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

EFFECT OF HYDROGEN SULFIDE ON HEMOGLOBIN-LIPID INTERACTIONS IN ATHEROSCLEROTIC PLAQUES

by László Potor

Supervisor:
Professor Dr József Balla

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF KÁLMÁN LAKI

DEBRECEN, 2022

Effect of hydrogen sulfide on hemoglobin-lipid interactions in atherosclerotic plaques

By László Potor, biologist MSc degree

Supervisor: József Balla, MD, PhD, DSc

Doctoral School of Kálmán Laki, University of Debrecen

Head of the **Examination Committee**:	Pál Soltész, MD, PhD, DSc
Members of the Examination Committee:	Tamás Forster, MD, PhD, DSc
	György Reusz, MD, PhD, DSc

The Examination takes place at the Library of Division of Nephrology, Faculty of Medicine, University of Debrecen, the 06th of June 2023, 10.00 AM.

Head of the **Defense Committee**:	Pál Soltész, MD, PhD, DSc
Reviewers:	Anikó Somogyi, MD, PhD, DSc
	Harjit Pal Bhattoa, MD, PhD
Members of the Defense Committee:	Tamás Forster, MD, PhD, DSc
	György Reusz, MD, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, the 06th of June 2023 1:00 PM

1. INTRODUCTION

Cardiovascular diseases cause approximately 18 million deaths every year, making them the leading cause of death worldwide, according to the World Health Organization (WHO). This number is 32% of all annual deaths. Cardiovascular diseases are the cause of every second death in Hungary. Arteriosclerosis plays a significant role in the development of this group of diseases. During atherosclerosis, fatty deposits called plaques form on the walls of blood vessels. During the process, the plaque hemorrhage occurs and destabilizes, which results in their rupture and the release of the fatty substance in them into the bloodstream. Arteriosclerosis also results in heart attack, stroke, embolism, and thrombosis.

The basis of my choice of topic is the research into the pathogenesis of the hemorrhages observed inside the plaques, as well as the pharmacological investigation of the progression of the hemorrhages. The development of hemorrhage greatly increases mortality, and the drug therapies used only slow down this process, but do not prevent it. For this reason, the social applicability of the results from the research topic is significant.

The research aims to explore in detail the processes taking place in human blood clots, as well as to test medicinal attack points, which can be used to stop and/or reverse the pathogenesis of atherosclerosis.

To test the hypotheses, I purified hemoglobin from the blood of healthy donors. In hemoglobin oxidation tests, I isolated and purified low-density lipoprotein (LDL) from the whole blood of healthy donors. Plaque lipids were separated from the atheroma of patients who underwent endarterectomy. In the cellular experiments, I tested the molecular biological processes in atherosclerosis on human vein endothelial cells. The healthy human carotid arteries were provided to us by the Institute of Forensic Medicine of the University of Debrecen. The human atheroma and hemorrhagic plaques were provided to us by the Vascular Surgery Department of the Department of Surgery of the University of Debrecen. I used 54 human carotid samples for my research, which is the basis of my Ph.D. thesis.

2. LITERATURE REVIEW

2.1 ATHEROSCLEROSIS

2.1.1 THE EPIDEMIOLOGY AND RISK FACTORS OF ATHEROSCLEROSIS

According to the report of the World Health Organization (WHO), cardiovascular diseases are the leading cause of death in the world (32% mortality; 17.9 million deaths/year). The background of the cases is heart attack and stroke caused by atherosclerosis (85%). Lifestyle-related risk factors for atherosclerosis are smoking, an unhealthy diet, a sedentary lifestyle, and excessive alcohol consumption. Physiological risk factors are high blood pressure, high blood cholesterol and glucose levels, chronic kidney disease, diabetes, overweight, and aging (see https://www.who.int/health-topics/cardiovascular-diseases/#tab=tab_1).

2.1.2 GENERAL STRUCTURE OF THE AORTA AND ARTERIES

Blood vessels form a closed system in which blood flows as liquid connective tissue. The interstitial part of the blood vessels, where the blood flows, is called the lumen. The aorta and arteries can be separated into three layers. The lumen is in direct contact with the inner layer of the vascular system, the intima (*tunica intima*). Towards the lumen, this layer is made up of a single layer of squamous cells, so-called endothelial cells, and the proteoglycan layer below. The middle layer (*tunica media*) consists mainly of elastic fibers and smooth muscle cells. The outer layer of connective tissue (*tunica adventitia*) of the vessel wall contains the vessels that nourish the vessel wall (*vasa vasorum*) and the nerves that supply the vessel wall (*nervi vasculares*). The structure of the arteries differs depending on the type (elastic arteries, muscular arteries, arterioles). In the thoracic and abdominal parts of the aorta, there are structural differences.

2.1.3 THE PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis is an inflammatory disease of the aorta and large arteries, during which the lumen of the vessel narrows due to the thickening of the intima. According to the recommendation of the AHA (American Heart Association), the course of the disease can be grouped into the following categories.

Type I lesion – initial lesion.

The formation of lesions begins in infancy. When the LDL leaving the blood system enters the subendothelial space, it is modified by reacting with proteoglycans, creating so-called minimally modified LDL, which after further oxidation steps turns into oxidized LDL (oxLDL). Lipid hydroperoxide (LOOH) and 4-hydroxy-nonenal (4-HNE), which are toxic to endothelial cells, should be highlighted among the products produced during the formation of oxLDL. Endothelial cells are activated due to their effect, and the expression of adhesion proteins increases. Monocytes circulating in the vasculature roll to the inflammatory area under the influence of selectins (e.g. E-selectin; P-selectin), and then the adhesion proteins (e.g. intracellular adhesion molecule-1: ICAM-1; vascular cell adhesion molecule-1: VCAM-1), they stick to the surface of the endothelial cells. Monocytes penetrate the subendothelial space. Subsequently, the endothelial cells produce macrophage colony-stimulating factor (M-CSF) in the presence of oxLDL, which causes monocytes to differentiate into macrophages. oxLDL polarizes macrophages, as a result of which lipids are taken up due to the increased expression of scavenger receptors (CD36; SRA). Macrophages saturated with lipid droplets are called foam cells. In the case of each type of lesion, microscopically observable small yellow dots can be seen on the surface of the aorta, and groups of foamy cells containing lipids can be seen in the tissue.

