BIOLOGICAL RESEARCH CENTER OF THE HUNGARIAN ACADEMY OF SCIENCES
SZEGED

PROTECTION AGAINST BLOOD-BRAIN BARRIER DAMAGES IN PATHOLOGICAL CONDITIONS: *IN VIVO* AND *IN VITRO* STUDIES

Ph.D. thesis

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CONTENTS

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. Veszelka S, Urbányi Z, Pázmány T, Németh L, Obál I, Dung NTK, Ábrahám CS, Szabó G, Deli MA, 2003. Human serum amyloid P component attenuates the bacterial lipopolysaccharide-induced increase in blood-brain barrier permeability in mice. *Neuroscience Letters,* 352, 57-60. (IF: 2.019)

II. Veszelka S, Pásztói M, Farkas AE, Krizbai I, Dung NTK, Niwa, M, Ábrahám CS, Deli MA, 2007. Pentosan polysulfate protects brain endothelial cells against bacterial lipopolysaccharide-induced damages. *Neurochemistry International,* 50, 219-228. (IF: 2.994)

III. Andras IE. Deli MA, Veszelka S, Hayashi K, Henning B, Toborek M, 2007. The NMDA and AMPA/KA receptors are involved in glutamate-induced alterations of occludin expression and phosphorylation in brain endothelial cells. *Journal of Cerebral Blood Flow and Metabolism*, Epub ahead of print. (IF: 4.786)

ABBREVIATIONS

AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor
ANOVA	analysis of variance
BBB	blood–brain barrier
CNS	central nervous system
DAF-FM	4-amino-5-methylamino-[2,7]-difluorofluorescein diacetate
DCFDA	chloromethyl-dichloro-dihydro-fluorescein diacetate
DME	Dulbecco's-modified Eagle's medium
DNQX	6,7-dinitroquinoxaline-2,3-dione
EBA	Evan's blue-labelled albumin
GLUT-1	glucose transporter-1
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon γ
IL-1β	interleukin-1β
iNOS	inducible NO synthase
ip.	intraperitoneal
iv.	intravenously
JAM	junctional adhesion molecules
KA	kainate receptor
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MK801	dizocilpine
MOF/MODS	multiple organ failure/dysfunction syndrome
MRP	multidrug resistance protein
NF-κB	nuclear factor κB
NMDA	N-methyl–D-aspartate receptor
NO	nitric oxide
P_e	endothelial permeability coefficient
PPS	pentosan polysulfate
PS_e	permeability surface area product value
ROS	reactive oxygen species
SAP	serum amyloid P component
SEM	standard error of mean
SF	sodium fluorescein
TEER	transendothelial electrical resistance
TF	tissue factor
TJ	tight junctions
TLR-4	Toll-like receptor 4
TNF-α	tumor necrosis factor-α:
ZO	zonula occludens protein

1. INTRODUCTION

1.1. The blood-brain barrier

The blood–brain barrier (BBB) plays an important role in the homeostatic regulation of the brain microenvironment necessary for the stable and co-ordinated activity of neurones. The barrier is formed by brain endothelial cells lining the cerebral microvasculature, and is an important mechanism for protecting the brain from fluctuations in plasma composition, and from circulating agents such as neurotransmitters and xenobiotics capable of disturbing neural function. Brain capillary endothelial cells have a dynamic interaction with other neighbouring cells, astroglia, pericytes, perivascular microglia, neurons and organized into well-structured neurovascular units, which are involved in the regulation of cerebral blood flow (Abbott *et al.*, 2006) (Fig. 1.).

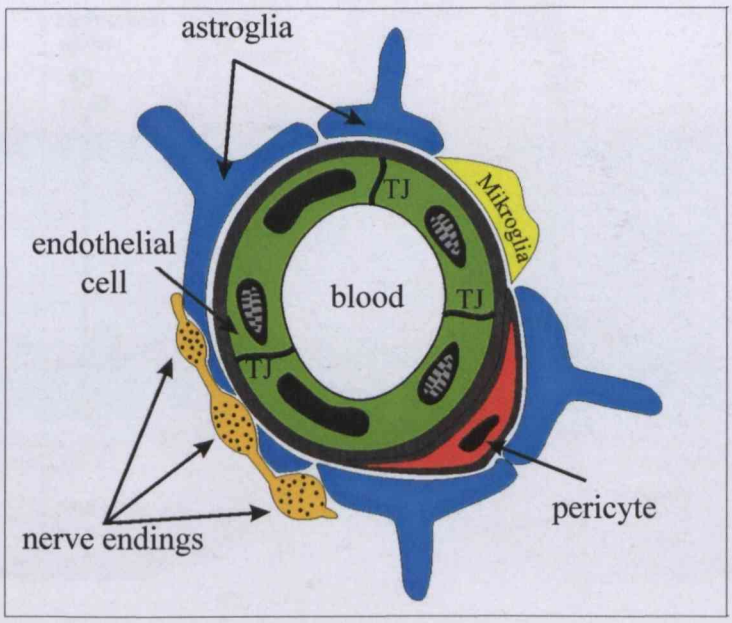

Fig. 1. Schematic drawing of the blood brain barrier.

The continuous layer of cerebral endothelial cells attached to each other by tight intercellular junctions (TJ) constitutes the morphological basis of the BBB. These junctions significantly restrict even the movement of small ions such as Na^+ and Cl^-, so that the transendothelial electrical resistance (TEER), which is typically 2–20 ohm x cm^2 in peripheral capillaries, can be >1000 ohm x cm^2 in brain endothelium *in vivo*. The TJ is built up by integral membrane proteins such as occludin, claudins and junctional adhesion molecules (JAM) which are connected to the actin cytoskeleton by peripheral membrane proteins.

Members of these peripheral proteins are the zonula occludens proteins (ZO-1, ZO-2, ZO-3), cingulin, and cathenins (Krizbai and Deli, 2003).

Cerebral endothelial cells share many general endothelial characteristics. They regulate blood coagulation and vasoreactivity by secreting mediators, such as endothelins, nitric oxide, angiotensin peptides (Joó, 1996), adrenomedullin (Kis *et al.*, 2001). In addition, brain endothelium has a unique BBB phenotype. Receptors, transporters and enzymes are localized in a polarized way in brain endothelial cells. These cells express a variety of transporters for nutrients, like glucose (glucose transporter-1 GLUT-1), neutral, acidic and basic amino acids (large neutral amino acid transporter LAT1), choline, nucleosides (sodium-coupled nucleoside transporter CNT2), vitamins, minerals, *etc.*, to feed neural cells (Abbott *et al.*, 2006) (Fig. 2).

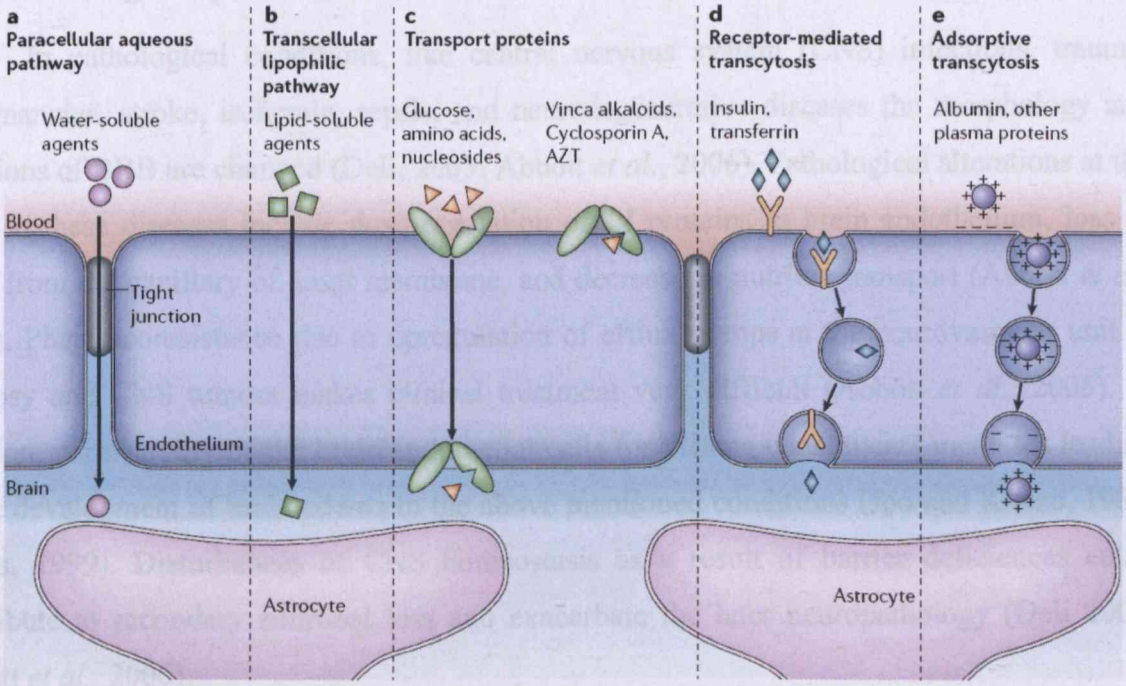

Fig. 2. Transport pathways at the blood brain barrier (Abbott et al., 2006).

Cerebral endothelium protects the nervous system from xenobiotics by efflux transporters. Some of them belong to the family of ABC transporters, like P-glycoprotein (ABCB1), multidrug resistance proteins MRP-1, -4, 5-, and -6 (ABCC1-6), and brain multidrug resistance protein (BMDP/ABCG2/BCRP), while others, *e.g.* organic anion-transporting polypeptide OATP2, organic acid transporters OAT1 and OAT3, or glutamate transporters EAAT1-3 do not (Kusuhara and Sugiyama, 2001).

Since the discovery of the BBB by Paul Ehrlich in 1885, several *in vivo* and *in vitro* models have been developed to quantify BBB permeability and transport. The *in vivo* intravenous administration technique, the brain perfusion technique, the indicator diffusion technique, or the brain uptake index technique are widely used (Smith, 1989). Because the majority of the brain endothelial cells resisted damage during isolation and remained viable, isolated brain microvessels, the first *in vitro* BBB model (Joó and Karnushina, 1973) proved to be an excellent model to describe and characterize signalling pathways in a direct way (Joó, 1993). The next generation of *in vitro* models using co-cultured brain endothelial cells has continuously evolved in the last 30 years, and greatly contributed to research on physiology, pathology and pharmacology of the BBB (Deli *et al.*, 2005).

1.2. BBB changes in pathological conditions

In pathological conditions, like central nervous system (CNS) infections, trauma, malignancies, stroke, ischemia, sepsis, and neurodegenerative diseases the morphology and functions of BBB are changed (Deli, 2005; Abbott *et al.*, 2006). Pathological alterations at the BBB in these diseases include downregulation of TJ proteins in brain endothelium, loss of agrin from the capillary of basal membrane, and decrease in nutrient transport (Abbott *et al.*, 2006). Pharmacoresistance due to upregulation of efflux pumps at the neurovascular unit in epilepsy and CNS tumors makes clinical treatment very difficult (Abbott *et al.*, 2006). In addition, permeability of the brain endothelial cells for plasma constituents increases leading to the development of brain edema in the above mentioned conditions (Joó and Klatzo, 1989; Banks, 1999). Disturbances of CNS homeostasis as a result of barrier deficiences could contribute to secondary neuronal loss and exacerbate the later neuropathology (Deli 2005; Abbott *et al.*, 2006).

1.2.1. Endotoxin-induced damage of the BBB

Sepsis is still associated with a high mortality rate despite recent progress in antibiotics and critical care therapy. In the USA alone there are 300 000–500 000 septic episodes each year, with mortality rates ranging from 20% to 40% (Karima *et al.*, 1999). Sepsis develops when the initial, appropriate host response to an infection becomes amplified, and then dysregulated. If untreated, the patient may develop respiratory or renal failure, abnormalities of coagulation, and profound and unresponsive hypotension. The main cause of

death is the refractory hypotension (septic shock) within a few days of the onset of sepsis. Later the sequential multiple organ failure/dysfunction syndrome (MOF/MODS) becomes the primary clinical problem and main cause of mortality. Once a patient develops septic shock or sequential MOF/MODS, the mortality rate increases to 60–70%. The Gram-negative bacterial infections are responsible for about 60% of sepsis cases, Gram-positive for the remainder (Cohen, 2002).

