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The impact of Hemoglobin oxidation in NLRP3 inflammasome activation upon intravascular hemolysis

by

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The impact of Hemoglobin oxidation in NLRP3 inflammasome activation upon intravascular hemolysis

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Theoretical medicine

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INTRODUCTION

Hemoglobin (Hb) protein characterizes the main properties of red blood cells (RBCs) as it forms 96% of RBC content and fundamentally involved in physiological respiration. Hb is a tetramer composed of four polypeptide chains (2β and 2α) with a prosthetic oxygen-binding heme tightly bound to each subunit and folded in a globular shape. RBCs are continuously exposed to high oxygen and Iron, a perfect recipe for oxidative stress, leading to the generation of reactive oxygen species (ROS). As a result, functional RBCs are equipped with an antioxidant system to counteract the deleterious effects of ROS. On the other hand, aged RBCs express surface markers such as flipped phosphatidylserine and phosphatidylethanolamine reduced expression of sialic acid and decreased expression of CD47 enabling the macrophages to phagocyte and process them in the liver and spleen. The interaction between aged cells and erythrophagocytes is accomplished through receptor-ligand binding, opsonization, and antigen-antibody mechanisms. In the macrophages, heme is broken down into Iron and majorly recycled, CO and biliverdin are further utilized in other cellular functions.

Hemolysis

Hemolysis is the breakdown of RBCs membrane leading to the release of cellular content, whenever the RBCs lyse in the blood vessels it is referred to as intravascular hemolysis and extravascular hemolysis occurs when the RBCs are destroyed in the spleen and liver. Intrinsic factors that can cause the immature destruction of RBCs include membrane abnormalities, hemoglobinopathies, and RBCs enzyme defects. Besides, extrinsic factors of hemolysis may be due to immune cross-reactions, mechanical stress, infections, malaria, and drugs.

Haptoglobin- hemopexin heme oxygenase-1 ferritin defense system

Extracellular Hb is susceptible to oxidation and subsequent release of heme. Cell-free Hb depletes Nitric Oxide (NO) which in turn compromises the vascular tone, oxidized Hb and heme potentially trigger pro-inflammatory, pro-oxidant, and pro-thrombotic reactions in the vasculature. The deleterious extracellular Hb requires a defense system to counteract its effects. Haptoglobin (Hp), a tetramer of 2α and 2β polypeptide chains connected through disulfide bridges, is the first molecule released to bind cell-free Hb. It is characterized by molecular heterogeneity of three subtypes (Hp1-1, Hp2-1, and Hp2-2) in humans. Hp bind Hb in a ratio of 1:1 and a very stable

noncovalent Hp-Hb association allowing the efficient clearance of cell-free Hb in a CD163 receptor-mediated endocytosis manner by macrophages. Moreover, CD163 receptors have been shown to bind extracellular Hb directly. Hp Polymorphisms are associated with the prevalence and clinical progression of many inflammatory diseases. This effect is influenced by phenotype-dependent modulation of oxidative stress and prostaglandin synthesis.

Massive hemolysis exhausts the binding capacity of Hp leading to the accumulation and oxidation of Hb and release of heme subsequently. The second phase of protective proteins in plasma is released to bind free heme. Hemopexin (Hx) is a major acute-phase protein with the affinity ($K_d < 10^{-12}$ M) in humans) that binds heme to suppress the prooxidant activity of extracellular Heme and facilitates its clearance. Hx-Hb complex is recognized by the CD91 receptor on macrophages and hepatocytes and taken up in a receptor-mediated endocytosis manner. Other proteins involved in the binding of cell-free heme are albumin, Lipoproteins, and α_1-microglobulin. Intracellularly, inducible heme oxygenase-1(HO-1), catalyzes the breakdown of heme into equimolar amounts of Fe, CO, and biliverdin (BV). Further, Fe is recycled and stored for the synthesis of RBCs, while BV is converted to

bilirubin by biliverdin reductase and CO acts as a gasotransmitter. BV and CO exhibit antioxidant and cytoprotective properties respectively.

