Blood-brain barrier changes in hemorrhagic shock and after cerebral ischemia-reperfusion. Possible protective mechanisms.

Ph.D. Thesis

Gábor Lenzsér M.D.

Semmelweis University
Doctoral School of Basic Medicine

Supervisor: Péter Sándor, M.D., Ph.D., DMSc

Official Reviewers: Violetta Kékesi, Ph.D.
    Ferenc Domoki, M.D., Ph.D.
Head of Comprehensive Exam Committee: Tibor Wenger, M.D., Ph.D., DMSc.
Members of Comprehensive Exam Committee: Mihály Boros, M.D., Ph.D., DMSc
    Ákos Zsembery, M.D. Ph.D.

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Introduction

For the proper neuronal functioning a well regulated extracellular milieu is a necessity, which is protected from the fluctuations in the concentration of plasma constituents. The diffusion and filtration of hydrophilic molecules and solutes is highly restricted between the plasma and the interstitial fluid of the central nervous system. The blood-brain barrier (BBB) is responsible for the strict regulation of transcapillary transport in the brain. The cerebral endothelial cells are the main elements of the BBB and mainly responsible for the barrier properties. They differ in several aspects from their systemic counterparts. They have less pinocytotic vescicles and they posses more developed, continous tight junctions, which seal the paracellular clefts between the neighbouring endothelial cells, hindering the free diffusion of lipid insoluble molecules.

In the event of BBB opening neuronal malfunctioning may occur. Furthermore, since there are no proteins in the extracellular space of the brain, the extravasation of proteins will cause vasogenic edema leading to death in severe cases. BBB damage and the consequent brain edema accompany and aggravate many central nervous system pathologies, such as cerebral hypoperfusion disorders. During reperfusion after cerebral ischemia, there is a biphasic opening of the BBB associated with brain edema and hemorrhagic transformation in severe cases.

Tight junctions are important in maintaining the low permeability of the BBB. Important elements of the tight junctions are the transmembrane proteins, the claudins and occludin. Occludin has two extracellular loops that span the intercellular cleft and is responsible for the closure of the paracellular space. It is essential for the low permeability as decreased expression of occludin is associated with disrupted barrier function. The other component of the
junctional complex is the adherens junction, it is responsible for stabilizing cell-cell interactions in the endothelium. Although it is primarily the tight junction that confers the low paracellular permeability, the disruption of the adherens junction can lead to increased permeability as well.

Preconditioning represents the phenomenon, when a sublethal insult induces resistance to a subsequent more deleterious insult. It is an effective way to induce ischemic tolerance in different organs. It is associated with two windows of protection: an early phase occurring immediately after the triggering stimulus and lasting 1–3 h, and a late phase providing a second window of protection 12–24 h latter and lasting for 2–3 days. It was demonstrated experimentally that the BBB can be preconditioned as well. Ischemic tolerance can be induced by diverse types of stressfull stimuli (for example: oxidative stress, heat shock) and by modification of intracellular signaling pathways involved in the development of the resistant phenotype. The activation of the mitochondrial ATP-sensitive potassium (mitoK<sub>ATP</sub>) channel has been proposed to play a pivotal role in preconditioning, both as a trigger and as a mediator. Diazoxide is an opener of the mitoK<sub>ATP</sub> channel, it has been reported to induce preconditioning in several preparations.

The nuclear enzyme poly (ADP-ribose) polymerase (PARP) recognizes DNA strand breaks caused by free radicals and upon binding to damaged DNA forms homodimers and catalyzes the cleavage of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) into nicotinamide and ADP-ribose. It uses the latter to synthesize branched nucleic acid-like polymers of poly(ADP-ribose), covalently attached to nuclear acceptor proteins. It has important function in DNA repair, but its overactivation induces widespread changes, exacerbating the cellular damage. These include the depletion of cellular NAD<sup>+</sup> and ATP pools resulting in cell death, the augmentation of pro-inflammatory gene transcription and the
apoptotic pathways. According to its substantial role in cell death, blocking of PARP has been proved to protect against ischemic damage in several tissue.
The aim of our investigations

The main goal of our investigations was to reveal new aspects about BBB changes during and after cerebral hypoperfusion states, such as hemorrhagic shock and global cerebral ischemia/reperfusion. We wanted to elucidate the possible mechanisms of the opening of the barrier, especially concerning possible alterations in the structure of the interendothelial tight and adherens junctions. Furthermore, we wanted to test possible protective mechanisms of the BBB, proved to be efficient against parenchymal damage in cerebral ischemia/reperfusion, but only sparsely or not at all tested against the injuries of the cerebral microvasculature and the BBB.

