SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of heme-induced endoplasmic reticulum (HIER) stress on resident cells of plaque progression

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DEBRECEN, 2025

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The Examination takes place at the Library of Division of Nephrology, Faculty of Medicine, University of Debrecen, the 11th of June 2025, 11.00 AM.

Head of the **Defense Committee**:	Pál Soltész, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Bldg. „A" Department of Internal Medicine, Faculty of Medicine, University of Debrecen, the 11th of June 2025 1:00 PM

I. Rewiew of Literature

1.1 The *atherosclerosis*

1.1.1 Population incidence of atherosclerosis

Atherosclerosis is a common disease in both developed and developing societies and is among the leading causes of death worldwide. According to the WHO report, more than 17.9 million new cases or comorbidities appear and are responsible for about 32% of all deaths. The incidence of atherosclerosis in the modern sense has increased exponentially with social development over the past 50-60 years. With the growing economy and the emergence of welfare societies resulting from this, a large part of the population has become a potential patient group. Changes in Western-style diets, behaviors and attitudes such as eating habits, smoking or decreased physical activity have brought with them higher rates of obesity, diabetes, high blood pressure and metabolic syndromes at the population level. The diseases listed are all comorbidities or early risk factors for the development of atherosclerosis. Environmental factors alone are sufficient for the progression of the disease, but genetic factors also play a role in its development. Atherosclerosis is not only a disease that occurs in adulthood, it also affects the child and young adult population. In the infant and toddler (29 days-9 years), young child (9-19 years), and young adult (19-25 years) population, the presence of the disease was proven in 20-50% of patients selected based on preliminary radiological examination and suspicion of atherosclerosis. Although atherosclerosis is a rare cause of death in children or young adults, the results indicate that vascular changes related to stroke are already present in childhood in older adults, and the incidence of these vascular changes increases with age. Therefore, the reduction of risk factors should be started as soon as possible.

1.1.2. History and discovery of atherosclerosis

In the case of atherosclerosis, we are not talking about a new disease. Vascular lesions were discovered in the mummified remains of people who lived 4000 years ago. 34% of the subjects examined showed lesions, which were also identified in various ancient cultures (Egyptian, Peruvian, etc.). The pathophysiological abnormalities affected the aortic region, the iliac/femoral arteries, the popliteal and tibial arteries, and the carotid arteries, which are also among the most common lesions today. The exact mechanism of atherosclerosis is not yet fully known, but the appearance of early lesions and the condition of the initial stages support the significance of the “lipid hypothesis”. The first experimental observations in this regard were described at the beginning of the 20th century by a Russian researcher, Alexander Ignatowski.

During the autopsy of rabbits fed with egg yolk, he discovered lesions in the vascular system. Later, another Russian experimental pathologist, Nikolai N. Anichkov, conducted a similar experiment, but supplemented the rabbits' diet with vegetable oil. His observations were identical to Ignatowski's results. These observations were the basis for the current study of atherosclerosis associated with hypercholesterolemia in the human population.

1.1.3 Background of the biology of atherosclerosis

It is now proven that persistently high levels of certain plasma lipoproteins, such as low-density lipoprotein (LDL) or lipoprotein small a (Lpa), have a proatherogenic effect, while high-density lipoprotein (HDL) may have an antiatherogenic protective function. According to the literature, the initiation step of the disease is the interaction between lipoproteins and cells in the vascular wall, during which the combined response of resident cells of the vascular wall, such as endothelial cells, smooth muscle cells, and monocytes (later macrophages and mast cells) infiltrating from the bloodstream, to high levels of lipoproteins initiates the process of plaque formation. Endothelial cells lining the inside of the vessel wall secrete adhesion molecules (MCP-1, VCAM-1, ICAM-1, SELE) in response to elevated lipoprotein levels, which trigger monocyte migration. Monocytes differentiate following subintimal infiltration according to the given signals. Their task is to eliminate the enriched lipids, but they are unable to break down lipids modified by the pH and other factors below the intima. The macrophage cells thus collected clump together and the process of so-called “foam cell” formation can be observed. Foam cells are large, in the vast majority of cases, cell clusters involving the fusion of several cells, which are embedded under the subendothelial layer, thereby initiating the progression of plaque.

1.1.4 Möckenberg's classification

All of the above-mentioned changes, regardless of size and organ location, are generally called arteriosclerosis, but from a pathological point of view it is important to distinguish certain groups. According to Möckenberg's classification, these changes differ in both morphology and extent. He calls arteriosclerosis the change that appears with age in the middle layer of the vascular wall (tunica media) with lime deposits. Arteriolosclerosis is considered to be changes in arteries and arterioles smaller than 200 μm. Such changes include loss of elasticity, elongation of the vessels and the development of luminal stenosis. Atherosclerosis actually corresponds to the description of arteriolosclerosis, but it is also associated with the appearance of atheromatous plaques in the arteries. In everyday terms, while arteriolosclerosis is a change

that can only be detected under a microscope, atherosclerosis can even be recognized with the naked eye. From the point of view of characterizing the disease, it is important to know that we are not talking about a systemic disease, but rather a local change. There are more susceptible (abdominal, external thoracic, coronary, carotid, cerebral and renal) and less susceptible (internal thoracic, hepatic and radial) arteries. The vessels that are susceptible do not show the changes in a uniform and diffuse manner, often even within a given vessel. The degree and speed of the change are determined by a number of hemodynamic factors, the presence of shear forces and other permeability factors. However, it is clear that in the early phase diffuse local intimal thickening occurs and the “fatty streak” condition develops.

1.1.5 Characterization of plaques

In the human body, it takes several decades for lesions to develop into fibrous plaques, but once this occurs, the process of atherosclerosis accelerates exponentially. At this time, an unstable fibrotic cap, a combination of lipid and necrotic cores, appears, which is called a complicated lesion. The properties of the lesions are very diverse based on the histological characteristics. Both early and advanced lesions are present within an aorta. The American Heart Association (AHA) has classified the lesions into a 6-member class. The so-called clinically silent class includes lesions I-IV, and the clinically expressed class includes lesions V-IV. Clinically silent lesions are characterized by plaque progression that has already started, but they do not yet cause systemic complaints in the function of the vessel. However, clinically expressed lesions are accompanied by noticeable symptoms due to the narrowing of the lumen. It is important to note that grade VI lesions are the most dangerous and lead to death. Here, the plaque may already be ruptured, and may also be hemorrhagic and thrombotic. Ruptured plaques can cause even greater monocyte infiltration through their cracks, thus contributing to further plaque growth. Thrombotic plaques can cause vascular occlusion by detachment of the fibrous cap, which in practice leads to myocardial infarction and in many cases death. Hemorrhagic plaques, during the interaction of hemoglobin, lipid and other substances inside the plaque with cells, initiate smooth muscle cell migration, pathological cell and vascular proliferation and other harmful processes from the tunica media through a self-exciting process. The changed internal pH and pressure differences destroy the immature, protruding vessels and cause the lysis of red blood cells (RBCs) released from them, initiating cell reactive processes. Macrophages with an eliminating function express peroxides, various lytic enzymes and matrix metalloproteinases into the plaque interior, which further weaken the entire, thus damaged structure.

1.2 Red blood cells (RBCs)

1.2.1 General characteristics of RBCs

The most abundant cell type in the human body is the RBC. Every second, 2 million newly formed RBCs enter the circulation from the bone marrow and approximately the same number leave via the reticuloendothelial system. Under physiological conditions, 4-5 million RBCs are present per microliter in whole blood. Their lifespan is 110-120 days in normal circulation and during natural aging, their constituent elements (iron, cell component proteins) are recirculated. Young RBCs have intracellular phosphatidylserine and phosphatidylethanolamine signals, which are transferred to the cell surface during aging, thus the aged cells are marked with a phagocytosis signal and eliminated. At birth, we already have a set of precursors and precursors, so formation is constantly ensured under physiological conditions. For 5 years after birth, the red bone marrow ensures the synthesis of RBCs, then the flat bones take over this role.

1.2.2 Structure of the VVT

VVTs occupy a special place among cells, as their structure is very different from other cells. They do not have a nucleus, ribosomes, or mitochondria. Although these organelles are essential for the survival of most eukaryotic cells, VVTs have several advantages due to the lack of these organelles. The lack of a nucleus freed up more cell space, which in turn allowed them to have a higher Hb storage capacity, allowing them to transport more oxygen and increase their lifespan. In the absence of the listed organelles, they have a more flexible cell morphology, allowing them to move freely in capillaries. In the absence of mitochondria, VVTs produce energy directly through glycolysis in the cytosol, where numerous uniporter-type glucose transporters provide the substrate to meet their energy needs.

