Short Thesis for the degree of doctor of philosophy (PhD)

Heme stress response in human podocytes

Emese Bányai, MD

Supervisor: Viktória Jeney, PhD

UNIVERSITY OF DEBRECEN

Kálmán Laki Doctoral School

Debrecen, 2018

Heme stress response in human podocytes

Author: Emese Bányai, MD

Kálmán Laki Doctoral School
Thrombosis, Haemostasis and Vascular Biology Programme

Supervisor: Viktória Jeney, PhD

Examination Committee:

Head: Soltész Pál, MD, PhD, DSc
Members: Csilla Csortos, PhD, DSc
Reusz György, MD, PhD, DSc

Date of Examination: 11 am, October 8, 2018
Place of Examination: Library of Building C, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

Reviewers: László Cervenák, PhD
Tamás Szabó, MD, PhD

Defense Committee:

Chairman: Soltész Pál, MD, PhD, DSc
Members: László Cervenák, PhD
Tamás Szabó, MD, PhD
Reusz György, MD, PhD, DSc
Csilla Csortos, PhD., DSc

Date of Defense: 1 pm, October 8, 2018
Place of Defense: Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

1. Introduction and literature review

The defensive intracellular milieu of red blood cells (RBCs) confers protection against the oxidative effect of contiouosly generated reactive oxygen species (ROS). During intravascular hemolysis Hb is released to the extracellular compartment, where it becomes rapidly oxidized creating Hb with different oxidation state of iron. Heme is easily released by these oxidized Hb forms.

Heme is a molecule with prooxidant and proinflammatory nature, that senzitizes endothelial cells against oxidative stress during intravascular hemolysis.

In massive intravascular hemolysis Hb and heme accumulated in the plasma is partially filtered to the urine by the kidney. These nephrotoxic molecules are supposed to contribute the development of acute kidney failure in hemolytic diseases. The podocyte is a special cell type with long duration of life and special function in the kidney. Little is known about heme stress tolerability in podocytes. The aim of our work was to investigate the response of podocytes to different forms of Hb and heme and correlate it with the well-known response of endothelial cells.

Excretory function of the kidney, the role of podocytes

Major function of the kidney is the clearance of metabolic endproducts from blood. The nephron is the area where primary ultrafiltrate of plasma is prepared. The glomerulus is a special subunit of the nephron. Primary ultrafiltration takes place across the semipermeable membrane established between the glomerular capillary and the Bowman's space. This filtration membrane comprises two layers of glomerular endothelial cells and podocytes (visceral epithelial cells) separated by a basement membrane. Multiple primary and secondary foot processes extend from the stellate cell body of the differentiated glomerular podocyte. These interdigitating foot processes cover the surface of the glomerular capillaries. Slit diaphragms are special intracellular structures connecting interdigitated podocyte secondary foot processes along the glomerular basement membrane. The slit diaphragm is a porous structure, which retains high molecular weight plasma

proteins from the ultrafiltrate. Detachment of foot processes is a definite sign of podocyte injury and tightly correlates with the presence of albuminuria and proteinuria.

Intravascular hemolysis

Intravascular hemolysis can be the primary or secondary pathogenic factors of several hematological diseases (sickle cell anemia, thalassaemia, PNH, G6PD deficiency, HUS, TTP). During a hemolytic episode the amount of Hb released to the plasma exeeds the capacity of Hb- and heme-binding proteins. In that case, a significant amount of free Hb and free heme accumulates in the plasma.,

Hb oxidation and dissociation of heme

Physiologically metHb constitutes around 1-2% of Hb in the red blood cells. When heme combines reversibly with oxygen a partial transfer of an electron occurs from the ferrous iron atom (Fe^{2+}) to the oxygen molecule. By surrendering its electron the iron atom has become ferric (Fe^{3+}) and, in accepting an electron, the ligated oxygen molecule becomes a superoxide anion. Hydrogen peroxide can react with hemoglobin by undergoing a two-electron oxidation and producing ferryl hemoglobin ($Fe^{4+}=O^{2-}$). FerrylHb is the complex of globin-radicals, porphyrin-radicals and cross-linked hemoglobin multimers. When heme iron is oxidized, heme loses its ability to bind oxygen. Due to the looser globin-iron binding, heme dissociates from the globin molecule.

Systemic and cellular defence against heme-containing moieties released during intravascular hemolysis

Intracellular ROS production is dependent on the function of the antioxidant systems. RBCs and also other cells contain many antioxidant enzymes in order to protect cells from ROS mediated injury and regulate redox-sensitive pathways. The major antioxidant enzymes in human are the superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPX). The RBC specific NADH-dependent methemoglobin reductase also functions as an antioxidant.