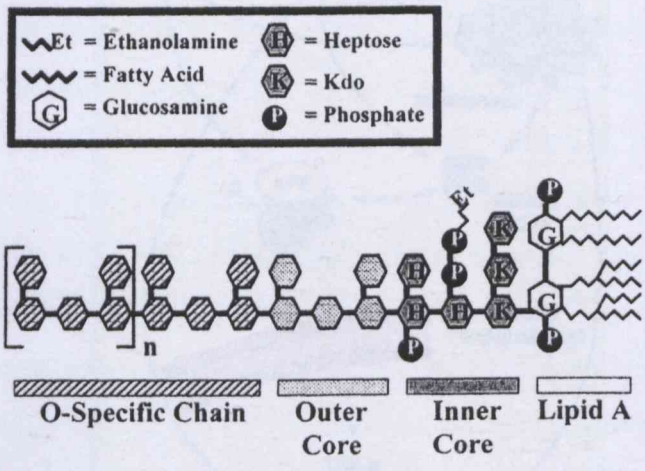

Fig. 3. Structure of lipopolysaccharide (Bannerman and Goldblum, 1999)

Lipopolysaccharide (LPS) or endotoxin plays a pivotal role in the initiation of a variety of host responses caused by Gram-negative bacterial infection. LPS is the constituent of the outer cell wall of the Gram-negative bacteria. It is an amphipathic molecule with a hydrophobic, well conservated part, called lipid A, and a hydrophilic sugar chain. The hydrophilic part consists of an oligosaccharide core and the O-antigen containing of repeating carbohydrate units and shows extreme diversity. The major biological activities of LPS are mainly attributed to a lipid A component (Fig. 3.).

Since 1980s, novel insights into the molecular pathogenesis of LPS-induced shock (endotoxic shock) and organ dysfunction have been gained. It has been revealed that LPS interacts with the CD14 receptor on macrophages, monocytes and neutrophils. LPS binding protein (LBP) might facilitate this interaction, and then signaling through the Toll-like receptor 4 (TLR-4). The LPS induced cell activation results in a rapid activations of several transcription factors such as nuclear factor-κB (NF-κB), which are involved in the gene transcription of numerous proinflammatory cytokines (tumor necrosis factor-α (TNF- α), interleukins IL -1β, IL-6, IL-8, interferon- γ (IFN-γ)) tissue factors (TF), adhesion molecules (ICAM-1) and inducible nitric oxide (NO) synthase. Peripheral endothelial cells which do not express CD14 are stimulated by LPS bound to soluble CD14 (Fig. 4.).

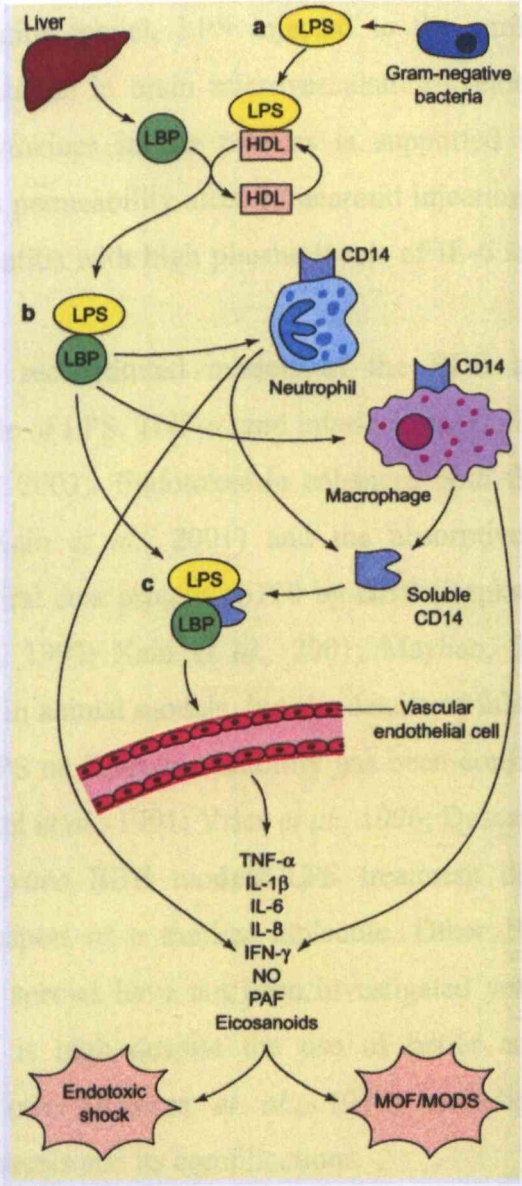

Fig. 4. Cd14-dependent cellular activation by LPS.(Karima et al., 1999)

The steps mentioned above are responsible for the circulatory failure, leukocyte-induced tissue injury and coagulation disorder and these are critical determinants in the development of sequential organ failure in endotoxemia. Brain endothelial cells express LPS receptors TLR-4, TLR-2 and CD14 and mediate the effects of peripheral LPS in the CNS (Singh and Jiang, 2004).

The permeability of the BBB is very low for water-soluble compounds and macromolecules (Pardridge, 2002; Deli *et al.,* 2005). LPS increases BBB permeability in different animal models, including newborn pigs, rats and mice (Temesvári *et al.,* 1993; Xaio *et al.,* 2001; Mayhan, 2001). Rodents are highly insensitive to LPS (Redl *et al.,* 1993). In studies using repeated high doses of LPS BBB opening was observed in mice (Xaio *et al.,* 2001), while 3 h after a single lower dose of LPS no effect was seen in rats (Vries *et al.,*

1995). In a porcine meningitis model, LPS injected to the cerebrospinal fluid induced permeability marker extravasation in brain microvasculature (Temesvári *et al.*, 1993). The role of proinflammatory cytokines in the process is supported by *in vivo* experiments demonstrating elevated BBB permeability after intracarotid injection of TNF- α (Ábrahám *et al.*, 1996) or in acute pancreatitis with high plasma levels of IL-6 and TNF-α (Farkas *et al.*, 1998).

Studies on *in vitro* reconstituted models of the BBB also provided important information regarding the role of LPS, TNF-α, and interleukins (Deli *et al.*, 1995; Vries *et al.*, 1996, 1997; Gaillard *et al.*, 2003). Endotoxemia enhances both the saturable transport of insulin across the BBB (Xaio *et al.*, 2001) and the absorptive endocytosis of human immunodeficiency virus-1 viral coat protein gp120 by BBB (Banks *et al.*, 1999). Data from literature (Temesvári *et al.*, 1993; Xaio *et al.*, 2001; Mayhan, 2001) indicate, that LPS increases BBB permeability in animal models, but the details of this effect has not been fully understood. The effect of LPS on BBB permeability has been confirmed on cultured bovine brain endothelial cells (Tunkel *et al.*, 1991; Vries *et al.*, 1996; Descamps *et al.*, 2003; Gaillard *et al.*, 2003). In these *in vitro* BBB models LPS treatment decreased transendothelial resistance or increased transport of a marker molecule. Other BBB parameters or brain endothelial cells from other species have not been investigated yet. Mortality rate of sepsis due to bacterial infections is high despite the use of better antibiotics and significant development in intensive care (Karima *et al.*, 1999) underlining the need for new therapeutical treatments for sepsis and its complications.

1.2.2. Glutamate-induced damage of the BBB

Neuronal death in stroke and neurotrauma mediate involve multiple interdependent molecular pathways. It has been suggested that these pathways are triggered following elevations in extracellular excitory amino acids, primarily glutamate. Glutamate is the major excitatory neurotransmitter in the mammalian CNS. It is continuously released from the cells and removed from the extracellular space in a dynamic equilibrium.The effects of glutamate can be mediated by two types of receptors: ionotropic and metabotropic receptors. The ionotropic glutamate receptors function like ligand-gated ion chanels and they can be classified into N-methyl–D-aspartate (NMDA) receptors, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (KA) receptors. Cerebral endothelial cells

express several types of glutamate receptors, namely GluR-1 (an AMPA receptor subunit) and NMDR-NR-1 and NMDA-2B (NMDA receptor subunits) (Krizbai *et al.,* 1998).

Ionotropic glutamate receptors appear to play a critical role in the pathology of cerebral infarction. During ischemia and stroke, a reduction in blood flow to the brain microvasculature induces a decrease in the delivery of oxygen and nutrients. The resulting ATP depletion contributes to increased leakage of glutamate from the cells (Sharp *at al.,* 2003). The high levels of extracellular glutamate can overstimulate the glutamate receptors, leading to an upregulated increase in intracellular calcium and neuronal death (Dempsey *et al.,* 2000); disruption of the BBB and development of vasogenic edema (Mayhan and Didion, 1996).

Alterations of tight junction proteins were demonstrated in hypoxia/reoxygenation modeling of transient ischemia. Specifically, hypoxia causes occludin, claudin-1, ZO-1, and ZO-2 redistribution; however, reoxygenation results in increased expression of occludin, ZO-1, ZO-2, claudin-1 protein levels (Brown *et al.,* 2003; Mark and Davis 2002). Discontinuous tight junctions have also been demonstrated in experimental cerebral infarction (Shibata *et al.,* 1988). The mechanisms of glutamate-induced disruption of the BBB integrity are not fully understood. We hypothesized that glutamate-induced alterations of tight junction protein expression can play a critical role in this process.

1.3. Potential therapeutical molecules against BBB damages

Clinical and research data support the involvement of BBB damage as an early event in many neurological conditions (Abbott *et al.,* 2006). Very recently the BBB has been considered as a therapeutic target in those diseases, where neuronal damage is secondary to, or exacerbated by BBB dysfunction (Deli, 2005; Abbott *et al.,* 2006). Beside current therapeutical and experimental neuroprotective strategies, prevention of BBB damage or protection of BBB functions could also be clinically valuable. The role of cerebral endothelium in brain edema was recognized decades ago, and new therapeutic means, like Ca^{2+} channel blockers, steroids, non-steroid antiinflammatory drugs, ROS scavengers acting on BBB were suggested for its prevention and treatment (Joó and Klatzo, 1989). Histamine is a well-known mediator of brain edema, and anti-histamines proved to be effective in preventing the BBB permeability increasing effect (Joó, 1993). Dexamethasone, a steroid used in clinical practice for the treatment of brain edema can improve barrier functions of the BBB (Deli *et al.,* 2005). Because of this shift of emphasis from the rescue of neurons to

treatment of the BBB research on potential therapeutical molecules becomes even more important.

1.3.1. Serum amyloid P component

Serum amyloid P component (SAP) belongs to the highly conserved lectin fold superfamily and within it to the smaller subgroup of pentraxins known for their pentagonal structure and calcium-dependent ligand binding. SAP is a 235 kDa plasma glycoprotein composed of two non-covalently associated pentameric rings. SAP ligands include glycosaminoglycans, DNA in chromatin, all types of amyloid deposits, LPS and Gram-negative bacteria, expressing short types of LPS (Haas, 1999). The physiological role of SAP through chromatin and LPS binding comprises protection against chromatin-induced autoimmunity and modulation of host defense during bacterial infections, participating in binding and clearance of host- or pathogen-derived cellular debris at sites of inflammation (Gewurz *et al.*, 1995)

SAP is a normal circulating protein in humans with a serum concentration of about 30 μg/ml. Serum SAP level increases with age, from 10 μg/ml in neonates to 60 μg/ml in persons over 80 years, and it is regulated by estrogen (Hashimoto *et al.*, 1997). SAP concentration in serum doubles during sepsis in humans, while in mice it grows from 100 μg/ml basal level to 4 mg/ml 24 h after endotoxin challenge, indicating that it is a major acute phase protein in this species (Taktak and Stenning, 1992). SAP is synthesized and catabolyzed in the liver, its half life in plasma is 7-8 h in mice (Baltz *et al.*, 1985). No SAP was detected in other tissues and organs in physiological conditions (Kalaria *et al.*, 1991; Haas, 1999).

It is debated if SAP can protect from LPS toxicity *in vivo*. SAP and LPS-binding short synthetic peptides neutralize toxic effects of LPS *in vitro* and a peptide could protect mice against LPS-induced septic shock (Haas *et al.*, 2000). However, $SAP^{-/-}$ knockout mice were only slightly more sensitive to lethal doses of LPS, and intraperitoneal injections of SAP did not affect survival in LPS injected wild type mice (Noursadeghy *et al.*, 2000). On the contrary, Soma *et al.* (2001) found that SAP-deficiency conferred resistance to lethality induced by high-dose LPS in mice.

1.3.2. Pentosan polysulfate

Pentosan polysulfate (PPS), a highly polyanionic sulphated semisynthetic polysaccharide (average MW: 3-6 kDa) obtained from beech tree (*Fagus silvatica*) shavings,

is structurally related to the glycosaminoglycans (GAGs) synthesized by cells (Fig. 5.). PPS has a wide range of pharmacological effects (Maffrand *et al.,* 1991; Ghosh, 1999) since it is interacting with GAGs such as heparin, heparan sulphate or dermatan sulphate, which are important regulators in many biologically diverse processes. High sulfation rate and charge density enables PPS to compete more effectively than other polyanions with endogenous GAGs (Ghosh, 1999).