1.2.3 Formation of RBCs

Since RBCs do not have a nucleus, they are unable to divide. Erythropoiesis, i.e. RBC formation, occurs in blood islands located in the bone marrow. The differentiation process is very long, approximately 1 week, and occurs in a well-regulated manner. During maturation, two processes occur simultaneously: the development of the nucleus and the maturation of the plasma membrane. When the nucleus is no longer needed, the RBCs contract with their actin ring and divide into two parts: one part corresponds to the nuclear fraction, which is rich in DNA, and the other is the RBCs that are already functioning according to their function. During plasma maturation, oxygen-carrying Hb appears in the cytosol. Tissue hypoxia is the primary

stimulus for the formation of RBCs. Under the influence of hypoxia, the kidney secretes a polypeptide called erythropoietin, which, thanks to its direct effect on the bone marrow, induces the differentiation of hemopoietic stem cells into erythrocytes. The first step of this is the maturation of proerythroblasts in the blood islets, which transform into macroblasts, and then Hb is incorporated in the normoblast phase. During the subsequent maturation phases, the final erythrocyte form is formed, which after a short time become active RBCs. Macrophages observed at the site of maturation have a supporting role. On the one hand, they eliminate the cell nuclei that are expelled during maturation, and on the other hand, they provide heme for the synthesis of active Hb.

1.2.4 Characterization of hemoglobin (Hb)

The function of mature circulating red blood cells is to transport oxygen from the lungs to the organs and carbon dioxide from the tissues to the lungs via the bloodstream. The Hb of red blood cells is responsible for binding oxygen. Hb constitutes approximately 30% of the red blood cells. Structurally, it is composed of four well-defined subunits: each subunit consists of a globular protein section and a heme prosthetic group. Iron protoporphyrin IX, or heme, is structurally composed of a four-membered heterocyclic ring centered on Fe2+. Fe2+ is bound at four points to the nitrogen atoms of the pyrrole rings and to one nitrogen of the histidine imidazole ring of globin. Reversible binding of oxygen occurs at the sixth coordination site.

1.2.5 Hemolysis

Hemolysis, i.e. the breakdown of red blood cells, is a common process under physiological conditions. Hemolysis in the circulation is considered normal at a rate of approximately 1-2%. In this case, haptoglobin, as an acute phase protein, binds Hb, which binds to the CD163 receptor of circulating macrophages and is degraded following endocytosis. Several proteins play a role in binding the toxic, free heme released during hemolysis. One such protein is hemopexin, which, after binding the heme, is taken up by macrophages via the CD91 receptor, which eliminate the heme bound by hemopexin. Albumin, which binds heme non-specifically, is also a heme-binding protein, and alpha-1-microglobulin also plays a role in detoxification. During significant hemolysis, the capacity of the above endogenous Hb/heme-binding proteins is exhausted, so the free Hb/heme complex initiates pathological processes.

1.2.6 Hemolysis-associated diseases

During the irregular lysis of VVT, a number of diseases associated with hemolytic and hemorrhagic processes may develop. We can talk about intra- and extravascular hemolysis, which most often develop as a side effect of various drugs, due to mechanical causes, and during infection. Inherited hemolytic conditions are most often hemorrhagic conditions associated with hemophilia. In acquired hemolytic conditions, hemorrhage occurring in plaques and subarachnoid hemorrhage affecting the nervous system are also significant at the population level.

1.2.7 Importance of the Heme Oxygenase-1/Ferritin System

During significant intravascular bleeding, free heme is taken up into the surrounding cells. Heme is highly lipophilic and easily moves either actively or passively through the cell membrane. The intracellular degradation of heme is catalyzed by heme oxygenase-1 (HO-1). The degradation of heme requires oxygen, during which iron, carbon monoxide and biliverdin are produced from the degraded heme in equimolar amounts. In order to avoid free radical formation, the cell either places the accumulated iron in the iron core of the ferritin molecule in an inactive form or transports it to the extracellular space via ferroportin transporters. Genetic or functional HO-1 deficiency may result in increased cytotoxicity, which may even induce apoptosis. Intracellular heme can affect cell division, lipid metabolism and cause several functional disorders at the cellular level.

1.3 Endoplasmic Reticulum (ER) Stress

1.3.1 Characteristics of the ER

One of the most unique features of eukaryotic cells is the presence of membrane-bound spaces within the cytosol. This so-called compartmentalization allows for simultaneous molecular processes to occur. The ER, which is responsible for the synthesis of secreted and transmembrane proteins, and the maturation of proteins, also takes place here. The rough-surfaced (DER) and smooth-surfaced (SER) ER are the sites of protein synthesis and maturation. The DER and its ribosomes are responsible for the production of large quantities of general proteins, which are then transported to the Golgi apparatus. The SER is similar in structure to the DER, but ribosomes are not attached to its cytoplasmic surface. It accounts for a small percentage of total protein synthesis within the cell and is found in significant numbers in specific steroid-producing cells and in the liver, where it synthesizes detoxification proteins.

1.3.2 The phenomenon of ER stress

Protein secretion exceeding physiological levels or ER protein folding errors cause the accumulation of unfolded or misfolded proteins in the ER lumen, which is called ER stress. The ER is characterized by a tightly regulated equilibrium state: if the amount of newly synthesized, misfolded or unfolded proteins exceeds the folding capacity of the ER, ER stress signaling pathways are activated. ER stress is also caused by changes in the ER calcium balance and oxidative stress.

1.3.3 The role of the UPR

The characteristic process of ER stress is the so-called Unfolded Protein Response (UPR), which is an evolutionarily highly conserved signaling cascade. In eukaryotic cells, the role of the UPR is to ensure the proper folding of proteins, maintain ER functions, protect against the harmful effects of ER stress, and maintain ER homeostasis. If the degree of stress is milder or shorter, and the degree of stress does not reach a threshold value, the UPR, which only repairs or degrades defective proteins, is activated, promoting cell survival. If the degree of ER stress is increased or persists for a longer period, the UPR is automatically activated together with the apoptotic cascade, which leads to cell death.

1.3.4 ER stress-associated diseases

Abnormal ER stress plays a role in many diseases. For example, certain metabolic diseases such as hepatic steatosis and hyperlipidemia, which are associated with IRE1 and ATF6. In cancer, the presence of elevated levels of UPR activation may also be the background of the alteration, since increased UPR function promotes the adaptation and survival mechanisms of cancer cells. Increased XPB1s expression has been found in various biopsy samples (brain, breast, lymphoma, multiple myeloma), which has been correlated with reduced survival rates and poor prognosis. Altered ER function is also common in several neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, prion-related disorders, and amyotrophic lateral sclerosis. These diseases are associated with the UPR, but the majority of the aggregates accumulate in the cytosol and not in the ER lumen.

1.3.5 Biochemical background of UPR pathways

During ER stress, adaptive signaling pathways are activated, thus improving protein folding capacity and reducing ER load. This is regulated by three sensor proteins located in the ER membrane: pancreatic ER kinase-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1),

and activating transcription factor-6 (ATF6). Under normal conditions, these proteins are bound to glucose-regulated protein 78/immunoglobulin-binding protein (Grp78/BiP) and kept in an inactive state. When the amount of misfolded or incompletely folded proteins increases, Grp78 dissociates from the proteins that activate the stress cascade, which is followed by the activation of stress signaling pathways. This is because Grp78/BiP, as a nanny protein, binds to these misfolded proteins with higher affinity than to sensor proteins.

1.3.5.1 PERK pathway

The activation of sensor proteins is complex and can be activated independently or simultaneously. PERK is activated by homomultimerization and autophosphorylation, then phosphorylates the α-subunit of eukaryotic translation initiation factor-2 (eIF2α), which leads to the cessation of general protein synthesis and selectively initiates the translation of stress proteins such as activating transcription factor 4 (ATF4). The transcription factor ATF4 transactivates elements of the UPR that are involved in protein folding, redox homeostasis, activates autophagy, regulates amino acid metabolism, and can induce cell death. Under prolonged and increased ER stress, persistent ATF4 induces the expression of proteins involved in apoptotic processes, such as C/EBP homologous protein (CHOP). CHOP can be activated by all three ER stress sensor proteins, but is primarily activated through PERK.

