Investigating the modulating role of HSPB1 in neuroinflammation using a transgenic mouse model

Summary of the Ph.D. thesis

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Introduction

Neuroinflammation, the inflammatory process of the central nervous system, plays a key role in the pathophysiology of most acute and chronic brain diseases. Inflammation is a complex process characterized by an increased level of cytokines, chemokines, and other inflammatory mediators with concomitant activation of microglia cells, reactive astrogliosis, and infiltration of peripheral immune cells. Inflammation of the central nervous system is primarily initiated in order to aid the repair of the damaged brain area. However, in case it becomes excessive, chronic or dysregulated, it can further aggravate the tissue damage and can lead to secondary damage.

Evolutionarily conserved heat-shock proteins function primarily as molecular chaperones within the cells and contribute to the maintenance of normal cellular protein homeostasis. In response to different stress conditions, heat-shock proteins show rapid induction, and they protect the cells from damaging effects. Moreover, heat-shock proteins have an effect on the regulation of the pro- and anti-inflammatory processes. The newly synthesized intracellular heat-shock proteins can modulate the release of inflammatory mediators by interacting with several components of the major signaling pathways. However, cellular stress promotes the release of heat-shock proteins into the extracellular space as well. Lethal stress leads to the passive secretion of heat-shock proteins from necrotic cells, which usually induce a strong pro-inflammatory response. In the case of mild stress, however, heat-shock proteins are released from the cell by active secretion with the help of lysosomes or exosomes. If these vesicles burst outside of the cell, heat-shock proteins can bind to various immunoreceptors on the cell surface, whereas if the vesicles fuse with the cell membrane, heat-shock proteins act on the intracellular pathways.

The small heat-shock protein, HSPB1, is constitutively expressed in the brain, but there are some differences in the expression pattern of the different cell types. Based on previous data, HSPB1 is the most abundant in astrocytes, while it is present to a smaller extent in neurons and shows only a low expression in microglia. In addition to its well-known chaperone function, HSPB1 is involved in several processes closely related to neuroinflammation. For example, HSPB1 helps maintain the integrity of cytoskeletal networks by which not only can it play a key role in the protection of cells under stress conditions, but can also influence the activation of glial cells. Inflammation is often accompanied by neurodegeneration, however, HSPB1 can interact with several components of the apoptotic pathways, thus playing an important anti-apoptotic role. In addition, numerous data suggest that HSPB1 can also affect the cytokine expression, but whether it has pro- or anti-inflammatory properties is not yet fully understood, as it induced diverse effects depending on the cell type or condition. Although an increasing number of studies have revealed the regulatory function of HSPB1 in inflammation, most of these data are either from peripheral inflammatory models or, in the case of neuroinflammation, most experiments were performed on cell cultures, so there is a dearth of comprehensive studies investigating the role of HSPB1 in neuroinflammation *in vivo*.

Aims of the Thesis

Using our previously established transgenic mouse strain overexpressing the human HSPB1, our group has demonstrated the protective effect of hHSPB1 in both acute and chronic neuronal damages. Since inflammatory processes play a central role in most neurological diseases, we aimed to investigate the role of hHSPB1 in neuroinflammation in the present study.

The following questions were raised during our experiments:

1. How does the overexpression of hHSPB1 affect the neuroinflammatory processes *in vivo*?

   Does it affect a) the gene expression of cytokines?

   b) the activation of microglia cells?

   c) the development of reactive astrogliosis?

   d) the level of cell death?

2. Which cell types of the brain are responsible for the effects observed in hHSPB1-overexpressing transgenic animals?

   a) Which cell type is the main source of cytokines?

   b) Is the extracellular or intracellular form of hHSPB1 responsible for the regulation of inflammatory processes?

   c) How does the expression pattern of hHSPB1 change in different cell types under stress conditions?

Prof. Dr. Mária Deli and her coworkers of the Biological Barriers Research Group (Institute of Biophysics, BRC) helped us to answer the second question.

