoxygenation inhibits reestablishment of both the permeability and the organisation of the actin-cytoskeleton in our system after 2 hours. Therefore, ERK1/2 activity may be necessary for proper (re)organisation of the actin-cytoskeleton with subsequent implications for the permeability characteristics of the monolayer. This data is further substantiated by the fact that incubations of the cells with U0126 for 6 hours leads to disruption of the cytoskeleton also under normoxic conditions. Inhibition of ERK1/2 during ischemia had no influence on the permeability rise. To further enlighten the role of ERK1/2 in the regulation of cytoskeletal organisation, systems counteracting the loss in ERK1/2 activity must be created, such as ERK1/2 overexpressing cells. ERK1/2 has been connected in several reports with the actin-cytoskeleton. Generally ERK1/2 has been shown to associate with the cytoskeleton (Reszka et al., 1995). Furthermore ERK1/2 bind to actin and actin-binding proteins via the calponin homology (CH) domain, which makes up the actin-binding domain of many cytoskeletal proteins (Leinweber et al., 1999). A translocation of ERK1/2 to actin rich regions has also been demonstrated in freshly isolated ferret aorta cells activated with phenylephrine (Khalil et al., 1995). In this case the activated ERK1/2 co-localised with filamentous actin bundles. However not only colocalisation but also functional influence of ERK1/2 on the cytoskeleton has been shown. Different cytoskeletal proteins have been shown to be phosphorylated by ERK1/2, such as caldesmon (Adam and Hathaway, 1993) or myosin light chain kinase (Morrison et al., 1996), influencing the functional state of the cytoskeleton. In our system, the distribution of activated ERK1/2 after 2 hours of reoxygenation suggests that a great part of the activated protein is located in the nucleus at this time point as shown previously following stimulation by agonists (Chen et al., 1992b) (Gonzalez et al., 1993) (Lenormand et al., 1993). A minor part is visible in the cytoplasm where it has direct access to cytoskeletal proteins. However, the cytoskeletal rearrangement is too fast to be dependent upon gene expression. Therefore rather the ERK1/2 activity in the cytoplasm is important for the detected cytoskeletal restoration. The remaining cytoskeletal part of the activated ERK1/2 may be enough for this function. However as the immunofluorescence pictures after 2 hours of reoxygenation only show a time point, it is also possible that activated ERK1/2 is first present in larger amounts in the cytoplasm and afterwards in the nucleus. It is known that ERK1/2 is activated in the cytoplasm before it is translocated into the nucleus (Lenormand et al., 1993). Further studies are necessary to clarify the
Research papers

temporal alterations of the location of activated ERK1/2 during reoxygenation and their implications in cytoskeletal reassembly. Regulation of many actin-dependent processes has been shown to be mediated by small GTPases belonging to the Rho family. Rho itself is known to be involved in the formation of actin stress fibres (Nobes and Hall, 1995). In a recent work Rho has been linked to the actin reassembly after ATP depletion in a chemical ischemia model (Raman and Atkinson, 1999). Furthermore Rho has been shown to be involved in ERK1/2-activation upon cell adhesion to their substrate via integrins (Renshaw et al., 1996) (Mainiero et al., 1997). These data suggest that ERK1/2 is a possible downstream molecule of Rho and support our finding that ERK1/2 is implicated in mediating actin reassembly during reoxygenation following ischemia.
References


Legends to figures

Figure 1

Effect of ischemia/reoxygenation on ERK1/2 phosphorylation and distribution of ppERK1/2 in HUVECs. Cells were exposed to ischemic conditions for various time periods between 0 and 6 hours with or without subsequent reoxygenation for 2 hours, as indicated. Panel A, immunoblot showing the effect of ischemia (I)/reoxygenation (R) on ERK1/2 and ppERK1/2. Panel B, graph showing the data from a densitometric measurement of the ppERK1/2 blot. Panel C, Confocal images in the basal region of immunofluorescent stainings of ppERK1/2 and phase-contrast images of the same cells to visualise the cells. Data are indicated for control cells (A,B), cells exposed to ischemia for 1 hour (C,D) or 5h (E,F) and cells exposed to ischemia for 5h followed by 2h of reoxygenation (G,H).

Figure 2

Effect of ischemia (I)/reoxygenation (R) on the permeability of HUVEC monolayers. HUVEC grown on transwell filters for 7 days were exposed to ischemia for 5 hours (I5) with or without subsequent reoxygenation for 2 hours (R2) or control conditions (N). Permeability of the monolayers to FITC-dextran [4.4 kDa] was then measured. Data is expressed as means±SD and represents triplicates.

Figure 3

Effect of the MEK1/2-inhibitor U0126 on the reestablishment of the permeability of ischemia-treated HUVEC monolayers during reoxygenation and on ERK1/2 phosphorylation. Panel A, HUVECs grown on transwell filters for 7 days were exposed to ischemia (black bars) or normoxia (hatched bars) for 5 hours. Cells were then processed immediately for permeability assay (C) or reoxygenated for 2 hours (R2) in presence or absence of different concentrations of U0126. Afterwards the permeability of FITC-dextran [4.4 kDa] was measured. Data is expressed as means±SD and represents triplicates. Panel B, the influence of U0126 on ERK1/2 phosphorylation was tested by westernblot with the anti-ppERK1/2 antibody. Cells were incubated with or without 60 μM U0126 for 2 hours and cell lysates were used for immunodetection of ERK1/2 and ppERK1/2.

Figure 4

Effect of ischemia on the distribution of different proteins of the adherens and tight junctions. Cells grown on coverslips were exposed to ischemia (B,D,F) or control
(A,C,E) conditions for 5 hours and immunostained for β-catenin (A,B), occludin (C,D) and ZO-1 (E,F).

