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

Ph.D. thesis

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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. 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), can be >1000 Ω 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. Cerebral endothelial cells regulate blood coagulation and vasoreactivity. 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, amino acids, nucleosides, *etc.* to feed neural cells. Cerebral endothelium protects the nervous system from xenobiotics by efflux transporters like P-glycoprotein, multidrug resistance proteins MRP-1, -4, 5-, and -6.

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. 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. Pharmacoresistance due to upregulation of efflux pumps at the neurovascular unit in epilepsy and CNS tumors makes clinical treatment very difficult. In addition, permeability of the brain endothelial cells for plasma constituents increases leading to the development of brain edema in the above mentioned conditions. Disturbances of CNS homeostasis as a result of barrier deficiences could contribute to secondary neuronal loss and exacerbate the later neuropathology.

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. 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. The Gram-negative bacterial infections are responsible for about 60% of sepsis cases. 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. Brain endothelial cells express LPS receptors (TLR-4, TLR-2) and mediate the effects of peripheral LPS in the CNS. Studies on *in vitro* reconstituted models of the BBB provided important information regarding the role of LPS. Data from literature indicate, that LPS increases BBB permeability in animal models, but the details of this effect has not been fully understood. Mortality rate of sepsis due to bacterial infections is high despite the use of better antibiotics and significant development in intensive care 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 involve multiple interdependent molecular pathways. It has been suggested that these pathways are triggered following elevations in extracellular excitory amino acids, primarily glutamate. Ionotropic glutamate receptors (NMDA, AMPA/KA) appear to play a critical role in the pathology of cerebral infarction. The high levels of extracellular glutamate can overstimulate the glutamate receptors, leading to an upregulated increase in intracellular calcium and neuronal death; disruption of the BBB and development of vasogenic edema. The mechanisms of glutamate-induced disruption of the BBB integrity are not clarified yet. 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. 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. Beside current therapeutical and experimental neuroprotective strategies, prevention of BBB damage or protection of BBB functions could also be clinically valuable.

1.3.1. Serum amyloid P component

Serum amyloid P component (SAP) is a 235 kDa plasma glycoprotein and belongs to pentraxins. SAP is a normal circulating serum protein in humans. SAP ligands include glycosaminoglycans, DNA in chromatin, all types of amyloid deposits, LPS and Gram-negative bacteria. SAP concentration in serum doubles during sepsis in humans. The physiological role of SAP through chromatin and LPS binding comprises protection against chromatin-induced autoimmunity and modulation of host defense during bacterial infections. It is debated if SAP can protect from LPS toxicity *in vivo*.

1.3.2. Pentosan polysulfate

Pentosan polysulfate (PPS) is a semisynthetic polysaccharide, structurally related to the glycosaminoglycans. Pentosan is used in clinical practice as an anticoagulant and as a treatment for interstitial cystitis. Results from the research group indicate that PPS can favorably regulate BBB phenotype in brain endothelial cells.

1.3.3. Glutamate antagonists

Glutamate receptor agonists and antagonists can alter barrier integrity and pharmacological studies may facilitate development of drugs effective in the prevention of postischemic brain edema. *In vivo* studies confirm the protective role of glutamate antagonists in postischemic conditions. The effect of glutamate antagonists has not been investigated on a co-cultured based rat BBB model previously.

1.4. Aims

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. METHODS

2.1. Reagents and animals

All reagents used in the study were purchased from Sigma-Aldrich Ltd., Budapest, Hungary, unless otherwise indicated. Male CBA/BL6 mice and Wistar rats were obtained from the animal facility of the Biological Research Center, Szeged, and kept under standard conditions. 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).

2.2. Cell culture

Brain capillary fragments were isolated from the forebrains of 2-week-old Wistar rats and seeded on cell culture inserts coated with collagen type IV and fibronectin. For immunofluorescent staining brain endothelial cells were cultured on glass coverslips. Brain microvascular endothelial cells migrated from isolated microvessels. 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.

2.3. *In vivo* LPS and SAP treatment

Mice (CBA/BL6) 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 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, 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.

2.4. *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. The doses of PPS (Cartrophen, Biopharm Australia Pty Ltd.) varied between 1 and 100 μg/ml.

2.5. *In vitro* glutamate treatment

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. 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.

2.6. *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. Mice were given a solution of both dyes (2%, 5 ml/kg) in an *iv*. injection to the tail vein for 1 h. At the end of the experiments the animals were perfused. Samples from four brain regions, right and left cerebral cortex, midbrain and cerebellum were homogenized in trichloroacetic acid and centrifuged. Dye concentrations were measured in supernatants, the absorbency of EBA at 620 nm, while the emission of SF at 525 nm after excitation at 440 nm. BBB permeability was expressed as ng tracer/g brain tissue.

2.7. *In vitro* measurement of brain 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, USA) using STX-2 electrodes, and it was expressed relative to the surface area of endothelial monolayer ($\Omega \times cm^2$). The concentrations of the marker molecules (SF, EBA) in samples from the upper and lower compartments of the cell culture inserts were determined and the transendothelial permeability coefficient value (P_e) was calculated, as described earlier.

2.8. Immunostaining

Brain endothelial cell monolayers 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 and was examined by a fluorescent microscopy.

2.9. Functional assay for P-glycoprotein activity

Activity of P-glycoprotein was determined by the measurement of cellular accumulation of rhodamine 123. Endothelial monolayers pretreated with LPS and/or PPS for 16 h were washed, and incubated with 10 μM rhodamine 123 for 1 h at 37 ˚C. The solution was quickly removed, endothelial cells were washed and rhodamine 123 content was determined by a fluorescent plate reader (excitation at 485 nm, emission at 538 nm). Verapamil (2 μM, 30 min preincubation) was used as a reference P-glycoprotein inhibitor.

2.10. Detection of reactive oxygen species and nitric oxide

Two probes were used for fluorometric detection of free radicals, both from Molecular Probes (USA): 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. 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. The endothelial cell layers were pretreated with LPS and/or PPS for 16 h, then washed, and incubated 1 μM DAF-FM diacetate or 1 μM CM-H_2DCFDA for 1 h at 37 ˚C. The plates were measured by a fluorescent plate reader (excitation at 485 nm, emission at 538 nm).

2.11. 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. 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. The LPS-induced elevation in fluorescein permeability was even higher in cerebellum (5-fold) and in midbrain (7-fold) than that in cortex. 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, similarly to the pattern of fluorescein data. 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. In LPS-treated mice, SAP treatment significantly decreased the extravasation of fluorescein in all brain regions tested, also attenuated the increase in albumin permeability in cerebral cortex and diminished clinical signs of endotoxemia.

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

The integrity of brain endothelial monolayers, measured by TEER has been affected by LPS-treatment in a dose- and time-dependent manner. The TEER value of controls

dropped already in the first hour of vehicle treatment, 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. 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.

After the endotoxin treatment the permeability of the monolayers to markers was also significantly increased. The paracellular flux of fluorescein was elevated two-fold or more after the higher dose of LPS. The permeability for albumin, indicating transendothelial transport, was elevated by five- and six-fold in monolayers. Pentosan could effectively block the increased flux of both markers after LPS treatment.

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

Treatment with LPS resulted in changes in immunostaining for junctional proteins in rat brain endothelial cells examined by fluorescent microscopy. 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, 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.

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. 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.

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. 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. The amount of ROS 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.

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

Treatment with glutamate significantly reduced TEER values, indicating disruption of brain endothelial barrier integrity. Most importantly, inhibition of the NMDA receptors by MK-801 fully protected against these effects. In contrast, pretreatment with DNQX (inhibitor of the AMPA/KA receptors) did not affect significantly glutamate induced alterations of TEER. Exposure to glutamate markedly increased brain endothelial permeability as determined by SF flux. The transendothelial permeability coefficient was elevated by more than 50 %. While, preexposure to MK-801 could significantly protect against the effect of glutamate, DNQX did not show a protective effect, which suggest the participation of NMDA receptors in the process.

4. SUMMARY

The major findings of our experiments:

(1) Intraperitoneally injected LPS induced typical signs of sickness behavior and increased several fold the permeability of BBB to marker molecules in mice. Human serum amyloid P component could decrease the LPS-induced elevation in BBB permeability and diminish clinical signs of endotoxemia *in vivo*.

(2) In accordance with the results of the *in vivo* study, *in vitro* LPS treatment damaged the integrity of brain endothelial monolayers, reduced transendothelial electrical resistance, increased permeability for marker molecules. Concomitantly, tight junction structural organization was injured, the activity of the efflux pump P-glycoprotein was inhibited. 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.

(3) A transient exposure of brain endothelial cells to extracellular glutamate resulted in an increased paracellular permeability. Glutamate-induced increase in brain endothelial monolayer permeability could be blocked by the NMDA receptor blocker MK-801.

Conclusion:

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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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: Human serum amyloid P component attenuates the bacterial lipopolysaccharide-induced increase in blood-brain barrier permeability in mice. *Neuroscience Letters,* 352: 57-60, 2003. IF: 2.019

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III. András IE. Deli MA, Veszelka S, Hayashi K, Henning B, Toborek M: 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, 2007. IF: 4.786