Hb oxidation

In a physiological setting, iron exists in the ferrous state in the heme pocket. However, oxygen binding may trigger an autoxidation converting ferrous iron to ferric iron accompanied by superoxide. Normally this reaction is controlled by the antioxidant system present in the RBCs. During hemolysis, cell-free Hb accumulates and undergoes a series of oxidation reactions as shown in equations below forming metHb, ferryHb, and their respective radicals. The termination of globin radicals is accomplished by covalent cross-linking of the globin chains.

(1) $Hb(Fe^{2+})O_2 \rightarrow Hb(Fe^{3+}) + O_2^{\bullet -}$

(2) $Hb(Fe^{2+})O_2 + H_2O_2 \rightarrow Hb(Fe^{4+}= O^{2-}) + H_2O + O_2$

(3) $Hb(Fe^{3+}) + H_2O_2 \rightarrow Hb^{\bullet +}(Fe^{4+}= O^{2-}) + H_2O$

(4) $Hb(Fe^{4+}= O^{2-}) + 2H^+ \rightarrow Hb^{\bullet +}(Fe^{3+}) + H_2O$

(5) $Hb^{\bullet +}(Fe^{3+}) + Hb^{\bullet +}(Fe^{3+}) \rightarrow (Fe^{3+})\ ^{+}Hb\text{-}Hb^{+}(Fe^{3+})$

Studies of Hb with H_2O_2 in the presence of spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO), have revealed that

βCys93, αTyr24, αTyr42, and αHis20 residues from both alpha and beta chains can be the target of globin radical formation.

Danger theory and DAMPs

Besides the classical pathogen-induced immune response, Poly Matzinger (1994) proposed that immune response can be triggered by 'danger signals' or 'alarm signals' released from the cells of an organism. The theory was later named as 'damage theory' to reflect the fundamental cause of this response as essentially cellular stress or tissue damage. Damage associated molecular patterns (DAMPs) are molecules released by a perturbed or dying cell with the potential to trigger an immune response. Several categories of molecules have been identified as DAMPs due to their ability to activate antigen-presenting cells. DAMPs may originate Extracellularly for example hyaluronan and proteoglycans, as well as intracellular molecules that can be released from all cellular compartments with the ability to act as DAMPs whenever the cell is damaged/stressed. Nucleus-originating DAMPs are histone proteins and nuclear proteins such as high mobility group box-1. The Mitochondria too releases ATP and mtDNA and cytosolic-originating DAMPs may be heat shock proteins and uric acid. Moreover, different forms of exogenous and endogenous crystals provoke an immune response in chronic

inflammation. The commonly studied crystals are Cholesterol crystals in atherosclerosis, monosodium urate crystal in gouts, calcium pyrophosphate dihydrate crystals in pseudogout, and amyloid fibrils in Alzheimer.

DAMPs sensors

Both immune and non-immune cells display a cluster of germline-encoded pattern recognition receptors (PRRs) for the recognition of PAMPs/ DAMPs. Among them are type 1 transmembrane receptors TLRs which are characterized by leucine-rich repeat (LRR) domain and cytosolic toll/IL-1 receptor (TIR) domain. Generally, receptor-agonist interaction triggers TLRs dimerization and subsequent binding of molecules that culminate in the translocation of nuclear factor kappa B (NF-κB) to the nucleus which in turn initiates the expression of target genes. Additionally, is the vast family of intracellular PRRs Nod-like receptors (NLRs) with a basic structure of C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding oligomerization domain NOD or NACHT, and N-terminal caspase recruitment (CARD) or pyrin (PYD) domain used to determine the subgroups of NLRs. NOD-, LRR- and pyrin domain-containing protein 3(NLRP3) is the most studied intracellular multiprotein complex molecular sensor that detects a broad range of DAMPs

and/or PAMPs resulting to the assembly of a cluster of protein known as inflammasomes. Two signals are required to activate NLRP3 inflammasome, the first signal activates NF-κB pathway, and the transcription of pro-IL-1β whereas the second signal comprises of a wide range of PAMPs and stress-related signals or DAMPs such as potassium efflux, ROS generation, and the release of cathepsin B that initiate the assembly of the inflammasome complex.