**Figure 5**

Effect of ischemia on the integrity of adherens junctions. Lysates from HUVECs exposed to ischemia (I) or control conditions (N) for 5 hours were used for immunoprecipitation of β-catenin, pan-cadherin or p120. Panel A, immunoprecipitates of β-catenin were used for detection of β-catenin, α-catenin and pan-cadherin. Panel B, immunoprecipitates of pan-cadherin were used for detection of pan-cadherin and β-catenin. Panel C, precipitates of p120 were used for detection of p120 and pan-cadherin.

**Figure 6**

Effect of ischemia/reoxygenation on the integrity of the actin-cytoskeleton. HUVECs grown on coverslips were exposed to different conditions as indicated, fixed and processed for staining of the actin-cytoskeleton with TRITC-phalloidin. Panel A, confocal images of basal regions (A,C,E) and apical regions (B,D,E) of the cells. Images A and B show control cells, images C and D show cells exposed to ischemia for 5 h (I5) and images E and F show cells reoxygenated for 2 h after ischemia for 5h (I5/R2). Panel 6B, influence of U0126 on the actin redistribution during reoxygenation. Confocal images of basal regions of the cells exposed to ischemia with (D,E) or without (B,C) subsequent reoxygenation in the presence (B,E) or absence of 60 µM U0126 (C,D).

**Figure 7**

Effect of ischemia on the distribution and tyrosine phosphorylation of FAK. HUVECs were exposed to ischemia (I) or control conditions (N) for 5h and used for immunofluorescent staining of FAK or preparation of cell lysates for immunoprecipitation of FAK. Panel A, immunofluorescent staining of FAK in control cells (control) or ischemic cells (I5). Panel B, immunoprecipitations of FAK from control (N) or ischemic(I) cells were immunoblotted and detected with antibodies against FAK (A) or phospho-tyrosine (B). Panel C, quantitation of the phospho-tyrosine signal in the FAK-immunoprecipitates. Data are means±SD from two experiments.

**Figure 8**

Effect of ischemia/reoxygenation on the ATP content of HUVE cells. HUVECs were exposed to ischemia for 1 hour (I1) or 5 hours (I5) with or without subsequent reoxygenation (R) and cell lysates were used to determine the ATP concentration with the ATP bioluminescence assay kit HS II. Data are means±SD of triplicates.
Figure 1

A

ERK1/2

ppERK1/2

I (h) 0 0.5 1 4 5 5
R (h) 0 0 0 0 0 2

B

control L1 L5 L5/R2

a c e g

b d f h
Figure 2
Figure 3

**A**

Bar graph showing the relative permeability of samples under different conditions:

- **C** (Control)
- **R2** with varying concentrations of U0126

Key:
- □ Normoxia [5h]
- ■ Ischemia [5h]

**B**

Western blot analysis showing the expression levels of ERK1/2 and ppERK1/2.

- 60μM UO126
Figure 5

A

<table>
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<tr>
<th>Blot</th>
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<tr>
<td>IP</td>
<td>β-catenin</td>
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<td>pan-cadherin</td>
<td>p120</td>
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</table>
Figure 6

A

control 15 I5/R2

b

d

lateral

I5

I5/R2

B

control N5/R2 I5 I5/R2 I5/R2

+60μM UO126 +60μM UO126
Figure 8

![Bar graph showing ATP (arbitrary units) levels in different conditions: N, I1, I5, I5/R2.](image)
5.3. Downregulation of occludin expression in astrocytes by TNF is mediated via TNF type 1 receptor and nuclear factor-κB activation

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Abbreviations:

BBB: blood-brain barrier; CAPE: caffeic acid phenetyl ester; IFN: interferon; NF-κB: nuclear factor-κB; TNF: tumor necrosis factor; TNFR1: TNF type 1 receptor; TNFR2: TNF type 2 receptor; TJ: tight junction.
Summary of major results

Abstract

Tight junctions (TJs) form the diffusion barrier of brain microcapillary endothelial cells and support cell polarity. Also astrocytes express TJ components such as occludin, claudin-1, ZO-1 and ZO-2, but do not establish a permeability barrier. However, little is known about function and regulation of these molecules in astrocytes. We studied the impact of tumor necrosis factor (TNF) on occludin and ZO-1 expression in astrocytes. TNF decreased occludin, but not ZO-1 expression. In brain microcapillary endothelial cells, as well as in epithelial cells, occludin expression was not influenced by TNF. Removal of TNF from astrocytes restored the basal level of occludin. Downregulation was inhibited by caffeic acid phenethyl ester, a specific inhibitor of nuclear factor-κB (NF-κB) activation. Exposure of astrocytes isolated from mice deficient in either TNF type 1 receptor (TNFR1), TNF type 2 receptor, or both, respectively, revealed that downregulation was entirely mediated by TNFR1. ZO-1, which can interact with occludin, coprecipitates connexin43, but not occludin. These findings demonstrate that TNF selectively downregulates occludin in astrocytes, but not in cells forming established TJs, through TNFR1 and suggest that NF-κB is involved as a negative regulator.

Key words: Occludin, TNF, astrocytes, tight junctions, NF-κB

Running title: TNF downregulates occludin in astrocytes
Introduction

Highly impermeable tight junctions (TJs) of CNS microvessels contribute to the blood-brain barrier (BBB) in higher vertebrates. In addition, these vessels are nearly completely covered by endfeet of astrocytes and these cells can induce BBB properties in non-CNS endothelial cells in vivo (Janzer and Raff, 1987). More recent studies using in vitro systems have confirmed this finding and furthermore suggested that also neurons play a role (Heller et al., 1997). It is, however, not clear, whether astrocytes act solely as inducers of endothelial barrier properties or whether, in addition, they can contribute to particular aspects of BBB function. Such an assumption is supported by the observation that the blood-nerve barrier of insects is anatomically formed by glial cells and genetically-induced disruption of inter-glial contact leads to a lethal phenotype (Auld et al., 1995).