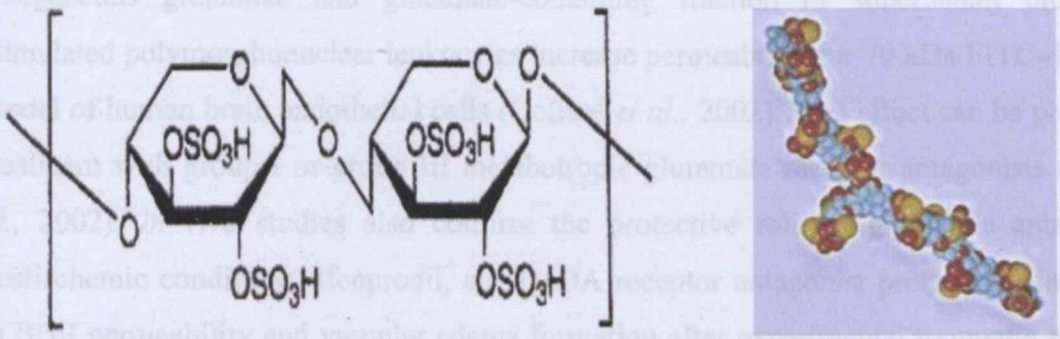

Fig. 5. Structure of pentosan polysulfate

PPS exhibits diverse pharmacological activities that may also have relevance in the control of different functions of endothelial cells forming the BBB. PPS can inhibit protein kinase A, protein kinase C, tyrosine protein kinase, serine proteases, matrix metalloproteinases, lysosomal enzymes, coagulation factors, complement factors, cytokines (Ghosh, 1999). It could stimulate the release of tissue-type plasminogen activator, superoxide dismutase, and lipase in different cells and tissues (Ghosh, 1999).

Pentosan is used in clinical practice as an anticoagulant (Maffrand *et al.,* 1991) and as a treatment for interstitial cystitis (Nordling, 2004). Experimental and clinical data suggest that PPS has a therapeutic efficacy in osteoarthritis (Ghosh, 1999) and in prion diseases (Deli *et al.,* 2000a,b). PPS can favorably regulate BBB phenotype in brain endothelial cells (Deli *et al.,* 2000a), and protect them in models of prion peptide treatment (Deli *et al.,* 2000b, 2005). PPS had no major effect on BBB parameters of TEER, permeability or fluid phase endocytosis (Deli *et al.,* 2000a,b, 2005).

1.3.3. Glutamate antagonists

Glutamate plays a crucial role in the pathology of ischemic brain injuries, and increases BBB permeability (Deli *et al.,* 2005). Glutamate receptor agonists and antagonists also alter barrier integrity and pharmacological studies may facilitate development of drugs

effective in the prevention of postischemic brain edema. NMDA or glutamate decrease TEER, while a specific metabotropic glutamate receptor agonist, trans-(±)1-amino-1,3-cyclo-pentanedicarboxylic acid, could even temporarily increase barrier properties in a study using human immortalized brain endothelial cells (Sharp *et al.*, 2003). NMDA antagonist MK-801, intracellular Ca^{2+} scavenger TMB-8 or antioxidant N-acetyl-L-cystein protects against glutamate-induced deterioration of barrier integrity in this model (Sharp *et al.*, 2003). Exogeneous glutamate and glutamate-containing fraction of supernatant produced by stimulated polymorphonuclear leukocytes increase permeability for 70 kDa FITC-dextran in a model of human brain endothelial cells (Collard *et al.*, 2002). This effect can be prevented by treatment with group I or group III metabotropic glutamate receptor antagonists (Collard *et al.*, 2002). *In vivo* studies also confirm the protective role of glutamate antagonists in postischemic conditions. Ifenprodil, an NMDA receptor antagonist protects against increase in BBB permeability and vascular edema formation after experimental traumatic brain injury in rats (Dempsey *et al.*, 2000).

1.4. Aims

The role of the BBB to protect the brain in physiological and pathological conditions is increasingly emphasized in the literature (Abbott *et al.*, 2006). Protective strategies at the level of BBB are in the focus of new studies. We have selected LPS and glutamate, two major pathological factors in human diseases causing BBB disturbances and brain edema to study potential protective molecules on *in vitro* and *in vivo* BBB models. The effects of SAP in protecting against LPS toxicity, especially in septic shock, are controversial in the literature. Furthermore, SAP and PPS have never been tested in LPS-related BBB studies before. The molecular mechanisms of glutamate-induced changes in barrier integrity have not been studied in detail yet.

The main aims of our studies were the following:

(1) To test the effect of human SAP on LPS-induced BBB changes and clinical symptoms in mice.

(2) To examine the effects of LPS on barrier properties using a rat co-culture based *in vitro* BBB model, to reveal the underlying mechanisms and to test if PPS has any protective action on LPS-induced changes.

(3) To study the effects of glutamate on barrier properties of rat brain endothelial monolayers, and to evaluate the effects of antagonists on glutamate-induced changes.

2. MATERIALS AND METHODS

2.1. Reagents

All reagents used in the study were purchased from Sigma (Sigma-Aldrich Ltd., Budapest, Hungary), unless otherwise indicated.

2.2. Animals

Male CBA/BL6 mice (3-month-old, 30–35 g) and Wistar rats (2-week-old) were obtained from the animal facility of the Biological Research Center, Szeged, kept under standard conditions, and given tap water and rat chow *ad libitum*. The experiments performed conform to European Communities "Council directive for the care and use of laboratory animals" and were approved by local authorities (XVI/72-45/a/2001).

2.3. Cell culture

Brain capillary fragments were isolated from the forebrains of 2-week-old Wistar rats and seeded on cell culture inserts (Transwell clear, 1 cm^2; pore size, 0.4 μm, Costar) coated with collagen type IV and fibronectin. For immunofluorescent staining brain endothelial cells were cultured on glass coverslips coated with a biological matrix derived from corneal endothelial cells (Dömötör *et al.*, 1998). Brain microvascular endothelial cells migrated from isolated microvessels to reach confluence 4 days after the seeding (Deli *et al.*, 1997; Perrière *et al.*, 2005). Cultures were maintained in Dulbecco's-modified Eagle's medium (DME) supplemented with 5 μg/ml gentamicin, 20% plasma-derived bovine serum (First Link, UK), 1 ng/ml basic fibroblast growth factor (Roche) and 100 μg/ml heparin. In the first 2 days, culture medium contained puromycin (4 μg/ml) to selectively remove P-glycoprotein negative contaminating cells (Perrière *et al.*, 2005). Cultures reached confluency within a week and were used for experiments. To induce BBB characteristics, brain endothelial cells were co-cultured with rat cerebral glial cells (Kis *et al.*, 2001) (Fig. 6.). Briefly, primary cultures of glial cells were prepared from newborn Wistar rats. Meninges were removed, and cortical pieces were mechanically dissociated in DME containing 5 μg/ml gentamicin and 10% fetal bovine serum and plated in poly-L-lysin coated 12-well dishes and kept for minimum 3 weeks before use. In confluent glia cultures 90% of cells were immunopositive for the astroglia cell

marker glial fibrillary acidic protein, while the remaining 10% was immunopositive for CD11b, a marker of microglia.

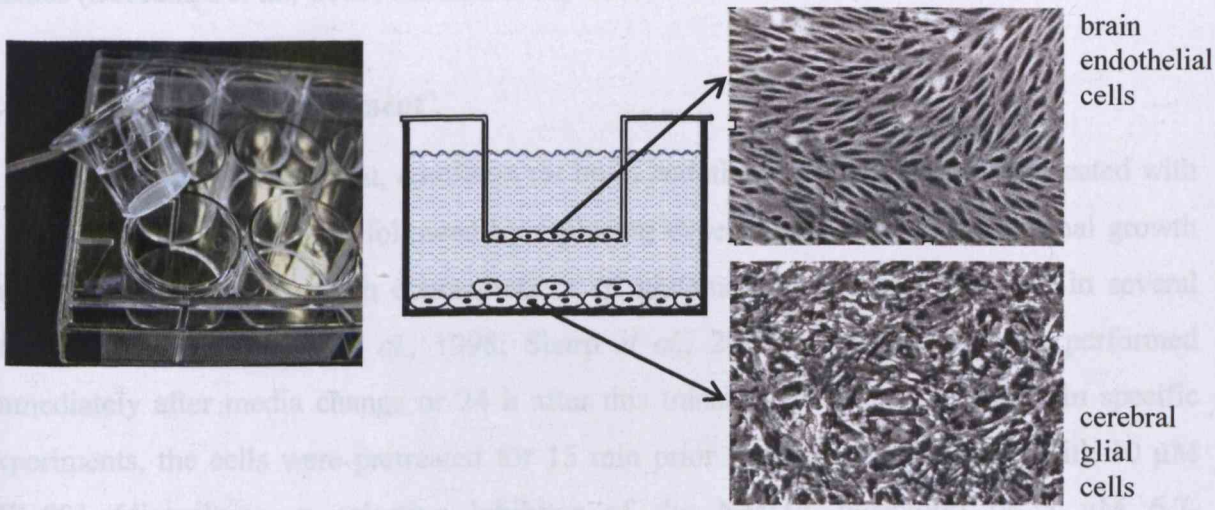

Fig.6. In vitro BBB model using co-cultured rat brain endothelial cells with rat cerebral glial cells

2.4. *In vivo* LPS and SAP treatment

Mice received three intraperitoneal (*ip.*) injections of *Salmonella typhimurium* LPS, 100 μg (3 mg/kg in 200 μl isotonic saline in each dose) at 0, 6, and 24 h according to the protocol of Xaio *et al.*, (2001) and after the last LPS injection the BBB permeability was determined at 6 h, 18 h and 24 h. Some groups also received intravenously (*iv.*) 250 μg human SAP (8 mg/kg, in 200 μl total volume, Calbiochem; La Jolla, CA, USA) 1 h before the permeability study. Seven animal groups (n = 5–12) were formed: (**1**) vehicle-treated control which received *ip.* injections of vehicle only; (**2**) LPS-treated mice 6 h, (**3**) 18 h, or (**4**) 24 h after the last LPS injection; (**5**) LPS 18 h + SAP 1 h treated mice; (**6**) LPS 24 h + SAP 1 h treated; and (**7**) only SAP-treated animals 1 h after the injection. SAP was reconstituted from lyophilized powder in sterile distilled water, protein content was 1.25 mg/ml, with physiological ion concentrations. This dose may correspond to the serum level of SAP (about 250 μg/ml) in acute phase response in mice.

2.5. *In vitro* LPS and PPS treatment

Several serotypes of LPS (*E. coli* O55:B5, O111:B4; *Salmonella typhimurium*) were tested at 0–10 μg/ml concentration for 0–48 h treatment period in brain endothelial cells and microvessels. The doses of PPS (Cartrophen, Biopharm Australia Pty Ltd.) varied between 1 and 100 μg/ml, as described in previous studies (Deli *et al.*, 2000a,b). The effective doses of

LPS and PPS were determined in preliminary experiments. Incubations of brain endothelial cells with LPS were performed in endothelial culture medium in accordance with other studies (Descamps *et al.*, 2003; Gaillard *et al.*, 2003).

2.6. *In vitro* glutamate treatment

In a typical experiment, confluent rat brain endothelial cell cultures were treated with 1 mM glutamate for 30 min followed by replacing experimental media with normal growth medium without serum. Such concentrations of glutamate have also been used in several literature reports (Krizbai *et al.*, 1998; Sharp *et al.*, 2003). Experiments were performed immediately after media change or 24 h after this transient glutamate exposure. In specific experiments, the cells were pretreated for 15 min prior to glutamate treatment with 10 μM MK-801 (dizocilpine, a selective inhibitor of the NMDA receptors) or 5 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX, an inhibitor of the AMPA and KA receptors). The inhibitors were left in cell culture media for the duration of glutamate treatment. These treatment conditions did not affect cell viability as determined by 3-[4,5] dimethylthiazol-2,5-diphenyltetrazolium bromide, MTT, conversion assay (data not shown).

