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The role of heme and oxidized hemoglobin forms in triggering proinflammatory response in endothelial cells

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1. Introduction and literature review

The far most abundant cell type in human body is red blood cell (RBC) with a life span of about 120 days. Hemoglobin (Hb) is the major component of RBCs, which is build by four subunits and each subunit is associated with an iron-protoporhyrin IX (heme) prosthetic group. RBCs are phagocytosed by macrophages at the end of their life, a process which occurs primarily in the spleen. In this mechanism Hb is broken down by a regulated process, giving a chance for heme iron to be recycled efficiently.

One-two percent of RBCs lyse within the vasculature. Released Hb and heme are removed from the circulation by specific and non-specific Hb- and heme-binding proteins respectively. Heme is a potent pro-oxidant molecule which largely enhances cell death induced by oxidative and inflammatory stimuli in many cell types. Recent studies showed that heme behaves as a danger / damage-associated molecular pattern (DAMP) and activates the toll-like receptor 4 (TLR4) and the nucleotide-binding domain, leucine-rich Repeat containing 3 (NLRP3) innate immune pathways in macrophages. Oxidation of Hb is a complex process in which the one- and two-electron oxidation of heme iron and the oxidation of globin can also take place. Oxidation of Hb give a rise of metHb, ferryl- and oxoferrylHb, covalently cross-linked oxidized Hb forms, and porphyrin-globin cross-linked forms, which have been detected in diseases accompanied increased hemolysis.

Hemolysis, hemorrhagia, RBCderived DAMPs

To the best of our knowledge, several diseases are associated with hemolysis and hemorrhagic episodes resulting from unregulated lysis of RBCs. Hemolysis has two type,- intra- or extravascular - and can be traced back to a number of hereditary or acquired causes. In addition, it is very important to mention the hemolysis associated with various infections, which may be due to a parasite (e.g. malaria), a virus (e.g. Dengue fever) or a bacterial infection.

Besides hemolytic diseases, hemorrhagic diseases are as much important. The most common form of cerebral hemorrhage is intracerebral hemorrhage (ICH), where a blood vessel inside the brain is injured and blood enter into the brain tissue. Subarachnoid hemorrhage (SAH), is bleeding into the subarachnoid space in the brain. In the case of intraventricular bleeding (IVH), the bleeding spreads to the ventricular system.

Outside of the protective environment, Hb is prone to oxidation and subsequent heme release. Cell free Hb is primarily scavenged by haptoglobin (Hp), an accute-phase protein. Hp-Hb complexes are bound to Hp receptor CD163 on machrophages initiating a receptor-mediated endocytosis. Extracellular heme is bound by a plasma protein, called hemopexin (Hx). The heme-Hx complex is recognized by CD91 receptors, initiating a receptor-mediated endocytosis.

Following its uptakek, heme is degraded in the cytosol, which is catalized by heme oxygenase-1 (HO-1).

In the case of massive intravascular hemolysis, Hb and heme scavenger pathways become overwhelmed and residual components from RBCs are enriched in plasma, tissue and CSF. Several components released from RBCs during hemolysis have been shown to cause sterile inflammation by acting as a DAMP including ATP, IL-33, heat-shock protein 70 (Hsp70), residual mitochondrial DNS (mtDNS), cyclophylin A and free heme as well.

Hemoglobin oxidation

Oxidation of hemoglobin is a complex process, which is triggered by several endogenous oxidative agents, such as hydrogen-peroxide (H_2O_2) and nitrogen-oxide (NO). In the first reaction oxiHb undergoes autooxidation, which results the formation of metHb. Two electron oxidation of oxiHb give rise of ferrylHb, where heme-iron is present in oxyferryl form ($Fe^{4+}=O$). Further oxidation of metHb results the formation of ferrylHb globin radical, in which oxoferryl ion is unstable. This ferryl-iron can oxidize the amino acid residues of globin chains which results the reduction of ferryl to ferri state (Fe^{3+}). This intramolecular electron transfer leads to the formation of metHb globin radicals, where the unpaired electrons can be found on the oxidized amino acid residues. Finally, reactions between globin radicals lead to the formation of globin-globin cross links, and stable Hb multimers are formed.

Heme-mediated TLR4 and NLRP3 activation

Heme-mediated TLR4 activation was first observed by Figueiredo et al. Heme has already been shown to induce TNF-α secretion in macrophages similarly to LPS. Furthermore, it has also been proved, that the integrity of the heme ring is required to elicit a proinflammatory effect. In addition, besides macrophages heme also activates endohelial cells via a TLR4-dependent pathway.

Dutra et. al demonstrated that heme induces IL-1β secretion in LPS-primed macrophages. In this work, it was identified, that heme exert this effect through activation of the NLRP3 inflammasome, as IL-1β secretion was not observed in macrophages isolated from caspase-1 or NLRP3-deficient mice.

Charactheristics and fuctions of endothelial cells

Endothelial cells (ECs) form a barrier between blood and tissues and they play a key role in several processes in the body. ECs regulate the tone of the vascular wall, and provide an anticoagulant and thromboresistant surface as well as ensure the uptake of dissolved nutrients from the blood to the tissues. ECs are able to activate and inactivate vasoactive agents and are in a dynamic contact with leukocytes. ECs regulates leukocyte adhesion and endothelial transmigration in the case of inflammation. ECs produce anti- and proinflammatory mediators that play a role in regulating inflammatory processes.

NLRP3 inflammasome activation in ECs

ECs express a number of receptors in the innate immune system that sense and respond to circulating PAMP and DAMP molecules.