TJs consist of transmembrane proteins such as occludin (Furuse et al., 1993) and members of the claudin family (Furuse et al., 1998). Intracellularly, these proteins are linked to ZO-1, ZO-2 and ZO-3, which form a scaffold to link TJ proteins to the cytoskeleton (for review see (Stevenson and Keon, 1998)). Several studies have demonstrated that primary astrocytes can express occludin, ZO-1 and ZO-2 in culture (Bauer et al., 1999; Duffy et al., 2000; Howarth et al., 1992). Members of the claudin family have been discovered in astrocytes after IL-1β exposure (claudin-1 (Duffy et al., 2000)) and in oligodendrocytes at interlamellar strands of myelin sheaths (claudin-11 (Morita et al., 1999)). Deletion of claudin-11 results in loss of these TJ-like structures and neuronal deficits (Gow et al., 1999). These results support the existence of TJs or TJ-like structures in glial cells. However, apart from myelin TJs, their precise location, functional role and regulation are currently unknown.

Tumor necrosis factor (TNF) can influence both endothelial and epithelial cells as well as astrocytes in several manners. For example, TNF increases endothelial (Deli et al., 1995) and epithelial (Schmitz et al., 1999) permeability and leads to chemokine production in astrocytes and endothelial cells (Otto et al., 2000). Since TNF has been shown to influence reporter gene expression from the occludin promoter in HT-29/B6 intestinal cells (Mankertz et al., 2000), we hypothesised that the occludin gene could be a target of TNF action in astrocytes. To test this assumption we exposed primary astrocytes to this cytokine. TNF downregulated occludin, but not ZO-1 production in astrocytes. In contrast to astrocytes, brain endothelial cells and epithelial cells revealed no change in the level of occludin after TNF stimulation. The downregulation in astrocytes was TNF type 1 receptor (TNFR1)-dependent and included activation of nuclear factor-κB (NF-κB).
Materials and Methods

Reagents

Mouse monoclonal (Oc-3F10) and polyclonal antibodies against occludin, the polyclonal antibody against ZO-1 and the polyclonal anti-Connexin43 antibody were from Zymed (Gebr. Mächler AG, Basel, Switzerland). Polyclonal antibodies against glial fibrillary acidic protein (GFAP) were from DAKOPATTS (Denmark). Polyclonal antibodies against von Willebrand factor were from DAKO (Denmark). Secondary antibodies for immunofluorescence (FITC- and Cy3-labelled) and for immunoblots (HRP-labelled) were from Sigma. Caffeic acid phenetyl ester (CAPE) and Genistein were from Sigma (Buchs, Switzerland). Calphostin C, KT 5720 and chelerythrine chloride were from Calbiochem (Juro, Switzerland). Recombinant TNF and recombinant interferon (IFN)-γ were both from Roche Molecular Biochemicals. Lipopolysaccharide (LPS) (E.coli 0127:serotype B8) was from Difco Laboratories. Tissue culture materials were from Life Technologies. Gel electrophoresis reagents and all standard laboratory chemicals were of the highest grade commercially available and purchased from Fluka (Buchs, Switzerland).

Mice

The generation of TNF receptor 1 (TNFR1)-deficient mice (Rothe et al., 1993) and TNFR2-deficient mice (Erickson et al., 1994) has been described previously. TNFR1, TNFR2-double deficient mice were generated by breeding from the single-deficient mice.

Cell culture conditions and cell lines

All cells were maintained at 37°C under 5% CO2 in humidified air. MDCK cells and the rat brain endothelial cell line GP8.3 (Greenwood et al., 1996) were cultured in DMEM/10% FCS.

Isolation and culture of primary astrocytes

For isolation of primary astrocytes the brains of newborn mice (wild type and TNFR-deficient) were removed and mechanically dissociated by sieving through a 100 μm cell strainer into culture medium with the help of the pistil of a syringe. Large cell aggregates were removed by sieving through a 70 μm cell strainer and the cell suspension was then seeded in DMEM/20% FCS in one 175 cm² tissue culture flask per 2 brains. After six days in culture, the medium was replaced by DMEM/10% FCS. After 14 days in culture, contaminating microglia were mechanically detached by shaking the flask for 20 minutes and aspirated. Then, astrocytes were trypsinised, replated at a
Summary of major results

density of $3-5\times10^4$/cm$^2$ and cultured for another 3-4 days before use. Culture purity was determined by immunofluorescence analysis with antibodies against GFAP. More than 95% of the cells stained positive for GFAP.

**Isolation of human brain microvascular endothelial cells**

Human brain endothelial cells were isolated as described (Andjelkovic et al., 2000; Gloor et al., 1997). Briefly, cortical tissue samples from epileptic patients undergoing surgical treatment were cleaned of meninges and superficial vessels. The samples were homogenised in DMEM/5% FCS with a syringe, stirred at 37°C for 30 minutes with collagenase type CSL (1.5 mg/ml in 10 ml HBSS), cooled on ice, gently homogenised with a fire-polished pipette, and then mixed with 10 ml 25% BSA in HBSS. After centrifugation (800xg, 20 minutes, 4°C), the pellet was resuspended in DMEM/5% FCS containing 1 mg/ml Collagenase/Dispase and 0.5 mg/ml DNAse I and incubated for 30 minutes at 37°C. The cells were pelleted at 380xg for 10 minutes at 4°C, washed with DMEM/5% FCS and plated on collagen-coated culture dishes in DMEM/F12/20% FCS containing 10 μg/ml endothelial cell growth factor, 1.2 mg/ml bovine brain extract, 0.1 ng/ml EGF, 1 ng/ml acidic FGF, 10 ng/ml hydrocortisone, 50 μg/ml gentamycin, and 16 U/ml heparin. After 2 weeks, cells were subcultured and cultured for another 7-10 days before use. Cell purity was determined by immunofluorescence analysis with antibodies against von Willebrand factor and against GFAP for detection of contaminating astrocytes. More than 95% of the cells stained positive for von Willebrand factor.