2.3.5.2 IRE1 pathway

During activation of the IRE1 pathway, IRE1 oligomerizes and autophosphorylates, and then, by a previously unknown cleavage mechanism, activates X-box binding protein 1 (XBP-1), the active form of which is XBP-1s. XBP-1s simultaneously activates ER chaperone proteins and the expression of ER-associated degradation (ERAD) proteins, which are responsible for the degradation of misfolded proteins. IRE-1, which also has kinase function, can phosphorylate c-Jun N-terminal kinase (JNK), thus triggering ER-dependent apoptosis. IRE1 activation is primarily regulated by the dissociation of Grp78 from IRE1, but based on literature data, certain secondary pathways can also lead to IRE1 activation, such as direct binding of IRE1 to misfolded proteins.

1.3.5.3 ATF-6 pathway

The ATF-6 protein is present in the ER in an inactive form. Following stress, it is transported from the ER to the Golgi apparatus during the activation process, where it is cleaved by intramembrane proteolysis. The cytosolic fragment of ATF-6, a leucine zipper-like

transcription factor, translocates to the nucleus and activates the expression of chaperones and enzymes that enhance ER protein folding, such as Grp78, Grp94, and calreticulin.

1.3.6 The relationship between oxidative stress and the UPR

Oxidative stress can negatively affect ER function, which is followed by the activation of ER stress cascades. In the event of oxidative damage, specific signaling pathways are activated, of which nuclear factor erythroid 2-related factor 2 (Nrf2) plays a central role. Under physiological conditions, Kelch-like ECH-associated protein-1 (Keap-1) and Nrf2 are present in the cytosol in heterodimeric form. In the presence of reactive oxygen radicals, Keap-1 dissociates from Nrf2, thus breaking the heterodimeric structure and Nrf2 translocating to the nucleus. The goal of this process is to activate the transcription of protective detoxifying and antioxidant genes, such as HO-1 and superoxide dismutase-1 (SOD-1). Antioxidant pathways and ER stress cascades interact at several points, such as Nrf2/HO-1 signaling with PERK and IRE1. It has been observed that exogenously administered carbon monoxide (CO) activates the Nrf2 signaling pathway through PERK phosphorylation, which leads to HO-1 expression. In addition, CO also reduces apoptosis by reducing CHOP expression. In light of this, the CO/HO-1 system may have a positive effect in the treatment of vascular diseases associated with ER stress. The transcription factor BTB and CNC homolog 1 (BACH1) plays an important role in the oxidative stress response, which, as a heterodimer, can inhibit the activation of genes regulated by Nrf2, including HO-1. The nuclear translocalization of Nrf2, which initiates the oxidative stress response, occurs simultaneously with its binding to the BACH1 promoter, thus creating a real-time competition between the two proteins. Nrf2 heterodimers initiate stress response cascades, including HO-1 expression. BACH1 that is not bound to the promoter is removed from the nucleus. Following activation of Nrf2 signaling, intracellular HO-1 levels increase and heme degradation produces free iron, thereby initiating ferritin production. Previous studies have shown that heavy chain ferritin (H-ferritin), which is formed during activation of the HO-1/Ferritin system in the presence of heme stress and radicals, functions as a cytoprotective protein, as its ferroxidase activity inhibits oxidant-mediated cytolysis by binding free iron.

1.3.7 Source of intraplaque heme

During plaque progression, characteristic pathophysiological conditions are observed as a result of hemorrhage and hemolysis, such as pH differences, activation of enzymatic processes, and pressure differences. Under the influence of these factors, the VVTs disintegrate and the

released Hb undergoes rapid oxidation (Figure 14). The naive oxyHb (Fe^{2+}) is converted to metHb (Fe^{3+}) by environmental factors, which is accompanied by heme release. This oxidation process is further enhanced by the strong oxidants produced by infiltrated monocyte/macrophage cells, such as hydrogen peroxide. The ferrylHb ($Fe^{4+=O2-}$) converted from metHb remains in this form only for a moment, although this time is enough for its protein structure to be severely deformed and additional heme to be released from its center. The resulting truncated Hb is highly cytotoxic and the large amount of heme released is also harmful to the surrounding cells. The central topic of my thesis is the heme-related observations of the pathophysiological processes taking place in atherosclerotic plaques and the demonstration of the presence of ER stress in resident cells.

II. Objectives

Physiological proteostasis is essential for normal cellular function. Normal proteostasis is maintained in the ER through the UPR. The primary function of the UPR is to repair or degrade misfolded proteins and to initiate cell death in response to stress that exceeds the capacity of repair mechanisms.

Previous studies have suggested a link between ER stress and vascular diseases, including atherosclerosis and oxidative stress. A direct link has been found between UPR-induced ER stress and atherosclerotic risk factors. Heme plays an important role in the generation of free radicals, which can lead to ER stress. Oxidized Hb forms and free heme are present in high amounts in hemorrhaged atherosclerotic plaques.

We hypothesized that heme present in plaques may induce ER stress, which may play a role in the pathology of atherosclerosis. Therefore, we aimed to investigate the ER stress response of resident cells present in atherosclerotic plaques to heme.

Our objectives were as follows:

I. We investigated the role of heme in the ER stress process in primary human aortic smooth muscle cell (HAoSMC) and human aortic endothelial (HAoEC) cell cultures. Our questions were as follows:

• If ER stress can be induced in the examined cells, which ER stress pathways are activated and to what extent during heme treatments?

• Can heme-induced endoplasmic reticulum (HIER) stress be inhibited by specific heme binding proteins and free radical scavengers?

• Can heme-induced endoplasmic reticulum (HIER) stress be inhibited by classical ER stress inhibitors?

II. As a second step, we investigate the role of the HO-1/Ferritin system, which plays a central role in heme catabolism, in the HIER stress process.

• In the absence of the HO-1/Ferritin system, what is the degree of HIER stress in resident cells?

• What is the course of HIER stress in resident cells during increased activation of the HO-1/Ferritin system?

• Do intermediate products generated during heme degradation affect HIER stress?

• Can the cytotoxicity caused by HO-1 depletion be prevented by using different cell death inhibitors in our system?

III. In the third step, we will study the presence of HIER stress in atherosclerotic plaques using transcriptomic and immunohistochemical studies from ex vivo human histological samples. In this process, we set the following goals:

• Collect and select patient samples from CEA to represent plaque progression groups according to the AHA classification,

• Examine the presence of ER stress markers at the RNA level in samples from non-hemorrhagic and hemorrhagic plaques by RNA sequencing,

• Examine the presence of ER stress markers at the protein level in samples from non-hemorrhagic and hemorrhagic plaques by immunohistochemical staining.

III. Materials and Methods

3.1 Reagents

Unless otherwise noted, reagents used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hemin chloride stock solution (2 mM) was prepared fresh in sterile filtered 20 mM NaOH on the day of treatment. Hem arginate was purchased from Orphan Europe Pharmaceutical (Puteaux, France). Human recombinant alpha-1-microglobulin (A1M) produced in E. coli and purified by ion exchange chromatography was provided by our collaboration partner Bo Akerström and Magnus Gram (Lund University, Sweden). Hemopexin (HPX) was a generous contribution from Ann Smith and colleagues (University of Missouri, Kansas, USA).

3.2 Cell Cultures

Human aortic smooth muscle cells (HAoSMC) were purchased from Cell Applications (San Diego, CA, USA) and Lonza (Allendale, NJ, USA). Human aortic endothelial cells (HAoEC) were also purchased from Lonza. Cell cultures were obtained from healthy, disease-free, Caucasian donors. HAoSMC cultures were grown in low-glucose (1g/L) DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antimicrobial cocktail (PSA: 100 U/ml penicillin, 100 µg/ml streptomycin and amphotericin B). HAoEC cultures were grown in endothelial growth factor supplemented Medium 199 (CM199) with the same supplements as DMEM. Treatments were performed after the cells reached 90% confluence. Heme treatments were performed in antibiotic- and serum-free media. HAoSMC cells were treated with heme for 1 hour and HAoEC cells for 2 hours. After treatment, the cells were washed twice with an equal volume of Ca2+ and Mg2+ containing Hank's saline solution (HBSS+). During the regeneration period (3, 6 and 16 hours), the cells received complete culture medium supplemented with FBS and PSA. 1µM Thapsigargin, a non-competitive inhibitor of sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) that induces ER stress, was used as a positive control. For A1M and HPX experiments, the proteins were incubated together in the heme-containing treatment solution, also without the addition of antibiotics and FBS. They were gently shaken at room temperature and protected from light for 30 minutes. The 25 µM heme-containing treatment solution was supplemented with 12.5 µM A1M (which has two active heme binding sites) and 25 µM HPX (which has one active heme binding site). For N-acetyl cysteine (NAC) treatments, cells were pretreated with 10 mM NAC in a treatment solution containing antibiotics and FBS for 1 hour. After that, the cells were washed twice with an equal volume of HBSS+. After the 1-hour heme