Accordingly, previous work has shown that ECs express components of the NLRP3 inflammasome, and several DAMPs have been shown to induce NLRP3 inflammasome activation and IL-1β secretion in ECs. NLRP3 inflammasome activation is associated with ROS production and some degree of EC necrosis, during which additional DAMPs e.g. high mobility group box-1 (HMGB1) or ATP are released from necrotizing cells. HMGB1 and ATP induce NLRP3 inflammasome activation, enhancing endothelial dysfunction. Endothelial dysfunction associated with NLRP3 inflammasome activation has been observed in several diseases including diabetes.

2. Aims

1. **Hypothesis**: Heme is a proinflammatory molecule that induces IL-1β secretion *in vivo* and in ECs *in vitro*.

Aim: To investigate the effect of heme on mRNA and protein expressions and IL-1β secretion in C57BL/6 mice, and *in vitro* in human umbilical vein endothelial cells (HUVECs).

2. **Hypothesis**: Heme induces IL-1β production through activation of the NLRP3 inflammasome.

Aim: To investigate the effect of heme on NLRP3 expression in HUVECs and the role of NLRP3 in heme-induced caspase-1 activation and IL-1β production *in vivo* in NLRP3-deficient mice.

3. **Hypothesis**: Heme-induced increased reactive oxygen species (ROS) production plays a role in NLRP3 inflammasome activation.

Aim: To investigate heme-induced NLRP3 inflammasome activation int he presence of ROS scavengers in HUVECs *in vitro*

4. **Hypothesis**: Heme-binding proteins inhibit the proinflammatory effect of heme.

Aim: To investigate the effect of albumin- heme complex on IL-1β production in HUVECs.

5. **Hypothesis**: Following IVH, Hb oxidation products accumulate in the cerebrospinal fluid (CSF).

Aim: Quantitative and qualitative analysis of different Hb forms in CSF samples obtained from preterm infants diagnosed with IVH.

6. **Hypothesis:** Oxidized Hb forms and free heme play a role in neuroinflammation following IVH.

Aim: To determine the levels of inflammatory cytokines and endothelial activation markers in CSF samples obtained after the onset of IVH. To investigate correlations between inflammatory markers and the levels of oxidized Hb forms in post-IVH CSF. To examine the effect of different oxidized Hb forms towards the activation of brain microvascular endothelial cells.

3. Materials and methods

Mice experiments

Forty C57BL/6 mice were used in our experiments. Mice were treated with different doses of heme, LPS and heme +LPS intraperitoneally. The number of leukocytes in the peritoneum was determined by flow cytometry.

Cell culture

Our experiments were performed on primary human umbilical vein endothelial cells (HUVEC) and human brain microvascular endothelial cells (HBEC).

Hemoglobin preparation

Hb was purified from fresh blood anti-coagulated with heparin. We used anion exchange chromatography on a DEAE Sepharose CL-6B packed column. MetHb was prepared by addition of 1.5 molar excess of $K_3[Fe(CN)_6]$ to Hb solution, and ferrylHb was prepared by adding 10 molar excess of H_2O_2. Following oxidation, samples were dialyzed and stored at -70°C until use.

Quantitative real time polymerase chain reaction (qRT-PCR)

RNA samples were solubilized with Trizol (RNA-STAT60, Tel-Test B Labs, Alvin, TX, USA) reagent and purified according to the manufacturer's protocol. Two μg of RNA were reverse transcribed into cDNA, and the amplification of target genes was performed using an iTaqTM Universal Probes Supermix master mix, a validated FAM fluorophore-conjugated TaqMan probe and primers designed. The PCR reaction was performed by using a real time qPCR instrument.

Western blot analysis

Whole cell lysates were run on a 10% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. After blocking the primary antibodies were added, and after washing steps we added secondary antibodies. Antige-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system and the signals were detected on X-ray film.

ROS measurement

ROS formation was detected by DCFDA-assay (5-(6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester) according to manufacturer's protocol. In some of our experiments, we inhibited ROS formation by N-acetylcysteine (NAC).

IL-1β secretion in HUVECs

Following treatment, the supernatants were aspirated and IL-1β levels was determined from 100 μL of undiluted supernatant was used for ELISA assay, according to the manufacturer's protocol.

Patient selection and CSF collection

The liquor samples used for our studies in each case were left over from diagnostic purposes, which were collected at the Neurosurgery Department, at University of Debrecen. Premature infants (n=20) was diagnosed with grade III IVH. After sampling, the samples were centrifuged and the supernatants were aliquoted and stored at -70 ° C until analysis.

Determination of Hb, metHb, ferrylHb, total heme, free heme and bilirubin levels in CSF

To determine the levels of Hb, metHb, and ferrylHb in the CSF samples, the absorption spectra of the CSF samples were taken and the concentrations of each Hb oxidation product were calculated from the absorbance values measured at 541, 576, and 630 nm. The total heme concentration of the CSF samples was determined with the QuantiChrom Heme Assay Kit according to the manufacturer's protocol. Bilirubin levels were determined colorimetrically.

Endothelial cell monolayer integrity assay

We used the ECIS (electric cell-substrate impedance sensing) method to examine the integrity of the monolayer. HBEC cells were cultured in a special 8-well ECIS plate. After reaching confluence, cells were treated with different Hb forms and the complex impedance spectrum was examined with an ECIS ZΘ instrument.

Determination of soluble VCAM-1, ICAM-1 and IL-8 levels in CSF samples

Soluble VCAM-1 and ICAM-1 levels were analyzed by ELISA analysis according to manufacturer's protocol.