**Electrophoresis and immunoblotting**

Western blot analysis was performed as described (Wachtel et al., 1999). Briefly, protein concentration in the extracts was measured by a Bradford assay (Bio Rad) and equal amounts were loaded on the gel. After SDS-PAGE proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The quality of the transfer was controlled by Ponceau S staining of the membrane. The blots were blocked in 5% non-fat dried milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) containing 0.05% Tween-20 for 45 minutes at room temperature. The filters were then incubated for 1 hour with the primary antibodies diluted in blocking buffer. After washing 3 times for 5 minutes in TBS containing 0.05% Tween-20, the primary antibody was reacted with a horseradish peroxidase-conjugated secondary antibody in blocking buffer for 30 minutes. After washing 3 times with TBS plus 0.05% Tween-20 and once with TBS, immunoreactive bands were detected by enhanced chemiluminescence (Amersham) following the manufacturer’s instructions. The chemiluminescent signals were scanned from autoradiographic films (Fuji RX) and imported into Molecular Analyst for densitometric measurements.
Summary of major results

Immunoprecipitation

For immunoprecipitation extracts were prepared with ice-cold lysisbuffer (50 mM Tris-Cl pH 7.2, 150 mM NaCl, 10 mM EDTA, 40 mM NaF, 1 mM Na vanadate, 1% NP-40, 0.1% Na deoxycholate, 1x complete® protease inhibitor mix). Extracts were incubated for 30 minutes on ice and homogenised by pressing several times through a 21g needle. After centrifugation at 10000xg for 20 minutes, the protein concentration in the supernatant was determined and adjusted to 0.5 mg/ml. 0.5 ml extracts were precleared with 25 μl of a 10% suspension of protein G-sepharose (Sigma) for 1 hour at 4°C on a rotating wheel. After centrifugation, the lysates were incubated over night at 4°C with 25 μl protein G-sepharose conjugated to anti-ZO-1 or anti-connexin43 antibodies, respectively (previously prepared by incubating 25 μl protein G-sepharose with 2 μg anti-ZO-1 or anti connexin43 antibodies for one hour at 4°C in 1 ml 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA). Immunoprecipitates were washed 5 times for 5 minutes with lysis buffer, resuspended and boiled for 8 minutes in 40 μl 2x SDS sample buffer and then used for SDS-PAGE analysis.

Immunofluorescence analysis

Cells grown on gelatine-coated glass coverslips were washed once with DPBS, fixed and permeabilised with precooled acetone/methanol (1:1) for 10 minutes at -20°C and air dried for 10 minutes at room temperature. Incubation with the primary antibody in PBS containing 3% BSA was for 1 hour at room temperature. The cells were then washed 3 times for 5 minutes with DPBS and incubated with a 1:200 dilution of FITC-or Cy3-conjugated secondary antibody in PBS containing 3% BSA for 1 hour at room temperature. After washing 3 times for 5 minutes with PBS, the coverslips were drained and mounted in 70% glycerol, 5% n-propyl-gallate in 30 mM Tris-HCl pH 9.5. The coverslips were sealed with nail polish and examined by immunofluorescence microscopy on a Leica inverted microscope.
Summary of major results

Results

Primary astrocytes expressed both occludin and ZO-1 in a circumcellular pattern (Fig. 1A,B,D,E), similar to the distribution of these two proteins in endothelial cells (Wachtel et al., 1999), suggesting their overlapping accumulation at sites of cell-cell contact. All astrocytes were positive for ZO-1, whereas occludin immunoreactivity was confined to clusters of astrocytes making up about 40-50% of all cells. To study whether occludin and ZO-1 were regulated by inflammatory cytokines, we exposed astrocytes to TNF for 48 h. Occludin immunoreactivity was strongly reduced in astrocytes, whereas no effect was visible with ZO-1 (Fig. 1C,F). The immunofluorescence results were confirmed by Western blot analysis. As shown in figure 2A, the amount of occludin in total extracts of astrocytes was reduced by TNF in a dose-dependent manner. Downregulation was also observed with LPS, whereas IFN-γ was without effect. Using 100 ng/ml TNF, a time course experiment revealed that occludin downregulation requiring at least 48 h to reach maximal reduction to 30% (Fig. 2A,C). Consistent with the immunofluorescence observation, ZO-1 expression was not influenced by TNF (Fig. 1B). In addition, also the expression of β-catenin and pan-cadherin, constituents of adherens junctions, was not altered by TNF under these conditions. To assess whether downregulation of occludin was specific for astrocytes we used brain microcapillary endothelial cells and MDCK epithelial cells. In both cell types TNF did not influence the expression level of occludin under the same conditions (Fig. 2B). From these results we conclude that TNF-induced downregulation of occludin is cell specific and does not involve other junction-associated proteins.

Since TNF is known to induce apoptotic processes in a variety of cell types we asked whether downregulation of occludin was reversible. After exposure to 100 ng/ml TNF for 48 h one half of the cultures was washed and incubated in the absence of TNF, whereas the remaining half was kept in the presence of TNF. 24 and 60 h after TNF removal occludin production was assessed by Western blot analysis. As shown in figure 3, removal of TNF led to resynthesis of occludin, whereas in the presence of the cytokine the amount further declined. Consistent with this reversibility, dye exclusion controls revealed that more than 95% of the cells remained viable both in the absence and presence of TNF (not shown).