Materials and methods

In vivo experiments

- Acute ethanol treatment: seven-day-old hHSPB1-overexpressing transgenic or wild-type mice were treated with subcutaneous injection of 2x2.5g/kg ethanol or physiological saline
- DNA purification from tail biopsies, and detecting the presence of the transgene by PCR
- Protein extraction and quantification of hHSPB1 protein by Western-blot analysis
- Total RNA isolation from whole brain homogenates which was followed by reverse transcription; RT-PCR was performed to analyze the level of HSPB1, cytokines, and glial markers
- Quantification of cell death using TUNEL-assay
- Investigating the cell-specific expression of hHSPB1 in the brain by fluorescent immunohistochemistry
- Studying the expression levels of hHSPB1 and the activation of microglia cells and astrocytes in the brain by peroxidase immunohistochemistry

In vitro experiments

- Preparation of primary cell cultures from the brain of hHSPB1-overexpressing transgenic and wild-type animals
- Determination of the purity of isolated primary cell cultures by fluorescent immunostaining
- Viability tests on the primary cell cultures (impedance measurement, Resazurin assay, quantification of cell nuclei)

- Treatment of primary cell cultures with ethanol and cytokines (TNFα and Il-1β)
- Analysis of the intracellular level of hHSPB1 by fluorescent immunostaining
- Quantifying the concentration of hHSPB1 and TNFα proteins released into the supernates of the primary cell cultures by ELISA

Results

In vivo experiments

1. High expression level of transgenic hHSPB1 was detected in the brains of control transgenic animals both at the protein and mRNA levels, however, we demonstrated that hHSPB1 expression was further increased 24 hours after ethanol treatment. We also found that the transgenic protein was primarily expressed by astrocytes and neurons, whereas double-labeled microglia was rarely detected.

2. In our model of early postnatal ethanol exposure, inflammatory processes began as early as 7 hours after ethanol treatment:

- *Tnf* expression showed a significant increase, the level of which was significantly higher in hHSPB1-overexpressing mice than in their wild-type littermates.

3. The largest inflammatory response was detected 24 hours after ethanol treatment, at which time point an intense inflammation and cell death could be observed:

- the expression level of pro-inflammatory cytokines (*Tnf, Il1b*) was nearly doubled in ethanol-treated wild-type animals, whereas hHSPB1 overexpression, in response to ethanol treatment, further increased the expression level of the pro-inflammatory cytokines, thus eliciting a more intense inflammatory reaction than ethanol treatment alone.
- in the brain of hHSPB1-overexpressing animals, there was a significantly greater microglia coverage in the frontal cortex, thalamus, and striatum after ethanol treatment, indicating an enhanced microglial response. This

was also supported by the expression changes of the M1 (*Cd68*) and M2 (*Arg1*) microglial markers, since, in response to ethanol treatment, the expression levels of both markers were significantly higher in hHSPB1-overexpressing animals compared to wild-type animals.

- the expression level of the astrocyte marker *Gfap* was also greatly elevated after ethanol treatment, showing significantly higher expression in ethanol-treated transgenic animals compared to ethanol-treated wild-type ones. However, at this time point, no substantial changes were found neither in the morphology of the astrocytes nor in the GFAP coverage.
- the enhanced inflammatory responses in the transgenic mice were not accompanied by increased cell death.

4. One week after ethanol treatment, the resolution of inflammation began:

- the level of pro-inflammatory cytokines, and the expression of microglia and astrocyte markers decreased to levels similar to those of the control groups in both genotypes.
- one week after the treatment, microglia cells returned to their resting state-like morphology and were evenly distributed throughout the brain.
- ethanol-induced morphological changes in astrocytes were detectable at this time point because more time is necessary for the formation of GFAP-positive filaments. Astrocyte activation, like microglia, was higher in the brains of hHSPB1-overexpressing animals, as significantly greater astrocyte coverage was observed in the parietal cortex and striatum compared to the ethanol-treated wild-type littermates.

In vitro experiments

- The purity of the primary cultures was assessed by immunohistochemistry: the microglia culture was 93%, the astrocyte culture was 78%, and the neuron culture was 85% pure.
- No detectable amount of hHSPB1 was released by any of the analyzed primary cell cultures upon ethanol/cytokine treatment or under control conditions.
- Of the brain cells, astrocytes and neurons proved to be the primary hHSPB1-expressing cell types:
  - intracellular hHSPB1 levels of primary neurons were not significantly altered by either ethanol or cytokine treatments.
  - in primary transgenic astrocytes a greatly elevated expression of hHSPB1 was detected, as the level of intracellular HSPB1 showed a significantly high, approximately five-fold increase after cytokine treatment, while a smaller, two-fold increase after ethanol treatment.
- Microglia cells proved to be the main sources of TNFα.
- Overexpression of hHSPB1 could significantly modulate the release of TNFα by glial cells under inflammatory conditions:
  - cytokine treatment significantly increased the release of TNFα from microglia cultures derived from hHSPB1 transgenic mice compared to non-treated transgenic cells; however, no such effect was observed in wild-type microglia.
  - contrarily, after cytokine treatment, TNFα production was significantly increased in wild-type astrocytes compared to the control group; however, it remained unchanged in the hHSPB1 transgenic cells.
  - there was a significant difference between the two genotypes after cytokine treatment in both primary microglia and astrocyte cultures.