Cell viability assay

Cell viability was assessed by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay, according to manufacturer's instructions.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis of the results was performed with GraphPad Prism 8.01 software. Comparison between more than two groups were carried out by ANOVA, followed by post hoc Tukey's multiple comparisons test. A value of P <0.05 was considered statistically significant. According to the correlation studies, a value of $r > 0,7$ considered strong correlation.

4. Results

The proinflammatory nature of heme and its effect on IL-1β secretion in vivo

In our first experiment we investigated, we investigated the pro-inflammatory effect of heme in vivo. Therefore, heme was injected into the peritoneal cavity of C57BL/6 mice at the dose of 75, 150, and 300 nM heme/peritoneal cavity. After 16 hours the peritoneum was washed out and the number of monocytes and neutrophil granulocytes infiltrated into the peritoneal cavity and the level of IL-1β were measured. We found, that heme induced peritoneal infiltration of monocytes and neutrophil granulocytes in a dose-dependent manner. We found a 25-fold elevation of IL-1β in the peritoneal cavity of mice injected with heme vs. vehicle (300 nmol heme or vehicle/peritoneal cavity).

Heme enhances NLRP3 expression and IL-1 secretion in HUVECs

Previouly, Dutra et al. showed that heme activates the inflammasome complex containing NLRP3 / ASC / caspase-1 and induces active IL-1β production in LPS-primed macrophages. In our experiment, HUVECs were pretreated with LPS (10 μg/mL, 24 h) or left untreated and exposed to different concentrations of heme (10, 25, 50 μmol/L). We found, that heme increased NLRP3 mRNA levels in a dose-dependent manner. Subsequently, the effect of heme on IL-1β mRNA and protein expression was examined. We have proved that heme at high concentrations (50 μmol/L) slightly increases the levels of IL-1β mRNA and IL-1β production in HUVECs.

Heme decreases the viability of HUVEC at high concentration

In our next experiment, we examined the toxic effect of heme in LPS-pretreated or untreated HUVECs. We demonstrated that heme at lower concentrations (10, 25 μmol/L) have not affected endothelial cell viability significantly, even if we applied LPS pretreatment. However, heme at a concentration of 50 μmol/L significantly reduced the viability of HUVEC, which was independent of LPS pretreatment.

Integrity of heme is critical in heme-mediated IL-1β production in HUVECs

In this experiment, we investigated the role structural motifs PPIX and Fe^{2+} of heme in inflammasome activation. We found that neither PPIX nor free iron caused increase in IL-1β mRNA levels in LPS-pretreated HUVECs. Consistent with this notion, PPIX and Fe^{2+} failed to increase IL-1β production in the supernatant of LPS-pretreated HUVECs.

Increased ROS production plays a role in heme-mediated IL-1β production in HUVECs

We terated HUVECs with heme (12.5 and 25 μmol/L) and found that heme at both concentrations induced a signficant increase in ROS production, which was further enhanced by LPS pre-treatment. NAC treatment significantly inhibited ROS production of HUVECs, and importantly inhibited heme-induced production of IL-1β.

Heme binding is associated with decreased proinflammatory effect

In our following experiments, we examined whether albumin is able to inhibit heme-induced pro-inflamamtory effects. First we examined the effect of heme-albumin complex (H-A) on IL-1β expression in LPS-pretreated and control HUVECs. Compared to heme, H-A did not increase IL-1β mRNA levels and IL-1β secretion in LPS-pretreated HUVECs. Subsequently, we checked the effect of albumin on the prooxidant nature of heme. H-A, unlike heme, could not increase ROS production in LPS-pretreated HUVECs. Finally, we examined whether heme-albumin inhibit hem-induced leukocyte infiltration. Heme and H-A were injected into the peritoneal cavity of C57BL/6 mice at a dose of 300 nmol/mouse. We found that, unlike heme, H-A did not induce peritoneal infiltration of leukocytes.

Oxidized Hb forms could not trigger NLRP3 inflammasome activation in ECs

We investigated the effect of different oxidized Hb forms on HO-1 induction. HUVECs were treated with Hb, metHb, ferrylHb and heme at a concentration of 25 μmol/L heme. As expected, heme induced the most significant increase in HO-1 mRNA level. At the same time, both metHb and ferrylHb significantly increased HO-1 mRNA levels too. We could obtain the same effect in the expression of HO-1 protein. Heme was the most effective inducer, followed by ferrylHb and metHb.

In our next experiment, we investigated the effect of different Hb forms in the formation of intracellular ROS. At low concentrations (25 μmol/L), in contrast to heme, none of the oxidized Hb forms increased ROS production in HUVECs. Although, using different oxidized Hb forms at high concentration (250 μmol/L), we observed an increase in ROS production in HUVECs treated with metHb and ferrylHb.

Afterwards we investigated whether different forms of Hb induce IL-1β production in HUVECs. Control and LPS-pretreated HUVECs were treated with different oxidized Hb forms at low (25 μmol / L) and high (250 μmol / L) concentrations. High concentrations of ferrylHb resulted a 2.2-fold increase in IL-1β mRNA levels, whereas Hb and metHb did not increase IL-1β mRNA expression. We repeated this experiment in LPS-pretreated cells and we found that Hb forms could not enhance the LPS-induced IL-1β secretion.

The role of NLRP3 in heme-mediated IL-1β production

We investigated the role of NLRP3 in heme-induced IL-1β production in NLRP3-deficient mice *in vivo*. Heme (300 nmol/mouse) or PBS (control) was injected into the abdominal cavity of WT and NLRP3 $^{-/-}$ mice. Upon the activation of the caspase-1 enzyme it is cleaved and active 20 kDa (p20) caspase-1 is formed. Caspase p20 levels were increased approximately 5-fold in heme-treated WT mice compared to control, whereas no caspase activation was observed in the liver of heme-treated NLRP3 $^{-/-}$ mice. In parallel, we also examined the expression of IL-1β in these liver samples. Due to the activation of the NLRP3 inflammasome complex, caspase-1 cleaves pro-IL-1β, resulting the formation of active IL-1β, which we could detect in the liver samples from heme-treated WT mice. However, we could not observe any IL-1β production in the liver of heme-treated NLRP3-deficient mice.