To gain first insight into the signalling cascade mediating occludin downregulation in astrocytes by TNF we preincubated the cells with the NF-κB inhibitor CAPE (Natarajan et al., 1996). After preincubation with 10 or 25 μg/ml CAPE, 100 ng/ml TNF was added to the cells and incubation continued for 48 h. CAPE was able to partially prevent downregulation of occludin in the presence of TNF (Fig. 4A). The inhibitor had no significant effect on the level of occludin in the absence of TNF, supporting that
downregulation of occludin was, to a great degree, mediated via NF-κB. Inhibitors against protein kinase A, protein kinase C, and protein tyrosine kinases were ineffective in antagonising the TNF-induced downregulation of occludin (Fig. 4B).

To assess which TNF receptor type mediated occludin downregulation, we used mice deficient in either TNFR1, or TNFR2, or TNFR1, TNFR2 double-deficient mice, respectively. Astrocytes prepared from wild type and receptor knockout mice were exposed to 100 ng/ml TNF during 48 h and then probed for occludin expression by Western blot analysis. As before, downregulation was found in astrocytes from wild-type animals and, in addition, in astrocytes from TNFR2-deficient animals, but not in TNFR1 mutants and in TNFR1, TNFR2 double mutants (Fig. 5). This result shows that downregulation of occludin in astrocytes by TNF was entirely mediated via TNFR1. This finding makes secondary effects of e.g. contaminating LPS in the TNF preparation unlikely, even though LPS was able to downregulate occludin in astrocytes (see Fig. 2A). Additionally, the ability of LPS to induce secretion of TNF from astrocytes resulted in levels not exceeding 0.5 ng/ml.

In epithelial and endothelial cells occludin and ZO-1 uniformly colocalise at the cell periphery and in vitro assembly studies demonstrated the ability of occludin to bind via its C-terminal end to the second PDZ domain of ZO-1. Since in astrocytes not all cells positive for ZO-1 were also positive for occludin we wondered whether ZO-1 bound occludin or proteins of other junctions present in astrocytes. Among these a possible candidate is connexin43, which forms gap junctions in astrocytes and has an overall membrane topology similar to the one of occludin (Giepmans and Moolenaar, 1998). To assess a possible interaction of ZO-1 with occludin and/or connexin43 in astrocytes we performed coimmunoprecipitation experiments. Detergent extracts were prepared under conditions previously shown to preserve the interactions between ZO-1 and connexin43 (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). Immunoprecipitations were performed with antibodies against ZO-1 and connexin43, respectively. The precipitates were then probed with anti connexin43, anti ZO-1 and anti occludin antibodies, respectively. As shown in figure 6, connexin43 was indeed coprecipitated with ZO-1, whereas no occludin immunoreactivity was discovered under these conditions. From this experiment we conclude that in astrocytes ZO-1 binds at least a fraction of connexin43, whereas under the same conditions no complex with occludin could be detected. This result suggests that TJ proteins in astrocytes may be organised distinctively from the array in endothelial and epithelial cells, respectively.
In this study we have investigated the TJ molecules occludin and ZO-1 in astrocytes. Both proteins are present at intercellular contact sites. Occludin, but not ZO-1 levels are strongly reduced upon exposure to TNF, whereas TNF has no effect on occludin and ZO-1 in brain endothelial cells and MDCK cells. Downregulation of occludin is mediated by TNFR1 and involves the transcription factor NF-κB. In addition, in astrocytes ZO-1 and connexin43, a constituent of gap junctions, form a detergent-resistant complex.

In endothelial cells forming a permeability barrier occludin and ZO-1 exhibit a uniform and overlapping distribution at sites of cell-cell contact (Wachtel et al., 1999). The peripheral location is similarly found in astrocytes, but in these cells only ZO-1 is generated by virtually all cells, suggesting that the structural and functional integration of occludin and ZO-1 in astrocytes may be distinct. To explore this idea we assessed the influence of TNF on occludin and ZO-1. A previous study showed that TNF, and in addition IFN-γ, strongly reduced occludin promoter activity in transfected HT-29/B6 intestinal cells (Mankertz et al., 2000). In the same cells endogenous occludin mRNA was decreased after 6 h of TNF incubation, but increased again after 24 h (Mankertz et al., 2000). In astrocytes we found downregulation of occludin to occur only after exposure to TNF, whereas endothelial and kidney epithelial cells showed no effect. Occludin protein levels continuously decreased during TNF incubation and did not recover before removal of the cytokine. Such differences are likely to reflect physiological differences among astrocytes, endothelia, as well as renal and intestinal epithelia. The non responsiveness of endothelial cells is unlikely to be caused by the absence of TNF receptors, as these cells are susceptible towards TNF treatment (Otto et al., 2000; Greenwood et al., 1996), and MDCK cells loose barrier properties after TNF incubation (M.W., unpublished observation). Of note, also IL-1β downregulates occludin in astrocytes, and at the same time leads to upregulation of claudin-1 (Duffy et al., 2000). Together with our finding the above data suggest that the proinflammatory cytokines TNF and IL-1β can lead to a dynamic change in the expression of TJ constituents in astrocytes. ZO-1, in contrast, is neither susceptible to TNF nor to IL-β action (Duffy et al., 2000). Since also β-catenin and pan-cadherin expression were not affected, occludin appears to be a critical target of cytokine action among junctional proteins in astrocytes.