Type II lesion - fatty streaks.

The accumulation of lipids in the intima can be seen with the naked eye, lipid droplets are already visible in the smooth muscle cells. Lesion types one and two are often referred to together as early lesions.

Type III lesion – pre-atheroma.

Lipids released from dead foam cells appear extracellularly.

Type VI lesion – atheroma.

The atheroma contains a high level of extracellular lipids, which together form a so-called lipid core. At this stage, the calcification of some smooth muscle cells can already be observed. Cholesterol crystals are found within the lipid core.

Type V lesion – advanced plaque.

It is characterized by the formation of fibrous connective tissue under the surface of the plaque, which is called a fibrous cap. This connective tissue consists of smooth muscle cells with an increased amount of collagen and a rough endoplasmic reticulum. Type V lesions can be divided into three subgroups. The V/a lesion is the fibroatheroma, which contains numerous lipid cores that are separated from each other by thin fibrous connective tissue. The V/b lesion is a calcified lesion that contains a large number of extracellular hydroxyapatite crystals, the formation of calcification is a well-regulated process. The V/c lesion is a fibrotic lesion, the characteristic of which is that the intima has been replaced by a thin layer of fibrous connective tissue. Lipids are absent or minimal in this lesion.

Type VI lesion – complicated lesion.

Mortality caused by atherosclerosis is mostly due to the development of complicated lesions. It can develop by bleeding on the surface of the plaque or inside the plaque itself. As a result of hemorrhage, the plaque becomes unstable, due to the narrowed lumen, increased shear forces, and proteolytic enzymes produced mainly by macrophages. After the unstable plaque ruptures, the fatty substances contained in it can enter the bloodstream and cause heart attacks, strokes, thrombosis, or embolism.

2.2 INTRAPLAQUE HEMORRHAGE

Due to the formation of lipid peroxidation products (LOOH, aldehydes, ketones, endoperoxides, malondialdehyde, 4-HNE), complicated lesions have a highly oxidative milieu. These intermediate and end products of lipid peroxidation are toxic to cells, especially to macrophages that have been penetrated and differentiated from monocytes. Dead macrophages contribute to the breakdown of the stability of the plaque, thus also to the formation of hemorrhages. Due to the peroxidation taking place in the plaques, hypoxic conditions develop, as a result of which neovascularized blood vessels grow in the plaque from the side of the *vasa vasorum*. Michel JB et al described that under the influence of oxLDL, the expression of vascular endothelial growth factor (VEGF) in smooth muscle cells located in the *tunica media* layer of the vessel wall increases via the peroxisome proliferator-activated receptor ((PPAR)-γ), which also results in neovascularization in the vasa vasorum. Endothelial cells of these small blood vessels die due to the highly toxic environment, so bleeding within the plaque can occur.

2.3 FORMATION AND PHYSIOLOGICAL EFFECTS OF OXIDIZED HEMOGLOBIN

During the formation of a complicated plaque, red blood cells (RBCs) appear inside the plaque. E. Nagy and his colleagues proved that oxLDL and lipid extract cause hemolysis of VVTs in human atheromatous plaques (PL) and the released hemoglobin (Hb) is also oxidized. Furthermore, it was shown that cumin hydroperoxide, an analog of lipid hydroperoxide (LOOH), also lyses VVT and oxidizes Hb. It has also been shown that glutathione/glutathione peroxidase (GSH/GPx) inhibits the lysis of oxLDL and PL-induced VVTs and the oxidation of Hb by converting LOOH to alcohol.

During the interaction between Hb and peroxides, ferrylHb (Fe^{4+}) and oxoferrylHb ($Fe^{4+}=O^{2-}$) are formed. During oxidation, radicals are formed on different amino acids of the α globin (α Tyr-24, α Tyr-42, α His-20) and β globin (β Tyr-36, β Tyr-130, β Cys-93) chains of Hb. These radicals can covalently form Hb dimers, tetramers, and multimers. As a result of oxLDL and oxPL, the two-electron oxidation of Hb takes place, resulting in the formation of ferrylHb. Elevated dityrosine levels in human complicated lesions demonstrate the presence of ferrylHb.

It was previously described that the oxidized form of Hb, metHb (Fe^{3+}), has a cytotoxic effect, by releasing its heme group and sensitizing human umbilical vein endothelial cells, causing them to die upon H_2O_2 treatment. The ferrylHb produced during the two-electron oxidation of hemoglobin has a unique pathophysiological effect. It can rupture the EC layer, during which holes are formed between the cells. At the same time, the expression of adhesion molecules also increases (VCAM-1, ICAM-1, E-selectin) in the EC. These processes suggest that ferrylHb has pro-inflammatory properties and may play a role in the progression of plaques, as neither Hb nor metHb causes the effects mentioned above.