Accumulation of different forms of Hb, free heme and bilirubin in the CSF upon IVH

In the second part of our work, we examined whether various Hb oxidation metabolits accumulate in CSF following IVH, and whether they contribute to the development of neuroinflammation. For that purpose, CSF samples were collected at the Department of Neurosurgery, University of Debrecen. We analyzed 20 CSF samples from patients diagnosed with grade III IVH. The samples were divided into three groups based on elapsed time between birth and sampling, 0-20 days, 21-40 days and 41-60 days. To evaluate Hb, metHb and ferrylHb levels in each sample we took the visible spectra of the CSF samples and we used the molar extinction coefficients described previously in literature. Hb levels levels significantly decreased according to the elapsed time between IVH and sampling, and in the 41–60 days sample we could not detect Hb. Similarly to Hb concentrations, metHb concentrations were high in the first group (0–20 days), which gradually decreased during our studies. Interestingly, we could detect ferrylHb only in two CSF samples, which were obtained in the first 20 days after the onset IVH.

Next, CSF samples were analyzed by western blot under reducing conditions. According to our studies, CSF samples contained different oxidized Hb forms, Hb monomer with a molecular weight of 16 kDa, Hb dimers (32 kDa) and tetramers (64 kDa). Densitometric analysis revealed that in CSF samples obtained from 0-20 days the Hb monomer form was the predominant and they contain less dimers and very low amounts of tetramers. Also, we observed a remarkable shift towards dimers and Hb tetramers by the time. We detected tetramers exclusively in CSF samples obtained between days 41 and 60.

Hb oxidation can lead to the dissociation of the heme group from the globin, giving a rise of the production of free heme (non-Hb bound heme). To check whether this occurs in the CSF after onset of IVH, first we determined the total heme content in CSF samples. In the 0-20 days group we measured the highest heme concentrations in the

21-40 days group we detected significantly lower heme concentrations, while in the 41-60 days group heme levels were below 1 µmol/L. Heme and Hb levels correlated strongly (r = 0.7296), which suggests that Hb is the main source of heme in the analyzed CSF samples.

In our next study, we calculated the free heme content of CSF samples. Similarly to the total heme levels, we found the highest levels of free heme in the samples taken 0-20 days after onset of IVH. In the CSF samples obtained between 21-40 days free heme content was significantly lower and in samples collected 41-60 days free heme was below 1 µmol/L. Bilirubin is a well-known degradation product of heme, therefore in our next experiment we determined the bilirubin levels of CSF samples. We identified the highest concentrations in the first group (days 0–20), which gradually decreased in time.

Prooxidant and proinflammatory effects of free heme and oxidized Hb forms in human brain microvascular endothelial cells (HBECs)

In our first experiment, we investigated whether different forms of Hb promote HO-1 in HBECs. Therefore, HBECs were treated with different forms of Hb and free heme (25 µmol/L). Oxidized Hb forms (metHb and ferrylHb) induced 18.2 ± 2.6-fold induction and 12.1 ± 3.1-fold induction in HO-1 mRNA levels, although Hb did not cause any increase in HO-1 mRNA level. Free heme revealed a much stronger inducer of HO-1 in HBEC, causing a nearly 1000-fold increase in HO-1 mRNA levels. In parallel, metHb and ferrylHb induced an almost 20-fold induction in HO-1 protein expression, whereas free heme induced an approximately 200-fold increase. Then we investigated the effect of heme and oxidized Hb forms on ROS production in HBEC. Cells were treated with Hb, metHb, ferrylHb and heme at concentrations of 10, 25, 50, 100 μmol/L. Heme provoked increasing ROS production on a dose-dependent manner in HBEC, whereas Hb forms did not affect ROS levels.

Afterwards, we investigated whether heme and different oxidized Hb forms impair HBEC monolayer integrity. Cells were cultured in 8-well electrode arrays. After reaching confluence, the cells were treated with Hb, metHb and ferrylHb solutions (50 μmol/L) and the changes of endothelial monolayer resistance was measured for 4 hours with an ECIS ZΘ instrument. The integrity of HBECs monolayer was significantly reduced by ferrylHb. In contrast Hb and metHb did not affect endothelial monolayer permeability.

In our next studies, we examined the effect of heme and oxidized Hb forms on the expression VCAM-1, ICAM-1, and IL-8 in HBECs. HBECs were treated with Hb, metHb, ferrylHb, and heme (25 μmol/L), and VCAM-1, ICAM-1, and IL-18 mRNA levels were measured after 4 hours. We observed, that free heme and ferrylHb caused significant increase in VCAM-1 mRNA levels, while Hb and metHb caused a minor increase, approximately 5-fold. We found the same trend in VCAM-1 protein expressions, heme and ferrylHb were the most potent inducers of VCAM-1 expression, compared to Hb and metHb. Additionally, heme and ferrylHb increased ICAM-1 mRNA expression and IL-8 secretion.