TNF elicits a variety of cellular responses including endothelial activation, cell proliferation, differentiation and apoptosis, suggesting the involvement of distinct signalling pathways. Our analysis showed that the effect of TNF on occludin expression in astrocytes is entirely mediated through TNFR1 and involves NF-κB. Earlier studies
demonstrated nuclear translocation of NF-κB in TNF-stimulated astrocytes (Kemler and Fontana, 1999) and NF-κB induction via TNFR1 (Adam et al., 1995). Also compatible with our findings is the fact that NF-κB activation can inhibit TNF-induced apoptosis (Liu et al., 1996). The ability of the NF-κB inhibitor CAPE to antagonise downregulation by TNF can be considered an argument that NF-κB could act as a negative regulator of occludin expression in astrocytes. Supporting such a mechanism is the presence in the occludin promoter of the motif GGGAGGAGGC (at position 1753; see (Mankertz et al., 2000)), which is nearly identical to the recently identified NF-κB binding motif GGGAGGAGTC (Wang et al., 2000). A repressive function of NF-κB proteins has been found for the choline acetyltransferase gene in PC-12 cells (Toliver-Kinsky et al., 2000), as well as for the bcl-2 gene in lymphocytes (Sohur et al., 1999). Alternatively, NF-κB activation could lead to the activation of a repressor which then acts on the occludin promoter. Our data furthermore suggest no requirement for protein kinase A or C, or tyrosine kinase, respectively. In endothelial cells TNF did not downregulate occludin but led to increased paracellular permeability through a mechanism including tyrosine kinase activity and excluding NF-κB (M.F.B. and S.M.G., unpublished observation). The involvement of tyrosine kinase activation has also been shown in TNF-mediated epithelial barrier breakdown (Schmitz et al., 1999). These results further underline the existence of distinct signalling pathways to transduce selective activities of TNF.

It has been demonstrated that the TJ proteins ZO-1 and occludin form a complex which together with other junctional proteins establishes the linkage to the actin cytoskeleton ((Furuse et al., 1994), for review see (Tsukita et al., 1999)). Our results do not suggest the existence of such a complex in astrocytes, but demonstrate that ZO-1 binds at least a fraction of the gap junction protein connexin43. The ability of ZO-1 to bind connexin43 has been demonstrated previously in other cell types (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). Differences in the pattern of proteins coprecipitating with ZO-1 between epithelial cells and astrocytes have been shown (Howarth and Stevenson, 1995), further supporting that the organisation of TJ proteins may be cell type-specific.

The interaction of ZO-1 with occludin and connexin43, respectively, has been suggested to be involved in targeting these proteins to the plasma membrane (Furuse et al., 1994; Toyofuku et al., 1998). Since our immunofluorescence analysis with permeabilised cells clearly showed that the predominant fraction of occludin is found at the cell periphery, we assume that occludin delivery to the plasma membrane does not depend on its ability to interact with ZO-1 exclusively. Indeed, deletion studies have shown that occludin mutant proteins with internal truncations but authentic C terminus were not found in TJs, although they bound ZO-1 in vitro (Furuse et al., 1994). Since ZO-1 also interacts with ZO-2 (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994), ZO-2, which is
expressed by astrocytes (Duffy et al., 2000), may be involved in occludin interaction and targeting in astrocytes.

Although astrocytes produce the TJ proteins occludin, claudin-1, ZO-1 and ZO-2 (Bauer et al., 1999; Duffy et al., 2000; Howarth et al., 1992), astrocytes in culture do not develop significant paracellular barrier to dextran tracer molecules (M.W. unpublished observation). From this result we assume that TJ proteins in astrocytes do not build the same barrier as in epithelia and endothelia. This raises the question of the functional role of TJ proteins in astrocytes. Beside regulation of paracellular permeability (gate function) TJ molecules are involved in several other functions. They take part in the separation of the membrane in subdomains (fence function), they are involved in signal transduction (Balda and Matter, 2000), and they are implicated in neutrophil and monocyte transmigration (Huber et al., 2000)(Martín-Padura et al., 1998). Astrocytes in vivo are morphologically subdivided in different domains, with endfeet in close contact to the blood vessels. Specific expression of the TJ proteins at the border of the endfeet could therefore help to separate the endfoot microdomain from the remaining astrocyte plasma membrane and play a role during cellular transmigration under normal and e.g. inflamed conditions, when cytokines such as TNF are elevated.
Acknowledgements

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Legends to Figures

Figure 1

Immunofluorescence localisation of occludin and ZO-1 in primary astrocytes. A, B: Occludin distribution under control conditions. D, E: ZO-1 distribution under control conditions. C: Downregulation of occludin expression after exposure to 100 ng/ml TNF for 48 h. F: ZO-1 expression after exposure to 100 ng/ml TNF for 48h. (B) and (E) are higher magnification images of a double labelling of occludin and ZO-1 to visualise their colocalisation. Scale bars in A, D, F are 62.5 μm, and in B, C, E 25 μm.

Figure 2

Analysis of expression of junctional proteins in various cell types under control and stimulated conditions. A: Western blot analysis of occludin expression in astrocytes. Lane 1: control; lane 2: 1 μg/ml LPS for 24h; lane 3: 100 ng/ml TNF for 24h; lane 4: 200 U/ml IFN-γ for 24h; lanes 5-9: dose response of occludin expression to TNF for 24 h; lanes 10-15: time course of occludin expression after exposure to 100 ng/ml TNF. B (upper panel): Western blot analysis of ZO-1, β-catenin and pan-cadherin expression in astrocytes under control conditions and after exposure to 100 ng/ml TNF for 48h. B (lower panel): Western blot analysis of occludin expression in human brain endothelial cells (EC), rat brain endothelial cells (GP8.3) and MDCK cells under control conditions and after exposure to 100 ng/ml TNF for 48h. C: Quantitative analysis of occludin expression in astrocytes after exposure to 100 ng/ml TNF for the times indicated in (A). Blots from 3 independent experiments were densitometrically analysed and the values normalised to the control, set to 100%.