Heme as TLR4 and NLRP3 ligand

Heme has been found to induce TNF-α secretion in macrophages but not iron or protoporphyrin IX, moreover, heme induces programmed necrosis on macrophages through TLR4/Myd88 dependent release of TNF-α and ROS production. In a mouse model of intracerebral hemorrhage-induced neuro-inflammation, TLR4 deficient mice exhibited less severe inflammation compared to wildtype mice. moreover, Heme-mediated IL-1β production is dependent on caspase-1 activation and NLRP3 as neither caspase-1 deficient, nor NLRP3 deficient mice respond to heme treatment.

Other RBCs derived DAMPs

Together with Hb-derived DAMPs, other RBCs components have been shown to trigger an immune response hence considered as potential DAMPs. For instance, ATP binds to P2-

purinergic receptors and triggers inflammatory responses in various immune and non-immune cells. IL-33 also activates innate immunity and contributes to inflammatory diseases, and finally, RBC-derived macroparticles deplete NO and augment systemic inflammation through thrombin-dependent activation of the complement system and endothelial activation via heme transfer.

AIMS

Non-Hb-bound heme is a DAMP, TRL4 ligand, and an inducer of NLRP3 inflammasome activator. However, Merle and colleagues showed that IVH-induced renal injury was largely heme independent since the injection of free heme alone could not trigger the same renal alterations. This observation leads to the assumption that hemolysis products upstream of heme are involved in the detrimental effects of massive hemolysis. This work aimed to provide a detailed analysis of extracellular heme forms following IVH and to investigate the contributions of these heme forms to hemolysis-associated inflammation, particularly NLRP3 inflammasome activation and subsequent production of IL-1β. We accomplished this by addressing the following questions.

- What kinds of Hb species can be detected in the mice plasma following PHZ-induced IVH?
- Does PHZ-induced IVH trigger IL-1β production *in vivo*?
- Which Hb redox forms induce IL-1β production in macrophages *in vitro*?
- Which Hb redox forms behave in a pro-inflammatory manner *in vivo*?
- Is IVH-induced IL-1β production dependent on NLRP3 inflammasome activation?
- If NLRP3 deficiency provides a survival advantage to mice is it associated with increased resistance or increased tolerance to IVH?
- In which manner and how fast does ferrylHb decompose after its formation?

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Hb preparation and measurement

Blood was drawn from a health volunteer and Hb was isolated by using ion-exchange chromatography on a DEAE Sepharose CL-6B column (Sigma-Aldrich). From the purified Hb we generated metHb and ferryHb by incubating with a 1.5-fold molar excess of $K_3Fe(CN)_6$ and 10-fold molar excess of H_2O_2 over heme respectively. The term ferrylHb does not reflect the real oxidation status of heme iron but rather how the molecules were formed. The Hb solutions were dialyzed, concentrated then aliquoted and snap-frozen in liquid nitrogen, afterward they were stored in -72°C for later use. The purity of Hb samples was evaluated by SDS-PAGE followed by silver staining, and the endotoxin content of Hb preparations was measured by Limulus amebocyte lysate assay. The purity of Hb was above 99.9%. To determine Hb forms concentrations from the full spectra absorption between (250-700), we used the method of Winterbourn (Winterbourne CC 1990) to determine Hb, metHb and hemichrome concentrations and the published method of Meng and Alayash to calculate Hb, metHb and ferrylHb concentrations. Finally, we applied the equation: [non Hb-bound heme] = [total heme] − [Hb heme] − [metHb heme] − [hemichrome heme] to calculate free heme.