Correlation between heme levels and pro-inflammatory markers in post-IVH CSF samples

Based on our *in vitro* data, we concluded that Hb-derived heme may play a role in eliciting the inflammatory response after the onset of IVH. Therefore, the levels of sVCAM-1, sICAM-1, and IL-8 were determined in in post-IVH CSF samples. We found the highest sVCAM-1 levels in CSF samples obtained 0-20 days after onset of IVH. In comparison, we measured significantly lower sVCAM-1 levels at 41-60 days after the onset of IVH. sICAM-1 levels showed a decreasing trend, but the differences were not significant. Finally, IL-8 levels were analyzed. We found the highest levels in CSF samples obtained at 0–20 days following IVH. In addition, we observed a gradual decrease in IL-8 levels at 21–40 and 41–60 days upon IVH. Finally, we found a positive correlation between all the three inflammatory marker levels and total heme concentration in CSF samples.

5. Discussion

Exposure of ECs to classical DAMPs, such as ATP or HMGB-1, induce IL-1β production through activating the NLRP3 inflammasome. EC activation of NLRP3 inflammasome was already described in hyperglycemia and hypercholesterolemia. It is also already observed, that NLRP3 inflammasome activation in ECs plays a role in the pathomechanism of hemorrhagic shock.

Activation of various innate immune receptors (NLRP1, NLRP3, NLRC4, AIM2) leads to the assembly of the inflammasome complex, which induces the activation of inflammatory caspases and the subsequent formation and secretion of IL-1β. Previous studies have shown that heme induces IL-1β production in macrophages through the activation of the NLRP3 inflammasome. Our results showed, that heme induced the NLRP3 inflammasome activation in ECs, suggesting that ECs contribute to the inflammatory response in diseases related to intravascular hemolysis.

It is known that activation of NLRP3 inflammasome in macrophages requires two signals. The first signal provided mainly by the TLR or TNF receptor 1 and 2, triggering NF-κB-mediated NLRP3 expression. The second stimuli is caused by PAMPs or DAMPs that activate NLRP3. This leads to the assembly of the inflammasome platform, activation of caspase-1, and subsequent cleavage of pro-IL-1β and secretion of the active cytokine. This phenomenon was also confirmed by Dutra et al., which showed that LPS pretreatment is essential for heme-mediated NLRP3 inflammasome activation in macrophages. According to our experiments, we found that LPS pretreatment significantly enhanced IL-1β secretion in HUVECs. However, heme-induced IL-1β production in ECs is much weaker compared to macrophages. Although, heme-induced endothelial response was comparable to the known inducers (ATP, HMGB1). We found that, high concentration of heme induces EC death. This gives the possibility that ATP and HMGB1 released upon heme-mediated cell death enter to the extracellular space during cell necrosis and further exacerbate heme-induced IL-1β formation.

Heme increased NLRP3 mRNA and protein expression in both the presence and absence of LPS in HUVECs. We investigated the role of NLRP3 in heme-mediated IL-1β production in NLRP3-deficient mouse model. We showed that heme induced caspase-1 activation and the cleavage of pro-IL-1β in wild-type mice, but the contrary heme failed to increase caspase-1 activation and pro-IL-1β cleavage in NLRP3-deficient mice. This suggests that NLRP3 plays a central role in heme-induced IL-1β production.

Heme consist of the PPIX ring and an central iron ion, which is stabilized by four N-Fe coordinate covalent bonds. Intracellularly, heme is broken down by the heme oxygenase enzyme family, yielding iron, biliverdin, and carbon monoxide. The iron released from heme contributes to labile iron pool of cells. Recently iron has been shown to induce NLRP3 inflammasome activation in human monocytes. Here we showed that, unlike monocytes, iron itself is unable to induce IL-1β production in HUVECs. Furthermore, we demonstrated that PPIX fail to induce NLRP3 inflammasome activation and IL-1β production in LPS-pretreated ECs. Overall, we concluded that the coordinated iron in heme plays a critical role in NLRP3 inflammasome activation and subsequent IL-1β formation and secretion.

The role of increased ROS in the heme-mediated activation of the NLRP3 inflammasome in macrophages is known, therefore, we investigated the role of ROS in heme-induced NLRP3 inflammasome activation in ECs. We showed that heme induces ROS production in HUVECs and observed that LPS pretreatment synergistically increased heme-mediated ROS production in HUVECs. When we applied the well-known ROS scavenger NAC, it decreased heme-induced ROS production as well as IL-1β secretion in HUVECs, suggesting that increased ROS production plays a central role in heme-mediated NLRP3 inflammasome activation. Besides increased ROS production NLRP3 inflammasome activation may be affected by other factors. Dutra et al. showed that heme-mediated inflammasome activation in macrophages was dependent on ROS production and K^+ ion efflux, but independent on lysosomal destabilization. Further studies are needed to check whether increased K^+ ion efflux and lysosomal destabilization play a role in heme-mediated NLRP3 inflammasome activation in HUVECs.

The pro-oxidant and pro-inflammatory effects of extracellular heme are controlled by specific and non-specific heme-binding plasma proteins. Hx is an acute-phase plasma protein that binds heme with the highest affinity of any known protein. Due to this, Hx is a key player in the defense system against extracellular heme. Besides Hx, several other plasma proteins have heme-binding capacity, which also play a role in the case of massive intravascular hemolysis. Given the amount of albumin, it is the major non-specific heme-binding protein in plasma. Here we demonstrated that albumin inhibited heme-mediated NLRP3 inflammasome activation in LPS-pretreated HUVECs *in vitro* and was able to block peritoneal leukocyte infiltration *in vivo* in C57BL/6 mice.