Figure 3

Reversibility of TNF-induced downregulation of occludin in astrocytes. Western blot analysis of astrocytes incubated without TNF (lane 1) and with 100 ng/ml TNF for 24 h (lane 2) and 48 h (lane 3). After 48 h one half of the cultures was washed and further incubated in the absence of TNF for another 24 h (lane 5) and 60 h (lane 6). The remaining half was incubated in the presence of TNF for a further 60 h period (lane 4, total incubation time with TNF 108 h).
Figure 4

Suppression of TNF-mediated downregulation of occludin in astrocytes. A (upper panel): Western blot analysis. 2 h before addition of 100 ng/ml TNF, astrocytes were incubated with 10 and 25 μg/ml CAPE, a specific inhibitor of NF-κB. The inhibitor was present during the entire incubation period with TNF. Control cells received CAPE, but not TNF; A (lower panel): quantitative analysis of the suppression of occludin downregulation by CAPE. Blots from two experiments were densitometrically analysed and the values normalised to the control, set to 100%. B: Western blot analysis of the impact of the protein kinase A inhibitor KT5720 (KT, 0.2 μM), the protein kinase C inhibitors calphostin C (Cal, 0.25 μM), chelerythrine chloride (Chel, 1 μM), and the tyrosine kinase inhibitor genistein (25 μg/ml) on TNF-induced downregulation of occludin.

Figure 5

Downregulation of occludin in astrocytes from mice deficient in TNF receptors. Astrocytes from wildtype (wt), TNF receptor 2 (TNFR2-/-), TNF receptor 1 (TNFR1 -/-) and double knockout (TNFR1/2 -/-) animals were exposed to 100 ng/ml TNF for 48h.

Figure 6

Coimmunoprecipitation of ZO-1 and connexin43 from astrocyte extracts. Cleared protein extracts were used for immunoprecipitation with antibodies against connexin43 and ZO-1, respectively. After SDS-PAGE and electrotransfer, the blots were probed with antibodies against connexin43 (C43, lanes 2, 3, 4), ZO-1 (lanes 5) and occludin (Occl., lane 6), respectively. Lane 1 shows a Western blot of connexin43 from whole astrocyte extracts. The position of ZO-1 and connexin43 are indicated at the left margin. Ab denotes the position of the heavy chains of the precipitating antibodies which are recognised by the secondary HRP-labelled antibody. Molecular masses are given in kDa at the right margin. IP: immunoprecipitation; WB: Western blot.
**Figure 3**

<table>
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<tr>
<th>100 ng/ml</th>
<th>TNF (h)</th>
<th>-</th>
<th>24</th>
<th>48</th>
<th>108</th>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>24</td>
<td>60</td>
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Figure 4

A

100 ng/ml
TNF [48h] - + + +
CAPE [μg/ml] - - 10 25 10 25

B

100 ng/ml
TNF - + + + +
Inhibitor - - KT Gen ChCl Cal
Figure 5

wt  TNFR2\textsuperscript{\textminus}  TNFR1\textsuperscript{\textminus}  TNFR1/2\textsuperscript{\textminus}  

100 ng/ml TNF (48h)
Figure 6

<table>
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<tr>
<th>IP</th>
<th>C43</th>
<th>ZO-1 no Ab</th>
<th>ZO-1</th>
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<td>WB</td>
<td>C43</td>
<td>ZO-1 Occl.</td>
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Summary of major results

6. Summary of major results

6.1. Influence of protein tyrosine phosphatase-inhibitors on TJs and vascular permeability.

In this study it could be demonstrated that inhibition of PTPs by the inhibitors pervanadate and phenylarsine oxide led to a time- and concentration-dependent opening of the paracellular permeability barrier to FITC-dextran tracer molecules of different sizes in endothelial cells. Barrier opening was accompanied by proteolysis of the 61 kDa protein occludin, resulting in formation of a 51 kDa fragment. This suggests a proteolytic cleavage in the first extracellular loop of occludin. In parallel, redistribution of the protein from its circumcellular localisation to a diffuse distribution was observed. This phenomenon could be detected in endothelia as well as in epithelia, suggesting that it is a widespread mechanism. Other junctional proteins such as ZO-1, cadherin and β-catenin were neither affected in their distribution nor their integrity. Occludin proteolysis, redistribution, and barrier opening, initiated by PTP-inhibition, could be prevented by preincubation of the cells with the metalloproteinase inhibitor 1,10-phenanthroline, but not by inhibitors against other types of proteases. These results suggest that PTP-inhibition leads to activation of a metalloproteinase, which cleaves occludin as a consequence of which occludin is redistributed and the barrier opened.

6.2. Influence of ischemia and reoxygenation on vascular permeability.

The paracellular permeability in HUVECs incubated for 5h under ischemic (1% O₂, no glucose), but not hypoxic conditions increased about 40%. Total cytoplasmic ATP dropped to about 50% during 5h ischemia. The rise in permeability was reversible after 2 hours of reoxygenation (21%O₂, complete medium). Only little changes could be detected in the distribution of AJ and TJ molecules after ischemic treatment. Instead, a significant redistribution of the actin cytoskeleton could be detected, with a complete loss of cell-crossing stress fibres. This redistribution was also reversible during reoxygenation and temporally correlated with the permeability change. Changes could also be detected at the focal adhesions. Focal adhesion kinase (FAK) was redistributed from its predominantly focal to a diffuse cytosolic location. Furthermore tyrosine phosphorylation of FAK decreased to about 50%. As a lot of environmental stresses such as UV light, osmotic shock, heat shock or cytokines transduce their effects in the cells via different MAP kinase pathways such as the ERK1/2 pathway, we investigated the role of ERK1/2 in the permeability modulation induced by ischemia and reoxygenation. Ischemia first led to activation of ERK1/2 after 1 hour, followed by a
Summary of major results