The aim of our investigations was:

1. to determine the opening of the BBB for low and high molecular weight tracers during cerebral hypoperfusion states, namely in different phases of hemorrhagic shock and in global cerebral ischemia/reperfusion.

2. to determine the development of cerebral edema in reperfusion

3. to determine the possible changes in the expression and localization of cerebral endothelial junctional proteins in hemorrhagic shock and in global cerebral ischemia/reperfusion.

4. to test the hypothesis that diazoxide preconditioning would reduce BBB permeability and decrease brain water content following
global cerebral ischemia/reperfusion and that mitoK\textsubscript{ATP} channel opening is involved in its action.

5. to determine the effect of pre- and post-ischemic treatment with the PARP enzyme inhibitor, PJ34, on functional and structural BBB alterations and on the brain water content changes after global cerebral ischemia/reperfusion.

6. to test the role of the anti-inflammatory effect of PARP inhibition in the supposed BBB protection in reperfusion
Materials and methods

To reach our goals three sets of experiments were conducted:

1. In a hemorrhagic shock model we investigated the BBB permeability changes and the structural alteration of the junctional komplex.
2. In a severe global cerebral ischemia/reperfusion model we checked early reperfusional BBB permeability alterations and the effect of diazoxide preconditioning on the BBB damage.
3. In a less severe global cerebral ischemia model we investigated late reperfusional BBB permeability alterations as well, tight junctional protein changes and the effect of PARP inhibition on the reperfused BBB.

Experimental animal models

Hemorrhagic shock model

The experiments were carried out in anesthetized male Wistar rats. Systemic mean arterial blood pressure (MAP) of the rats was decreased to 40 mmHg by blood withdrawal from the femoral artery. Experiments were performed in three groups: Group 1: control, normotensive rats; Group 2: 40 mmHg MAP was kept constant for 15 min to achieve the compensated phase of hemorrhagic shock; and Group 3: 40 mmHg MAP was maintained until one-half of the total amount of the shed blood returned from the reservoir to the animal’s systemic circulation spontaneously. This signaled the achievement of the irreversible phase of the shock.

Global cerebral ischemia/reperfusion models

Experiments were carried out in anesthetized male Wistar rats. The animals were exposed to global cerebral ischemia by using combined bilateral common
carotid artery occlusion and hemorrhage induced arterial hypotension. In the severe ischemia protocol, MAP was lowered to 35–40 mmHg by blood withdrawal and both common carotid arteries were occluded by microaneurysm clips for 30 min. Reperfusion (30min, 4h) was achieved by removing the clips and giving back the shed blood. In the less severe ischemia protocol MAP was lowered to 45–50 mmHg and the carotid arteries were occluded for 20min. Reperfusion was allowed for different durations (40min, 24h, 48h).

**Drug treatments**

**Diazoxide preconditioning**

Diazoxide was administered to the animals intraperitoneally in a dose of 6, 20, or 40 mg/kg on three consecutive days. The final diazoxide or vehicle injection was given 24 h before the global cerebral ischemia. Two groups of animals were treated by the mitoK$_{ATP}$ channel antagonist 5-hydroxydecanoic acid (5-HD) in a dose of 40 or 100 mg/kg 20 min before the diazoxide (40 mg/kg) injection as well.

**Inhibition of the PARP enzym**

Animals were treated with saline or the PARP inhibitor, PJ34 (10mg/kg, i.v.) 40min before the start of ischemia. One set of animals was treated with the drug at the start of reperfusion and 6h latter again (10mg/kg, i.p.).

**Quantitation of BBB opening**

30 min before the end of the experiments (in the global cerebral ischemia/reperfusion studies 30 min before the end of the given reperfusion period) the animals received an intravenous injection of 4 ml/kg 2-2 % Evans Blue albumin (EB) (molecular weight of 67,000 Da) and sodium fluorescein
(Na-F) (molecular weight of 376 Da). The rats were perfused with isotonic saline and samples were taken from different brain regions. The samples were homogenized in 50% trichloroacetic acid and centrifuged. For the EB measurement, the supernatant was diluted with ethanol (1:3), and its fluorescence was determined (excitation at 620 nm and emission at 680 nm). For the Na-F measurement, the supernatant was diluted with 5 M NaOH (1:0.8) and its fluorescence was determined (excitation 440 nm and emission at 525 nm).