PHZ IVH-induced and peritoneal mice models

All the mice were kept in the conventional university experimental house. Two injections of PHZ were administered intraperitoneal (i.p) to the mice. First, 50mg/kg of PHZ dissolved in PBS was injected and 16h later the second injection of PHZ was administered at the dosage of 30 mg/kg body weight. The control received PBS and the mice were sacrificed at 4h, 16h, and 20h time points. In a peritonitis mouse model, WT mice (n=25, male and female, 8-10 weeks old, 5 mice/group sex-matched) were injected with heme, Hb, metHb, and ferrylHb (300 nmol heme/cavity) or LPS (100 µg/ cavity) in a volume of 200 µl using PBS as the vehicle. The control mice were injected with 200 µl of sterile PBS. After 16h of the injection, The mice were euthanized by CO_2, and infiltrated peritoneal leukocytes were collected by peritoneal lavage using 5 mL of ice-cold PBS containing 2% FCS. The collected cells were analyzed by flow cytometry.

Cell culture and treatment

Murine RAW 264.7 macrophage cell line was purchased from ATCC (Manassas, VA, USA). The RAW cells were cultured in supplemented DMEM (10% heat-inactivated fetal bovine serum (Gibco, Waltham, MA, USA), L-glutamine, and 1% penicillin/streptomycin) and incubated in 5% CO_2 humidified

atmosphere at 37°C. on the other hand, Primary mice bone marrow-derived macrophages (BMMs) were isolated from tibia and femur of 8-12 weeks old WT mice. The cells were counted by hemocytometry and re-suspended in complete medium supplemented with 50ng/ml M-CSF and seeded at the density of 3×10^5 cell/well in 96-well tissue culture plates. The BMMs differentiated after 7days and non-adherent cells were discarded. Both cells were LPS- primed for 4h and later they were treated with heme (25 μmol/L) and different Hb redox forms (25-150 μmol/L heme groups) at different duration in respect to the experiment.

Molecular analysis

Cell viability was determined using MTT assay. RNA isolation was done using (RNA-STAT60, Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's protocol and the cDNA was obtained by reverse transcribing of RNA by High-Capacity cDNA Reverse Transcription Kit. Quantitative RT-PCR of IL-1β, HO-1, and GAPDH was performed. Protein from the liver and whole-cell lysates were probed for caspase-1 IL-1β and HO-1 using their respective primary antibodies and detected by secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit (NA931) and anti-mouse (NA934) Amersham Biosciences Corp., Piscataway, NJ,

USA). HRP-conjugated anti-β-actin antibody was used to probe for housekeeping gene β-actin. We detected Hb by the use of an HRP-conjugated polyclonal anti-Hb antibody (ab-19362, Abcam, Cambridge, UK). Secreted IL-1β in cellular supernatant was determined by ELISA.

RESULTS

Different forms of heme accumulate in plasma upon IVH

To establish an IVH model, we injected (i.p) WT mice with PHZ (50mg/kg bodyweight and 30mg/kg body weight 16h later) while the control mice were injected with sterile PBS. A brownish/yellow discoloration of plasma, enlargement of the spleen, and decreased hematocrit levels were observed in PHZ-injected mice. Total heme levels were strikingly higher compared to the controls and increased further after the second injection given at 16h. next, we determined the concentration Hb forms (Hb, metHb, and hemichrome) in the plasma and detected higher levels of hemichrome at 4h time point and in later time points, Oxidized Hb forms were dominant as plasma levels of non-Hb bound heme increased steadily with time after the PHZ injection.

PHZ-triggered IVH induces caspase-1 activation and processing of IL-1β in mice liver

We measured plasma levels of IL-1β in PHZ treated mice and detected increased levels of IL-1β at 16h and 20h post-injection. At the same time, HO-1 was upregulated in the liver samples indicating that extracellular Hb was taken up and catabolized by the liver cells. We detected a time-dependent increase of IL-1β in the liver that corresponded with processed caspase-1 levels in PHZ-injected mice as compared to the control group. Suggesting the involvement of NLRP3 in the release of mature IL-1β.