treatment, 10 mM NAC was also present in the media during the regeneration period. These experiments were performed only on HAoSMC cultures. In some experiments, HAoEC cells were pretreated with phenylbutyric acid (4-PBA) and sodium valproate (VPA) at 5 mM and then incubated in complete culture media for 1 h. This was followed by 2 h of heme treatment and two different regeneration times (3 and 6 h), during which they were present in the media throughout. In the heme-arginate (HA) experiments, cells were preconditioned overnight with 10 μM HA, followed by 2 h of heme treatment and the given regeneration time. Bilirubin (BR) dose curve treatment was performed in reduced media containing 1% FBS supplemented with antibiotics (to minimize non-specific BR binding of albumin) for 16 hours. After that, the cells were treated with 25 μM heme. For carbon monoxide (CO) donor studies, after appropriate solubilization (CORM-2-DMSO, CORMA1-cell culture water), the cells were preincubated in CM199 containing 2% FBS and 1% PSA at different doses (20 and 40 μM) for 6 hours, followed by 25 μM heme treatments and subsequent regeneration.

3.3 RNA Isolation and Cleavage XBP1 PCR

Cells grown in 6-well culture dishes were treated according to the experimental conditions and total RNA was isolated using Tri-reagent (Zymo Research). RNA concentrations were determined using an Implen NanoPhotometer 50 (Biotech Hungary) and reverse transcription was performed to produce cDNA final products using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, CA, United States). cDNA samples previously amplified with XBP1 and GAPDH primers were run on horizontal 2-D gel electrophoresis and the resulting cleaved variant pattern was recorded. Image evaluation was performed using ImageJ software and normalized to GAPDH.

3.4 RT qPCR

After the treatments, RNA was isolated and cDNA was transcribed using the above-mentioned methods. The mRNA expression patterns of CHOP (Hs00358796_g1), Grp78 (Hs00607129_gH), ATF4 (Hs00909569_g1), HO-1 (Hs01110250_m1), BVRA (Hs00167599_m1) and GAPDH (Hs02758991_g1) were determined using TaqMan Gene Expression Assays (Applied Biosystems Inc., CA, United States) designed for the studied gene and the values of the studied genes normalized to GAPDH were shown. The studies were performed on a CFX 96 Real-Time PCR system (Bio-Rad, Hercules, CA, United States).

3.5 Small interfering RNA (siRNA) transfection

Cells grown in 6-well culture dishes were transfected with small interfering RNAs specific to the gene and gene silencing was performed. Heme oxygenase 1 (HO-1), ferritin heavy chain (FT-H), biliverdin reductase (BVRA) and non-coding control (NC) siRNAs were purchased from Ambion (Thermo Fisher Scientific, Waltham, MA, USA). Using the manufacturer's protocol, 10 nmol/L siRNA was added to the cells in the presence of oligofectamine for 24 hours in FBS and PSA-free OptiMEM (Thermo Fisher Scientific, Waltham, MA, USA). After that, the transfection media was replaced and the previously mentioned treatments were performed, followed by ER stress markers.

3.6 Cytotoxicity assay

The cells were plated in 96-well black culture dishes used for fluorescent assays. After reaching the appropriate confluence level (90 %), the cells were silenced with 10 nmol/L small interfering selective RNA (FT-H, HO-1 and BVRA siRNAs) in the presence of oligofectamine. After 24 hours of transfection, the cells were treated with heme and well-known cell death inhibitors in the literature: necroptosis (necrostatin), apoptosis (ZVAD) and ferroptosis (ferrostatin). In addition, previously used ER stress inhibitors: 4-PBA and VPA. After 24 hours of treatment, the cells were washed with HBSS+ and incubated with 1μM calcein AM for 30 minutes. Fluorescence was measured at 485/530 nm.

3.7. Cell lysis for protein-based measurements and Western blot

Cells treated in 6-well culture dishes were washed with HBSS (pH 7.4) and then solubilized with RIPA buffer (50 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1% Igepal CA-630, 1% SDC, 0.1% SDS, protease and phosphatase inhibitors) for 30 min on ice. The collected lysates were centrifuged at 16,000 x g at 4˚C for 15 min. After centrifugation, the supernatant was collected, which contained the whole cell lysate excluding cell membrane fragments and other components. The protein content of the samples was determined using a BCA protein assay kit (Pierce, Thermo Fisher Scientific, Waltham, MA, United States of America) and the same amount of protein (30 μg protein) was injected onto 10 or 12 % reducing Tris-Glycine gels per sample. After electrophoresis, the gels were transferred to a 0.22 μm PVDF membrane (Advansta Inc., Menlo Park, CA, United States) or a 0.45 μm nitrocellulose membrane (GE Healthcare, Chicago, IL, United States). Nonspecific antibody binding sites were blocked with 5% BSA (bovine serum albumin) or 5% nonfat dry milk for 60 min at room temperature. After blocking, the membranes were incubated with the following primary antibodies for a minimum

of 16 h at 4˚C with shaking: antibodies against human ATF4, ATF6, BVRA, CHOP, eIF2α, peIF2α (Ser451), FT-H, Hgb, HO-1, GAPDH and XBP1 proteins. CHOP, HO-1 and GAPDH antibodies were purchased from ProteinTech (Manchester, M33WF, United Kingdom). The above-mentioned antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). The target antibodies were used at a dilution of 1:1000 and the normalizing protein was used at a dilution of 1:5000. Secondary antibodies, coupled to the primary antibodies, were applied to the membrane after 16 hours for 60 minutes at room temperature. Antigen-antibody binding was detected by chemiluminescent assay (Westernbright ECL HRP substrate - Advansta). The quantitative emission of the obtained signals was normalized to the expression of the investigated housekeeping protein using ImageJ software.

3.8 Confocal microscopy

For immunofluorescence staining, cells were treated in a 24-well culture dish containing a 12mm glass disc. After cell treatment, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were washed 3 times with PBS in an equal volume of culture medium. Nonspecific binding sites were blocked in PBS containing 0.3% Triton-X and 5% goat serum for 60 minutes. Subsequently, the samples were incubated with primary antibodies (anti-human Grp78 monoclonal antibody – Thermo Fisher Scientific and anti-human HO-1 antibody – Protein Tech) for 16 hours. The next day, fluorophore-labeled secondary antibodies (Grp78-green-Alexa Fluor 488 mouse anti-human IgG and HO-1-red-Alexa Fluor 568 rabbit anti-human IgG) were applied to the samples for 60 minutes at room temperature. Hoechst stain was used to visualize the cell nucleus with a 10-minute incubation after incubation with the secondary antibody. Images of the samples were taken with a Leica Microsystems high-resolution confocal microscope.

3.9 Tissue samples

The study samples are not based on individual patient histories, but rather groups were selected where we could examine the HIER stress process in healthy subjects and in patients undergoing CEA due to atherosclerosis. The CEA samples were provided by the Department of Vascular Surgery, Department of Surgery, University of Debrecen. The sample collection was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen, Clinical Center under the following permit number: DE OEC RKEB/IKEB 3712-2012. Donors were informed in writing about the sample collection and gave their consent for their use. The

samples were classified by a pathologist according to the AHA guidelines: type I (healthy), type IV (atheroma) and type VI (complicated lesion) groups.

3.10 Immunohistochemistry

The CEA artery samples used for the study were fixed in 4% formaldehyde solution for 1-3 days depending on the size of the sample. This was followed by a decalcification process, and then further immunostaining was performed by the colleagues of the Institute of Pathology. CHOP, Grp78/BIP, SMA and H&E staining were performed on the 3-5 μm thick fixed artery sections upon our request. The images were recorded with a light microscope and a connected camera system. The relevance of the stainings on the samples was determined by a pathologist colleague.

3.11 RNA Sequencing

Total RNA was extracted from human CEA samples (n=5) using the above-mentioned method. The quality validation of the samples was performed on an Agilent Bioanalyzer using the Eukaryotic Total RNA Nano Kit (Agilent Technologies, Santa Clara, CA, United States of America). Samples with an RNA integrity number greater than eight were included for library preparation. The sequencing process was performed by our collaboration partner using the Illumina NextSeq500 with a 75-cycle protocol. The raw sequencing results are available in the NCBI SRA database under the accession number PRJNA594843. Heat maps and dot plots of the genes relevant to us were provided by our collaboration partner after statistical analysis.