2.7. *In vivo* measurement of BBB permeability

Permeability for sodium fluorescein (SF; mw: 376 Da), a marker of paracellular flux, and Evan's blue-labelled albumin (EBA; mw: 67 kDa), a tracer for transendothelial transport, was measured as it was described in details (Ábrahám *et al.*, 1996). Mice were given a solution of both dyes (2%, 5 ml/kg) in an *iv.* injection to the tail vein for 1 h, and at the end of the experiments the animals were perfused with 50 ml phosphate-buffered saline for 15 min. Samples from four brain regions (right and left cerebral cortex, midbrain, cerebellum) were collected, weighed and stored at -20 °C. Tissue pieces were homogenized in 1.5 ml of cold, 15% w/v, freshly prepared, trichloroacetic acid and centrifuged with 10,000 g for 10 min. Dye concentrations were measured in supernatants by a Polarstar Galaxy fluorescent microplate reader (BMG Labtechnologies), the absorbency of Evan's blue at 620 nm, while the emission of sodium fluorescein at 525 nm after excitation at 440 nm. BBB permeability was expressed as ng tracer/g brain tissue.

2.8. *In vitro* measurement of endothelial monolayer resistance and permeability

Transendothelial electrical resistance (TEER), representing the permeability of tight junctions for sodium ions, was measured by an EVOM resistance meter (World Precision Instruments) using STX-2 electrodes, and it was expressed relative to the surface area of endothelial monolayer ($\Omega \times cm^2$) (Fig. 7). The TEER of cell-free inserts (90–100 $\Omega \times cm^2$) were subtracted from the values. The TEER of rat primary brain endothelial monolayers in co-culture varied between 250 and 700 $\Omega \times cm^2$.

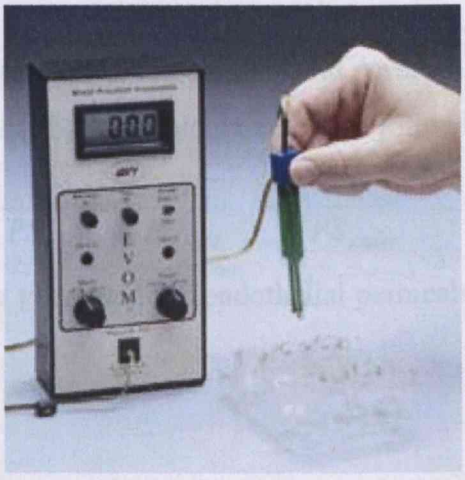

Fig. 7. TEER measurement by an EVOM resistance meter using STX-2 electrodes.

The flux of fluorescein and albumin across endothelial monolayers was determined as previously described (Kis *et al.*, 2001) (Fig. 8). Cell culture inserts, following treatment and measurement of TEER, were transferred to 12-well plates containing 1.5 ml Ringer–Hepes solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.5 mM D-glucose, 20 mM Hepes, pH 7.4) in the basolateral compartments. In apical chambers culture medium was replaced by 500 µl Ringer–Hepes containing 10 µg/ml SF and 165 µg/ml Evan's blue bound to 0.1% BSA. The inserts were transferred at 20, 40 and 60 min to a new well containing Ringer–Hepes solution.

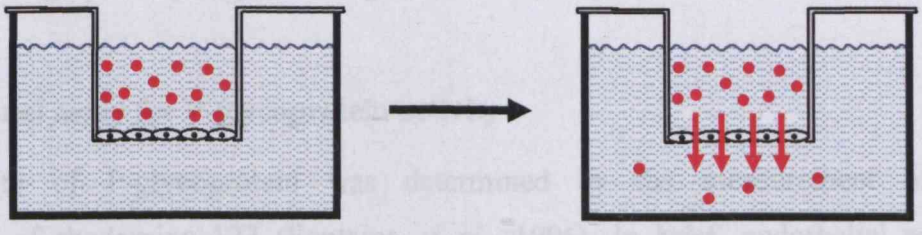

Fig. 8. Schematic drawing of permeability measurement of brain endothelial monolayers

The concentrations of the marker molecules in samples from the upper and lower compartments were determined. Evan's blue concentration was measured by a Labsystems Multiscan plate reader (absorbency: 620 nm), and fluorescein levels by a Polarstar Galaxy fluorescent plate reader (BMG Labtechnologies; emission: 525 nm, excitation: 440 nm). Flux across cell-free inserts was also measured. Transport was expressed as 'µl' of donor (luminal) compartment volume from which the tracer is completely cleared and calculated from the abluminal and luminal concentration ratio and the abluminal volume (Deli *et al.*, 1995):

$$\text{Cleared volume}\,(\mu l) = \frac{C_{abluminal} \times V_{abluminal}}{C_{luminal}}$$

The average cleared volume was plotted versus time, and permeability × surface area product value for endothelial monolayer (PS_e) was calculated by the following formula:

$$\frac{1}{PS_e} = \frac{1}{PS_{total}} - \frac{1}{PS_{insert}}$$

PS_e divided by the surface area generated the endothelial permeability coefficient (P_e, in 10^{-6} cm/s).

2.9. Immunostaining

Brain endothelial cell monolayers cultured on fibronectin- and collagen coated glass coverslips and treated with LPS and/or PPS were stained for ZO-1, claudin-5 and β-catenin junctional proteins. The cultures were washed in PBS and fixed with ethanol (95 vol.%) – acetic acid (5 vol.%) for 10 min at - 20 °C (ZO-1 and β -catenin) or with ethanol for 30 min at 4 °C (claudin-5). Cells were blocked with 3% BSA and incubated with primary antibodies (Zymed, USA) anti-ZO-1, anti-claudin-5, anti-β-catenin for 1 h 30 min. Incubation with secondary antibody Cy3-labelled anti-rabbit IgG lasted for 1 h. Between incubations cells were washed three times with PBS. Coverslips were mounted in Gel Mount (Biomeda, USA) and staining was examined by a Nikon Eclipse TE2000 fluorescent microscope (Nikon, Japan) and photographed by a Spot RT digital camera (Diagnostic Instruments, USA).

2.10. Functional assay for P-glycoprotein activity

Activity of P-glycoprotein was determined by the measurement of cellular accumulation of rhodamine 123 (Fontaine *et al.*, 1996). In brief, endothelial monolayers pretreated with LPS and/or PPS for 16 h in 24-well plates were washed, and incubated with Ringer–Hepes buffer containing 10 µM rhodamine 123 for 1 h at 37 °C. The solution was

quickly removed, endothelial cells were washed three times with ice-cold PBS, and solubilized in 0.2 M NaOH. Rhodamine 123 content was determined by Polarstar Galaxy fluorescent plate reader (BMG Labtechnologies; excitation at 485 nm, emission at 538 nm). Verapamil (2 μM, 30 min preincubation) was used as a reference P-glycoprotein inhibitor.

2.11. Detection of reactive oxygen species and nitric oxide

Two probes were used for fluorometric detection of free radicals, both from Molecular Probes: chloromethyl-dichloro-dihydro-fluorescein diacetate (CM-H_2DCFDA) to measure reactive oxygen intermediates, and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate) to measure intracellular nitric oxide (Kojima *et al.*, 1998). The indicators penetrate the cells by diffusion and become deacetylated by intracellular esterases. Oxidation of CM-H_2DCFDA by reactive oxygen species yields a fluorescent molecule. DAF-FM diacetate reacts with nitrosonium cation and forms a fluorescent heterocycle trapped in the cytoplasm. Confluent brain endothelial cell layers cultured in 96-well plates were pretreated with LPS and/or PPS for 16 h, then washed, and incubated with Ringer–Hepes buffer containing 1 μM DAF-FM diacetate or 1 μM CM-H_2DCFDA for 1 h at 37 °C. The plates were measured by Polarstar Galaxy fluorescent plate reader (BMG Labtechnologies; excitation at 485 nm, emission at 538 nm).

2.12. Statistical analysis

All data presented are means ± S.E.M. The values were compared using the analysis of variance (ANOVA) followed by Dunnett, Bonferroni or Newman-Keuls post hoc tests. Changes were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1. Effects of LPS on the blood-brain barrier integrity

3.1.1. BBB permeability changes after LPS treatment in mice and effect of SAP

BBB permeability for both fluorescein and albumin was significantly elevated after the administration of three consecutive intraperitoneal *S. typhimurium* LPS injections in mice. The extravasation of the tracers was already evident macroscopically by the green coloration in cerebral cortex, midbrain and cerebellum, the brain regions studied during the experiment. The brains of LPS-treated animals were macroscopically different from vehicle-treated ones: petecchias, small haemorrhages could be seen in cerebellum and midbrain. In the cerebral cortex of LPS-treated mice extravasation of fluorescein increased about 4-fold compared to that in vehicle treated animals 6 h after the last injection and remained elevated at 18 h and 24 h timepoints (Fig. 9A). The LPS-induced elevation in fluorescein permeability was even higher in cerebellum (5-fold) and in midbrain (7-fold) (Figs. 10A, 11A) than that in cortex.

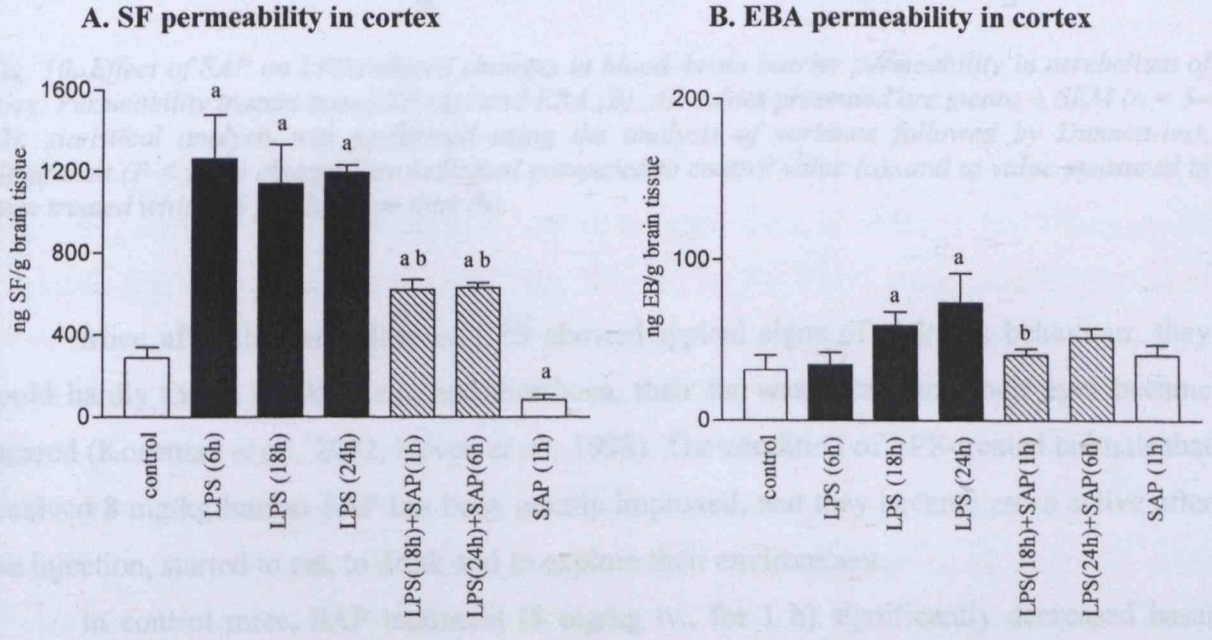

Fig. 9. Effect of SAP on LPS-induced changes in blood–brain barrier permeability in cerebral cortex of mice. Permeability tracers tested SF (A); and EBA (B). All values presented are means ± SEM (n = 5–12), statistical analysis was performed using the analysis of variance followed by Dunnett-test. Significant (P < 0.05) changes are indicated compared to control value (a), and to value measured in mice treated with LPS for the same time (b).

LPS treatment induced a significant increase in cortical EBA extravasation 18 h and 24 h after the third injection compared to permeability measured in vehicle treated animals. The increase in albumin permeability was the highest in midbrain, about 6-fold compared to control, it was 2.8-fold in cerebellum and 1.8-fold in cortex (Figs. 9B, 10B, 11B), similarly to the pattern of fluorescein data. Extravasation of fluorescein was about ten times higher than that of albumin.