2.4 PRODUCTION OF ENDOGENOUS HYDROGEN SULPHIDE

Hydrogen sulfide (H_2S) is the newest member of the endogenous gas transmitters, along with nitric oxide (NO) and carbon monoxide (CO). The three main H_2S-producing enzymes are cystathion-γ-lyase (CSE), cystathion-β-synthetase (CBS) and 3-mercaptopyruvate (3-MST). Methionine and cysteine taken in during meals are the substrates of the transsulfuration pathway. The homocysteine produced during the methionine-homocysteine cycle is converted into cystathionine by CBS, which is converted into L-cysteine by CSE. Both CSE and CBS

can convert the resulting L-cysteine into H_2S. From L-cysteine, 3-MST results in the production of H_2S together with an intermediate reaction.

2.5 THE ROLE OF HYDROGEN SULPHIDE IN THE PROGRESSION OF ATHEROSCLEROSIS

In the early 2000s, several publications were published on the effect of H_2S on atherosclerosis. One of the main initial steps in atherosclerosis is the oxidation of LDL in the subendothelial space. It has been shown that hydrogen sulfide can inhibit the induced oxidative modification of LDL by heme under *in vitro* conditions. It has been described that H_2S slows down the process of atherosclerosis, which has been demonstrated in various animal models, but due to the lack of the exact mechanism of protection, the cellular response, and the lack of clinical tests, intensive research in this area is necessary. Wang et al showed that sodium hydrosulfide (NaSH) inhibited the formation of atherosclerotic plaques in Apolipoprotein E deficient mice ($ApoE^{-/-}$) without an atherogenic diet. Furthermore, it was demonstrated that the progression of atherosclerosis in $ApoE^{-/-}$ mice was more advanced when the CSE inhibitor was used compared to control $ApoE^{-/-}$ mice. Using CSE-deficient mice, Mani et al showed that the lack of the CSE protein causes increased atherosclerosis and, in parallel, reduced H_2S production.

2.6 THE RELATIONSHIP BETWEEN HYDROGEN SULPHIDE AND INFLAMMATION

Pro-inflammatory cytokines and proteases expressed during arteriosclerosis contribute to the bleeding of plaques and the formation of thrombus. It has been shown that the expression of CSE, the main enzyme responsible for the production of hydrogen sulfide, is increased in mice inflamed with lipopolysaccharide (LPS). From their results, they concluded that H_2S has a pro-inflammatory effect in case of septic shock. Another research group detected elevated CSE expression in chondrocytes and mesenchymal stem cells treated with pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and LPS. During pharmacological and small interfering RNA inhibition of CSE and CBS, cell death was detected in cells treated with pro-inflammatory cytokines and LPS. From their results, they concluded that H_2S performs a protective function in cells during inflammation. Furthermore, it was shown that oxidative stress induced by hydrogen peroxide (H_2O_2) increases the expression of CSE in embryonic kidney and renal

fibroblast cells. Their results show that the increased endogenous H_2S radical scavenging effect due to CSE expression has a protective function.

Due to the growing number of contradictory articles, investigating the relationship between hydrogen sulfide and inflammation is an important and urgent challenge for the scientific world.

3. THE AIMS OF THE STUDY

The study aims to explore in detail the processes taking place in the human hemorrhaged plaque of the carotid artery, as well as to test medicinal attack points, which can be used to stop and/or reverse the pathogenesis of atherosclerosis.

1. I will examine whether ferrylHb, similar to heme, induces the oxidative modification of LDL, thereby destroying EC cells.

2. The expression of heme catabolism proteins – heme oxygenase-1 (HO-1) and H-ferritin (HFT) – is increased by heme treatment on EC cells, so I thought that ferrylHb also increases the level of these proteins.

3. OxLDL and reactive lipids are proven constituents of atherosclerotic plaques. I assumed that during the bleeding of the plaques, in contact with oxLDL and reactive lipids, hemoglobin is oxidized to ferrylHb.

4. I assumed that haptoglobin (Hp) due to its ability to bind hemoglobin and the antioxidant glutathione (GSH)/glutathione-peroxidase (GPx) system can inhibit ferrylHb formation induced by H_2O_2 and oxidized plaque lipids.

5. FerrylHb (but not heme or Hb) ruptures the layer of EC cells. For this reason, I hypothesized that the formed holes trigger the adhesion of monocytes to the EC.

6. The expression of CSE levels is increased in animal models of atherosclerosis. Therefore, I examined the expression of CSE in human healthy, atheroma, and complicated lesions, as well as in $ApoE^{-/-}$ mice fed a high fat and cholesterol diet.

7. Hydrogen sulfide inhibits lipid peroxidation. Knowing this, I tested the effect of various H_2S-releasing molecules on the inhibition of lipid peroxidation under *in vitro* conditions. I examined the effect of H_2S in our atherosclerotic mouse model.

8. I assume that H_2S can inhibit lipid peroxidation in human complicated lesions.

9. Since we know that H_2S can quickly bind to heme proteins, I thought that H_2S can also inhibit the formation of ferrylHb.

10. FerrylHb has a pro-inflammatory effect, induces the expression of adhesion molecules, and ruptures the layer of endothelial cells. I examined whether the effect of ferrylHb formed during Hb-lipid interaction on endothelial cells can be eliminated with H_2S.

11. Certain cytokines, LPS and H_2O_2 have also been shown to induce the expression of CSE, the main enzyme responsible for H_2S production. Consequently, I also examined the effect of IL-1β, TNF-α, H_2O_2, oxidized and non-oxidized LDL, and non-oxidized and oxidized lipids isolated from human carotid tissue on CSE expression on human aortic endothelial cells (HAoEC), RAW264.7 macrophages and on human aortic smooth muscle cells (HAOSMC).