3.12 Statistical analysis

The statistical analysis of the data was performed using GraphPad Prism 5.13 software. The results were presented as mean±standard error. The one-way ANOVA posthoc Bonferroni algorithm was used for statistical analysis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

IV. Results

4.1 The presence of endoplasmic reticulum stress in the pathophysiology of atherosclerosis

The relationship between ER stress and hemorrhage was investigated in samples from CEA, representing different stages of plaque progression (atheroma and hemorrhaged plaque), which were compared with healthy control samples. During immunohistochemical staining, smooth muscle actin (SMA), Grp78 and CHOP proteins were examined. Based on our studies, the healthy vessel wall shows low levels of Grp78 and CHOP staining. The smooth muscle cell layer mobilized around the lipid deposits is clearly visible in the atheroma section, but the expression of ER stress markers is similar to that of the control vessel wall. In the samples of ruptured, hemorrhaged complicated lesions, high immunopositivity is seen for both Grp78 and CHOP. In the higher magnification image, activated smooth muscle cells show cytosolic Grp78 staining and CHOP staining showing cytosolic and nuclear translocation.

4.2 Significant amounts of free heme are observed in the bleeding plaques

We extracted proteins from CEA samples for Western blot and spectrophotometric measurements. During the studies, we detected that, compared to healthy samples, cross-linked hemoglobin forms are present in complicated lesions, which strengthen the process of hemoglobin oxidation. Heme is released during oxidation. Based on the spectrophotometric measurements, significantly higher ferriHb and heme content can be measured in complicated lesions. In light of this, we proved that a high heme concentration is present inside the plaque.

4.3 Heme activates the PERK arm of ER stress in a time- and dose-dependent manner in human aortic smooth muscle cells

The activation of the PERK arm of ER stress by heme in HAoSMC cells was first investigated by monitoring CHOP expression. We showed that heme results in a dose- and time-dependent activation of CHOP mRNA. A significant increase is most significant at the earliest time point, 3 hours, and then the heme-induced CHOP expression shows a decreasing trend as a function of time. Since CHOP expression can also be induced by the activation of the IRE1 and ATF6 arms of ER stress, we therefore examined the post-translational and gene expression changes of two proteins regulated by PERK. Activation of the PERK arm is followed by phosphorylation of eIF2α Ser51 and increased ATF4 expression. We showed that ATF4 is expressed at both the RNA and protein levels in a time- and dose-dependent manner. ATF4 expression is expressed 3 and 6 hours after heme treatment, while it decreases to the level of

the untreated control after 16 hours in heme-treated cells. Phosphorylation of eIF2α is increased in the early stage of the cascade (3 hours) and then decreases to the level of the control.

4.4 Time- and dose-dependent activation of the ER stress IRE1 arm by heme in human aortic smooth muscle cells

We monitored the activation of the IRE1 arm by heme at the RNA and protein levels by the appearance of cleaved XBP1 (XBP1s) in HAoSMC cells. We showed that the amount of XPB1s RNA changes in a time- and dose-dependent manner in response to heme at both the RNA and protein levels. Increased XBP1s expression is highest 3 hours after heme treatment and then decreases to control levels.

4.5 Heme induces the ATF6 arm of ER stress in a time- and dose-dependent manner in human aortic smooth muscle cells

ATF6 is activated by protein cleavage, which was monitored by measuring the expression of full-length ATF6 protein by immunoblotting. We showed that heme enhances ATF6 cleavage in a time- and dose-dependent manner in HAoSMC cells, indicating activation of the ATF6 arm. ATF6 cleavage is most pronounced after 3 hours and is still significant after 6 hours. After 16 hours, protein expression returns to near basal levels, but some loss is still seen at the highest concentration of heme.

4.6 Heme induces the ER "master regulator" Grp78 and the expression of HO-1/Ferritin involved in heme catabolism in human aortic smooth muscle cells in a time- and dose-dependent manner

Grp78 is a central master regulator of ER stress, so we investigated how heme affects Grp78 expression in HAoSMC cells. Significant gene expression was observed as early as 3 hours after heme treatment, even at the lowest heme dose (1 μM). The highest Grp78 expression was detected at the RNA level 6 hours after heme treatment, and the expression of Grp78 at the RNA level decreased thereafter. At the protein level, Grp78 expression was elevated both after 6 and 16 hours. We then examined the expression of two proteins involved in heme catabolism, HO-1 and ferritin heavy chain (H-ferritin). Heme activated HO-1 gene expression and HO-1/H-ferritin protein expression in a time- and dose-dependent manner at all tested concentrations.

4.7 Hemopexin and alpha-1-microglobulin heme-binding proteins reduce HIER stress in human aortic smooth muscle cells

The protection of alpha-1-microglobulin (A1M) and hemopexin (Hpx) lies in their chelating properties with heme. The chemical reaction between heme and hemoproteins allows extracellular heme to not interact with the cell. We examined the effect of heme-binding proteins against heme with regard to the activation of UPR pathways. For these experiments, cells were treated with heme-protein complexes, where equimolar amounts of the given acute phase proteins were used in addition to heme, corrected for the heme binding site (25 μM heme + 12.5 μM A1M / 25 μM Hpx). We examined the extent to which the participants of the IRE1, PERK and ATF6 pathways change upon treatment with heme-binding proteins. The first half of the section represents mRNA-based changes, which were examined at two time points, in the presence of both heme-binding proteins. The measurements show that two elements of the IRE1 pathway: ATF4, CHOP and the gene expressions of Grp78 and HO-1 involved in the adaptation mechanism show a marked decrease compared to the response of cells treated with heme alone. The protection of hemoproteins remained significant at both the early 3-hour and late 16-hour time points. In the second half of the section, the protein expression pattern was examined with regard to PERK/IRE1/ATF6, and the IRE1-mediated XPB1 cleavage under the above-mentioned conditions. The pattern of cleaved XBP1 shows that the pathway is not activated by heme bound to hemoproteins. Both proteins provided equal protection against the heme-induced effect. Similar to the gene expression patterns, protein expression also followed the mechanism of inhibition in the case of ATF4 /Grp78/XBP1 and HO-1 proteins. Phosphorylation of the eIF2α protein and proteolytic cleavage of the ATF6 protein did not occur in the presence of A1M and Hpx. Overall, it can be said that A1M and Hpx are able to inhibit the ER-provoking effect of heme on the tested cells.

4.8 Preventing the effect of heme as a free radical generating compound with N-acetylcysteine

According to literature data, abnormally enriched free heme causes the release of free radicals, thereby inducing cell death in extreme cases. N-acetylcysteine (NAC) is a widely used antioxidant, which, thanks to its radical scavenging effect, is able to inhibit the harmful free radical effect caused by heme. In our work, we investigated whether NAC is able to inhibit HIER stress. Our results show that NAC treatment effectively reduced the expression of the examined ER stress markers.

4.9. Plaque hemorrhage enhances ER stress in CEA-derived samples

Following our in vitro studies, we extended our work to human vascular samples. Thus, we examined healthy CEA-derived vessel samples, samples from atheroma and hemorrhaged plaques by RNA sequencing. From the resulting heat map, we can see that ER stress genes are highly expressed (ATF5, CALR, CANX, ATF6, CHOP, Grp78, HSP90B1), while others that are activated early in the signaling pathway (DNAJB9, XBP1, EIF2AKA3, ATF4) show reduced expression. These results suggest that the ER stress process changes dynamically during plaque progression and hemorrhage and the heme accumulation there markedly modulate the ER stress process.

4.10 ER stress markers are upregulated in endothelial cells during plaque progression

Following our RNA-level studies, by monitoring CHOP and Grp78 markers, we investigated whether the expression of ER stress markers changes during plaque progression in another resident cell of the vascular wall, endothelial cells. We demonstrated by immunohistochemistry that increased expression can be observed in both fresh and chronic hemorrhage compared to healthy and non-hemorrhaged atheroma for both Grp78 and CHOP. Moreover, endothelial cells of neovascularized small vessels formed at the site of fresh and chronic hemorrhage also show positive staining. Our research group demonstrated that hemorrhaged plaques contain large amounts of oxidized Hb forms, and that a huge amount of heme is released from the disintegration of this Hb. In this regard, we further investigated the phenomenon of HIER stress in vitro on human aortic endothelial cells (HAOEC). We demonstrated by immunofluorescence staining that heme increases the expression of HO-1 and the ER stress marker Grp78 in HAOEC cells, which supports our observations on HAoSMC cells in this cell type as well.