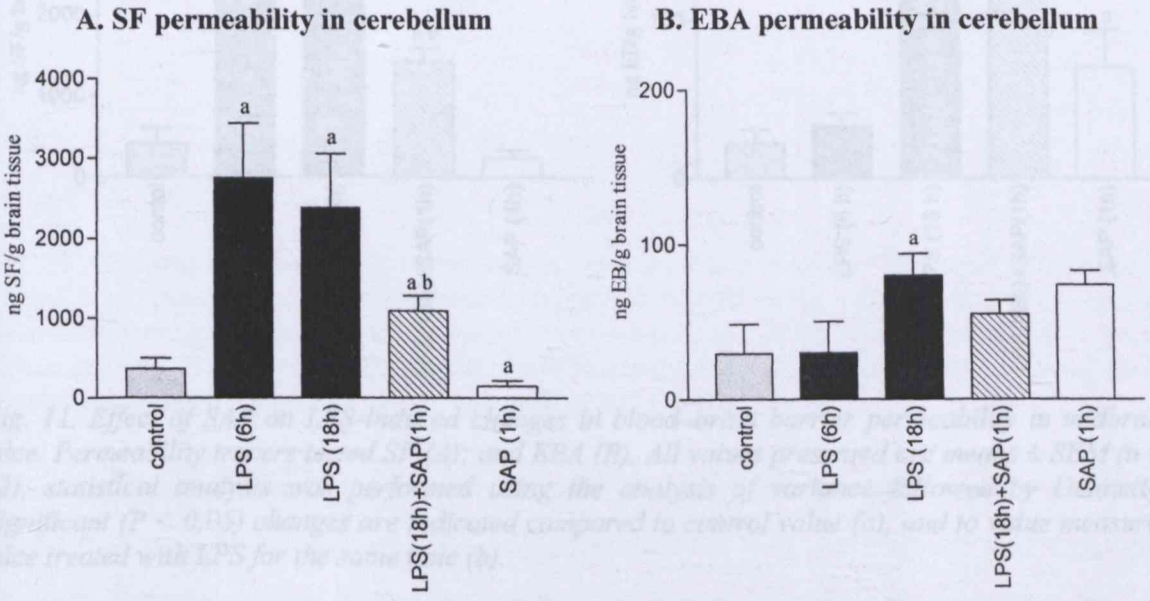

Fig. 10. Effect of SAP on LPS-induced changes in blood–brain barrier permeability in cerebellum of mice. Permeability tracers tested SF (A); and EBA (B). All values presented are means ± SEM (n = 5–12), statistical analysis was performed using the analysis of variance followed by Dunnett-test. Significant (P < 0.05) changes are indicated compared to control value (a), and to value measured in mice treated with LPS for the same time (b).

Mice after the third dose of LPS showed typical signs of sickness behaviour, they could hardly move, drink or eat, had diarrhoea, their fur was fuzzy, and their eyes became bleared (Konsman *et al.,* 2002, Neveu *et al.,* 1998). The condition of LPS-treated animals that received 8 mg/kg human SAP has been greatly improved, and they became more active after the injection, started to eat, to drink and to explore their environment.

In control mice, SAP treatment (8 mg/kg iv., for 1 h) significantly decreased basal BBB permeability in all brain regions for fluorescein (Figs. 9A, 10A, 11A). It did not change the extravasation of albumin (Figs. 9B, 10B), except in midbrain (Fig. 11B), where a higher permeability was measured. In LPS-treated mice, SAP treatment (8 mg/kg iv., for 1 or 6 h) given 17 h after the last LPS injection significantly decreased the extravasation of fluorescein in all brain regions tested (Figs. 9A, 10A, 11A) and also attenuated the increase in albumin

permeability in cerebral cortex (Fig. 9B). No significant protective effect of SAP was observed in the cerebellum, or in the midbrain (Figs. 10B, 11B).

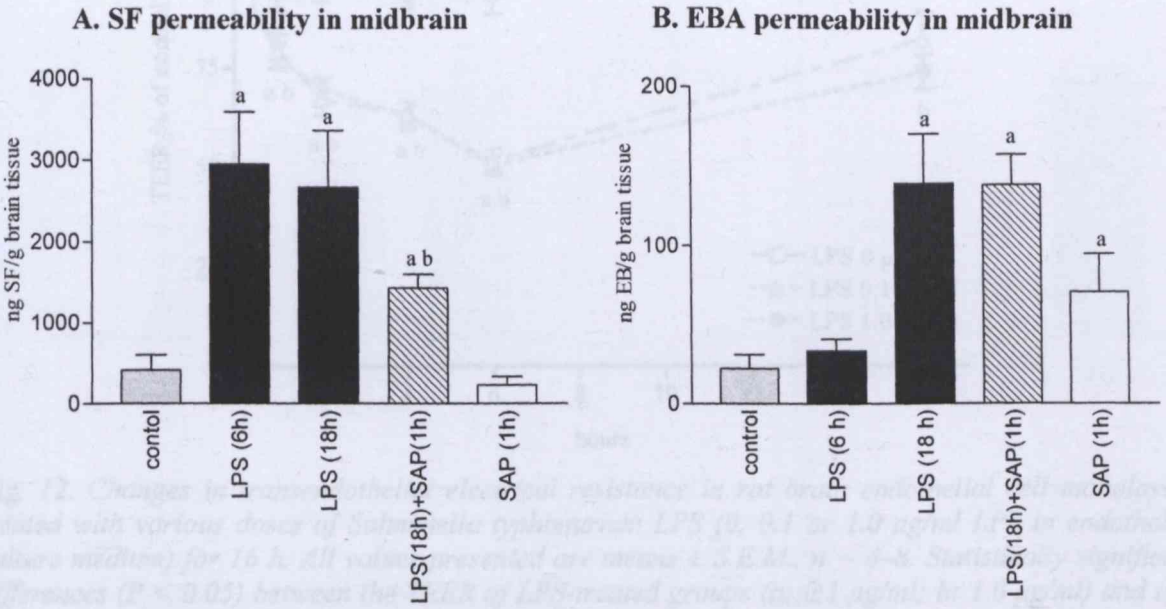

Fig. 11. Effect of SAP on LPS-induced changes in blood–brain barrier permeability in midbrain of mice. Permeability tracers tested SF (A); and EBA (B). All values presented are means ± SEM (n = 5–12), statistical analysis was performed using the analysis of variance followed by Dunnett-test. Significant (P < 0.05) changes are indicated compared to control value (a), and to value measured in mice treated with LPS for the same time (b).

3.1.2. Permeability changes after LPS treatment on an *in vitro* BBB model

The integrity of brain endothelial monolayers, measured by transendothelial electrical resistance, representing the paracellular sodium ion flux, has been affected by LPS-treatment in a dose- and time-dependent manner (Figs. 12 and 13). The TEER value dropped already in the first hour of the treatment (Figs. 12 and 13), then continuously decreased and reached a minimum at 6 h, followed by a recovery close to the level of the original TEER by 16 h (Fig. 12). The kinetics of TEER changes in monolayers treated by 0.1 or 1 μg/ml LPS showed similar pattern, although at 16 h the resistance remained significantly decreased only in monolayers exposed to the higher LPS dose. During the 16 h of experiments the TEER value of control monolayers treated with vehicle did not differ significantly from its basal value of 302.6 ± 10.9 $\Omega \times cm^2$ (n = 16) at the start of the experiment; (Fig. 12).

We found no changes between the effects of LPS from different bacteria on brain endothelial TEER. A similar decrease and kinetics was observed in experiments with LPS from *S. typhimurium* or *E. coli* 0111:B4 serotype (Figs. 12 and 13).

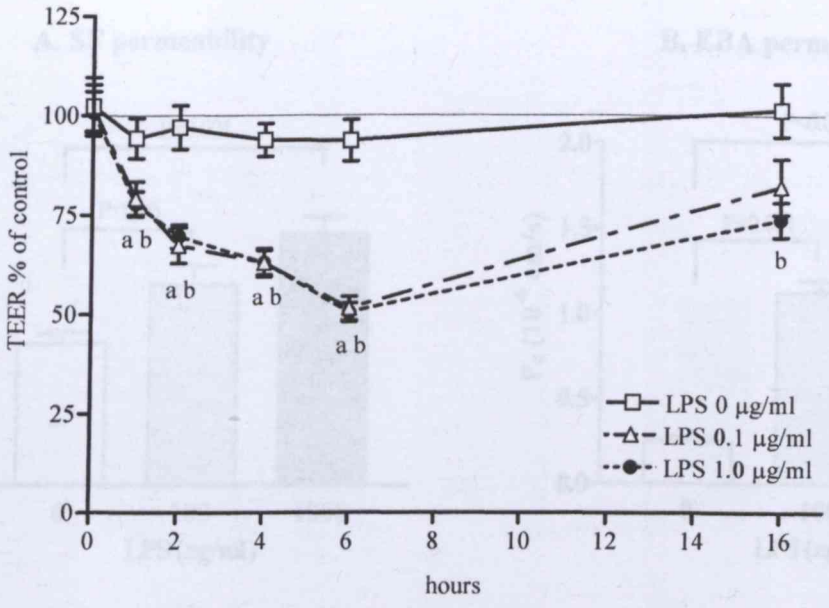

Fig. 12. Changes in transendothelial electrical resistance in rat brain endothelial cell monolayers treated with various doses of Salmonella typhimurium LPS (0, 0.1 or 1.0 µg/ml LPS in endothelial culture medium) for 16 h. All values presented are means ± S.E.M., n = 4–8. Statistically significant differences (P < 0.05) between the TEER of LPS-treated groups (a: 0.1 µg/ml; b: 1.0 µg/ml) and that of culture medium treated controls are indicated at different time-points.

After the endotoxin treatment (0.1 and 1 µg/ml LPS) the permeability of the brain endothelial monolayers was significantly increased for both paracellular and transcellular markers. The paracellular flux of fluorescein was elevated two-fold or more after the higher dose of LPS (Figs. 14A and 15A). The permeability for albumin, indicating transendothelial transport, was elevated by five- and six-fold in monolayers (Figs. 14B and 15B).

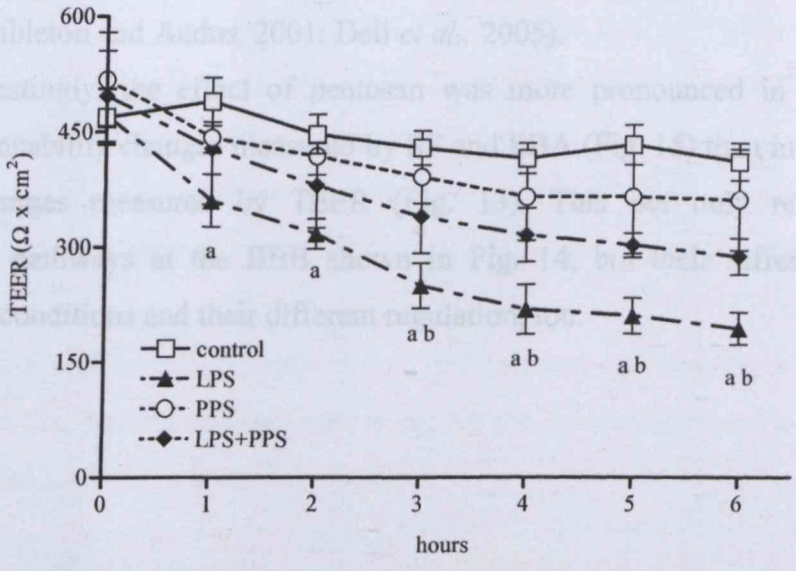

Fig. 13. Changes in TEER in rat brain endothelial cell monolayers exposed to E. coli O111:B4 LPS (0, 0.1 or 1.0 µg/ml LPS in endothelial culture medium) for 6 h. Values presented are means ± S.E.M., n = 6. Statistically significant differences (P < 0.05) between the TEER of LPS-treated groups and that of culture medium treated controls (a), and LPS + PPS treated groups (b) are indicated at different time-points.