Hb and heme-binding proteins in the plasma

Haptoglobin (Hp) binds free Hb in the plasma and facilitates its removal by endocytosis to the macrophages by CD163. In the macrophages heme oxygenase-1 breaks down the heme group. When the amount of Hb released during hemolysis exeeds the binding capacity of plasma Hp, free Hb is partilly excreeted by the kidney. The 32-kDa αß-dimer of hemoglobin is small in size, therefore it readily reaches the kidney and the vessel wall, passes the endothelial layer of the vessel wall to the subendohelial and perivascular region. The major role of hemopexin (Hx) is the binding of free heme released from Hb during intravascular hemolysis. LDLR1/CD91 mediates the endocytosis and internalzation of the Hx-heme complex, heme is degraded by HO-1 and iron is sequestered by ferritin.

Heme-oxigenase/ferritin system

Heme is a porphyrin complex containing a tetrapyole ring and a ferrous iron(II).. The HO/ferritin system protects cells against heme-mediated cellular damage. HO catalyzes the degradation of heme, which produces biliverdin, ferrous iron, and carbon monoxide. Heme oxygenase-1 isoform is an inducible 32-kD heat shock protein possessing cytoprotective properties. HO-1 has several inductors, particularly regarding heme and Hb forms within the framework of this paper. Ferritin in an intracellular iron storing protein consisting of a protein shell (apoferritin) having the iron micelles firmly bound. This multimeric protein consists of subunits of heavy (H) and light (L) chains. H chain has ferroxidase activity. The major role of ferritin is the sequestration of free iron from availability to participate in free radical production.

Hemolysis and acute kidney failure (AKF)

Heme containing proteins are able to cause acute kidney failure due to their direct cytotoxic effect. Recurrent acute kidney injury predisposes the kidney to chronic kidney failure.

Heme as a prooxidant, heme as a Fenton reagent

70% of whole iron store of the human body is present in the heme prostetic group of the Hb. Heme iron plays an important role in oxygen transport, binding and dissociation. Reactive hydroxyl radicals are formed in the Fenton reaction with the participation of iron and oxygen.

Heme as a proinflammatory molecule, heme as danger-associated molecular pattern (DAMP)

Heme also serves as a danger-associated molecular pattern (DAMP). Heme activates the innate immune system through the activation of the NLRP3 inflammasome and consequetively contibutes to podocyte damage and the formation of glomerulosclerosis.

Prooxidant and proinflammatory effects of oxidized Hb forms

ROS converts Hb released from red blood cells to metHb. Like heme, metHb is able to cause oxidative damage in endothelial cells, induces HO-1 and ferritin synthesis. Sensitizing endothelial cells by heme by means of the induction of HO-1/ferritin system may help to prevent acute organ injury caused by oxidative stress. The redox state of ferryl iron (Fe^{4+}) is not stable, it readily transforms to the more stabe state of ferric iron (Fe^{3+}) while reacting with amino acids of the globin chains. The formed globin radicals bind covalently and form coss-linked Hb multimers, thereby producing ferrylHb species. FerrylHb has proinflammatory properties in endothelial cells, destroys the integrity of the endothelial monolayer and increases its permeability.

2. Aims

The aims of my scientific research were the followings:

- To quantify extracellular Hb, oxidized Hb forms and free heme in the plasma in a mouse model of sterile hemolysis.
- To quantify extracellular Hb, oxidized Hb forms and free heme in the urine in a mouse model of sterile hemolysis.
- To investigate the cytotoxic effect of oxidized Hb forms and free heme and explore their effect on ROS production in human podocytes *in vitro*.
  Compare the results of podocyte experiments with former results of human endothelial cell experiments.
- To investigate the effect of extracellular Hb, oxidized Hb forms and free heme on HO-1 and ferritin expression in podocytes *in vitro*, and analyze the role of these proteins in the protection of human podocytes against heme stress.
  Compare the results of podocyte experiments with former results of human endothelial cell experiments.

3. Materials and methods

3.1. Culturing the AB 8/13 human podocyte cell line

Human podocytes were kindly provided by Moin Saleem (University of Bristol). The AB 8/13 cell line was propagated at 33°C under permissive condition in RPMI 1640. At ~50–60% confluence, the cells were transferred to 37°C (nonpermissive condition) for 14 days.

3.2. Culturing human umbilical vein endothelial cells (HUVEC)

We separated endothelial cells from the umbilical vein of neonates and cultured them in Complete Medium.

3.3. Immunfluorescent staining

The podocytes were stained with anti-synaptopodin antibody. For the visualization of the cytoskeleton FITC-labelled phalloidin was applied. The samples were covered with a DAPI-containing EverBrite® mounting medium.