4. MATERIALS AND METHODS

4.1. Materials

All chemicals were analytical reagent grade or better and purchased from Sigma-Aldrich (St. Louis, MO, USA). The sulfide donor molecules used in this study—GYY4137 (P-(4-methoxyphenyl)-P-4 morpholinylphosphinodithioic acid morpholine salt), AP67 (4-methoxyphenyl)(pyrrolidin-1 yl)phosphinodithioc acid), and AP72 (4-methoxyphenyl)(piperidin-1-yl)phosphinodithioc acid)— were synthesized in-house. Sulfide stock solutions were prepared fresh daily in water and used immediately.

4.2. Human Tissue Samples

For the study, we used 54 carotid artery specimens collected from 54 human patients who underwent carotid endarterectomy surgery. 15 samples were used for immunohistochemistry analysis, and 39 carotid arteries were used for the in vitro experiments. Written informed consent was received from the participants according to the Declaration of Helsinki. A pathologist examined the samples and classified them according to AHA guidelines. Type I (healthy), IV (atheromatous), and VI (complicated) lesions were selected for the study.

4.3 Animal model

All the animal experiments were approved by the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Animal experiments performed in this study were approved by the Scientific and Research Ethics Committee of the Scientific Council of Health of the Hungarian Government under the registration number DE MÁB/157-5/2010 and are reported by the ARRIVE guidelines. C57BL/6 ApoE$^{-/-}$ mice were maintained at the University of Debrecen under specific pathogen-free conditions by guidelines from Institutional Ethical Committee. To induce atherosclerotic plaque formation, the standard chow diet was changed to an atherogenic diet (15% fat, 1.25% cholesterol, ssniff Spezialdiäten GmbH, Soest, Germany) at the age of 8 weeks. Mice were randomly divided into three groups and parallel with the atherogenic diet mice were injected intraperitoneally with NaSH (56 μmol/kg body weight; N=9), PPG (50 mg/kg; N=5) or vehicle (PBS; N=21) in every other day as previously described. Aortas were harvested after 8 weeks of treatment. All mice were euthanized by a predictable and controllable administering slow-fill compressed CO_2 asphyxiation.

4.4 Cell culture

Human aortic endothelial cells (HAoECs) (PromoCell, Heidelberg, Germany) were cultured in medium 199 containing 15% FBS, antibiotics, L-glutamine, sodium pyruvate, and EC growth factor as described previously. HAoECs was used at passage 2 and 3 within 2 days post-confluence. Human aortic smooth muscle cells (HASMCs) (PromoCell, Heidelberg, Germany) were cultured in DMEM supplemented with 10% FBS, L-glutamine, sodium pyruvate, and antibiotics. RAW264.7 murine macrophages (ATCC) were grown in RPMI supplemented with L-glutamine, sodium pyruvate, and antibiotics.

4.5 Immunohistochemistry

Immunohistochemistry from the carotical arteries was performed on formalin-fixed, paraffin-embedded tissue sections. 4 μm slides were then deparaffinated using xylol and ethanol.
Samples were incubated with the anti-CSE primary monoclonal antibody (12217-1-AP Proteintech, Chicago, IL, USA) at a dilution of 1:200. Other slides of the same samples were incubated with anti-hemoglobin (clone: goat polyclonal HRP - ab19362 Proteintech Group, Rosemont, IL 60018, USA) primary monoclonal antibody at a dilution of 1:100; with HO-1 (clone: rabbit polyclonal 10701-1-AP Proteintech Group, Rosemont, IL 60018, USA) primary monoclonal antibody at a dilution of 1:200; and with HO-2 (clone: rabbit polyclonal 14817-1-AP Proteintech Group, Rosemont, IL 60018, USA) primary monoclonal antibody at a dilution of 1:400. Specific antibody binding was visualized by the Dako EnVision FLEX/HRP and FLEX DAB3 Chromogen detection system (Dako, Glostrup, Danmark) followed by hematoxylin counterstaining and coverage. The intensity and distribution of proteins immunostaining were assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera, and Leica Application Suite V3 software, Leica).

4.6 Hemoglobin preparation

Hb of different redox states, i.e. (Fe^{2+}) oxyHb, (Fe^{3+}) metHb, and ferrylHb, were prepared as described. Briefly, Hb was isolated from fresh blood drawn from healthy volunteers using ion-exchange chromatography on a DEAE Sepharose CL-6B column. MetHb was generated by incubation (30 min, 25°C) of purified Hb with a 1.5-fold molar excess of $K_3Fe(CN)_6$ over heme. FerrylHb was obtained by incubation (1 h, 37°C) of Hb with a 10:1 ratio of H2O2 to heme. After oxidation, both metHb and ferrylHb were dialyzed against saline (3 times for 3 hours at 4°C) and concentrated using Amicon Ultra centrifugal filter tubes (10,000 MWCO, Millipore Corp., Billerica, MA, USA). Aliquots were snap-frozen in liquid nitrogen, and

stored at -80°C until use. The purity of each Hb preparation was evaluated by SDS-PAGE followed by silver staining. The purity of Hb preparations was above 99.9%. Hb concentrations were calculated as described by Winterbourn.

4.7 Isolation and oxidation of LDL

LDL was isolated from the plasma of EDTA-anticoagulated venous blood of healthy volunteers by gradient ultracentrifugation (Beckman Coulter Inc., Brea, CA, USA). The density of plasma was adjusted to 1.3 g/mL with KBr and a two-layer gradient was made in a Quick-Seal ultracentrifuge tube by layering saline on 10 mL plasma. Ultracentrifugation was performed at 302,000 g for 2 hours at 4°C (VTi 50.2 rotor). LDL samples were kept at -70°C until use and the protein concentration was determined by Pierce BCA protein assay Kit (Pierce Biotechnology, Rockford, IL, USA). LDL oxidation was carried out at 37°C in a reaction mixture containing LDL (200 μg/mL) heme (5 μmol/L) and H_2O_2 (75 μmol/L).