In previous studies it was shown that metHb and ferrylHb act as heme sources for ECs. In addition, ferrylHb activates the NF-κB pathway, and enhances the expression of adhesion molecules in ECs and increases monolayer permeability too. These pro-inflammatory effect of ferrylHb leading to the activation of ECs and the impairment of monolayer integrity are independent of heme release, and only linked to ferrylHb as neither Hb, nor metHb exhibit such activities. ECs exposed to heme upregulate HO-1, an enzyme which break down heme. In our work, we demonstrated that the oxidized forms of Hb, like metHb and ferrylHb, release their heme prosthetic group leading to increased HO-1 mRNA and protein expression in HUVECs. Our data suggest, that metHb and ferrylHb are much weaker inducers of HO-1 compared to heme.

Free heme enhances ROS formation in HUVECs. We showed that oxidized Hb forms, metHb and ferrylHb, also increase ROS production in ECs but this effect was significantly weaker compared to the effect of heme. Considering that increased ROS production is associated with NLRP3 inflammasome activation, we investigated whether oxidized Hb forms are able to induce NLRP3 inflammasome activation and IL-1β production in HUVECs. We found, that neither metHb nor ferrylHb induced NLRP3 inflammasome activation in HUVECs.

In the second part of my dissertation, we analyzed CSF samples obtained from preterm infants following IVH. Here we investigated whether different oxidized Hb forms play a role in neuroinflammatory response after onset of IVH, as IVH is a common complication in preterm infants.

Our primary goal was to perform qualitative and quantitative analysis of CSF samples collected from preterm infants after IVH. We proposed to the better understanding the kinetics of Hb accumulation and the oxidized products formed during Hb oxidation. It is important to highlight that the CSF samples studies here were taken for diagnostic purposes, so we could not influence the time of sampling. Samples were divided into three groups based on the elapsed time between IVH and sampling. The three groups were the fllowing: 0-20 days, 21-40 days, 41-60 days after onset of IVH. We confimerd here, that upon IVH, Hb accumulates in the CSF and during its oxidation metHb, ferrylHb, and covalently cross-linked oxidized globin multimers are formed. In the CSF samples which were analyzed, the level of metHb at 0-20 days was 80.51 ± 77.65 μmol/L. In agreement with previous studies in CSF samples obtained between 21-40 days, we found similar levels of metHb (~40 μmol/L).

Two-electron oxidation of Hb by peroxides leads to the formation of ferrylHb, in which the oxidation state of iron is +4, which is unstable. Interestingly, it has previously been detected in blood under physiological and pathophysiological conditions, but has not been studied in the CFS. In two CSF samples out of twenty we detected ferrylHb at 0-20 days.

The high-valent iron (Fe^{4+}) in ferrylHb triggers the oxidation of specific amino acids of the globin chains following intermolecular cross-linking of the globin-units. The covalently cross-linked oxidized

Hb metabolites have been detected already in plasma, urine, and hemorrhagic atherosclerotic plaques. In this study, we were the first to detect covalently cross-linked oxidized Hb multimers in CSF samples at different time points following IVH.

Following intravascular hemolysis, extracellular Hb is eliminated from plasma through the Hp-CD163 pathway. Hp is present in the CSF as well, but in a much lower concentration compared to plasma. Regarding preterm infants, the Hp content and Hb-binding capacity of the CSF are still unknown, but our data suggest that after IVH the Hb-binding capacity of the CSF is overwhelmed leading to Hb accumulation and oxidation. Thus, Hb and and oxidized Hb species may enter the periventricular white matter from the intraventricular space, contributing to the development of neuroinflammation following IVH.

Total and free heme content in the liquor samples were also determined. We found a wide range in free -non-Hb-associated- heme levels in samples, obtained 0-20 days after IVH (42-717 µmol/L). The source of free heme is probably metHb and ferrylHb, which bind the hem prosthetic group with weaker affinity.

During intravascular hemolysis, free heme which enters to the plasma is bound by Hx and removed from the circulation via the CD91 receptor. The Hx-CD91 system is exist in the central nervous system and plays a key role in heme detoxification after subarachnodial hemorrhage. However, we are lack of information on the defense system of the central nervous system in preterm infants against heme stress and Hx levels in their CSF. Our results suggest that in preterm infants the Hx-CD91 pathway is overwhelmed, leading to accumulation of heme in the CSF after IVH.

Hb, metHb, ferrylHb, heme and free heme levels were also lower at 21-40 days compared to samples obtained at 0-20 days. Furthermore, we could hardly detect any heme in samples obtained 41-60 days. This suggests an active or passive relatively slow removal mechanism of heme from the CSF.

We also examined the presence of covalently crosslinked Hb multimers in CSF samples after IVH and we found a shift towards the formation of higher multimers at later time points. At 0-20 days Hb monomers and dimers were dominant, furthermore in samples collected between 41-60 days we detected Hb tetramers exclusively. Taking into consideration, that we could not detect any heme in these CSF samples, we can conclude that these covalently-cross linked Hb multimers have already released their heme prosthetic groups. The presence of this form of Hb at 41-60 days after the onset of IVH, suggests that there is no efficient mechanism in the central nervous system to eliminate these oxidized Hb forms or this mechanism remains very slow.

The blood-brain barrier prevents blood cells and pathogens from entering the brain parenchyma and regulates the transport of molecules between plasma and central nervous system. The brain microvascular EC monolayer is an important component of the blood-brain barrier and contributes to the neuroinflammatory response, mainly inducing leukocyte adhesion cascade which facilitate the transmigration of inflammatory cells into the central nervous system.

Therefore, we investigated the effect of different Hb forms detected in CSF samples from preterm infants in HBECs.