3.4. Time-lapse imaging videomicroscopy

Long-term time-lapse microscopy analysis was performed on the culture under nonpermissive condition from day 0 to day 14 to follow the process of podocyte differentiation.

3.5. Immunohistochemistry of Human Kidney Specimen

Podocytes in the formalin-fixed paraffin-embedded tissue from the human kidney cortex were stained with Wilms tumor 1 (WT-1) antibody, for FtH visualization in podocytes monoclonal antibody from Paolo Arosio was used. To visualize antigens of interest Vectastain Elite ABC dual detection system was used.

3.6. Giemsa staining

The monolayer of cells was fixed with methanol and stained with freshly prepared Giemsa solution.

3.7. Isolation of Nuclei and Visualization of Chromatin Structures

After permeabilization podocytes were treated with colcemid to prevent them from entering a new cell cycle. Nuclei were isolated by fixed ratio of acetic acid and

methanol (1:1). Preparation of nuclei were spread over glass slides dropwise and covered with DAPI containing EverBrite mounting medium.

3.8. Activities of antioxidant enzymes

Podocytes were harvested in KH_2PO_4 buffer. Cells were lysed by sonication on ice and cell lysate was obtained by centrifugation. Enzyme activities of SOD, GPX and catalase were measured from cell lysates.

3.9. Western blot analysis

After treatment, podocytes and endothelial cells were harvested and solubilized. For the detection of FtH, samples were subjected to 6% nondenaturing PAGE and human FtH monoclonal antibody from Paolo Arosio was used at a dilution of 1:1000. For the detection of HO-1, samples were subjected to 12% SDS-PAGE and anti-human HO-1 monoclonal antibody was used at a dilution of 1:2500. Covalently cross-linked Hb forms were detected from urine samples subjected to 12% SDS-PAGE with a horseradish peroxidase conjugated antibody.

3.10. Quantitative real time polymerase chain reaction (qRT-PCR)

Endothelial cells and differentiated podocytes were solubilized in RNA-STAT60 reagent and RNA was isolated from samples. RNA was transcribed to cDNA by SuperScript™ II reverse transcriptase. cDNA was used as a template in the qPCR system. For HO-1 gene amplification we used iTaqTM polimerase, validated TaqMan probe conjugated with FAM and self designed primers. We analysed GAPDH as a housekeeping gene. $\Delta\Delta C_t$ method was applied to determine relative changes in gene expression.

3.11. ECLIA method for human L-ferritin measurement

Endothelial cells ad podocytes were harvested and solubilized. Electrochemiluminescence sandwich immunoassay (ECLIA) was used to quantitatively determine ferritin by a diagnostic analyser.

3.12. Podocyte cytotoxicity assay

Podocytes were exposed to heme or H_2O_2 or the combination of heme and H_2O_2. When used in combination, cells were pretreated with heme for 1 hour prior to

H_2O_2 treatment. Cell viability was determined by MTT-reduction (3-2,5-diphenyl-tetrazolium-bromide) measured at 590 nm.

3.13. TBARS measurement

Cells were scraped in KH_2PO_4 buffer containing SDS (6%). To each sample TBARS reagent (2-thiobarbituric acid, HCl, trichloroacetic acid) was added and boiled for 10 minutes. Samples were cooled down and extracted with n-butanol. Optical density of the organic phase was measured at 532 nm.

3.14. ROS production

Endothelial cells and differentiated podocytes were exposed to H_2DCFDA, then we treated cells with heme (5 μmol/l) or H_2O_2 (100 μmol/l). Intracellular esterases converted H_2DCFDA to fluorescent DCF. Fluorescence intensity of the samples was detected.

3.15. C57BL/6 mice

Twelve C57BL/6 mice were randomly divided into 2 groups: in the PHZ group group mice were injected intraperitoneally (i.p.) with PHZ to induce sterile hemolysis. Control mice received PBS only.

3.16. Spectrophotometric determination of hemoglobin concentration from mouse serum and urine samples

In C57BL/6 mice we induced hemolysis with intraperitoneally injected PHZ, then collected urine an serum samples to quantitatively determine Hb, metHb and hemichrome content of the samples from optical density values.

3.17. Hemoglobin preparálás

Hemoglobin was purified from red blood cells (RBC) from blood anti-coagulated with heparin. RBSs were diluted and washed with isotonic saline, lysed by Sodium Phosphate Buffer, then Tris-base (1 mol/L) was added to the lysate in 1:20 volume ratio. The RBC lysate was loaded onto the column packed with DEAE Sepharose CL-6B resin. Hb was eluated from the coloumn by Tris-base buffer. The concentration of Hb was determined using the following method: $[Hb]=66xA_{574}-80xA_{630}$; $[metHb]=279xA_{630}-3xA_{574}$. MetHb was was prepared by the addition of a

1.5-molar excess of $K_3[Fe(CN)_6]$ to Hb. FerrylHb was prepared by the addition of a 10-molar excess of H_2O_2 to Hb. Finally, hemoglobin samples were dialyzed.