4.8 Oxidation of LDL

LDL (200 μg/mL) was oxidized with heme (5 μmol/L) and H_2O_2 (75 μmol/L) in the presence or absence of the sulfide donors NaSH, GYY4137, AP67, and AP72 at concentrations of 20 and 200 μmol/L at 37 °C. Conjugated diene formation was monitored continuously for 1 hour at 234 nm. Delta OD234 nm was calculated by subtracting the optical density measured at the 0-time point from the optical density measured at 1 hour. The formations of lipid hydroperoxides (LOOH) and thiobarbituric-acid reactive substances (TBARS) were measured at 60 minutes following the initiation of lipid peroxidation. The method of Wolf was used to evaluate LOOH content in the LDL samples [14]. For the TBARS measurement, 50 μL of a 200 μg protein/mL LDL sample was mixed with 100 μL of thiobarbituric acid reagent (0.375 g 2-thiobarbituric acid, 2.08 mL HCl, 15 mL 10% trichloroacetic acid to a final volume of 100 mL). After heating at 90°C for 20 minutes, the samples were cooled and extracted with 200 μL n-butanol. The upper phase was measured spectrophotometrically at 532 nm. Results were calculated using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ and are expressed as nmol TBARs/mg protein.

4.9 Plaque lipid oxidation

Lipids were extracted from human carotid artery plaques as described previously. Plaque lipids (0.5 mg/mL) were incubated with Hb (100 μmol/L) in the presence or absence of the sulfide donors NaSH, GYY4137, AP67, and AP72 at 200 μmol/L concentration for 4 days at

37 °C. In other cases, complicated lesions containing intraplaque hemorrhage were homogenized in saline. These samples (0.5 mg/mL) were incubated at 37 °C for 3 days in the presence or absence of the sulfide donors NaSH, GYY4137, AP67, and AP72. Lipid peroxidation was assessed by measuring LOOH and TBARs.

4.10 Hb oxidation and detection of covalently cross-linked Hb species

Purified Hb (5 μmol/L heme) was incubated with H_2O_2 (25 μmol/L) or oxidized LDL (50 μg protein/mL) in the presence or absence of the sulfide donors NaSH, GYY4137, AP67, and AP72 at 37 °C for 1 hour. For the detection of the covalently cross-linked Hb species, 0.5 μg of Hb samples were applied to 12.5% SDS-PAGE gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, NJ, USA) and Hb was identified using an HRP-conjugated goat anti-human Hb polyclonal antibody (ab19362-1 Abcam, Cambridge, UK) at a dilution of 1:15000.

4.11 Endothelial cell cytotoxicity assay

LDL (200 μg/mL) was oxidized with heme (5 μmol/L) and H_2O_2 (75 μmol/L). Oxidized LDL was incubated at 37°C overnight with the sulfide donors NaSH, GYY4137, AP67, and AP72 at concentrations of 20 and 200 μmol/L. Confluent HAoECs grown in 96-well tissue culture plates were washed twice with PBS and exposed to oxLDL samples for 6 hours. Cell viability was assessed by MTT assay as described previously.

4.12 Endothelial cell monolayer integrity assay

Electric Cell Substrate Impedance Sensing method was used to measure endothelial monolayer integrity. HAoECs were cultured on 8-well electrode arrays (8W10E, Applied BioPhysics Inc., Troy, NY, USA). Upon confluence, cells were challenged with Hb (10 μ mol/L) oxidized with H_2O_2 (50 μmol/L) in the presence of sulfide donor molecules at the concentration of 200 μmol/L. The complex impedance spectrum was monitored with an ECIS Zθinstrument (Applied BioPhysics Inc., Troy, NY, USA) for 3 hours every minute. Intercellular gap formation was calculated based on the difference between monolayer resistance at 4000 Hz at the 0-time point and 3 hours. In other experiments, HAoECs were treated with Hb (20 μmol/L) oxidized with plaque lipids (400 μg/mL) in the presence of sulfide donor molecules (200 μmol/L), and the impedance spectrum was monitored for 12

hours. Intercellular gap formation was calculated based on the difference between monolayer resistance at 4000 Hz at the 0-time point and 12 hours.

4.13 Western blot

The cells were cultured in 6-well plates, and upon reaching the confluence of the cells were treated with different triggers. After 8 hours of treatment the cells were solubilized in protein lysis buffer containing 10 mmol/L Tris-HCl, 5 mmol/L EDTA, 150 mmol/L NaCl (pH 7.2), 1% Triton X-100, 0.5% Nonidet P-40 and protease inhibitors (Complete Mini, F. Hoffmann-La Roche Ltd., Basel, Switzerland). In other experiments, tissue samples were homogenized under liquid nitrogen and solubilized in protein lysis buffer. Proteins (10-20 μg) were applied to 12.5% SDS-PAGE gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, NJ, USA). Proteins were identified using the following antibodies: mouse anti-human HO-1 antibody (Calbiochem, San Diego, CA, USA, 374087, dilution: 1:2500), rabbit anti-human HO-2 antibody (Proteintech, Chicago, IL, USA, 14817-1-AP), rabbit anti-human CSE antibody (Proteintech, Chicago, IL, USA, 12217-1-AP, dilution: 1:1000), rabbit anti-human VCAM-1 (Santa Cruz Biotechnology Inc., Dallas, TX, USA, sc8304, dilution: 1:200), mouse anti-human GAPDH (Novus Biologicals, Littleton, CO, USA NB-300-221, dilution: 1:1000), anti-rabbit IgG HRP-conjugate (GE Healthcare Life Sciences, Piscataway, NJ, USA, NA934, dilution 1:15000), and anti-mouse IgG HRP-conjugate (GE Healthcare Life Sciences, Piscataway, NJ, USA, NA931, dilution 1:15000). Antigen-antibody complex was detected by a horseradish peroxidase chemiluminescence system according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ, USA,). Quantification was performed using video densitometry (AlphaDigiDoc RT, Alpha Innotech Corp., San Leandro, CA, USA).