We demonstrated that heme at concentration above 50 µmol/L significantly enhances ROS production in HBECs and induces cell death. However, Hb and oxidized Hb forms, could not increase ROS production and were not toxic for HBECs. The free heme concentration of the liquor samples obtained at 0-20 days was high, between 50 and 250 µmol/L. We studied also the integrity of the HBEC monolayer and we showed that ferrylHb which contains covalently cross-linked Hb forms largely increase the permeability of the monolayer.

HO-1, an enzyme that catalyzes the breakdown of heme, is induced following ICH in various cells types of the central nervous system, such as astrocytes, microglia, and ECs. In our experiments, we showed that besides heme, oxidized forms of Hb (metHb, ferrylHb) also induce HO-1 expression in HBECs.

Adhesion molecules play a central role in the inflammatory response through adherence to the endothelial surface and transmigration. Under normal conditions, the vascular endothelium is characterized by low expression of adhesion molecules. In cerebral hemorrhage, a local inflammatory response develops and the levels of soluble adhesion molecules (E-selectin, ICAM-1, VCAM-1, and L-selectin) and inflammatory cytokines become elevated in the CSF. The inflammatory response is presumably associated with the damage of the white matter.

Heme is known to induce EC activation characterized by increased expression of adhesion molecules and increased production of proinflammatory cytokines (IL-6, IL-8). Here we showed, that levels of VCAM-1, ICAM-1, and IL-8 correlated to heme levels in post-IVH CSF samples. We found that besides heme, ferrylHb may also contribute to the activation of brain microvascular endothelial cells.

6. Summary

In the pathogenesis of hemolysis-associated diseases inflammation often occurs, even in the absence of pathogens. This is believed to be caused by RBC-derived DAMPs, which enter to the bloodstream. Upon RBC lysis Hb is prone to oxidation, resulting oxidized Hb species, metHb and ferrylHb. These Hb forms can easily release their heme prosthetic group. The pro-inflammatory nature of heme is already well-described and there is an evidence for heme-mediated NLRP3 inflammasome activation and IL-1β secretion in macrophages.

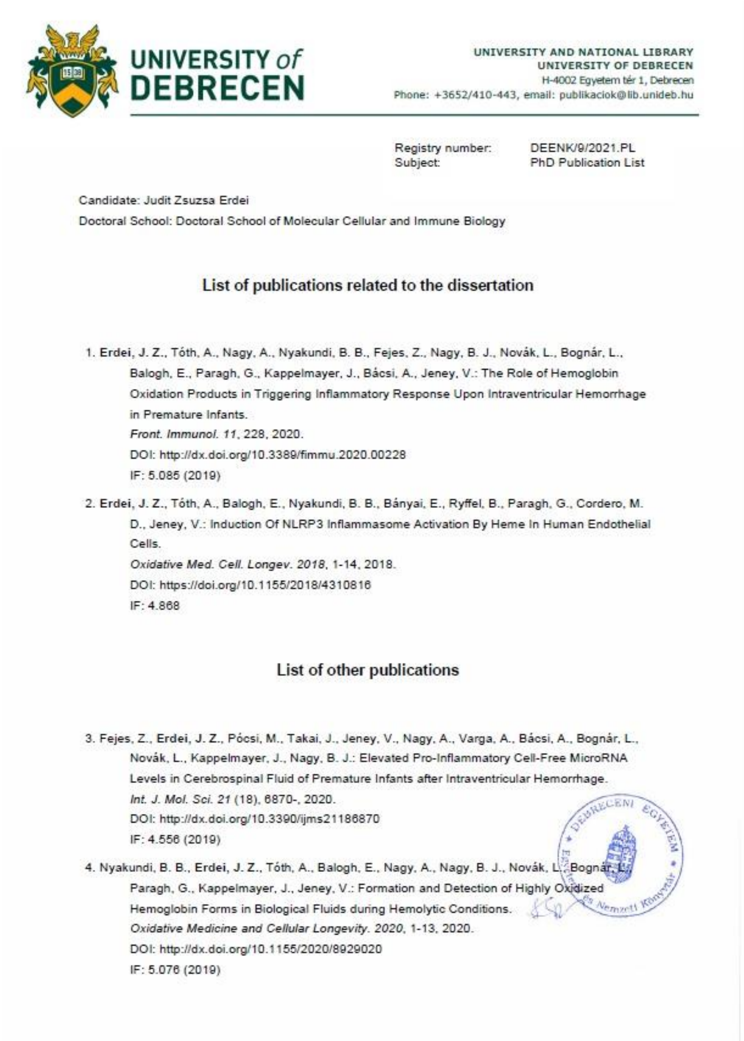
ECs play a crucial role in inflammatory response, thus we wanted to examine the effect of heme and the oxidized Hb forms in EC activation. Here we demonstrated, that heme induces IL-1β secretion in LPS-primed HUVECs. We showed, that heme triggers caspase-1 activation and active IL-1β secretion in an NLRP3-dependent manner *in vivo*. Furthermore, we proved that ROS have a key role in heme-induced pro-inflammatory response, as the ROS scavenger NAC inhibited IL-1β formation. Moreover, we showed that albumin prevents heme-mediated IL-1β secretion in HUVECs *in vivo*.

In addition albumin blocked heme-induced peritoneal leukocite infiltration in C57BL/6 mice.

In our work, we also studied the inflammatory role of heme and oxidized Hb forms after onset of IVH. We presented that upon IVH heme, Hb, metHb, ferrylHb and the covalently cross-linked globin multimers accumulate in the CSF. However, ferrylHb revealed the strongest pro-inflammatory effect, therefore induced the expression of adhesion molecules and pro-inflammatory citokines in HBECs, as well as impaired the integrity of the monolayer.