3.17. Statistical analysis

All data were analyzed using the GraphPad Prism 5 software. Differences between two groups were analyzed by unpaired *t*-test. For multiple comparisons ANOVA method followed by post hoc Tukey's test was used. *P* values <0.05 were considered statistically significant.

4. Results

4.1. Presence of extracellular Hb, oxidized Hb forms and free heme in mouse plasma and urine after hemolysis

In C57BL/6 mouse model we induced sterile hemolysis by intraperitoneal injection of phenylhidrazin (PHZ). 4, 16, and 20 hours after the first injection we measured the concentration of heme, Hb, metHb and hemichrome in the plasma. Based on these data, we calculated the concentration of free heme in the plasma. We defined free heme as heme not associated with hemoglobin. In PHZ treated mice during hemolysis the concentration of extracellular Hb, metHb, hemichrome and free heme increased significantly in the plasma. Considering all the measured parameters, changes could be detected as early as 4 hours after treatment, and became significant 16 hours later. 20 hours after inducing hemolysis, we determined the concentration of Hb, metHb and hemichrome in the urine of PHZ and PBS treated C57BL/6 mice. Hb concentration of PHZ-treated mice did not differ from that of the vehicle, but the concentration of oxidized Hb forms like metHb and hemichrome were much higher in the urine of PHZ treated mice.

In the following experiments we were looking for the covalently crosslinked ferrylHb in the urine of PHZ-treated mice after a sterile hemolysis. We collected urine samples 4, 16 and 20 hours after the induction of hemolysis and detected their Hb concentration by Western blot under reducing conditions. 16 and 20 hours after treatment we have demonstrated the presence of Hb monomers (16 kDa) and dimers (32 kDa) in the urine of PHZ-treated mice but not in the vehicle.

In the following part of the work we investigated *in vitro* the reaction of podocytes to hemolysis and hem stress related to the divisional and differentiational characteristics of the immortalized human podocyte cell line.

4.2. Replication and terminal differentiation of the podocyte cell line

At 50–60% confluence, nondifferentiated cells were placed from 33°C to 37°C to induce differentiation which was followed by videomicroscopy for 14 days. By analysing the growth curve we observed that the initial growth phase lasted for 9

days. After cell number reached its peak between days 9 and 10, we saw a slight decrease in cell number followed by stabilization of the culture (days 10–14) when we could hardly detect any mitotic event.

When the temperature was shifted to 37°C the morphology of nondifferentiated podocytes gradually transformed into that of differentiated cells. At permissive temperature (33°C) nondifferentiated podocytes showed typical epithelial cobblestone morphology. In contrast, differentiated podocytes are characterized by enlarged cell bodies with an irregular shape and the formation of processes involving shorter and rounded as well as longer and spindle-like projections. Formations of spindle-like projections were often preceded by partial retraction of cytoplasmatic protrusions. Many differentiated cells developed cellular hypertrophy, some of the cells died, and others started to arborize. We detected numerous binucleated but nonproliferating cells. After day 9 we registered a reduction of cell number, but the motility of cells remained high even after differentiation, and the culture proved capability of migration until all bare fields of the culturing vessel were completely covered.

To prove that differentiation of podocytes was complete during the 14 days, we evaluated synaptopodin expression in undifferentiated and differentiated cells by immunostaining. We observed that synaptopodin expression was missing in nondifferentiated podocytes, while differentiated cells showed obvious expression of this molecule. The actin-based foot processes of kidney podocytes with the interposed slit diaphragms form the final barrier to proteinuria. We used the actin cytoskeleton marker phalloidin to visualize cytoskeletal rearrangement during differentiation.

We observed significant increase both in cellular size and in nuclear size of differentiated podocytes relative to the cell body and nuclei of nondifferentiated cells. The differentiated nucleus is large, while the non differentiated nucleus is small. To further examine whether changes in nuclear size are associated with a different chromatin structure, we stained chromatin with DAPI after lysing the

nuclei of nondifferentiated and differentiated podocytes. In the nucleoplasm of undifferentiated podocytes there are several dispersed foci of heterochromatin where the transcription of genes is almost inactive. Images also show visible chromosomes referring to a more condensed state of the chromatin, which indicates high mitotic activity. In comparison, in differentiated podocytes the organization of the chromatin structure is more homogeneous; fewer foci of heterochromatin are visible as actively transcribed genes are more loosely packaged.