4.11 Investigation of ER stress arms in HAoEC cells in case of HIER stress

After we were convinced of the clinical relevance of the topic, that the ER stress process induced by hemorrhage has a role in the progression of atherosclerosis, we modeled the phenomenon of HIER stress in vitro in HAOEC cells. Under hemolytic conditions, heme is released into the plasma in high concentrations (up to 100 μM). Our in vitro studies in HAOEC cells supported our observations in vascular samples, since in a cell culture model even 10 μM of heme can induce the ER stress mechanism. The cells were treated with increasing doses of heme (10, 25 and 50 μM). The gene expression pattern gave a similar response to HAoSMC cells, and a dose-time curve can be observed for CHOP and Grp78 already at the early time point. Based on the protein expression pattern, a dose-time curve can be observed for all ER

stress markers. By examining a characteristic marker of the ER stress arms (Grp78, XBP1s, CHOP), we can see the activation of the signaling pathway and the activation of the HO-1/FT-H system. In another experimental setting, we were interested in whether HIER stress is a serum-dependent process. Serum itself contains direct and indirect heme-binding proteins, such as albumin or alpha-1-microglobulin. The varying serum content conditions shed light on whether ER stress can operate in an ischemic state or in a hemolytic region with reduced blood supply. HAOEC cells were treated with 50 μM heme and time curves were recorded for 3, 6 and 24 hours in media with a low (1%) and a higher (5%) serum content. Based on gene expression studies, the HO-1/FT-H system gave a nearly identical response regardless of serum concentration. However, for ER stress markers, we can see that the expression levels of CHOP and Grp78 differ significantly. In the low serum medium, multiple inductions of CHOP, which is responsible for cell death, are observed. Grp78 induction is still active after 24 hours, suggesting that the protein fights the stress caused by high levels of heme through its protective nanny function. In the medium with higher serum content, the degree of HIER stress is lower, with higher activity only observed at 6 hours. After that, the expression returns to basal levels. The protein expression pattern showed a similar trend as the PCR results. Surprisingly, in the presence of serum, the activation of XBP1, thus the cleaved XBP1s, was significantly reduced compared to CHOP expression, which is still detectable after 24 hours in the continuous presence of heme.

4.12. Inhibition of HIER stress with classical ER stress inhibitors

According to literature data, ER stress can be prevented by the use of certain chemical chaperones. Phenylbutyric acid (4-PBA) and sodium valproate (VPA) are widely used inhibitors. Based on our assumption, they may also exert their inhibitory effect in the process of HIER stress. In our experiments, HAOEC cells were preincubated overnight with the aforementioned chemical chaperones: the complete culture media (CM199 containing 10 % FBS and 1 % PSA) was supplemented with 5 mM VPA and 4-PBA. Subsequently, HIER stress was induced in serum- and antibiotic-free media with 25 μM heme for 2 hours, and the cells were dissected after 3 and 6 hours of complete media regeneration. Phenylbutyric acid, a low molecular weight chemical chaperone, can prevent protein misfolding, thereby protecting the ER from stress. Sodium valproate is a widely used stress inhibitor against a variety of stressors. Our results show that neither VPA nor 4-PBA was able to completely protect against HIER stress after 3 hours. Gene and protein level analysis revealed that the tested chaperones are not able to reduce the activation of CHOP leading to cell death, but they significantly reduce the

induction of protective Grp78 and HO-1. Our latter observation is supported by the literature data that VPA reduces the activation of the Nrf2-Keap1 pathway. Similar to the 3-hour measurements, HO-1 and Grp78 induction were also reduced by classical ER stress inhibitors, but they increased the induction of CHOP at both gene and protein levels at the 6-hour time point. Overall, it can be said that VPA and 4-PBA reduced the induction of protective Grp78. VPA seems to be more effective at activating cleaved XBP1, but both inhibitors resulted in significantly higher CHOP expression.

4.13 Study of the HO-1/Ferritin system in HIER stress on HAOEC cells

In addition to heme-binding proteins, the HO-1/H-ferritin system, which degrades heme, plays a central role in protection against heme stress. In our next experiments, we monitored the changes in the absence of the HO-1/H-ferritin system by selectively silencing HO-1 and H-ferritin. Our experiments showed that 3 hours after heme treatment, the absence of the HO-1 protein showed a drastic increase in ER stress markers compared to wild-type cells, while knocking out H-ferritin silencing did not result in a significant increase compared to control cells. Examining a later time point, we observed that, in contrast to the 16-hour regeneration time examined in HIER stress, the cellular stress response did not decrease, but rather started to increase. Overall, we can say that we can see marked ER stress in both early and late phases. Interestingly, only the lack of HO-1 caused such an increase, there was no change in the lack of H-ferritin. We then investigated whether the induction of the HO-1/H-ferritin system with the non-toxic, clinically used heme-arginate is protective against HIER stress. We showed that heme-arginate pretreatment significantly protected both in the early and late phases of HIER stress.

4.14 Investigation of heme degradation intermediates in HIER stress in HAOEC cells

From the existing experimental results, it is clear that heme-induced ER stress is enhanced in the absence of HO-1 and can be prevented by an elevated HO-1 level. We were further interested in whether any of the intermediates released during heme catabolism have an influence on the course of HIER stress. First, we examined the other central enzyme of heme degradation, biliverdin reductase (BVRA). After gene silencing with small interfering RNA, we treated the cells with heme and examined ER stress markers. The experiments were performed simultaneously with HO-1 gene silencing, comparing the two depletions. We showed that, in contrast to HO-1 deficiency, BVR deficiency did not increase the increase in stress markers. Consequently, the amount of active HO-1 is the limiting factor in our system,

not the lack of BVR. Bilirubin, as an intermediate in heme catabolism, broadly inhibits several stress mechanisms affecting endothelial cells. Since the absence of BVR has no influence on the course of HIER stress, we investigated whether exogenously administered bilirubin has a dose-dependent effect on the process. In our experiments, we saw the same effect as when silencing BVR. Bilirubin had neither a positive nor a negative effect on the course of HIER stress. The effects of carbon monoxide (CO), released during heme degradation, were last examined in our system. According to literature data, CO, as a gas transmitter, is a molecule involved in several signaling pathways, which has anti-inflammatory and anti-apoptotic properties. In this experiment, we wanted to represent the CO-induced HO-1 induction, which is likely to be able to successfully prevent the development of HIER stress. We used two different CO-donor molecules, one of which has a fast (CORM-2) and the other a slow (CORMA1) gas release kinetics. Comparing the two different CO-donor molecules, we can say that CORM-2, which works with a faster release rate, proved to be more effective against HIER stress than CORMA1. Both molecules show a significant difference, although CORM2 gave a more effective response. To our surprise, despite the literature data, the CO donor molecules only caused minimal HO-1 induction in the cells, and during co-treatment with heme, the HO-1 response was slightly reduced.

4.15 Inhibition of cell death caused by HO-1 deficiency

HO-1 deficiency - which can be congenital or a decrease in functional enzyme activity - dramatically increases the sensitivity of cells to heme, thereby reducing cell viability and in extreme cases can even cause cell death. The protective HO-1 system can be activated not only by heme, but also by BVR responsible for bilirubin synthesis, and by ferritin activated by iron. In the following experiments, we examined the role of these two enzymes in cell viability. Whether BVR and H-ferritin activated in HO-1 gene-silenced cells can compensate for the negative effects caused by the enriched free heme. In addition, we added specific cell death inducers to HO-1 depleted cells. As a result, we found that HO-1 silencing results in approximately 40% cell death after heme exposure. BVR and FT-H silencing did not cause any change in cell viability. In order to eliminate cell death caused by HO-1 depletion, known cell death inhibitors such as necroptosis (Necrostatin), apoptosis (ZVAD) and ferroptosis (Ferrostatin) inhibitors did not live up to their expectations. We also tried 2 classical ER stress inhibitors (VPA/4-PBA), but they could not affect the decrease of ER stress markers and they could not prevent cell death either. The results suggest that there is no known inhibitor against heme toxicity arising in HO-1 deficiency.

V. New conclusions

Our research investigates the processes of ER stress in atherosclerotic plaques under in vitro and ex vivo conditions. Our focus was on the investigation of the effect of pathological amounts of heme released during plaque hemorrhage on ER stress in resident cells of the vasculature. We examined using cell and molecular biological methods whether PERK, IRE1, ATF6 of the UPR pathway are activated.