Determination of brain water content

After decapitation, cortical samples were obtained and weighed immediately to obtain the wet weight. The tissue was then dried in an oven at 100 ºC for 24 h and reweighed to obtain the dry weight. Brain water content was calculated as (wet weight - dry weight) / wet weight x 100%.

Western-blot

Isolated capillaries or cortical samples were homogenized in PBS and proteinase inhibitors were added. Next, SDS buffer was added. Equal amounts of protein for each sample were separated by 4-20% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane. Blots were incubated with rabbit anti-cadherin, b-catenin, and occludin antibodies. The bound antibodies were visualized using enhanced chemiluminescence and recorded on X-ray film.

Myeloperoxidase activity assay

Inflammatory cell infiltration was determined at the end of the less severe global cerebral ischemia/reperfusion, PARP inhibition experiments using an assay for myeloperoxidase (MPO), an enzyme found within the azurophilic granules of neutrophil leukocytes. Following homogenization and centrifugation of cortical samples the pellet was suspended in 0.5%
hexadecyltrimethylammonium bromide. The samples were frozen immediately and subjected to freeze-thaw cycles and sonication. Thereafter the samples were centrifuged. Supernatant MPO activity was assayed by mixing it with o-dianisidine dihydrochloride and hydrogen peroxide. The rate at which a colored product formed during the MPO-dependent reaction of o-dianisidine dihydrochloride was measured. The change in absorbance at 460 nm was recorded at 15s intervals over 2min.

Real-Time PCR

Total cellular RNA was extracted from homogenates of the cortical samples using an SVtotal RNA isolation system (Promega, Madison, WI, USA). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using an ABI Prism 7700 Sequence Detection System and Taqman probe and primer set for ICAM-1.
Results

BBB permeability changes

Hemorrhagic shock

We have revealed a 2–3-fold increase in the extravasation of Na-F in the decompensated stage of hemorrhagic shock in the brain. No significant changes in the BBB permeability for sodium fluorescein were observed during the compensated phase and extravasation of EB did not change either in the compensated or in the decompensated phase of the shock.

Severe global cerebral ischemia/reperfusion, diazoxide preconditioning

BBB permeability was measured in early reperfusion. The amount of EB increased dramatically following ischemia/reperfusion in the vehicle treated animals. Similar results were obtained for Na-F. Diazoxide administration reduced the amount of the extravasated EB in a dose-dependent manner. Similar results were obtained for Na-F but the effect of diazoxide was not as great as with the EB. 5-HD, a mitoK\textsubscript{ATP} channel blocker, administered before diazoxide treatment failed to increase either EB or Na-F permeability in the cortex.

Less severe global cerebral ischemia/reperfusion, PARP inhibition

EB extravasation showed a tendency towards higher values at 40min reperfusion although the difference was not significant in this case. However, extravasation of EB measured at 24 and 48h after ischemia/reperfusion increased dramatically compared to the sham operated animals. Na-F extravasation was higher in all examined reperfusion time points and showed an increase with the elapsed time. PJ34 treatment significantly lowered EB extravasation in the 48h group. Pre-treatment with PJ34 significantly decreased
Na-F permeability 48h after reperfusion and post-treatment with PJ34 resulted in a significant reduction of the Na-F permeability 24 hours after reperfusion as well. At 40min the permeability of the barrier in the cortex was almost identical in the saline and PJ34 treated groups.

**Brain water content**

**Severe global cerebral ischemia/reperfusion, diazoxide preconditioning**

After ischemia/reperfusion, the water content of the cortical brain tissue elevated both in the vehicle and diazoxide-treated animals. However, diazoxide pretreatment significantly decreased the cortical edema.

**Less severe global cerebral ischemia/reperfusion, PARP inhibition**

After ischemia6reperfusion, there was no statistically significant difference in the brain water content to the sham animals at 24h. However, the water content of the brains was elevated after 48h reperfusion. In the PJ34 pre-treated animals the water content increase was significantly smaller after 48h reperfusion.