4.3. Heme influences the oxidative resistance and ROS production of endothelial cells and podocytes

Heme alone is not cytotoxic for endothelial cells, but even in low doses significantly potentiates the cytotoxic effect of ROS. We investigated the oxidative stress response elicited by heme and H_2O_2 in podocytes and compared their responses with that of endothelial cells. Heme and H_2O_2 alone did not evoke cytotoxicity in endothelial cells, but 50 μmol/L H_2O_2 given to the cells after heme pretreatment caused 40% of cell death, while 50 μmol/L H_2O_2 caused 50% of cell death. Conversely, neither heme or H_2O_2 alone, nor H_2O_2 given after heme pretreatment were toxic to podocytes. Podocytes were extremely resistant to oxidative stress.

Endothelial cells and podocytes were treated with 5μmol/L heme, or 100 μmol/L H_2O_2 or with H_2O_2 after heme pretreatment. ROS poduction was measured with the DCFDA method. In endohelial cells H_2O_2 slightly increased, while heme remarkably enhanced ROS production. H_2O_2 given after heme pretretment elicited ROS production similar to heme, or in certain timepoints in an even greater extent. Considering ROS production, we saw a tendency to increase in all cases, but significantly higher values were measured only in the first hour. Whereas in podocytes heme increased ROS production, but H_2O_2 not. H_2O_2 after heme pretreatment induced an increasing ROS production in tendency. Comparing

endothelial cells and podocytes, we concluded that podocytes produce less ROS than endothelial cells under all studied circumstances.

We treated endothelial cells and differentiated podocytes with heme (10-50 μmol/L, serum free conditions for 1 hour), then we harvested cells to collect RNS samples after 4 hours and to collect protein lysates after 8 hours. Both in endothelial cells and in podocytes heme induced HO-1 mRNS and protein expression. These effects were dose dependent in endothelial cells, while in podocytes 10 μM heme induced maximal HO-1 protein expression and no dose dependence could be seen.

In our experiments in an *in vivo* mouse model we observed that heme is primarily present in the plasma bound to different forms of oxidized Hb. Therefore we investigated whether these Hb forms are able to serve as heme source to endothelial cells and podoytes.

There were slight differences between the two cell types in HO-1 protein and mRNS induction caused by different Hb forms. Hb slightly induced HO-1 mRNS induction in podocytes, but not in endothelial cells. MetHb and ferrylHb induced HO-1 mRNS expression in both cell types and in both cell types ferrylHb was the more potent inducer. In podocytes and endothelial cells metHb and ferrylHb induced HO-1 protein expression.

We also explored the effects of oxidized Hb forms to the two subunits of ferritin both in endothelial cells and in podocytes. Under basal conditions endothelial cells expressed the H-subunit of ferritin (FtH) only in a small amount, but heme treatment notably induced FtH expression. On the contrary, in podocytes the basal FtH expression was high and heme treatment did not increase it. In endothelial cells FtH expression is induced by metHb leniently while by ferryl Hb more vigorously. The extent of FtH expression in podocytes exeeds that of endothelial cells and ferrylHb further increases it. In endothelial cells FtL expression was induced significantly by heme and ferrylHb. Under basal conditions in podocytes the expression of FtL is higher compared to endothelial cells, similar to the

expression of FtH. In podocytes the expression of FtL is induced by not only heme but also by ferrylHb.

To compare oxidative resistance of undifferentiated and mature podocytes, we tested the effect of H_2O_2 on cell viability. Nondifferentiated cells were sensitive to H_2O_2 that caused about 50% of cell death at doses between 125 and 500 μmol/L.

In contrast, no cytotoxicity was observed in differentiated podocytes using the same doses of H_2O_2. Heme alone caused death of nondifferentiated podocytes in a dose-dependent manner, while the same doses did not exert any cytotoxic effect on differentiated cells. Treatment of nondifferentiated podocytes with heme prior to H_2O_2 challenge led to decreased cell viability (53.3% versus 25.3%). In contrast, mature podocytes survived this lethal combination of heme and H_2O_2.

We found that nondifferentiated podocytes responded by a dose-dependent elevation of TBARS levels to both H_2O_2 and heme stress, whereas the same triggers did not cause a significant increase in TBARS level in differentiated podocytes. Activities of all the tested antioxidant enzymes were significantly higher in mature podocytes when compared to nondifferentiated cells.

Differentiated podocytes express about 4 times more FtH than nondifferentiated cells and endothelial cells. Upon heme treatment, nondifferentiated podocytes upregulated FtH expression dose dependently. In contrast, in differentiated podocytes only the highest dose of heme triggered slightly the induction of FtH. Adult human kidney sections were stained with WT-1 antibody to localize podocytes in the glomerulus. For functional assessment, the sections were counterstained with FtH antibody. Glomeruli in the cortex contained several podocytes with strong FtH staining, which supports our *in vitro* findings of high FtH content and iron sequestering capacity of kidney podocytes.