Based on our results, we made the following new conclusions:

1. In our in vitro experiments, ER stress can be induced in the examined cells and in the presence of heme, the PERK, IRE1, ATF6 branches of the UPR pathway are activated, which was supported both at the transcriptional and translational levels.

2. Heme-induced endoplasmic reticulum (HIER) stress can be inhibited by binding the inducing agent, i.e. heme, with heme-binding proteins (hemopexin, alpha-1-microglobulin), but classical ER stress inhibitors (VPA, 4-PBA) only partially inhibited the process.

3. The absence of the HO-1/Ferritin system increases, and its activation reduces, the degree of HIER stress in the examined cells.

4. Of the heme degradation intermediates, only CO had a positive effect on reducing HIER stress.

5. The inhibitors we used were unable to inhibit heme stress-mediated cell death caused by HO-1 depletion.

6. Transcriptomic analysis and immunohistochemical staining of ex vivo human tissue samples confirmed that in the sixth stage of the plaque, hemorrhagic plaques, the presence of ER stress was increased compared to earlier stages without bleeding.

VI. Summary

The ER plays a key role in the proteostasis of eukaryotic cells. Its normal function is essential for the functioning of intracellular processes. The accumulation of defective proteins, such as misfolded, misfolded, or partially folded proteins, in the ER lumen leads to ER stress. This accumulation activates the UPR, or the three branches of the "junk protein response": the IRE1, PERK, and ATF6 pathways. Following the activation of sensor proteins, the UPR is responsible for activating repair mechanisms and initiating apoptosis in response to stress.

Increased and/or persistent ER stress plays a role in many diseases. In metabolic diseases such as hepatic steatosis and hyperlipidemia (IRE1 and ATF6 mediated processes), in cancer diseases, where increased levels of XPB1s expression have been detected in tumor biopsy samples due to elevated UPR activation (brain, breast, lymphoma, multiple myeloma), and in neurodegenerative diseases (Alzheimer disease, Huntington disease, Parkinson disease), where elements of the ERAD and ATF6 signaling cascades interact abnormally with enriched protein aggregates.

In pathological hemolytic conditions, extracellular free Hb undergoes a rapid oxidation process as the eliminating acute phase proteins become saturated. During massive intravascular hemorrhage, free heme is taken up by surrounding cells. Because it is highly lipophilic, it readily moves either actively or passively across the cell membrane. The surrounding resident cells are exposed to high levels of stress, even hundreds of micromoles of heme. Heme stress is a direct or indirect etiological factor in many human diseases, such as: organ and tissue damage, malaria, subarachnoid hemorrhage, rhabdomyolysis with renal failure, complications during surgery or transfusion, inherited hemolytic syndromes, sickle cell anemia, hemolysis due to sepsis, and atherosclerotic plaque rupture. In all these diseases, heme is present in high concentrations. Although heme is essential for oxygen and electron transport systems as a prosthetic group in hemoproteins (Hb, myoglobin, cytochromes), high levels of free heme cause prooxidant, proinflammatory and cytotoxic effects. Evolutionarily well-conserved extracellular (hemopexin, alpha-1-microglobin) and intracellular (HO-1/Ferritin system) detoxification mechanisms against high free heme are known. Several previous studies have suggested a close relationship between ER stress and vascular diseases, including atherosclerosis and oxidative stress. These studies found a direct link between UPR-induced ER stress and atherosclerotic risk factors. Free cholesterol, 7-ketocholesterol, LDL, and oxidized LDL increased the expression of ER stress in resident cells of the vasculature (macrophages, endothelial cells, and smooth muscle cells).

Inside the hemorrhagic plaque, the altered internal pH and pressure differences destroy the immature inflowing vessels, and cause the lysis of the incoming VVT, releasing a huge amount of heme. The high heme accumulation appearing under the subendothelial layer is primarily exposed to endothelial cells and smooth muscle cells migrating there from the tunica media. In the publications serving as the basis for this thesis, we investigated the ER stress observed in human aortic endothelial cells and human aortic smooth muscle cells in the presence of heme under experimental conditions, which we named heme-induced endoplasmic reticulum stress (HIER stress).

Cells were examined in vitro in response to heme dose and time. The central proteins of the three arms of the UPR were examined at both transcriptional and translational levels. When examining the IRE1, PERK and ATF6 pathways, we observed that the arms are activated at both the RNA and protein levels. Simultaneous activation of all three arms was observed upon heme treatment, where the central effector proteins of the PERK pathway: CHOP and ATF4 appeared at elevated levels. Following the XBP1 cleavage pattern of the IRE1 pathway, we detected a significantly high level of the cleaved form of XBP1s. In the ATF6 pathway, we examined the proteolytic cleavage of ATF6 itself, which, after cleavage, its translocation to the cell nucleus triggers the expression of the nurse proteins responsible for folding (Grp78, Grp94, calreticulin). Grp78 normally binds to these sensor proteins and inhibits their expression. Given that the binding affinity of Grp78 is higher for disordered proteins than for sensor proteins, we also examined Grp78 expression. We detected high Grp78 expression in a dose- and time-dependent manner. Overall, it can be said that the presence of increased ER stress initiated by the UPR can be detected and the mechanisms improving the folding capacity of the ER have been initiated. HIER stress itself, as a phenomenon, was proven with these experiments in both cell types. After detecting HIER stress, we tried to achieve inhibition with acute phase proteins that also function in the body. Hemopexin, which is stoichiometrically 1:1 in a ratio and alpha-1-microglobulin, which is able to bind heme exogenously in a ratio of 1:2, may be able to inhibit HIER stress. During our experiments, the two acute phase proteins successfully inhibited the activation of the IRE1, PERK and ATF6 pathways, as well as the expression of Grp78. This leads to the conclusion that the UPR cascade was not initiated without the presence of the inducing agent.

Heme, as a free radical generating compound, has been reported in several studies. Oxidative damage to cells can negatively affect ER function, thus, with an abnormal increase in folding capacity, ER stress cascades can be initiated. The antioxidant pathways and ER stress cascades

themselves interact at several points, for example with Nrf2/HO-1 signaling via PERK and IRE1. We also investigated the role of NAC as a radical scavenger molecule in HIER stress. Our results show that, similar to acute phase proteins, it inhibits the expression of core proteins of the UPR pathways.

According to literature data, classical ER stress can be reduced or completely inhibited by the use of certain chemical chaperones. The phenylbutyric acid (4-PBA) and sodium valproate (VPA) we chose could only partially or slightly inhibit the expression of HIER stress. In contrast to the inhibitions with heme proteins, chemical chaperones could only inhibit one point of the stress arms or to a small extent the expression of the other target proteins tested. The presence of CHOP, which indicates the direction of apoptosis activated by PERK, was still high in the treated cells, on the other hand, the levels of HO-1 responsible for heme catabolism and Grp78 responsible for normal folding were strongly reduced. This suggests that classical ER stress and HIER stress exert their effects in cells via different pathways.

Our studies suggest that the HO-1/H-ferritin system plays a central role in the course of HIER stress. To prove this, we inactivated the system by siRNA-based gene silencing and examined the ER stress pattern. In the absence of the HO-1/H-ferritin system, the examined proteins of the ER stress arms showed significantly higher expression compared to wild-type cells. Interestingly, the absence of HO-1 caused only a drastic response, while the absence of H-ferritin did not change.

After HO-1 gene silencing, we saw that the presence of HO-1 is crucial in the defense against HIER stress. After conditioning the cells with heme-arginate, cells with a higher HO-1 pool before the stress did not show the presence of ER stress. We did not detect UPR sensor protein expression in either the early or late phases. In light of this, heme-arginate used in the clinic could be a potential inducer before interventions involving extra- and intravascular hemorrhages, which prepares the cells for the expected HIER stress. Our results show that HIER stress is enhanced in the absence of HO-1 and can be prevented with elevated HO-1 levels.

We further investigated the effects of the processes and intermediate products following HO-1-induced heme catabolism. Biliverdin reductase deficiency and bilirubin treatments had neither a positive nor a negative effect on the course of HIER stress. During the degradation, CO is released, a gas transmitter molecule involved in several signaling pathways according to literature data. It was observed that exogenously administered CO can activate the Nrf2 protein through the phosphorylation of PERK, which initiates HO-1 expression. In addition, CO also

counteracts the degree of apoptosis by reducing the expression of CHOP. Based on our measurements, only exogenously administered CO donors were able to inhibit HIER stress. However, they did not cause significant HO-1 expression. In light of this, the CO/HO-1 system may have a positive effect in the treatment of vascular diseases associated with ER stress.