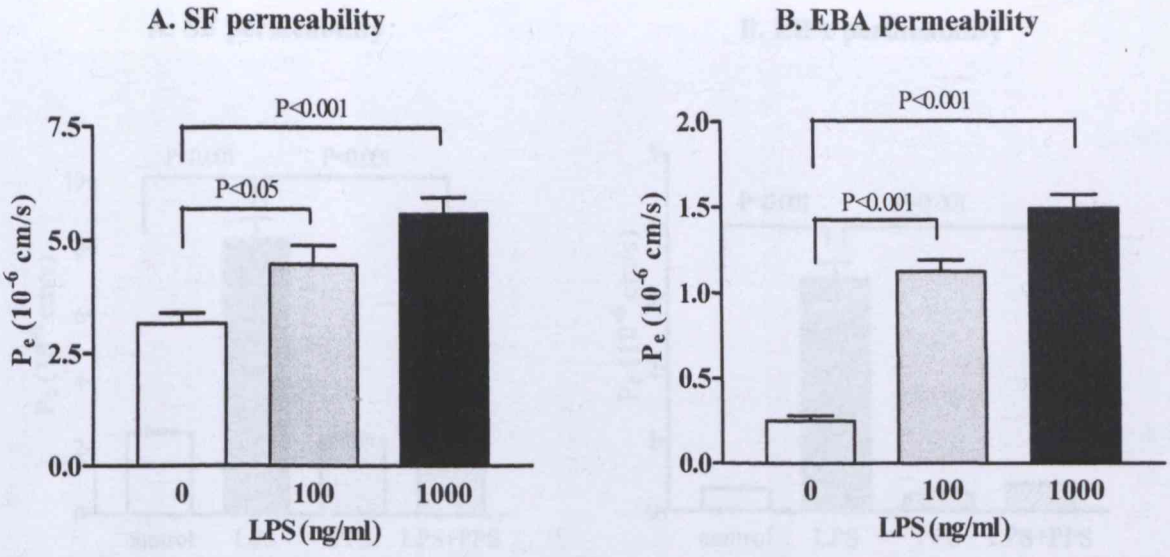

Fig. 14. Changes in transendothelial permeability (P_e) for SF (A) and EBA (B) in rat brain endothelial cell monolayers treated with various doses of Salmonella typhimurium LPS (0, 0.1 or 1.0 μg/ml LPS in endothelial culture medium) for 16 h. All values presented are means ± S.E.M., n = 4–8. Statistically significant differences (P < 0.05) between the LPS-treated groups (a: 0.1 μg/ml; b: 1.0 μg/ml) and that of culture medium treated controls are indicated at different time-points.

Pentosan (100 μg/ml) alone had no significant effect on TEER, while it could attenuate the effect of 1 μg/ml LPS (Fig. 13). Pentosan could also effectively block the increased flux of both fluorescein and albumin through brain endothelial monolayers after LPS treatment (Fig.15A and 15B). The transendothelial permeability coefficient for SF was higher with one order of magnitude than the value for EBA, in agreement with literature data review (Gumbleton and Audus, 2001; Deli *et al.*, 2005).

Interestingly, the effect of pentosan was more pronounced in reversing the LPS-induced permeability changes measured by SF and EBA (Fig. 15) than in blocking the barrier integrity changes measured by TEER (Fig. 13). This not only reflects the different permeability pathways at the BBB shown in Fig. 14, but their different participation in pathological conditions and their different regulation, too.

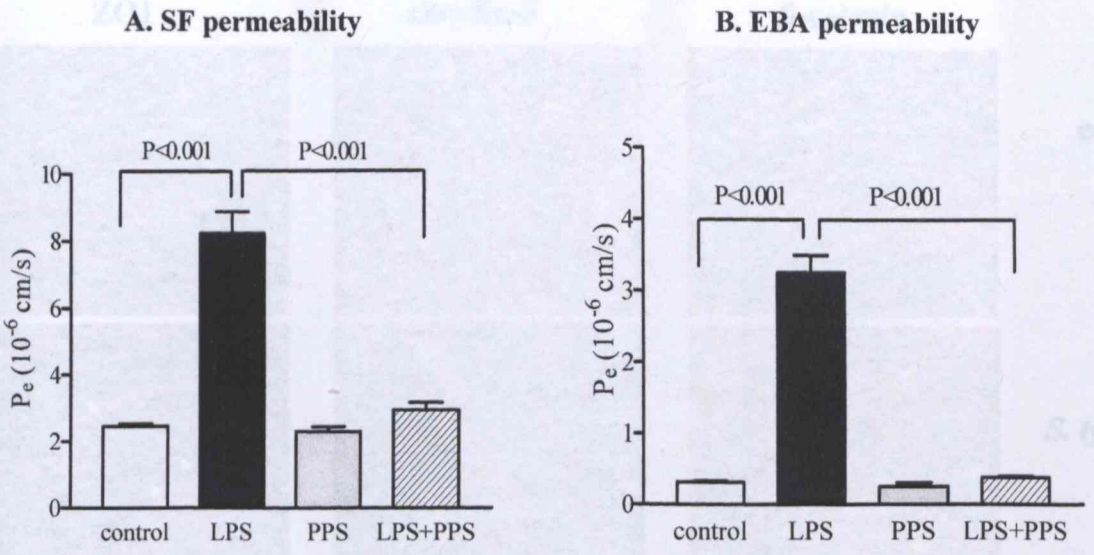

Fig. 15. Changes in transendothelial permeability (P_e) for SF (A) and EBA (B) in rat brain endothelial cell monolayers exposed to E. coli O111:B4 lipopolysaccharide (0,0.1 or 1.0 μg/ml LPS in endothelial culture medium) and/or PPS (100 μg/ml in culture medium) for 6 h. Values presented are means ± S.E.M., n = 6. Statistically significant differences (P < 0.05) between the LPS-treated groups and that of culture medium treated controls (a), and LPS + PPS treated groups (b) are indicated at different time-points.

3.1.3. Effect of LPS on immunostaining for junctional proteins in brain endothelial cells

Treatment with LPS, originated either from *S. typhimurium* or *E. coli* 0111:B4 serotype, resulted in changes in immunostaining for junctional proteins in rat brain endothelial cells examined by fluorescent microscopy (Fig. 16). The intensity of immunostaining for TJ proteins ZO-1 and claudin-5, as well as for junctional protein β-catenin became weaker in LPS-treated brain endothelial cells and the pattern of the staining has also been changed. The continuous cortical staining pattern became fragmented or has been lost in several areas and intercellular gaps appeared as indicated by arrows on Fig. 16, which are clear signs of injured barrier integrity.

Co-administration of PPS inhibited these changes, the monolayer integrity was better preserved and the immunostaining pattern for all the junctional proteins tested resembled to the control ones.

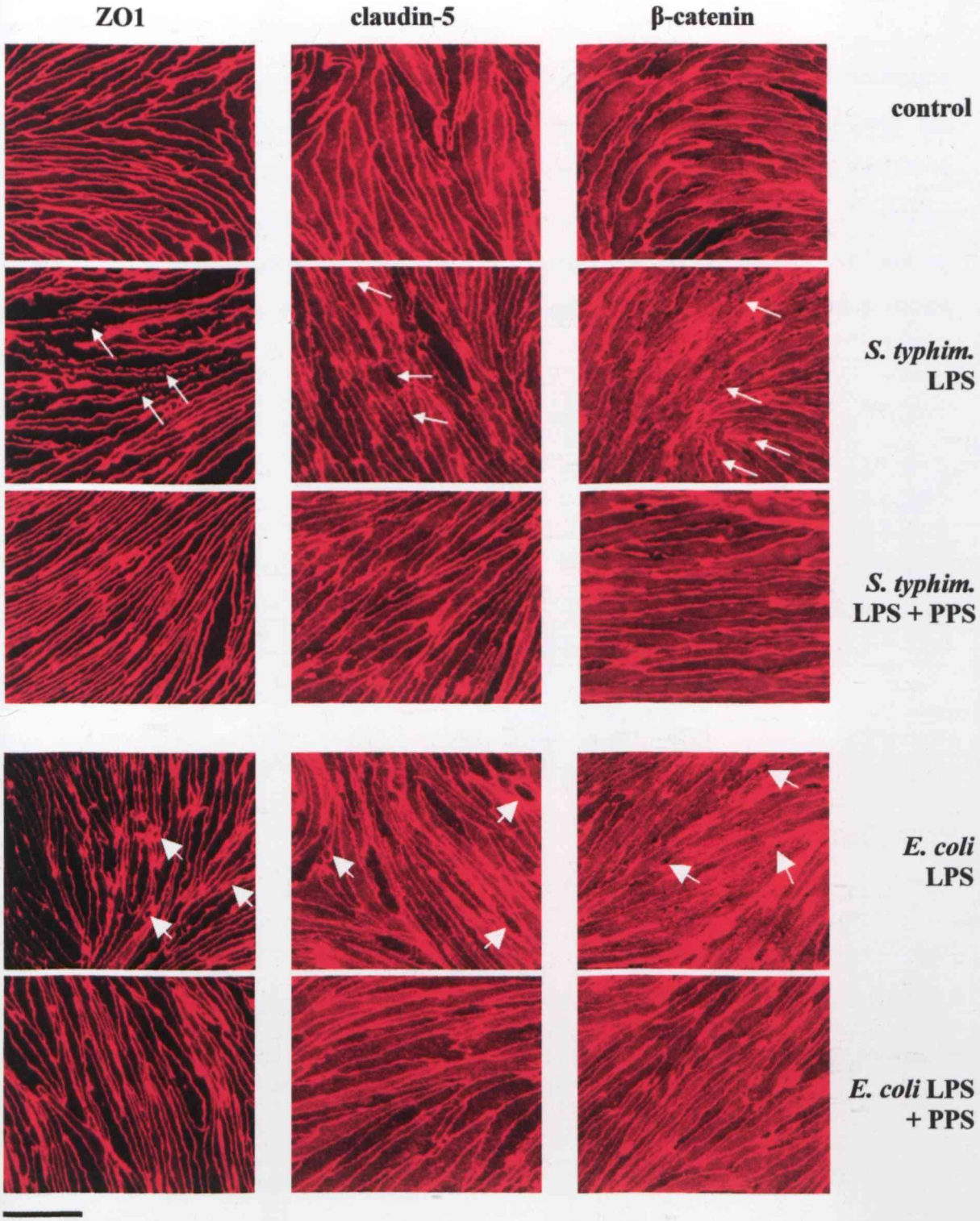

Fig. 16. Immunofluorescent staining for junctional proteins in rat brain endothelial cells treated with bacterial lipopolysaccharide (1.0 μg/ml LPS) and pentosan polysulfate (100 μg/ml PPS) for 6 h. Arrows show holes formed between endothelial cells, fragmentation or loss of junctional immunostaining (bar = 50 μm)

3.1.4. Effect of LPS on P-glycoprotein activity

P-glycoprotein efflux pump activity of brain endothelial cells, measured by rhodamine 123 accumulation, was dose-dependently decreased by LPS (Fig.17). However, only the highest dose of LPS (10 μg/ml) could significantly inhibit the pump causing a more than 50 % increase of the uptake. Pentosan could prevent this action of LPS, and the rhodamine accumulation returned to the control level. The Ca^{2+} channel blocker verapamil, a well-known inhibitor of P-glycoprotein used as a reference blocker in this assay, showed a robust inhibitory effect.

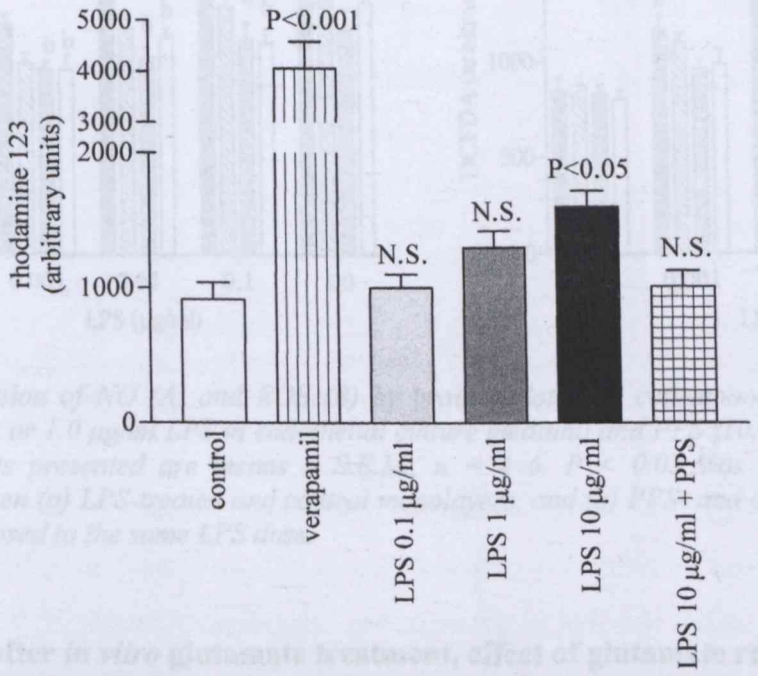

Fig. 17. P-glycoprotein activity measured by rhodamine 123 accumulation in LPS (0.1, 1.0 or 10.0 μg/ml LPS in endothelial culture medium) and PPS (30 μg/ml PPS) treated brain endothelial cells for 16 h. Values presented are means ± S.E.M., n = 5–6. Statistically significant changes in rhodamine accumulation compared to culture medium treated control are indicated (N.S. = P > 0.05).