Involvement of Hb forms with different redox states in IVH induced IL-1β formation

We observed IL-1β upregulation when oxidized Hb forms occupied the larger portion of the total heme. Therefore, we hypothesized that these oxidized Hb forms may play a role in the formation of IL-1β. We treated the LPS-primed RAW macrophages with heme and Hb forms. In agreement with previous data, heme conspicuously induced both mRNA and protein levels of mature IL-1β. Hb forms induced IL-1β mRNA upregulation in a concentration-dependent manner. However, among the Hb forms, ferrylHb induced both mRNA and protein levels of IL-1β as heme did in the cellular supernatant. We

confirmed these results by observing the same trend in the BMMs.

Heme and oxidized Hb forms induce peritoneal infiltration of neutrophils and monocytes/macrophages

We investigated the pro-inflammatory effects of heme and Hb forms in vivo by administering 300nm/mol heme/cavity into the peritoneal cavity of WT mice. The peritoneal cavity was rinsed 16h after the injection and the number of neutrophils and inflammatory monocytes was determined. Heme and ferrylHb exceptionally induced the infiltration of neutrophils and monocytes while metHb induced only neutrophils and Hb induced neither of the leukocytes.

Heme and oxidized Hb forms induce caspase-1 activation and IL-1β processing in the liver of C57BL/6 mice

After we had observed caspase-1 activation and IL-1β release in the liver samples of PHZ-induced hemolysis mice, we further investigated the oxidized Hb form responsible for this action in the liver. Therefore, we analyzed the liver samples of the mice that were injected with heme and Hb forms. FerrylHb was the most potent inducer of caspase-1 activation, triggering an 8-fold Casp-1 p20 induction. At the same time, we detected a significantly increased level of active IL-1β (IL-1β p20) in the liver samples of ferrylHb and heme-treated mice.

NLRP3 deficiency confers a survival advantage against IVH-mediated lethality

To address the role of NLRP3 in severe hemolysis, we conducted a survival experiment in a PHZ-induced IVH and compared survival rates of WT and NLRP3 deficient mice. Between days 2-6 after the experiment, 75% of WT mice died while 33% of PHZ injected NLRP3 deficient mice succumbed within the same period. We then examined if there was any difference in the degree of hemolysis between the two genotypes by comparing the hematocrit and levels of Hb form. There were no differences in the hematocrit or plasma Hb forms (Hb, metHb, and hemichrome) levels in WT and NLRP3$^{-/-}$ mice.

NLRP3 plays a key role in hemolysis-associated IL-1β production

Our previous results showed that IVH induces IL-1β processing in the liver samples of WT mice. We further investigated the role of NLRP3 in IL-1β release by injecting PHZ to WT and NLRP3$^{-/-}$ mice and analyzed their respective liver samples for IL-1β. The processing of IL-1β in the liver samples of NLRP3$^{-/-}$ did not occur, unlike WT mice where we observed an increased expression of IL-1β p20. similarly, the

injection of ferryHb did not induce IL-1β processing in the liver of NLRP3$^{-/-}$ mice.

Detection challenge of ferrylHb in biological samples

We calculated plasma levels of Hb, metHb, and hemichrome using the winterbourne method but not ferrylHb. However, we detected covalently crosslinked Hb dimers in the plasma and urine samples of PHZ treated mice and assumed the dimers may have been a result of intermolecular electron transfer between ferryl ion and the susceptible amino acid of the globin chain. We could not detect ferryHb in a biological sample either by using Meng and Alayash method.

Kinetic of ferrylHb formation, decomposition, and cross-linked formation *in vitro*