HO-1 deficiency, whether congenital or functional, dramatically increases the sensitivity of cells to heme and, in the case of sufficiently long-term heme toxicity, also causes cell death. Presumably, in the presence of inert HO-1, the degree of ER stress is milder during short-term heme stress, the stress level does not reach a threshold value, so only the UPR is induced, promoting cell survival. In the case of longer-term heme stress, in the HO-1 deficient state, the degree of ER stress is strong enough that the stress exceeds the threshold value, so that in addition to the UPR, apoptotic signaling becomes irreversible. In our cytotoxicity experiments, in addition to inducing a HO-1 deficient state (HO-1 gene silencing), we applied several cell death inhibitors to the cells. We showed that neither apoptosis, nor necroptosis, nor ferroptosis inhibitors were able to prevent cell death.

In addition to our cellular experiments, we performed RNA sequencing and immunohistochemistry staining of human ex vivo CEA endarterectomy specimens. RNA sequencing supported the results obtained in our cell models. ER stress (ATF5, ATF6, CANX, CHOP, Grp78, HSP90B1) genes were expressed at higher levels in samples from complicated hemorrhaged lesions than in samples obtained from healthy tissue. Based on this, during plaque progression, after hemorrhage, when free heme is enriched, high levels of ER stress are expressed in the interior of the plaque.

Tissue sections were also prepared from the same samples from which RNA sequencing was performed. Healthy, atheroma, complicated lesions (with fresh and chronic hemorrhage) represented the stages of plaque progression. CHOP and Grp78 staining confirmed that increased expression was observed in fresh and chronic hemorrhage samples compared to healthy and non-hemorrhage atheroma samples. SMA staining represents the presence of smooth muscles colocalizing with CHOP and Grp78 staining, and endothelial cells of small vessels caused by neovascularization at the site of fresh and chronic hemorrhage also show positive staining.

Overall, our studies can be said that ER stress is present at high levels in vitro and ex vivo under hemolytic conditions and atherosclerotic conditions with hemorrhage.

VII. Acknowledgements

First of all, I would like to thank my supervisor, Dr. Tamás Gáll, for his high-quality professional guidance, guidance and unwavering support. I am infinitely grateful for his patience and willingness to teach. In recent years, I have been able to boldly turn to him for advice and support, whether in connection with the research topic or with other professional matters.

I would like to thank Professors Dr. György Balla and Dr. József Balla for providing me with a high-quality professional and financial background for the tasks and works tailored to me, from my time as a research fellow until now, under the auspices of their research group and the institutes they lead.

I also gratefully thank the Vascular Biology Research Laboratory for its former and current colleagues, with a special mention to Dr. Annamária Nagy. I am grateful for her indispensable help, support and friendship over the years.

Special thanks to the Hungarian and foreign co-authors of publications, whose extremely valuable work and professional support helped me in my work.

Last but not least, I would like to thank my family and friends for their love and encouragement.

Grants supporting the research work:

OTKA-K112333, GINOP-2.3.2-15-2016-00043, HUN-REN (11003), OTKA-K132828, EFOP-3.6.2-16-2017-00006, ED_18-1-2019-0028, TKP2020-NKA-04, EFOP-3.6.3-VEKOP-16-2017-00009, KKM/33659-2/2024/Adm.

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Registry number: DEENK/495/2024.PL
Subject: PhD Publication List

Candidate: Dávid Pethő
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Pethő, D.**, Hendrik, Z., Nagy, A., Beke, L., Patsalos, A., Nagy, L., Póliska, S., Méhes, G., Tóth, C., Potor, L., Eaton, J. W., Jacob, H. S., Balla, G., Balla, J., Gáll, T.: Heme cytotoxicity is the consequence of endoplasmic reticulum stress in atherosclerotic plaque progression.
Sci. Rep. 11 (1), 2021.
DOI: http://dx.doi.org/10.1038/s41598-021-89713-3
IF: 4.996

2. Gáll, T., **Pethő, D.**, Nagy, A., Hendrik, Z., Méhes, G., Potor, L., Gram, M., Akerström, B., Smith, A., Nagy, P. F., Balla, G., Balla, J.: Heme Induces Endoplasmic Reticulum Stress (HIER Stress) in Human Aortic Smooth Muscle Cells.
Front. Physiol. 9, 1-25, 2018.
DOI: http://dx.doi.org/10.3389/fphys.2018.01595
IF: 3.201

List of other publications

3. Gáll, T., **Pethő, D.**, Erdélyi, K., Egri, V., Balla, G. J., Nagy, A., Nagy, A., Póliska, S., Gram, M., Gábriel, R., Nagy, P., Balla, J., Balla, G.: Heme: a link between hemorrhage and retinopathy of prematurity progression.
Redox Biol. 76, 1-24, 2024.
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IF: 10.7 (2023)

4. Somodi, L., Horváth, E., Bárdos, H., Baráth, B., **Pethő, D.**, Katona, É., Balla, J., Mutch, N. J., Muszbek, L.: Cellular FXIII in Human Macrophage-Derived Foam Cells.
Int. J. Mol. Sci. 24 (5), 1-12, 2023.
DOI: http://dx.doi.org/10.3390/ijms24054802
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5. Nagy, A., **Pethő, D.**, Gesztelyi, R., Juhász, B., Balla, G., Szilvássy, Z., Balla, J., Gáll, T.: BGP-15 Inhibits Hyperglycemia-Aggravated VSMC Calcification Induced by High Phosphate.
Int. J. Mol. Sci. 22 (17), 1-23, 2021.
DOI: http://dx.doi.org/10.3390/ijms22179263
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6. **Pethő, D.**, Gáll, T., Hendrik, Z., Nagy, A., Beke, L., Gergely, P., Méhes, G., Tóth, C., Gram, M., Akerström, B., Balla, G., Balla, J.: Ferryl Hemoglobin and Heme Induce A1-Microglobulin in Hemorrhaged Atherosclerotic Lesions with Inhibitory Function against Hemoglobin and Lipid Oxidation.
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Lab. Invest. 100 (7), 986-1002, 2020.
DOI: http://dx.doi.org/10.1038/s41374-020-0403-x
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9. Potor, L., Sikura, K. É., Hegedűs, H., **Pethő, D.**, Szabó, Z., Máthéné Szigeti, Z., Pócsi, I., Trencsényi, G., Szikra, D. P., Garai, I., Gáll, T., Combi, Z., Kappelmayer, J., Balla, G., Balla, J.: The Fungal Iron Chelator Desferricoprogen Inhibits Atherosclerotic Plaque Formation.
Int. J. Mol. Sci. 21 (13), 1-23, 2020.
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10. Gáll, T., **Pethő, D.**, Nagy, A., Balla, G., Balla, J.: Therapeutic Potential of Carbon Monoxide (CO) and Hydrogen Sulfide (H2S) in Hemolytic and Hemorrhagic Vascular Disorders-Interaction between the Heme Oxygenase and H2S-Producing Systems.
Int. J. Mol. Sci. 22 (1), 1-24, 2020.
DOI: http://dx.doi.org/10.3390/ijms22010047
IF: 5.924

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11. Nagy, A., **Pethő, D.**, Gáll, T., Zavaczki, E., Nyitrai, M., Posta, J., Zarjou, A., Agarwal, A., Balla, G., Balla, J.: Zinc Inhibits HIF-Prolyl Hydroxylase Inhibitor-Aggravated VSMC Calcification Induced by High Phosphate.
Front. Physiol. 10, 1-15, 2020.
DOI: http://dx.doi.org/10.3389/fphys.2019.01584
IF: 4.566

12. Potor, L., Nagy, P., Méhes, G., Hendrik, Z., Jeney, V., **Pethő, D.**, Vasas, A., Pálinkás, Z., Balogh, E., Gyetvai, Á., Whiteman, M., Torregrossa, R., Wood, M. E., Olvasztó, S., Nagy, P. F., Balla, G., Balla, J.: Hydrogen Sulfide Abrogates Hemoglobin-Lipid Interaction In Atherosclerotic Lesion.
Oxidative Med. Cell. Longev. 2018, 1-16, 2018.
DOI: https://doi.org/10.1155/2018/3812568
IF: 4.868

Total IF of journals (all publications): 70,625
Total IF of journals (publications related to the dissertation): 8,197

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

30 September, 2024

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