5. Discussion

In our work we induced steril intravascular hemolysis in C57BL/6 mice by the intraperitoneal injection of PHZ. We detected extracellular Hb, metHb, hemichrome and free heme in the plasma as early as 4 hours after treatment. In diseases with intravascular hemolysis acute kidney injury emerges few hour after the hemolysis, whilst chronic kidney failure becomes evident only after months or years. Intravascular hemolytic episodes may worsen impaired kidney function, on which more attention should be payed due to the wordwide epidemic of chronic kidney disease (CKD). The exact mechanism of irreversible podocyte injury in CKD is not yet explored.

In subsequent research we studied changes in podocytes elicited by hemolysis and heme stress. Podocytes are highly specialized cells with an exquisite cell structure underlying their function. Their foot processes extend far from the cell bodies and resemble an interdigitated scaffolding around the glomerular capillaries. The interdigitated foot processes in between with filtration slits are bridged by a slit diaphragm, which plays a major role in establishing the selective permeability of the glomerular filtration barrier. Injury to podocytes leads to protein leakage, a common mechanism present in most glomerular diseases. An immortalized podocyte cell line, which has been established by Moin Saleem et al. allowed us to follow the process of differentation of living podocytes by time-lapse microscopy imaging. Having undergone several mitotic events and a complete differentiation process, podocytes gained a different phenotype with huge cell bodies and longer lamellipodia. The cytoskeletal architecture of the cells became more prominently visible even under light microscopy, with voluminous longitudinal bundles of actin filaments in the cell bodies and in the processes of the podocytes. Synaptopodin is a podocyte specific protein that has an important role in the maintenance of cytoskeletal integrity, preserves the dynamic plasticity of foot processes, and thereby provides protection against proteinuria. Synaptopodin appears only in differentiated podocytes. The motility of cells remained high even after

differentiation. A previous report comparing four cultured podocyte cell lines from different species also published consistent data on the high migration rate and motility of human podocytes. As cultured podocytes progressively exited the cell cycle, parallelly they lost the ability to multiply themselves, that is, retrieve the function of damaged or dead podocytes. In conformity with the study by Liapis et al. terminal differentiation implied permanent exit from the cell cycle; thus maturation process was accompanied by loss of mitotic activity and termination of podocyte replication. This may serve as explanation for our observation that the undifferentiated culture showed a higher proliferation rate than the differentiated one.

To date, few studies have investigated and compared nuclear properties of glomerular podocytes. Consistent with those described in the literature, we also found that the nuclear architecture, including the chromatin, is distinct in undifferentiated cells from differentiated cells in many respects. In conformity with previous observations, heterochromatin foci were larger and dispersed in undifferentiated cells. We reported that the nuclei of undifferentiated podocytes contain several foci of heterochromatin and also contain visible chromosomes beside the more condensed state of the chromatin, which refers to high mitotic activity. On the contrary, in differentiated podocytes the organization of the chromatin structure itself is more homogeneous; fewer foci of heterochromatin are visible as actively transcribed genes are more loosely packaged. This also supports the fact that there is a high gene expression rate in differentiated cells. We did not observe tight chromatin packages and visible chromosomes in mature cells reflecting a weak tendency of differentiated podocytes to go through the cell cycle.

Oxidative stress resistance and the exact mechanisms by which these cells survive are yet uncovered.

Balla et al reported previously that heme alone is not cytotoxic to endothelial cells, but even in low doses notably increases the cytotoxic effects of ROS. We investigated the oxidative stress response related to heme and H_2O_2 in comparison

in endothelial cells and podocytes living in tight contact in the glomerulus. As we expected, heme and H_2O_2 alone were not cytotoxic to endothelial cells, but 100 μmol/L H_2O_2 after heme pretreatment resulted in 50% of cell death. In contrast, no cytotoxicity was observed in differentiated podocytes using the same doses of heme or H_2O_2 alone, or even after heme pretreatment. Podocytes were particularly resistant to oxidative stress. ROS production played an essential role in oxidative cell death after heme sensitizing in endothelial cells. In our experiments ROS production was increased slightly by H_2O_2, while it was increased significantly by heme in endothelial cells. H_2O_2 after heme pretreatment induced ROS production in an even greater extent than heme alone. Considering ROS production we saw a tendency to increase in all cases, but significantly higher values were measured only in the first hour. Whereas in podocytes heme increased ROS production, but H_2O_2 not. H_2O_2 after heme pretreatment induced an increasing ROS production in tendency. Comparing endothelial cells and podocytes, we concluded that podocytes produce less ROS than endothelial cells under all studied circumstances. HO enzyme is responsible for the intracellular degradation of heme forming biliverdin, CO and iron. In accordance with literature, heme induced HO-1 mRNS and protein expression both in endothelial cells and in podocytes. These effects were dose dependent in endothelial cells, while in podocytes 10 μM heme induced maximal HO-1 protein expression and no dose dependence could be seen. Iron is formed during the breakdown of heme and ferritin sequesters it in a biologically inactive form in the cytosol. Hemoglobin released from red blood cells during hemolysis is present in the plasma in Hb dimer, which is small in size therefore able to pass the glomeruli. Under those reactive circumstances Hb is oxidized and becomes nephrotoxic. In our experiments endothelial cells expressed the H subunit of ferritin only in a small amount, but heme induced FtH expression in a great extent. In contrast, podocytes expressed heme aboundantly even under basic conditions, and heme treatment did not induced FtH expression in podocytes.

We also investigated the effects of Hb forms to FtH expression. In endothelial cells FtH expression is induced by metHb leniently while by ferryl Hb more vigorously. The extent of FtH expression in podocytes exeeds that of endothelial cells and ferrylHb increase FtH expression further. In endothelial cells FtL expression was induced significantly by heme and ferrylHb.. Under basal conditions in podocytes the expression of FtL is higher compared with endothelial cells, similar to the expression of FtH. In podocytes the expression of FtL is induced by not only heme but also by ferrylHb. High content of FtH in podocytes and consecutively their high oxidative resistance may explain their long life duration and enables podocytes to survive in an oxidative environment.

Here we report that the oxidative resistance of differentiated podocytes is much higher than that of undifferentiated cells against H_2O_2-induced oxidative stress. Heme alone caused a strongly significant decrease in cell viability on nondifferentiated podocytes in a dose-dependent manner, while the same doses did not exert any cytotoxic effect on differentiated cells. Treatment of nondifferentiated podocytes with heme prior to H_2O_2 challenge resulted in an even lower rate of cell viability. In contrast, mature podocytes survived this lethal combination of heme and H_2O_2. H_2O_2 and heme-provoked cell death of nondifferentiated podocytes were accompanied by elevated TBARS levels, whereas the same triggers did not cause significant increases in TBARS levels in differentiated podocytes. Moreover, increased oxidative resistance of mature podocytes is associated with elevated activities of GPX, catalase, and SOD compared to nondifferentiated cells.

FtH has been shown to sequester iron from Fenton-like reactions. FtH provides protection to tissue damage, the conditional deletion of FtH increases nephrotoxicity in the experimental mouse models of acute kidney injury. This finding is in agreement with our hypothesis that FtH may play a role in protecting podocytes from deleterious injuries. Differentiated podocytes that are more resistant to oxidative stress express more FtH than nondifferentiated cells. To

assess the *in vivo* relevance of our observation, we examined the FtH content of human kidney specimens. Sections of kidney cortex rich in glomeruli were stained immunohistochemically. In support of our theory, we detected a massive FtH store in the cytoplasm of podocytes surrounding the glomerular capillaries that might be implicated in long-term survival of podocytes.

6. Conclusion

Podocyte is an unique cell type with special functions in the glomerulus. Their interdigitating foot processes covering the surface of the glomerular capillaries form the slit diaphragm.

During embryonic development podocytes loose their ability to proliferate. Podocytes are long lived cells but restorative replacement of injured podocytes is not readily solved. Effacement of foot processes from the glomerular capillary surface is an explicit sign of podocyte injury.

In hemolytic diseases (PNH, HUS, malaria, hemoglobinopathies, etc.) free heme released from hemoglobin causes injury of the arterial wall and the kidney due to its proinflammatory and prooxidative effects. Besides heme, oxidative forms of hemoglobin are also descibed to be able to senzitize endothelial cells against oxidative agents and trigger cytotoxicity acutely. Chronically, when endothelial cells are exposed to heme or oxidized hemoglobin forms for a longer period, these agents induce HO-1/ferritin system to protect cells against oxidative injury.

The aim of our work was to investigate and compare the reaction of glomerular podocytes to oxidative stress with the formerly published reactions of vascular endothelial cells. We also studied whether the HO-1/ferritin system plays an important role in the survival of podocytes. We have shown evidence that podocytes go through essential functional and morphological changes during the process of terminal differentiation. These changes result in the strengthening of their defence mechanisms against oxidative stress making podocytes resistant and long-lived. Antioxidant enzymes and FtH also belong to the defence mechanisms of podocytes against oxidative stress and highlight the role of this important but so far not well studied protective system in podocytes.

7. List of publications

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRAY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/88/2018.PL
Subject: PhD Publikációs Lista

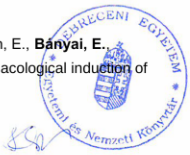
Candidate: Emese Bányai
Neptun ID: ITU3VF
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. Erdei, J., 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.
   IF: 4.593 (2016)
2. **Bányai, E.**, Balogh, E., Fagyas, M., Arosio, P., Hendrik, Z., Király, G., Szemán-Nagy, G., Tánczos, B., Pócsi, I., Balla, G., Balla, J., Bánfalvi, G., Jeney, V.: Novel functional changes during podocyte differentiation: increase of oxidative resistance and H-ferritin expression.
   Oxid. Med. Cell. Longev. 2014, 1-10, 2014.
   DOI: http://dx.doi.org/10.1155/2014/976394
   IF: 3.516

List of other publications

3. Balogh, E., Tóth, A., Tolnai, E., Bodó, T., **Bányai, E.**, Szabó, D. J., Petrovski, G., Jeney, V.: Osteogenic differentiation of human lens epithelial cells might contribute to lens calcification.
   Biochim. Biophys. Acta-Mol. Basis Dis. 1862, 1724-1731, 2016.
   DOI: http://dx.doi.org/10.1016/j.bbadis.2016.06.012
   IF: 5.476
4. Becs, G., Zarjou, A., Agarwal, A., Sikura, K. É., Becs, Á., Nyitrai, M., Balogh, E., **Bányai, E.**, Eaton, J. W., Arosio, P., Poli, M., Jeney, V., Balla, J., Balla, G.: Pharmacological induction of ferritin prevents osteoblastic transformation of smooth muscle cells.
   J. Cell. Mol. Med. 20 (2), 217-230, 2016.
   DOI: http://dx.doi.org/10.1111/jcmm.12682
   IF: 4.499



UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRAY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

5. Fagyas, M., Úri, K., Mányiné Siket, I., Daragó, A., Boczán, J., **Bányai, E.**, Édes, I., Papp, Z., Tóth, A.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) I: endogenous angiotensin converting enzyme (ACE) inhibition.
   PLoS One. 9 (4), 1-29, 2014.
   DOI: http://dx.doi.org/10.1371/journal.pone.0087843
   IF: 3.234

6. Fagyas, M., Úri, K., Mányiné Siket, I., Fülöp, G. Á., Csató, V., Daragó, A., Boczán, J., **Bányai, E.**, Szentkirályi, I., Maros, T. M., Szerafin, T., Édes, I., Papp, Z., Tóth, A.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) II: albumin suppresses angiotensin converting enzyme (ACE) activity in human.
   PLoS One. 9 (4), 1-28, 2014.
   DOI: http://dx.doi.org/10.1371/journal.pone.0087844
   IF: 3.234

7. Fagyas, M., Úri, K., Mányiné Siket, I., Daragó, A., Boczán, J., **Bányai, E.**, Édes, I., Papp, Z., Tóth, A.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) III: endogenous inhibition of angiotensin converting enzyme (ACE) provides protection against cardiovascular diseases.
   PLoS One. 9 (4), 1-29, 2014.
   IF: 3.234

8. Potor, L., **Bányai, E.**, Becs, G., Soares, M. P., Balla, G., Balla, J., Jeney, V.: Atherogenesis May Involve the Prooxidant and Proinflammatory Effects of Ferryl Hemoglobin.
   Oxid. Med. Cell. Longev. 2013, [1-13], 2013.
   DOI: http://dx.doi.org/10.1155/2013/676425
   IF: 3.363

Total IF of journals (all publications): 31,149
Total IF of journals (publications related to the dissertation): 8,109

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

11 April, 2018

8. Acknowledgement

I wish to thank to my consultant Viktória Jeney, Ph.D., who helped my research with his useful advices, encouraged my ideas, sponsored my research and aided the writing of my publications with his advantageous noticing.

I am grateful to Professor József Balla, M.D., who ensured a background for my research and supported my professional objectives.

I also thanks to all members of the laboratory with special thanks to Enikő Balogh for assisting the time-lapse videomicroscopic experiments and to my friend and colleague Gergely Becs, M.D. for aiding and facilitating my laboratory work.

Thanks to my family for their patience, love and encouragement also in the least successful times.

This work was supported by grant from the National Research, Development and Innovation Office (NKFIH) Grant no. K116024. The work was cofinanced by the European Union and the European Social Fund under the GINOP-2.3.2-15- 2016-00005 project.

I recommend this work to my husband, Miklós and my children, Lilla and Levente.