**Expression of junctional proteins**

**Hemorrhagic shock**

The expression of occludin was reduced in response to hemorrhagic shock, and the decrease of occludin was more pronounced in the decompensated phase. A similar expression pattern was shown by the transmembrane adherens junction protein cadherin as well. However, no changes in the expression of β-catenin could be observed.

Our results obtained by Western-blot analysis of the expression of occludin in isolated brain microvessels were confirmed by immunofluorescent studies.
The characteristic continuous vascular staining observed in the control animals became disrupted after decompensated hemorrhagic shock.

**Less severe global cerebral ischemia/reperfusion, PARP inhibition**

The amount of occludin did not change at 40min reperfusion but significantly decreased to less than half of the original amount at 24h reperfusion and was still lower at 48h reperfusion. The occludin content correlated well with EB and Na-F extravasation at 48h reperfusion.

Pre-treatment with PJ34 resulted in a trend for higher occludin content at 24h (not significant), but on the second day the occludin content was significantly higher in the drug treated animals. The post-treatment protocol protected against the loss of occludin at 24h reperfusion.

In the 24h reperfusion group there was a prominent band on the Western-bLOTS detected at ~25 kDa which was inversely related to the occludin band located at ~65 kDa.

**Myeloperoxidase levels**

At 40min reperfusion there were no MPO activity changes. The MPO activity was significantly elevated in the saline treated ischemic animals at 24 and 48h of reperfusion. Pre-treatment with PJ34 signficantly reduced MPO to near baseline values at 48h reperfusion.

In saline treated rats MPO activity correlated well with the extravasation of EB and Na-F.

**Real-Time PCR**

The transcription of the endothelial adhesion molecule ICAM-1 markedly increased at 24 and 48h after the start of reperfusion in the cortical samples
compared to the sham operated animals. PJ34 pre-treatment decreased the changes at both time points.
Conclusion

We have found the opening of the BBB for a low molecular weight tracer during the decompensated phase of the hemorrhagic shock. Small molecules usually pass through the endothelium on the paracellular route, between the endothelial cells, which is sealed by the junctional complex (tight and adherens junctions) in the intact blood-brain barrier. The expression of both, the tight junction protein, occludin and the adherens junction protein, cadherin significantly decreased in the walls of the cerebral microvessels in hemorrhagic shock. Our study revealed the first time in the literature that the BBB disturbance in the decompensated phase of hemorrhagic shock affects mainly the paracellular route and diminished occludin and cadherin contents could be responsible for it.

We have observed increased BBB permeability for low, as well as, for high molecular weight tracers during the early phase of reperfusion following severe global cerebral ischemia and the development of cerebral edema. After a less severe ischemia we have demonstrated delayed BBB opening during reperfusion (24h and 48h) accompanied by considerable cerebral edema. Occludin contents were significantly reduced during late reperfusion as well.

The mitoK$_{\text{ATP}}$ channel opener diazoxide used in a preconditioning protocol reduced the extent of the permeability increase of the barrier and the adjacent cerebral edema, although blocking the mitoK$_{\text{ATP}}$ channels could not diminish its effect. However, to the best of our knowledge, this is the first study in the literature in which chemical preconditioning resulted in BBB protection.

The poly (ADP-ribose) polymerase (PARP) enzyme inhibitor PJ34 used in a pre- and post-treatment protocol reduced the barrier permeability and the brain edema seen in late reperfusion. PJ34 treatment significantly hindered the decline
of the tight junction protein occludin during late reperfusion. The increase of protein degradation is a possible mechanism behind the decreased occludin levels deduced from the negative correlation between occludin and its byproduct in reperfusion. We have demonstrated the inhibition of inflammation after PJ34 treatment in reperfusion. The transcription of the pro-inflammatory adhesion molecule ICAM-1 and the leukocyte infiltration in the cerebral cortex were both highly elevated after 24 and 48h reperfusion and following inhibition of the PARP enzyme they were both attenuated. The permeability of the BBB was proportionate to the leukocyte infiltration after reperfusion, suggesting a major contribution of the inflammation processes in the barrier disturbance. The results of our studies indicate that the activation of the PARP enzyme after ischemia/reperfusion is involved in the BBB damage, it contributes to the increased BBB opening, to the disintegration of the endothelial tight junction complex and its effect is mainly driven by the augmentation of the inflammatory pathway.
Publications

Publications related to the thesis:


Other publications:


