SUMMARY OF PH.D. THESIS

MOLECULAR MECHANISMS OF DYSFUNCTION CAUSED BY TOLL-LIKE RECEPTORS AND TOBACCO SMOKE COMPONENTS IN CEREBRAL ENDOTHELIAL CELLS

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INTRODUCTION AND AIMS

The blood-brain barrier (BBB) plays a key role in the maintenance of the homeostasis of the central nervous system (CNS). The morphological basis of the BBB is formed by cerebral endothelial cells (CECs) which come in contact with pericytes and astrocytes and restrict free exchanges between the blood and the neural tissue. CECs form a single cell layer lining the blood vessels, and are sealed with a continuous belt of tight junctions. The permeability of the endothelial barrier is largely determined by the integrity of the tight junctions and adherens junctions between endothelial cells.

It is well established that under pathological conditions (e.g., inflammatory disorders, cerebral ischemia and subsequent reperfusion, neurodegenerative disorders, brain tumors, trauma) an increase in BBB permeability may occur, which may lead to a disturbed homeostasis of the CNS with severe consequences. Moreover, infections are often associated with systemic symptoms and may compromise the functional integrity of the BBB as well.

As an active part of the interface between blood and neural tissue the endothelial cells are extremely important in sensing and responding to stress factors. The Toll-like receptors, which are expressed on endothelial cells as well, recognize a broad range of exogenous and endogenous molecules and initiate inflammatory reactions.

Smoking also causes serious stress load in our body resulting in inflammatory reactions. Many components of tobacco smoke can reach most of the cells through the bloodstream. Thousands of different compounds get into the body with cigarette smoke including nicotine and polycyclic aromatic hydrocarbons. The high number of smokers suffering from neurological disorders shows that one of the main targets of tobacco smoke components is the central nervous system. Despite the high incidence of stroke among smokers and the key role of the blood-brain barrier in the pathogenesis of this disease very little information is available about the direct effect of smoking on the blood-brain barrier. One of the most controversial issues is the

extent of nicotine's contribution to the development of CNS disorders and the role of other tobacco smoke components in these processes.

The main aims of our studies were to elucidate the molecular changes appearing in cerebral endothelial cells in response to Toll-like receptor activation and tobacco smoke components. The following specific aims were addressed:

1. Which Toll-like receptors are expressed in cerebral endothelial cells? How do the effects mediated by Toll-like receptors affect the blood-brain barrier properties of cerebral endothelial cells?

How do tobacco smoke components, namely nicotine and polycyclic aromatic hydrocarbons affect the function of cerebral endothelial cells?

MATERIALS AND METHODS

CELL CULTURE

Human cerebral endothelial cell line hCMEC/D3 was grown on rat tail collagen-coated plates or glass coverslips in EBM-2 medium supplemented with EGM-2 Bullet Kit and 2.5% foetal bovine serum. Primary rat cerebral endothelial cells were isolated from 2-weeks old rats. Cerebral cortices were cut into small pieces and digested in two steps with collagenase and collagenase/dispase followed by centrifugation on Percoll gradient. The microvessel fragments were plated on collagen/fibronectin coated surfaces and endothelial cells were selected using 4 μg/ml puromycin.

TREATMENTS

Confluent monolayers of cerebral endothelial cells were treated in serum-free culture medium with 10, 50 or 100 μg/ml zymosan alone or in combination with 10 μM U0126, 100 μM PDTC or 5 μM DMNQ for 24 hours. Cells were also treated with 1 nM, 10 nM, 1 μM, 10 μM nicotine for different time periods and 30 μM

phenanthrene or 1-methylantracene for 24 hours. Finally, the 24 hours long 10 µM nicotine was also combined with 10 µM DMNQ.

PERMEABILITY MEASUREMENTS

For permeability measurements primary rat brain endothelial cells were passed onto filter inserts. After reaching confluency endothelial cells were cocultured with astrocytes for 24 h, reaching high transendothelial resistance values. The barrier function was evaluated by measuring the permeability of the cells to sodium fluorescein and Evan's blue labeled albumin. Ringer-Hepes was added to the abluminal side of the filter. The luminal side was loaded with Ringer-Hepes containing 10 µg/ml SF, 170 µg/ml Evan's blue and 10 mg/ml BSA. The cells were incubated at 37°C for 1 h with gentle shaking, and samples were removed from the abluminal side. The permeability coefficients were calculated using the concentration of SF and EBA measured by a fluorescent microplate reader.

MEASUREMENT OF TRANSENDOTHELIAL ELECTRICAL RESISTANCE

For the measurement of transendothelial electrical resistance cells were grown on filter inserts and cocultured with astrocytes. The electrical resistance was measured using a chopstick electrode and an EVOM epithelial voltohmmeter. With this method resistance values above 100 $\Omega \times cm^2$ could be measured.

REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated using TRIzol reagent. RNA was transcribed into cDNA according to standard protocol. The amplification was performed on a BioRad iQ5 instrument using FastStart SYBR Green Mix. Determination of threshold cycle was performed using the software of the instrument. The ΔΔCt method was used to

evaluate the changes in gene expression and the results were plotted using Microsoft Excel 2000.

WESTERN BLOT ANALYSIS

After the experiments, cells were scrapped into ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0,5% NP-40 (Nonidet P-40), 2 mM $CaCl_2$,5 mM NaF, 1 mM Na orthovanadate, and 1 mM Pefabloc). Lysates were clarified by centrifugation at 10.000 g for 10 min on 4°C, and the supernatant was used as the Triton X-100-soluble fraction. Then the protein concentration was determined with the bicinchoninic acid (BCA) method. The supernatant was mixed with Laemmli sample buffer and denatured for 3 minutes.

For the preparation of the Triton X-100 soluble fraction, cells were scrapped into lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM Na orthovanadate and 1 mM Pefabloc). Homogenates were centrifuged and the supernatant was used as the Triton X-100 soluble fraction. The pellet was resuspended in Laemmli sample buffer and used as the Triton X-100 insoluble fraction.

Proteins were electrophoresed with standard denaturing SDS-PAGE procedures and blotted on polyvinylidene difluoride (PVDF) or nitrocellulose membranes. After blocking the blots were incubated with the primary antibodies. After washing with TBS-T the blots were incubated with the secondary antibodies. The immunoreaction was visualized using Immobilon Western Chemiluminescent HRP Substrate on x-ray film.

IMMUNOPRECIPITATION

For immunoprecipitation, cells were homogenized and centrifuged as described above and the supernatant was subjected to immunoprecipitation. Supernatants were incubated with 2-5 µg primary antibody (β-catenin). The formed immunocomplexes

were precipitated by incubating the samples overnight with protein G-Sepharose beads. The precipitates were washed 4 times with lysis buffer, boiled in sample buffer, and subjected to SDS-PAGE and immunoblotting.

IMMUNOFLUORESCENCE

For immunofluorescent studies primary rat brain endothelial cells were cultured on coverslips. Brain endothelial monolayers were fixed using a mixture of ice-cold ethanol:acetic acid (95:5) for 10 min and then washed in PBS. After blocking with 1% BSA, coverslips were incubated with primary antibodies. The staining was visualized using Cy3- or Cy5-conjugated secondary antibodies followed by washing in PBS. Coverslips were mounted in anti-fading embedding medium and the distribution of the signal was studied using a photomicroscope connected to a digital camera.

STATISTICAL ANALYSIS

Statistical analysis was performed using means and standard error of means of three independent experiments. The values were compared using analysis of variance followed by Bonferroni's post hoc test. Changes were considered statistically significant at $P < 0.05$.

RESULTS

1. EXPRESSION OF TOLL-LIKE RECEPTORS IN CEREBRAL ENDOTHELIAL CELLS

Our results showed that cells of a human brain endothelial cell line (hCMEC/D3) express TLR2, 3, 4 and 6 while primary rat brain endothelial cells express TLR2, 3 and 6 under basal conditions. The mRNA expression of all identified human Toll-like receptors was induced by oxidative stress elicited by DMNQ. Zymosan, a TLR2/6 agonist elevated the expression of these two receptors while having no effect on the expression of TLR3 and 4.

2. EFFECT OF ZYMOSAN A TLR2/6 AGONIST ON CEREBRAL ENDOTHELIAL CELLS

Zymosan increased the permeability of the endothelial cell cultures. This phenomenon can be explained by the effect of zymosan on cell junctions: zymosan treatment resulted in a decrease in occludin and claudin-5 expression and the loss of membrane staining of these two transmembrane components of the tight junctions. U0126, the ERK 1/2 kinase inhibitor was able to prevent the changes of occludin caused by zymosan. However, neither PDTC (an NF-κB inhibitor) nor U0126 was able to prevent the decrease of claudin-5 expression. Zymosan combined with oxidative stress had an even more pronounced effect on the junctions because occludin completely disappeared from the cell contact sites.

3. EFFECT OF NICOTINE ON CEREBRAL ENDOTHELIAL CELLS

We investigated the effects of some tobacco smoke components, namely nicotine and two polycyclic aromatic hydrocarbons, phenanthrene and 1-methylanthracene on intercellular junctions of cerebral endothelial cells. Our Western

blot studies showed that short treatments with low concentrations of nicotine did not affect the transmembrane proteins of the tight junctions. Concentrations only above peak plasma levels of nicotine led to a decrease in occludin, ZO-1 and cadherin expression, members of the tight and adherens junctions, respectively. Results of the immunofluorescent analysis confirmed Western blot data showing that the most sensitive tight junction protein to nicotine was ZO-1. A similar, but less pronounced effect of nicotine was observed on the localization of occludin, ZO-2 and cadherin. Localization of junctional proteins after a combined nicotine and phenanthrene treatment was comparable to that seen after nicotine treatment alone. Nicotine alone had no effect on the transendothelial electrical resistance of the cell cultures. However, combination of nicotine treatment with oxidative stress induced by DMNQ led to a significant decrease in the transendothelial electrical resistance indicating damage of the gate function of endothelial cells.

4. EFFECT OF POLYCYCLIC AROMATIC HYDROCARBONS ON CEREBRAL ENDOTHELIAL CELLS

Phenanthrene treatment caused a redistribution of occludin from the Triton X-100 insoluble to the Triton X-100 soluble fraction. A similar but less pronounced effect was seen in the case of claudin-5. 1-methylanthracene caused a slight decrease in claudin-5 expression in the Triton X-100 insoluble fraction. Treatment with phenanthrene did not cause significant changes in ZO-1 or claudin-5 localization. In the case of occludin and ZO-2, a slight decrease in the continuity of the staining was detected. Neither phenanthrene nor methylanthracene had effect on adherens junction proteins, moreover, phenanthrene did not cause changes in the β-catenin-cadherin and β-catenin-α-catenin interactions of the adherens junction. Similar to nicotine, phenanthrene alone had no effect on the transendothelial electrical resistance of the cell cultures indicating that this polycyclic aromatic hydrocarbon had no significant effect on the gate function of endothelial cells.

SUMMARY

We are the first to identify the expression of TLR6 in cerebral endothelial cells. We also showed that after activation of TLR2/6 the amount of occludin decreased in the cells and the protein disappeared from the tight junctions. This process was mediated by ERK 1/2 kinases. Claudin-5 also disappeared from cell-cell junctions. The mechanisms of the latter process are under investigation.

The results of our complex investigation on the effect of nicotine and polycyclic aromatic hydrocarbons on cerebral endothelial cells suggest that tobacco smoke components do not cause acute alterations in the principal functional properties of the cerebral endothelial cells. However, in combination with other damaging effects like oxidative stress, these cigarette smoke compounds may cause a significantly impaired BBB function.

Taken together, our results give deeper insight into the processes mediated by cerebral endothelial Toll-like receptors and the endothelial damage caused by certain tobacco smoke components.

LIST OF PUBLICATIONS

Publications related to the thesis

Nagyőszi P., Wilhelm I., Farkas A.E., Fazakas C., Dung N.T., Haskó J., Krizbai I.A. (2010) Expression and regulation of Toll-like receptors in cerebral endothelial cells. Neurochem. Int. IF_{2009}: 3,541

Hutamekalin P., Farkas A.E., Orbók A., Wilhelm I., **Nagyőszi P.**, Veszelka S., Deli M.A., Buzás K., Hunyadi-Gulyás E., Medzihradszky K.F., Meksuriyen D., Krizbai I.A. (2008) Effect of nicotine and polyaromtic hydrocarbons on cerebral endothelial cells. Cell Biol. Int. 32, 198-209. IF_{2008}: 1,619

Publications not related to the thesis

Wilhelm I., **Nagyőszi P.**, Farkas A.E., Couraud P.O., Romero I.A., Weksler B., Fazakas C., Dung N.T., Bottka S., Bauer H., Bauer H.C., Krizbai I.A. (2008) Hyperosmotic stress induces Axl activation and cleavage in cerebral endothelial cells. J. Neurochem. 107, 116-126. IF_{2008}: 4,500

Wilhelm I., Farkas A.E., **Nagyőszi P.**,Váró G., Bálint Z., Végh G.A., Couraud P.O., Romero I.A., Weksler B., Krizbai I.A. (2007) Regulation of cerebral endothelial cell morphology by extracellular calcium. Phys. Med. Biol. 52, 6261-6174. IF_{2007}: 2.528

Farkas A., Szatmári E., Orbók A., Wilhelm I., Wejksza K., **Nagyőszi P.**, Hutamekalin P., Bauer H., Bauer H.C., Traweger A., Krizbai I.A. (2005) Hyperosmotic mannitol induces Src kinase-dependent phosphorylation of beta-catenin in cerebral endothelial cells. J. Neurosci. Res. 80, 855-861. IF_{2005}: 3.239

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