deactivation to about 15% of the control level after 5 hours. Reoxygenation led to a rapid reactivation of ERK1/2 above control levels. Inhibition of ERK1/2 activation with the specific MEK1/2 inhibitor U0126 had no influence on the changes during the ischemic phase, but inhibited both cytoskeletal reestablishment and reestablishment of the normal endothelial barrier function during the reoxygenation phase. Together these results show that the actin cytoskeleton, rather than TJ and AJ, is a major target during ischemia-induced endothelial barrier failure, and they suggest that the activities of ERK (and probably FAK) are required to maintain and/or restore barrier properties.

6.3. Influence of TNF-α on occludin expression in astrocytes.

As a side aspect of this study it could be demonstrated that occludin expression in astrocytes is reversibly downregulated by TNF in a concentration- and time-dependent way. The expression of other junctional proteins such as ZO-1, cadherin and β-catenin in astrocytes however is not influenced by TNF. In other cell types such as endothelial cells and epithelial cells TNF had no effect on occludin expression. Specific inhibition of the transcription factor NF-κB partially prevented the TNF induced occludin downregulation, whereas inhibitors against PKA, PKC and PTK were ineffective. Exposure of astrocytes isolated from mice deficient in either TNF type 1 receptor (TNFR1), TNF type 2 receptor (TNFR2), or both, respectively, revealed that downregulation was entirely mediated by TNFR1. These findings demonstrate that TNF selectively downregulates occludin in astrocytes, but not in cells forming established TJs, through TNFR1 and suggests that NF-κB is involved as a negative regulator.
Conclusions/outlook

7. Conclusions/Outlook

The results of this study call the readers attention to several points:

(1) The identity of the occludin-cleaving metalloproteinase. A first step in a search of the protease would be the determination of the cleavage site in occludin. Purification by immunoprecipitation and N-terminal sequencing of the fragment (or MALDI/MS) should allow to answer this point. With the knowledge of the cleavage site a screening assay for metalloproteinase identification could be established. Peptides containing the cleavage site would be used as competitors. When effective, they would be modified with a fluorophore side group and a quencher, allowing the establishment of a fluorometric assay to detect proteolytic activity able to cleave this peptide. Purified metalloproteinases would then be incubated with the peptide to identify those, which are able to cleave. These enzymes would then be used with endothelial cells. Such an assay would be the basis to screen for a novel protease by expression cloning using an endothelial cDNA library.

(2) The identity of the PTK involved in metalloproteinase activation. As it was already excluded in this study that direct substrate tyrosine phosphorylation was the reason for occludin proteolysis, tyrosine phosphorylation steps maybe lie more upstream of occludin and lead to another type of modification of occludin (e.g. Ser/Thr phosphorylation) or maybe affect the activity of the protease. As in the former case the direct substrate of the PTK is not known, it might be very complex to isolate the PTK directly. Agonists of well-known signalling cascades might be used to check whether the PTK belongs to such a signalling cascade. However, known agonists such as cytokines, growth factors, and tumour promoters, respectively, have already been tested in this study without success. In the latter case the first step in a search of the PTK would be the identification of the protease. Antibodies against this protease used for immunoprecipitation would help to confirm tyrosine phosphorylation of the protein induced by PTP inhibitors. If this phosphorylation can be confirmed, cells stably expressing the protease but not endogenously expressing the activating PTK could be transfected with an endothelial cDNA library and activation of the protease by phosphorylation could be used for a screen for the PTK. If no phosphorylation of the protease can be detected, the activation maybe is indirect and involves a different modification of the protease.

(3) The linkage between ERK and actin. In a first step dominant negative ERK mutants could be used for further substantiating the ERK-actin linkage shown in this study with
Conclusions/outlook

ERK-inhibitors. Cells expressing such mutants could be exposed to ischemia and reoxygenation and their effect on the remodelling of the cytoskeleton could be tested. Furthermore, to learn more about the proteins lying in a cascade between ERK and actin, ERK immunoprecipitates could be tested for known actin binding proteins. As many cytoskeletal regulations are mediated by small G-proteins belonging to the Rho-family (Rho, Rac, Cdc42), an investigation of the ERK-actin linkage could also include a test for a functional linkage between ERK and rho small G-proteins. Especially Rho itself, known to be involved in the regulation of stress fibre formation (Nobes and Hall, 1995) and focal adhesion formation (Ridley and Hall, 1992), would be an interesting candidate. Rho-Inhibitors or cells expressing dominant negative mutant forms of Rho could be used during reoxygenation, conditions known to lead to ERK-activation and ERK-mediated cytoskeletal remodelling under control conditions. This could help to test whether ERK lies downstream of this small G-protein.

(4) A possible linkage between ERK and FAK. As FAK is known to be involved in the formation of focal adhesions and as focal adhesions are known to initiate stress fibre formation, there is maybe a linkage between FAK and ERK. A possible test for such a linkage has to take into account both the possibilities of FAK lying upstream or downstream of ERK in an activation cascade. To test the former possibility, dominant negative FAK mutants could be used to test for their ability to suppress either ERK activation or ERK mediated actin reestablishment during reoxygenation. In the latter case, dominant negative ERK mutants or ERK inhibitors such as U0126 could be used and tested for their ability to suppress FAK phosphorylation and activation during reoxygenation.
8. References


References


References


References


References


References


References


References


Curriculum vitae

9. Curriculum Vitae

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Curriculum vitae

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