To bridge the gap between ferrylHb formation and the detection of covalently cross-linked Hb species, we reacted Hb and metHb with H_2O_2. The reaction of Hb forms with H_2O_2 triggers 2-electron oxidation of heme iron. Then we measured the concentrations of Hb, metHb, and ferrylHb. We observed a dose-dependent formation of metHb when we reacted Hb with different concentrations (125-500 µmol/L) of H_2O_2. But also, a low amount of ferryHb was formed in a dose-dependent manner. On the other hand, when we reacted metHb with H_2O_2 we detected a significant formation of ferryHb in all

concentrations. However, ferrylHb formation inversely correlated with the concentration of H_2O_2. Because ferryHb oxidation status is transitory, we hypothesized that ferryHb might have been converted to a covalently cross-linked form which was detected as metHb in the absorption spectra scan. Therefore, we subjected the products generated in the reactions between Hb and metHb with H_2O_2 to denaturing SDS-PAGE and detected Hb through immunoblotting. We observed a dose-dependent Hb dimer formation in a reaction between Hb and H_2O_2. Moreover, in the reaction between metHb and H_2O_2, we observed more pronounced Hb dimers and tetramers formation. In a similar set of the experiment, we analyzed the formation and decomposition of ferrylHb by taking the absorption spectra in every minute for 10 minutes in a reaction between metHb and different concentrations of H_2O_2 (125-500 µmol/L). FerrylHb formation peaked at two minutes after the reaction regardless of H_2O_2 concentration, and the decomposition rate positively correlated with the concentration of H_2O_2. Besides, the concentration of metHb and ferrylHb were inversely proportional. Finally, we found out that the cross-linked Hb forms formation took place in a time and dose-dependent manner.

DISCUSSION

DAMPs are molecules released by damaged cells and tissues with the potential to trigger or modify the innate immune response. Heme is the most characterized hemolysis-derived DAMP targeting immune and non-immune cells. However, the role of other hemolytic products upstream of free heme in hemolytic-mediated damage is unclear. Studies have shown that IVH-induced renal alterations are largely heme-independent since the administration of heme or heme scavenging does not mimic or prevent the damage. We aimed to provide a detailed analysis of extracellular heme forms following IVH and to investigate the contributions of these heme forms to hemolysis-associated inflammation through the NLRP3 inflammasome activation and IL-1β release. We induced IVH in the mice by injecting PHZ intraperitoneally. In agreement with the literature, we observed enlargement of the spleen, reduced hematocrit levels, and detected extracellular heme. We also detected a large amount of hemichrome four hours after PHZ injection that was attributed to the strong oxidative effect of PHZ drug. MetHb was the second most abundant Hb form and naïve Hb was the least. Although metHb easily releases heme we could not detect free heme probably due to Hx-Heme binding activity. However, 20h post-PHZ

injection we detected accumulation of free heme and this could be attributed to the exhaustion of the scavenging molecules Hp and Hx. We detected a higher amount of free heme than the levels previously measured in the literature. Non-specific heme-binding proteins and RBC-derived microparticles in the plasma may have been influenced the heme levels in our system. We detected caspase-1 activation and processed IL-1β in the liver samples and secreted IL-1β in the plasma of PHZ treated mice compared to the control group, suggesting that acute hemolysis triggers systemic inflammation. As demonstrated by Dutra et al. heme triggers the activation of NLRP3 inflammasome and IL-1β production in PHZ-induced IVH. Mononuclear cells are majorly the source of pro-inflammatory cytokines such as IL-1β. We detected IL-1β in the plasma of PHZ injected mice at the time when oxidized Hb forms occupied the larger portion of the total heme. Therefore, we investigated the proinflammatory activity of Hb forms upstream of heme by purifying and oxidizing Hb from human blood. We treated LPS primed RAW macrophage or primary BMMs with Hb forms. LPS priming was necessary because IL-1β release requires two signals to be activated as already established in the literature. Heme and ferrylHb induced the maturation and release of IL-1β *in vitro,* furthermore, ferryHb was the strongest inducer of neutrophils and monocytes

infiltration. The release of IL-1β *In vivo* in our PHZ-induced hemolysis model may have been triggered by gut-derived LPS as a first signal while the hemolytic product played the role of a second signal. FerrylHb and MetHb are potential sources of heme therefore we reasoned that ferrylHb-triggered inflammatory response may not have been mediated by heme since metHb could have triggered the same response. Therefore, we concluded that ferrylHb is distinctively a proinflammatory oxidized Hb molecule.