Conclusion

These results indicate that a single ethanol treatment induces intense inflammation in the brains of early postnatal mice and provide evidence that hHSPB1 overexpression has an inflammation-regulating effect after ethanol treatment, as it increased the expression level of pro-inflammatory cytokines and resulted in a higher activation of microglia and astrocyte cells. The results presented here suggest that hHSPB1 plays a complex role in the regulation of neuroinflammation, since the significantly higher level of inflammation described in the transgenic mice was not associated with increased neuronal damage, suggesting that hHSPB1 can counteract the deleterious effects of inflammatory cytokines. Increased expression of the anti-inflammatory microglial marker in transgenic animals also assumes that hHSPB1 induces beneficial anti-inflammatory processes as well. In addition, it is particularly important that one week after ethanol treatment, both wild-type and hHSPB1-overexpressing animals showed the resolution of inflammation, indicating that hHSPB1 overexpression amplifies the acute phase of inflammation and does not turn it into one of a chronic nature. The *in vitro* results demonstrated that, in our model, the intracellular form of hHSPB1 was responsible for the observed inflammation-regulating effects. Of the brain cells, astrocytes and neurons proved to be the primary hHSPB1-expressing cell types, while the microglia cells were the main sources of TNFα. We hypothesize that hHSPB1 exerts its modulating effects primarily through the astrocyte cells, since the high intracellular hHSPB1 levels in primary astrocytes can influence their TNFα release directly, whereas the differences between the wild-type and transgenic microglia cultures could be the result of a potential indirect effect of hHSPB1. Overall, the data presented in this study contribute to a better understanding of the regulatory role of hHSPB1 in the inflammatory processes

of the central nervous system, which may thus become a potential therapeutic target in the treatment of inflammation-related neurological disorders.

List of publications

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Publications related to the thesis:

1. **Brigitta Dukay**, Fruzsina R. Walter, Judit P. Vigh, Beáta Barabási, Petra Hajdu, Tamás Balassa, Ede Migh, András Kincses, Zsófia Hoyk, Titanilla Szögi, Emőke Borbély, Bálint Csoboz, Péter Horváth, Lívia Fülöp, Botond Penke, László Vígh, Mária A. Deli, Miklós Sántha* and Melinda E. Tóth*. (2021) Neuroinflammatory processes are augmented in mice overexpressing human heat-shock protein B1 following ethanol-induced brain injury. *Journal of Neuroinflammation* 18, 22 https://doi.org/10.1186/s12974-020-02070-2
   $IF_{2019/20}$: 5.793

2. **Brigitta Dukay***, Bálint Csoboz*, Melinda E. Tóth. (2019) Heat Shock Proteins in Neuroinflammation. *Frontiers in Pharmacology* 10:920. DOI: 10.3389/fphar.2019.00920.
   IF_{2019}: 4.225
   * these authors contributed equally to this work

Other publications:

1. Melinda E Tóth, **Brigitta Dukay**, Zsófia Hoyk, Miklós Sántha. (2020) Cerebrovascular Changes and Neurodegeneration Related to Hyperlipidemia: Characteristics of the Human ApoB-100 Transgenic Mice. *Current Pharmaceutical Design* 26(13):1486-1494. DOI: 10.2174/1381612826666200218101818.
   $IF_{2019/20}$: 2.208

2. Zsófia Hoyk, Melinda E Tóth, Nikolett Lénárt, Dóra Nagy, **Brigitta Dukay**, Alexandra Csefova, Ágnes Zvara, György Seprényi, András Kincses, Fruzsina R Walter, Szilvia Veszelka, Judit Vígh, Beáta Barabási, András Harazin, Agnes Kittel, László G Puskás, Botond Penke, László Vígh, Maria A Deli, Miklos Santha. (2018) Cerebrovascular pathology in hypertriglyceridemic APOB-100 transgenic mice. *Frontiers in Cellular Neuroscience* 12: 380. DOI: [10.3389/fncel.2018.00380]
   IF_{2018}: 3.900

3. Melinda E. Tóth*, **Brigitta Dukay***, Mária Péter, Gábor Balogh, Gergő Szűcs, Ágnes Zvara, Gábor J. Szebeni, Petra Hajdu, Márta Sárközy, László G. Puskás, Zsolt Török, Tamás Csont, László Vígh and Miklós Sántha. Male and female animals respond differently to high-fat diet and regular exercise training in a mouse model of hyperlipidemia. *Under submission.*
   * these authors contributed equally to this work