3.1.5. Effect of LPS on oxygen free radical production in brain endothelial cells

The NO production in cultured rat brain endothelial cells was enhanced by overnight LPS treatment compared to that in control cells (Fig. 18A). The effect was dose-dependent. While no significant change was seen at 0.001 μg/ml LPS, exposure to 0.01–1 μg/ml LPS resulted in a gradual increase of NO synthesis, that could be reduced by PPS administration. Similarly, reactive oxygen species (ROS) production was also elevated by LPS (Fig. 18B). The amount of ROS, measured by CM-H_2DCFDA assay was doubled at the highest dose of

endotoxin. PPS was effective in inhibiting the LPS-induced change in ROS synthesis, the two higher doses (30 and 100 μg/ml) proved to be more potent. There was no change in the inhibitory potential of 30 μg/ml PPS for NO or ROS release by rat brain endothelial cells exposed to 0.1 μg/ml LPS when PPS was added 30 min before, concomitantly with, or 30 min after LPS administration at the beginning of the 16 h incubation period (data not shown).

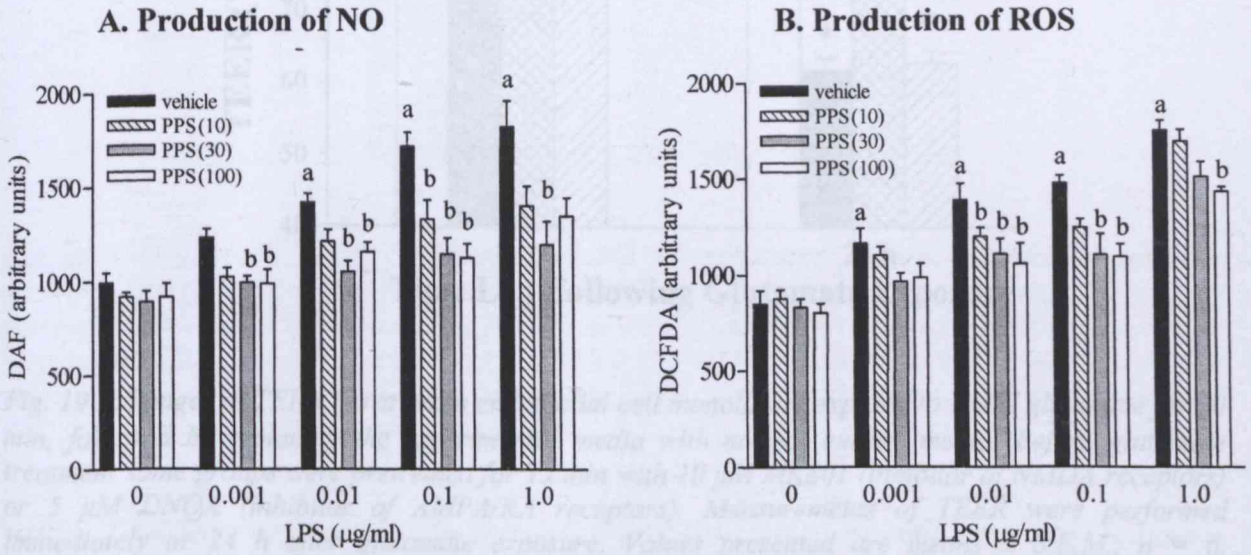

Fig. 18. Production of NO (A) and ROS (B) by brain endothelial cell monolayers treated by LPS (0.001, 0.01, 0.1 or 1.0 μg/ml LPS in endothelial culture medium) and PPS (10, 30 or 100 μg/ml PPS) for 16 h. Values presented are means ± S.E.M., n = 4–6. P < 0.05 was considered significant difference between (a) LPS-treated and control monolayers, and (b) PPS- and culture medium-treated monolayers exposed to the same LPS dose.

3.2. Changes after *in vitro* glutamate treatment, effect of glutamate receptor antagonists

The effects of glutamate and the NMDA and AMPA/KA receptor inhibitors dizocilpine (MK-801) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were examined on the barrier functions of brain endothelial cells using our co-culture model. The baseline TEER values of brain endothelial monolayers after co-culture with astrocytes varied between 182 and 268 $\Omega \times cm^2$, and decreased as a result of a 24 h maintenance in serum-free environment. However, independent of culture conditions, treatment with glutamate significantly reduced TEER values, indicating disruption of brain endothelial barrier integrity (Fig. 19.). Most importantly, inhibition of the NMDA receptors by MK-801 fully protected against these effects. In contrast, pretreatment with DNQX did not affect significantly glutamate induced alterations of TEER.

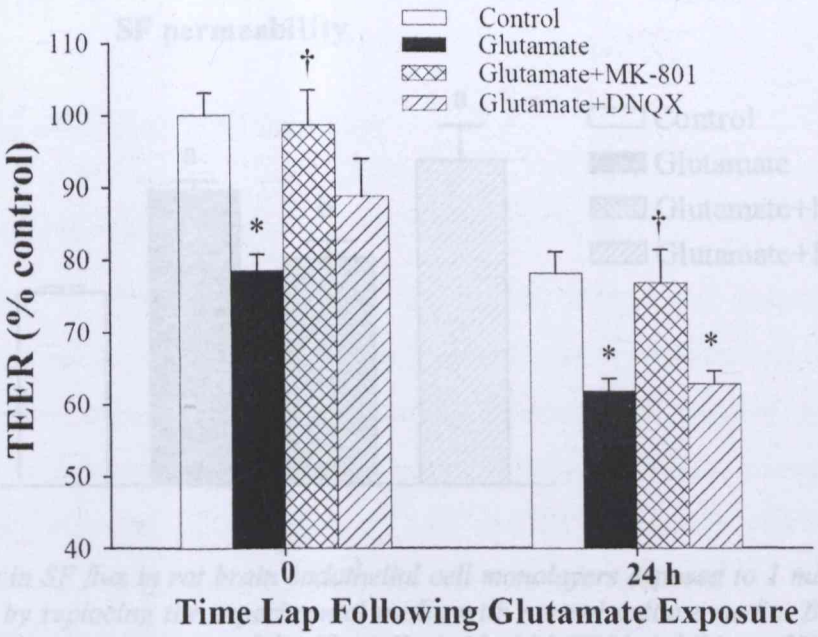

*Fig. 19. Changes in TEER in rat brain endothelial cell monolayers exposed to 1 mM glutamate for 30 min, followed by replacing the experimental media with normal culture media. Before glutamate treatment some groups were pretreated for 15 min with 10 μM MK801 (inhibitor of NMDA receptors) or 5 μM DNQX (inhibitor of AMPA/KA receptors). Measurements of TEER were performed immediately or 24 h after glutamate exposure. Values presented are means ± S.E.M., n = 6. *Statistically significant as compared with control. †Values in the glutamate plus MK-801 group are statistically different from those in the glutamate group.*

Exposure to glutamate markedly increased brain endothelial permeability as determined by SF flux (Fig. 20.). The transendothelial permeability coefficient was elevated by more than 50 %. Most interestingly, preexposure to MK-801 could significantly protect against the effect of glutamate, while DNQX did not show a protective effect, which suggest the participation of NMDA receptors in the process.

These functional data were supported by the results of further experiments on TJ protein occludin (Publication III, see in Appendix). Transient exposure to glutamate resulted in cellular redistribution of occludin, followed by a decrease in the total level of this protein in brain endothelial cells. Inhibition of the NMDA or AMPA/KA receptors attenuated the glutamate-induced changes in occludin redistribution but not in the total protein levels.

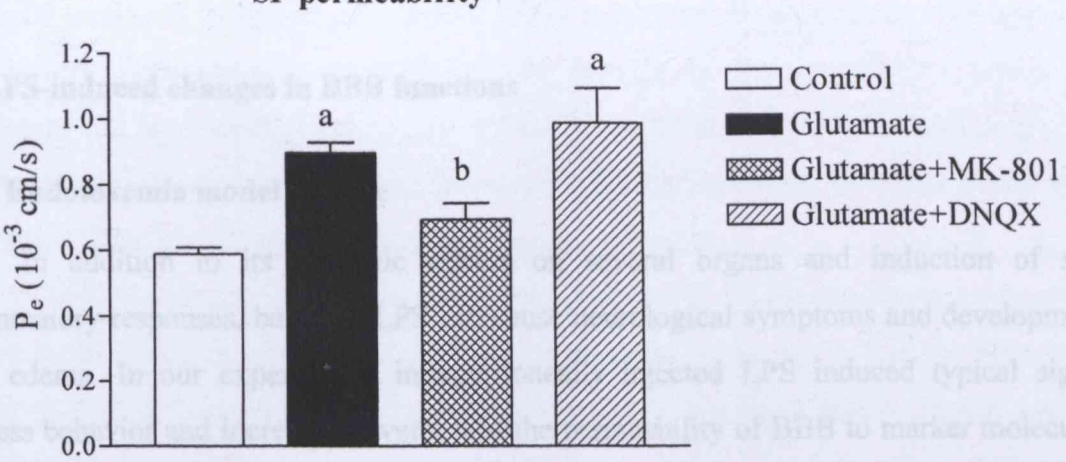

Fig. 20. Changes in SF flux in rat brain endothelial cell monolayers exposed to 1 mM glutamate for 30 min, followed by replacing the experimental media with normal culture media. Before glutamate treatment some groups were pretreated for 15 min with 10 μM MK801 (inhibitor of NMDA receptors) or 5 μM DNQX (inhibitor of AMPA/KA receptors).SF flux was determined 24 h after the transient glutamate exposure. Values presented are means ± S.E.M., n = 6. Statistically significant as compared with control (a) or with glutamate group (b).

Treatment with glutamate also increased tyrosine phosphorylation and decreased threonine phosphorylation of occludin. Inhibition of the NMDA receptors by MK-801 partially protected against glutamate-induced elevation of occludin tyrosine phosphorylation, while blocking of the AMPA/KA receptors by DNQX protected against hypophosphorylation of threonine residues of occludin.

4. DISCUSSION

4.1. LPS-induced changes in BBB functions

4.1.1. Endotoxemia model in mice

In addition to its dramatic effects on several organs and induction of severe inflammatory responses, bacterial LPS can cause neurological symptoms and development of brain edema. In our experiments intraperitoneally injected LPS induced typical signs of sickness behavior and increased several fold the permeability of BBB to marker molecules in mice. The greatly elevated extravasation of both sodium fluorescein and albumin to the cortex, midbrain and cerebellum indicates the involvement of both the paracellular and transcellular transport pathways (Fig. 2.). These findings on LPS-induced BBB permeability changes are in accordance with previous data obtained on animal studies (Temesvári *et al.*, 1993; Xaio *et al.*, 2001). BBB opening for sodium fluorescein in pial microvessels was seen after intracisternal injection of LPS in newborn pigs (Temesvári *et al.*, 1993), whereas Xaio *et al.*, (2001) found an about 50% increase in BBB permeability for albumin in cerebral cortex of LPS-treated CD-1 mice. A recent study demonstrated that abrogation of the BBB by treatment with LPS made hippocampal neurons vulnerable to neurotoxic antibodies and caused cell damage and memory impairment (Huerta *et al.*, 2006).

4.1.2. LPS-induced changes in brain endothelial cells

In vitro application of the bacterial LPS could directly damage the morphological and functional integrity of the BBB. LPS treatment resulted in a dose and time-dependent injury of brain endothelial cell monolayers.

TEER values indicate the permeability of the monolayer to ions and correspond to the function of interendothelial TJs. In our *in vitro* experiments LPS treatment caused a significant decrease in TEER (i.e. increased paracellular ion permeability) in the first 6 h, with a tendency to recovery in the following 10 h. A concomitant LPS-induced increase in the permeability of brain endothelial cell monolayers for sodium fluorescein, a small molecular weight tracer, confirm the LPS-induced induction of TJ damage. The 6–10-fold increase in the permeability to the macromolecule albumin, as compared to the 2- to 3-fold increase in small molecule fluorescein flux may indicate that beside increased passive paracellular

permeability transendothelial transport pathways could also be activated (Abbott *et al.*, 2006) after LPS treatment in our model system. These results are in accordance with previous *in vitro* studies demonstrating the LPS-induced damage to the integrity of bovine brain endothelial cell monolayers (Vries *et al.*, 1996; Descamps *et al.*, 2003; Gaillard *et al.*, 2003), and *in vivo* mouse studies indicating increased BBB permeability for albumin and sodium fluorescein (Xaio *et al.*, 2001; Publication I, see in Appendix).

The LPS-induced increase in paracellular permeability of monolayers is supported by the results of immunostaining. This is the first study to demonstrate that the intensity and pattern of immunohistochemistry for TJ proteins ZO-1, claudin-5 and β-catenin have been considerably changed in LPS-treated cultured brain endothelial cells. The appearance of intercellular gaps indicates the damaged integrity of monolayers. In a recent study, robust changes in F-actin pattern and appearance of stress fibers were demonstrated in bovine brain endothelial cells after incubation with LPS (Descamps *et al.*, 2003). These endotoxin-induced morphological changes both in TJ-specific and structural proteins may contribute to the increased endothelial permeability found in our *in vitro* BBB model.

Beside changes in barrier function, LPS incubation inhibited the activity of P-glycoprotein, an important efflux pump at the BBB. P-glycoprotein and other efflux pumps limit the flux of drugs and xenobiotics to brain (Pardridge, 2002). Our new *in vitro* observation on cultured brain endothelial cells suggest an increased entry of P-glycoprotein ligands into the brain during endotoxemia. The relevance of our data is confirmed by a recent study in which LPS reduced P-glycoprotein activity in freshly isolated rat brain microvessels (Hartz *et al.*, 2006). Our observation is further supported by findings on male rats injected by LPS to the cerebrospinal fluid (Goralski *et al.*, 2003) CNS inflammation induced by LPS resulted in downregulation of mdr1a mRNA expression in both brain and liver with maximal loss at 6 h, and increased the brain penetration of digoxin, a ligand of P-glycoprotein (Goralski *et al.*, 2003). Endotoxin-induced reduction in the intestinal expression and activity of P-glycoprotein, multidrug resistance-associated protein 2, and cytochrome P450 3A (Kalitsky-Szirtes *et al.*, 2004) may suggest that other efflux transporters and drug metabolizing systems located at the BBB might be affected by LPS, too.

4.1.3. Possible mechanism of LPS-induced changes in brain endothelial cells

NO is an important vasoactive mediator of brain endothelial cells participating in regulation of blood pressure and BBB permeability (Mayhan, 2001; Christov *et al.*, 2004;

Deli *et al.*, 2005). While the basal endothelial release of NO is an important physiological protective mechanism, overproduction of NO by inducible NO synthase (iNOS) can be harmful. LPS is one of the strongest inducer of iNOS. ROS, like superoxide, peroxides and hydroperoxides are formed in different physiological and pathological events, including sepsis. Peroxynitrite anion, a complex formed by NO and superoxides is a cytotoxic substance produced and released in sepsis, inflammation and ischemia (Karima *et al.*, 1999). Oxidative damage of the BBB impairs the energy supply of endothelial cells thus inhibiting the energy-dependent nutrient transport systems, like amino acid transport system A (Cardelli *et al.*, 2002). In a model of neurodegeneration induced by impairment of oxidative metabolism (Ke and Gibson, 2004; Kruse *et al.*, 2004) brain endothelial cells are the major site of NO production leading to neuronal cell loss and aggravation of neurodegeneration.

In LPS- treated cultured rat brain endothelial cells both ROS and NO productions were increased in a dose-dependent way. Our finding is in agreement with the results of Christov *et al.*, (2004), who also found that treatment of cultured brain endothelial cells with LPS and other inflammatory proteins result in a significant increase in intracellular levels of ROS. LPS exposure also increased NO and superoxide anion levels in isolated rat cerebral arteries (Hernanz *et al.*, 2004). The LPS induced concentration and time-dependent decrease in TEER in bovine brain endothelial cells could be reduced by free radical inhibitors, suggesting that ROS can influence brain endothelial junctions (Gaillard *et al.*, 2003). These data taken together with our observations further indicate a role for excessive free radical production in LPS-induced pathological changes in brain endothelial cells.

4.2. Protection against the LPS-induced BBB changes

Endotoxemia and sepsis are pathological conditions with high mortality rates despite the best available treatments. New, effective inhibitors of LPS, would be important for the clinical practice. In our experiments SAP and pentosan treatment could reduce the deleterious effects of LPS on BBB permeability and efflux pump activity.

4.2.1. Serum amyloid P component

The effect of human SAP was studied both on basal and LPS-stimulated BBB permeability. Interestingly, SAP injected to mice could decrease basal BBB permeability to sodium fluorescein. It should be also considered that SAP, or complexes formed by SAP and

blood constituents, can either interact with the luminal surface of brain endothelial cells (*e.g.* cell surface glycosaminoglycans) thereby reducing fluorescein permeability or can bind circulating fluorescein.

Our data are the first to demonstrate that intravenously injected human SAP can attenuate increased BBB permeability and diminish clinical signs of endotoxemia in LPS-treated mice. Although previous data indicated only marginal, if any, beneficial effects of SAP in LPS toxicity (Noursadeghi *et al.,* 2000), human SAP could diminish clinical signs of endotoxemia in the present experiments. The effect is probably mediated through binding circulating LPS in serum (Haas, 1999), but additional effects on complement cascade (Noursadeghi *et al.,* 2000), or on cerebral endothelium should be also considered. However, human SAP (100 mg/ kg) given *ip.* shortly before the challenge with a lethal dose of LPS (10 mg/kg) did not confer any protection against mortality (Noursadeghi *et al.,* 2000). Differences in the way of SAP administration, in LPS doses used, and in the endpoints of the experiments may explain the discrepancy between our present results and those published by Noursadeghi *et al.,* (2000). In the present experiment, we have used *iv.* SAP treatment for mice challenged with non-lethal LPS doses, and our main endpoint was assessment of BBB permeability, a specific physiological function.

4.2.2. Pentosan polysulfate

In the present study PPS treatment attenuated both the permeability barrier impairment and the P-glycoprotein inhibition in LPS-exposed brain endothelial cells. It was beneficial in reducing the LPS-induced TJ changes, revealed by TEER and permeability measurements and TJ protein immunostaining, and the free radical production.

The mode of action of PPS in brain endothelial cells has not been studied yet. We hypothesize, that PPS can interfere with LPS-induced changes in cerebral endothelium at several levels. PPS may exert a direct effect through blocking the interaction of LPS with Toll like receptors, inhibiting LPS-induced signaling or effector molecules of LPS-action, like protein kinase C (Shen *et al.,* 2005) or NO (Syed *et al.,* 2006) in endothelial cells. An indirect action of PPS can happen through its interaction with the luminal surface of brain endothelial cells, thus protecting the negative luminal charge contributing to the permeability barrier. Our observation, that PPS given 30 min after LPS treatment could also prevent endotoxin-induced increase in NO and ROS production indicates that the effect of this polyanionic compound is not (or not exclusively) related to its mechanical interaction with LPS-binding sites. Further

experiments are needed to prove which of the mentioned possibilities participate in the protective effect of pentosan in brain endothelial cells. Despite the unknown mode of action of PPS on the BBB, the importance of our finding is emphasized by the fact that PPS is a clinically used drug. Our new observation may suggest a potential therapeutical application of PPS in sepsis caused by Gram-negative bacteria.

4.3. Glutamate-induced changes in BBB permeability

Ischemic stroke is the second leading cause of death worldwide and the main disabilitating disease in the United States (Rymer and Thrutchley, 2005). One of the major complications of stroke is cytotoxic and/or vasogenic brain edema. Cytotoxic edema is caused by the loss of membrane ionic pumps and by cell swelling owing to cerebral ischemia. In addition, BBB leakage appears to be responsible for vasogenic edema (Joó and Klatzo, 1989; Rosenberg, 1999).

In the present study, a 30 min exposure to glutamate resulted in a significant decrease in TEER values and increase in fluorescein permeabilty in cultured rat brain endothelial cells indicating a rapid effect of the excitatory neurotransmitter on the barrier integrity of monolayers. The effect on paracellular permeability measured by TEER and SF flux was even more pronounced at 24 h.

The time course of these alterations is supported by the observation that cytotoxic and vasogenic edema can reach maximum intensity 24 to 72 h after the ischemic event (Rosenberg, 1999). Global cerebral ischemia resulted in a marked BBB permeability increase 24 h later (Preston and Webster, 2004), and the glutamate analogue kainate-induced BBB breakdown with plasma leakage into brain tissue could be also observed 24 h after the intrahippocampal injection (Chen *et al.*, 1999).

The underlying mechanism can be linked to alterations of junctional protein expression induced by high levels of extracellular glutamate. Changes in tight junction proteins were shown in hypoxia/reoxygenation modeling of transient ischemia using an in vitro BBB model. Specifically, hypoxia resulted in redistribution of occludin, claudin-1, ZO-1, and ZO-2 proteins (Brown *et al.*, 2003; Mark and Davis, 2002).

In agreement with these findings, a transient exposure to glutamate resulted in progressive changes in occludin immunoreactivity in brain endothelial cells (Publication III, Appendix). At 30 min occludin redistribution, at 24 h decrease or loss of this junctional protein was observed at cell–cell border segments. Such alterations can markedly affect the

BBB function. Redistribution of tight junction particles in the internal/external membrane leaflets can lead to a disturbed fence function, with the polarity change of the glucose transporter GLUT-1 (Lippoldt *et al.*, 2000).

Glutamate-induced alterations of occludin immunoreactivity were associated with significant disturbances in phosphorylation patterns. Increased phosphorylation of tyrosine residues and decreased phosphorylation of threonine residues were observed (Publication III). Alterations of phosphorylation status of junctional proteins can contribute to decreased barrier function of the BBB. A correlation was shown between tyrosine phosphorylation of occludin and the disruption of tight junctions (Basuroy *et al.*, 2006).

4.4. Protection against the glutamate-induced BBB changes

To distinguish the effects of individual ionotropic glutamate receptors on brain endothelial permeability and to test potential therapeutical molecules, the monolayers were exposed to glutamate in the presence of MK-801, a pharmacological inhibitor of NMDA receptors, or DNQX, a blocker of AMPA/KA receptors.

NMDA receptor antagonist MK801 could successfully inhibit the glutamate-induced decrease in TEER and increase in permeability for SF. DNQX did not have a protective effect on TEER changes at 30 min, and was not able to block the elevated SF flux after glutamate treatment. These results suggest that the effect of glutamate on paracellular permeability in our BBB model is primarily NMDA receptor-mediated.

The functional results are in agreement with the data of the experiments on occludin changes. Blockage of the NMDA receptors, which selectively protected against glutamate-induced disruption of brain endothelial monolayer integrity also effectively inhibited glutamate-induced tyrosine phosphorylation of occludin (Publication III). These results are supported by earlier observations on the involvement of the NMDA receptors in the regulation of the BBB functions and endothelial permeability (Sharp *et al.*, 2003).

Memantine, a clinically used NMDA receptor antagonist, restored BBB integrity and reduced symptoms in experimental allergic encephalomyelitis, a model of multiple sclerosis (Paul and Bolton, 2002). It also could attenuate brain edema formation and increased BBB permeability after cerebral ischemia and reperfusion in rats (Gorgulu *et al.*, 2000). These data and our results suggest that inhibition of NMDA receptors at the BBB might be therapeutically effective in preventing increased permeability in neuropathological conditions.

5. SUMMARY

Clinical and research data support the involvement of BBB damage as an early event in many neurological conditions. The role of the BBB to protect the brain in physiological and pathological conditions is increasigly emphasized in the literature. Protective strategies at the level of BBB are in the focus of new studies. We have selected LPS and glutamate, two major pathological factors in human diseases causing BBB disturbances and brain edema to study potential protective molecules on *in vitro* and *in vivo* BBB models.

LPS treatment resulted in damage to the integrity and functions of BBB on two different models. Intraperitoneally injected LPS induced typical signs of sickness behavior and increased several fold the permeability of BBB to marker molecules in CBA/BL6 mice. Human serum amyloid P component decreased the LPS induced elevation in BBB permeability and diminished clinical signs of endotoxemia *in vivo*.

In accordance with the results of our *in vivo* study, LPS treatment damaged the integrity of monolayers, reduced transendothelial electrical resistance and increased permeability in cultured brain endothelial cells. The LPS-induced injury to tight junction structural organization corresponded to the permeability changes observed. LPS also inhibited the activity of the efflux pump P-glycoprotein. The LPS-induced increase in reactive oxygen species and nitric oxide production can participate in the observed changes in BBB functions. Pentosan could reduce the deleterious effects of LPS and this new observation may suggest a potential therapeutical application of PPS in sepsis caused by Gram-negative bacteria.

A transient exposure of brain endothelial cells to extracellular glutamate resulted in an increased paracellular permeability. The functional changes were accompanied by redistribution and decreased expression of occludin, an important tight junction protein. Treatment with glutamate also induced excessive phosphorylation of the tyrosine residues of occludin. Glutamate-induced increase in brain endothelial monolayer permeability and hyperphosphorylation of occludin could be blocked by the NMDA receptor blocker MK-801.

Recently the BBB has been considered as a therapeutic target in those diseases, where neuronal damage is secondary to, or exacerbated by BBB dysfunction. Our findings may contribute to the development of new strategies for the prevention of BBB damage or protection of BBB functions in diseases.

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