Overall, our study may contribute to the understanding the role of Hb-derived DAMPs in NLRP3 inflammasome activation. In addition, it increase our knowledge about the role of Hb-associated DAMPs in the pathogenesis of IVH.

7. List of publications



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Registry number: DEENK/9/2021.PL
Subject: PhD Publication List

Candidate: Judit Zsuzsa Erdei
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Erdei**, J. Z., Tóth, A., Nagy, A., Nyakundi, B. B., Fejes, Z., Nagy, B. J., Novák, L., Bognár, L., Balogh, E., Paragh, G., Kappelmayer, J., Bácsi, A., Jeney, V.: The Role of Hemoglobin Oxidation Products in Triggering Inflammatory Response Upon Intraventricular Hemorrhage in Premature Infants.
   Front. Immunol. 11, 228, 2020.
   DOI: http://dx.doi.org/10.3389/fimmu.2020.00228
   IF: 5.085 (2019)
2. **Erdei**, J. Z., Tóth, A., Balogh, E., Nyakundi, B. B., Bányai, E., Ryffel, B., Paragh, G., Cordero, M. D., Jeney, V.: Induction Of NLRP3 Inflammasome Activation By Heme In Human Endothelial Cells.
   Oxidative Med. Cell. Longev. 2018, 1-14, 2018.
   DOI: https://doi.org/10.1155/2018/4310816
   IF: 4.868

List of other publications

3. Fejes, Z., **Erdei**, J. Z., Pócsi, M., Takai, J., Jeney, V., Nagy, A., Varga, A., Bácsi, A., Bognár, L., Novák, L., Kappelmayer, J., Nagy, B. J.: Elevated Pro-Inflammatory Cell-Free MicroRNA Levels in Cerebrospinal Fluid of Premature Infants after Intraventricular Hemorrhage.
   Int. J. Mol. Sci. 21 (18), 6870-, 2020.
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   IF: 4.556 (2019)
4. Nyakundi, B. B., **Erdei**, J. Z., Tóth, A., Balogh, E., Nagy, A., Nagy, B. J., Novák, L., Bognár, L., Paragh, G., Kappelmayer, J., Jeney, V.: Formation and Detection of Highly Oxidized Hemoglobin Forms in Biological Fluids during Hemolytic Conditions.
   Oxidative Medicine and Cellular Longevity. 2020, 1-13, 2020.
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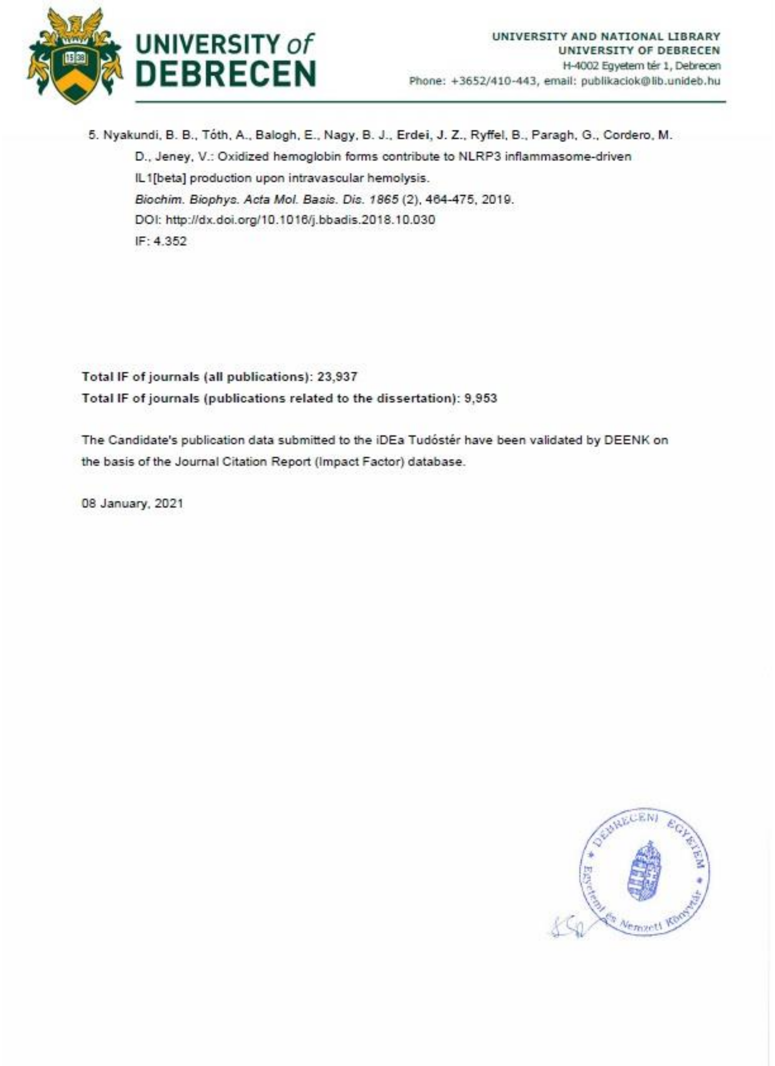
   IF: 5.076 (2019)



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5. Nyakundi, B. B., Tóth, A., Balogh, E., Nagy, B. J., Erdei, J. Z., Ryffel, B., Paragh, G., Cordero, M. D., Jeney, V.: Oxidized hemoglobin forms contribute to NLRP3 inflammasome-driven IL1[beta] production upon intravascular hemolysis.
Biochim. Biophys. Acta Mol. Basis. Dis. 1865 (2), 464-475, 2019.
DOI: http://dx.doi.org/10.1016/j.bbadis.2018.10.030
IF: 4.352

Total IF of journals (all publications): 23,937
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