We established that NLRP3 deficiency confers the mice a survival advantage over the wildtype in PHZ-induced severe hemolysis. We ruled out resistance as a reason for the survival we observed because the levels of hematocrit and Hb forms were similar in the WT and NLRP3$^{-/}$ mice. However, various studies have shown that disease tolerance is achieved by controlling tissue damage in the host upon infection or sterile inflammation, consequently improving the survival chances of the host. Our results revealed that neither PHZ nor ferrylHb injection could induce active IL-1β formation in NLRP3$^{-/-}$ mice which most likely may have contributed to the survival advantage of the NLRP3 deficient mice upon PHZ-induced hemolysis.

We identified ferrylHb as a potent pro-inflammatory oxidized Hb form that induces the production of active IL-1β. Nevertheless, we could not detect ferrylHb using the available spectrophotometric methods in plasma and urine samples rather we detected covalently cross-linked Hb dimers in the PHZ treated mice. We assumed that covalently crosslinked Hb dimers formed during the stabilization of the ferryl ion. We investigated the kinetics of ferrylHb and the covalently crosslinked Hb forms in a reaction between H_2O_2 and Hb or metHb. We made the following observations, (1) The reaction between metHb and H_2O_2 yielded more ferrylHb than the reaction between Hb and H_2O_2 (2) At two minutes after the reaction between metHb and H_2O_2 ferrylHb formation reached the peak and the rate of decomposition was dependent of H_2O_2 concentration. Studies have shown that decomposition of ferryHb takes place through intermolecular electron transfer between ferryl iron and specific amino acid residue of globin subunit resulting in ferric iron and globin-centered radicals. The reaction between globin-globin radicals produces covalently cross-linked Hb multimers which are detected as metHb in the visible spectra scan. In line with this, the concentration of metHb and ferryHb was inverse in the reaction between H_2O_2 and metHb. Parallel, we detected the formation

of covalently cross-linked Hb multimers in a time- and dose-dependent manner.

The covalently cross-linked Hb lacks a specific name since much of the literature refers to it as metHb because the two-electron oxidation of Hb or metHb ultimately forms the cross-linked Hb multimers hence the oxidation state of heme iron in these Hb forms is +3 (ferric). The name metHb ignores the modification of the globin subunit which gives this form of Hb unique features. On the other hand, calling this form as ferrylHb is untenable because the ferryl oxidation state is transitory. Therefore, we proposed to name the Hb forms produced in the two-electron oxidation of Hb or metHb as globin modified oxidized Hb (gmoxHb).

In this work, we demonstrated that following intravascular hemolysis different Hb oxidation products are formed which we assume contribute to the sterile hemolysis-induced inflammatory response. We proposed the name of gmoxHb for the oxidized Hb form that is produced via ferrylHb formation. We showed that Western blot is the appropriate tool for detecting gmoxHb in biological samples. We identified gmoxHb as a potent pro-inflammatory trigger that induces formation and maturation of IL-1β both *in vitro* and *in vivo*. The gmoxHb-mediated inflammatory response is mediated

through NLRP3 inflammasome activation and caspase-1 activation.

SUMMARY

In this work, we demonstrated that following intravascular hemolysis Hb oxidation leads to the formation of hemichrome, metHb, covalently cross-linked Hb multimers, and free heme. We showed that PHZ-induced IVH triggers IL-1 production via the NLRP3 inflammasome activation. We demonstrated that covalently cross-linked Hb forms during the decomposition of transient ferrylHb the molecules we proposed to name as gmoxHb. We Identified gmoxHb as a potent pro-inflammatory Hb form which induced the active production of IL-1 in vivo and in vitro. We confirmed that NLRP3 deficiency confers a survival advantage to mice in PHZ-induced IVH via increased tolerance and improved tissue damage control mechanisms. Identification of RBC-derived DAMPs and understanding the signaling mechanisms involved in the pathophysiology might provide new approaches for the treatment of IVH-related pathological conditions.

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List of publications related to the dissertation

1. 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.
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2. 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.
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List of other publications

3. 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: 4.716 (2018)
4. 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.
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