4.14 Quantitative Real-Time PCR (qRT-PCR)

$ApoE^{-/-}$ mice have intraperitoneally injected with NaSH (56 μmol/kg body weight) or vehicle (PBS) every other day over 8 weeks. Parallel with the treatment the mice were fed an atherogenic diet. Control mice were fed a standard chow diet. After the mice were sacrificed and the aortas were harvested. Total RNA was isolated using RNAzol STAT-60 according to the manufacturer's instructions (Cat. No. Tl-4120, TEL-TEST Inc., Friendswood, TX, USA). RNA concentration was measured with NanoDropTM 2000c spectrophotometer (Cat. No. S06497c, Thermo Scientific Inc. Waltham, MA, U.S.A.). After that, cDNA synthesis was

performed using a high-capacity cDNA kit (Cat. No. 43-688-13, Applied Biosystems, Foster City, CA). We used real-time PCR technique for quantification of mRNA levels of HO-1 (Mm00516005_m1, Thermo Fisher Scientific Inc.) and Beta-Actin (Mm02619580_g1, Thermo Fisher Scientific Inc.). TaqMan Universal PCR Master Mix was purchased from Applied Biosystems (Cat. No. 4269510, Applied Biosystems, Foster City, CA). Finally, we performed TaqMan quantitative PCR (40 cycles at 95°C for 15 Sec. and 60°C for 1 min.) in 96-well plates with the Bio-rad CFX96 (Bio-Rad Laboratories, Inc. Hercules, California, U.S.A.) detection system. Results were expressed as mRNA expression normalized to Beta-Actin.

4.15 Determination of sulfide level from tissue with zinc precipitation assay

Sulfide levels were measured with the zinc precipitation method based upon developed by Gil-boa-Garber and modified by A. D. Ang et al. The human carotid artery was homogenized under liquid nitrogen in 7.4 pH PBS and sonicated it. After that, the sample was centrifuged at 12. 000 G for 15 min and the lipids-free clear supernatant was collected. 200 µL sample was mixed with 350 µL 1% zinc acetate and 50 µL 1.5 mol/L sodium hydroxide and incubated for 60 minutes on a shaker. The incubation step was followed by centrifugation at 2000 G for 5 minutes to pellet the generated zinc sulfide. The supernatant was then removed, and the pellet was washed with 1 mL of distilled water by vortexing extensively, followed by centrifugation at 2000 G for 5 minutes. The supernatant was then aspirated off and the pellet reconstituted with 160 µL of distilled water and mixed with 40 µL of pre-mixed dye (20 µL of 20 mmol/L dimethyl-p-phenylenediamine dihydrochloride (NNDP) in 7.2 mol/L hydrochloric acid (HCl) and 20 µl of 30 mmol/L Iron(III) chloride ($FeCl_3$) in 1.2 mol/L HCl). After 10 min the absorbance of the generated methylene blue (MB) was measured with a spectrophotometer at 667 nm. Since during the reaction 1 mol/L MB formed from 1 mol/L sulfide, the concentration was determined by the MB's extinction coefficient (30 200 $M^{-1}cm^{-1}$). Samples were normalized for protein concentration.

4.16 DETERMINATION OF HYDROGEN SULFIDE LEVEL FROM TISSUE

We determined hydrogen sulfide levels in tissue using the zinc precipitation method first described by Garber and modified by A.D. Ang et al. Human carotid vessels (healthy, atheroma, complicated lesions) were homogenized under liquid nitrogen and then taken up in PBS with a pH of 7.4, followed by sonication of the samples 3 times for 5 seconds on ice.

After that, the samples were centrifuged at 12,000 G for 15 minutes. The lipid-free middle phase was collected (upper phase – lipid layer, middle phase – proteins, lower phase – tissue debris). 200 μL samples were incubated with 350 μL of 1% zinc acetate and 50 μL of 1.5 mol/L NaOH for 60 minutes on a mixer at room temperature. After incubation, the resulting zinc sulfide was centrifuged at 2000 G for 5 minutes. The supernatant was removed, then the pellet was washed with 1 mL of ultrapure distilled water with intensive stirring. After that, the samples were centrifuged again at 2000 G for 5 minutes. The supernatant was removed, and 160 μL of distilled water and 40 μL of premixed dye (20 μL of 20 mmol/L dimethyl-p-phenylenediamine dihydrochloride (NNDP) dissolved in 7.2 mol/L hydrochloric acid (HCl) and 20 μL of 30 mmol/L iron III chloride (FeCl3) dissolved in HCL with a concentration of 1.2 mol/L) was added. After 10 minutes, the absorbance of the generated methylene blue was measured with a spectrophotometer (Beckman DU-800) at 667 nm. During the reaction, 1 mol/L of methylene blue is produced from 1 mol/L of sulfide. To determine the concentration, we calculated the extinction coefficient of methylene blue (30,200 M-1cm-1). The resulting hydrogen sulfide concentrations were normalized to the protein content of the samples.

4.17 SILVER STAINING

The silver staining was performed according to the following protocol on samples injected into 12 % SDS-PAGE and electrophoresed. After electrophoresis, I washed the gel in distilled water (DW) for 10 minutes. After that, I fixed the gel in 12.5 mL ethanol + 5.37 mL formaldehyde + 32.125 mL DW solution for 16 hours at 4^{o}C. Then I washed the gel 4 times for 10 minutes in DW. After the washing step, the gel was incubated in a solution of 0.025 g of naphthalene dissolved in 50 mL of DW for 1 day on a shaking rotor at 4^{o}C. The next day, I washed the gel 4 times for 20 minutes in DW, and finally incubated it with a silver solution (30 mL DW + 468.75 μL 1M NaOH + 493.75 μL 5M ammonium hydroxide + 750 μL 1M $AgNO_3$) for 20 minutes protected from light at 4^{o}C. The brownish precipitate observed in the silver solution can be dissolved with a few drops of ammonium hydroxide. After incubation with the silver solution, wash the gel with DW for 20 minutes. Development of the proteins on the gel consists of a mixture of 50 μL formaldehyde, 9 μL 2M citric acid, and DW in a final volume of 50 mL. After development, the reaction was stopped with a solution consisting of a mixture of 1 mL case acid, 250 μL ethylnolamine and DW in a final volume of 50 mL. After washing 3 times for 5 minutes, the gel was placed in a drying solution for 2.5 hours (17.5 mL ethanol + 1 mL glycerol + 37.5 mL DW). After drying, I photographed the gels using a gel documentation tool.

4.18 DETERMINATION OF LIPID HYDROPEROXIDE USING THE IODOMETRICAL METHOD

100 μL of PL or bloody plaque suspension was extracted in 1 μL of 10 mmol/L EDTA and 200 μL of chloroform and 100 μL of methanol. I removed the chloroform phase and added 36 μL of formic acid, 24 μL of chloroform, and 40 μL of a 1.2 mg/mL potassium iodide solution. After 5 minutes of incubation, I stopped the reaction with 180 μL of 20 mmo/L cadmium acetate. I centrifuged the suspension at 120,000G for 15 minutes, then measured the OD value of the samples from the supernatant at 353 nm and 700 nm with a spectrophotometer. The obtained OD values at 353 nm were subtracted from the OD values measured at 700 nm. LOOH was calculated using the extinction molar coefficient (2.19X104 M^{-1} cm^{-1}) given for the triiodide (I-3) ion. LOOH was measured from PL and a hemorrhaged plaque was given as nmol LOOH/mg tissue.

4.19 PREPARATION OF SODIUM SULFIDE

The pipes to be used must be saturated with nitrogen gas, and all materials must be kept on ice under a chemical cabinet. I washed 3-4 larger pieces of sodium sulfide (Na_2S) crystals with DW 3 times to remove the polysulfides formed on their surface. After that, I dissolved the crystals in 4 mL of DW by stirring in a centrifuge tube wrapped around with aluminum foil. I diluted the Na_2S solution 100 times with DW. The resulting solution was measured at 230 nm using a spectrophotometer. I further diluted the stock solution so that the OD of 1 mL of the 100-fold diluted Na_2S solution was between 0.4 and 0.5. The concentration of the Na_2S solution at 230 nm was calculated using the extinction molar coefficient (7700 M^{-1} cm^{-1}) given for HS^-. After that, I pipetted another 100 μL of 10 mmol/L 5,5′-Dithiobis 2-nitrobenzoic acid (DTNB) into the cuvette and then measured the OD value at 412 nm. From the absorbance value obtained in this way, I calculated the extinction molar coefficient of 28,200 M^{-1} cm^{-1} to obtain the concentration of hydrogen sulfide. I averaged the concentration of Na_2S calculated with the two extinction coefficients.

4.20 PREPARATION OF POLYSULFIDE (HSX^-) SOLUTION

The concentrated sodium hypochlorite solution was diluted 10 times with 50 mmol/L PBS, and then the absorbance of the solution was measured at 292 nm. The concentration of the diluted sodium hypochlorite solution was calculated using the extinction molar coefficient of

350 M^{-1} cm^{-1}. A 10 mmol/L Na_2S solution was mixed dropwise with a 2 mmol/L sodium hypochlorite solution while stirring. I carried out the entire process on ice and in the dark and used up the finished polysulfide within 30 minutes.

4.21 HYDROGEN SULfIDE REMOVAL OF SULFIDE DONORS

I dissolved the sulfide donors in PBS and then allowed their entire hydrogen sulfide content to leave the base compound. The H_2S content of the solution was monitored using a spectrophotometer at 620 nm. I had to measure the H_2S level over a month to remove all the H_2S **.**

4.16 Experimental units

"N" represents the number of tissue samples used in each group. The "n" denotes the number of replications of the independent results.

4.22 Study approval

A collection of carotid artery plaques from patients who underwent carotid endarterectomy surgery was approved by the Scientific and Research Ethics Committee of the Scientific Council of Health of the Hungarian Government under the registration number DE OEC RKEB/IKEB 3712-2012.

4.23 Statistics

I analyzed the data using GraphPad Prism 5.02 software. The statistical analysis in the figures shows the standard error of the average of data from at least three independent experiments. The significance levels were determined using Student's t-test when comparing two groups. When comparing the averages of several groups with the same standard deviation, normally distributed, I used one-way ANOVA in combination with Dunett's post-test. I marked the following levels of significance: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), the NS sign shows the non-significant result.

5. RESULTS

5.1 PART ONE

In the process of arteriosclerosis, the formation of complicated lesions is considered a life-threatening event. Hemorrhage within the plaque initiates processes that lead to the loss of plaque stability. During the process, the surface of the plaque breaks open, and the fatty, bloody mass contained in it enters the bloodstream and leads to the development of heart attacks, strokes, and embolisms. In the first part of my research, I modeled the events following a hemorrhage. I examined the physiological effects of ferrylHb, which is created during the interaction of the lipids in the plaque and Hb.

5.1.1 OXIDIZED HEMOGLOBIN SPECIES INDUCE OXIDATIVE MODIFICATION OF LDL

To model the possible interactions that could take place inside a complicated atherosclerotic lesion between lipids and different Hb species, we purified Hb from human blood and generatedmetHb and ferrylHb. We should note that ferrylHb is not a homogenous chemical entity but is a mixture of globin- and porphyrin-centered radicals (which can be very short-lived) and covalently cross-linked Hb multimers. Human EDTA-anticoagulated plasma was incubated with heme and the three different Hb species, that is, Hb, metHb, and ferrylHb (100 μmol/L heme). After 1 hour of incubation at 37^{o}C LDL was isolated by ultracentrifugation and oxidative modification of LDL was monitored by the formation of conjugated dienes, lipid hydroperoxides, and TBARs in samples incubated at 4^{o}C for 15 days. Lipid peroxidation did not occur in LDL samples derived from nontreated or Hb-treated plasma samples. Starting on day 2 following isolation, heme treatment caused an extensive and rapid increase in conjugated dienes, LOOH, and TBARs content of LDL. MetHb and ferrylHb also initiated oxidative modification of LDL and increased the levels of lipid peroxidation products at days 7–10 after isolation. The kinetics of the formation of lipid peroxidation products in the LDL was strictly dependent on the dose of ferrylHb. Heme released from oxidized Hb in plasma preferentially associates with LDL and is degraded shortly thereafter in the course of lipid peroxidation. Therefore we assessed whether the heme moiety of ferrylHb is released and eventually taken up by LDL and degraded during lipid peroxidation. Plasma was incubated with heme or ferrylHb for 1 hour at 37^{o}C followed by LDL separation and measurement of LDL-associated heme. FerrylHb treatment dose-dependently increased the concentration of LDL-associated heme in the LDL. To determine whether similar events occurred in whole

plasma, we treated fresh plasma with heme, Hb, metHb, or ferrylHb. Following the isolation of LDL, the concentration of LDL-associated heme was measured on the day of LDL isolation and 15 days later. We observed that LDL-associated heme underwent degradation when plasma was treated with heme, metHb, or ferrylHb. In contrast, the heme content of LDL derived from Hb-treated plasma did not change over a 15-day incubation period. These results suggest that ferrylHb, likemetHb, readily releases heme, following which iron is released upon oxidative scission of heme and serves to catalyze the process of lipid peroxidation.

5.1.2 OXIDIZED HEMOGLOBIN FORMS CAUSE ENDOTHELIAL CELL DESTRUCTION THROUGH OXIDATIVE STRESS

It was previously shown that heme and metHb cause oxidative stress in endothelial cells by the fact that the redox-active iron released from their released heme group in response to H_2O_2 promotes the formation of reactive oxygen radicals. As a result, we tested whether ferrylHb, similar to metHb, sensitizes endothelial cells through oxidative stress. To prove this, I pretreated confluent EC cells with heme (5 μmol/L), Hb (5 μmol/L), metHb (5 μmol/L), and ferrylHb (5 μmol/L) for 1 hour in HBSS+ solution. After one hour, the solutions containing heme, Hb, metHb, and ferrylHb were replaced with a solution containing H_2O_2 (75 μmol/L). After another 4 hours of incubation, I performed a cell survival test. Heme, Hb, metHb, ferrylHb, and H_2O_2 used as internal controls did not alter EC viability by themselves. On the other hand, EC viability decreased to 18% (±2%) during H_2O_2 treatment following heme pretreatment. During H_2O_2 treatment following metHb pretreatment, EC viability decreased to 62% (±4%). During H_2O_2 treatment following ferrylHb pretreatment, EC viability decreased to 78% (±3%).

Heme and metHb have previously been shown to be toxic to EC by producing toxic lipid peroxidation products during the oxidative modification of LDL. Consequently, I investigated whether ferrylHb is also toxic to EC through the oxidation of LDL. During the experiments, I incubated LDL (250 μg protein/mL) with heme (10 μmol/L), Hb (10 μmol/L), metHb (10 μmol/L) and ferrylHb (10 μmol/L) for 1 hour in HBSS+ solution. After that, I treated the EC with the experimental solutions for 24 hours. Finally, I performed a cell survival test. The results of the survival test showed that the viability of the cells treated with heme, metHb, and ferrylHb was decreased. EC viability decreased to 5% (±3%) in the case of heme, 27% (±5%)

in the case of metHb, and 9% (±2%) in the case of ferrylHb. In the case of EC cells treated with LDL and Hb, I did not measure any significant changes in viability.

5.1.3 OXIDIZED HEMOGLOBIN FORMS INDUCE HEMEOXYGENASE-1 AND FERRITIN EXPRESSION IN ENDOTHELIAL CELLS

HO-1 breaks down the framework of heme pyrrole structure and turns it into biliverdin, while the released iron is stored by ferritin. It has been shown that the expression of heme catabolism proteins, HO-1 and HFT, is increased by heme treatment on EC cells. Furthermore, it was also demonstrated that native Hb does not induce the expression of HO-1 and HFT in EC cells since Hb is unable to release heme groups. For this reason, I tested the effect of ferrylHb on the expression of HO-1 and HFT on EC cells. Confluent EC cells were treated with increasing concentrations of heme (25-50-100 μmol/L), Hb (100 μmol/L), metHb (100 μmol/L) and increasing concentrations of ferrylHb (25-50-100 μmol/ L) was treated in CM199 culture medium containing 15% FBS. For the qRT-PCR experiments, the cells were treated for 4 hours, for the protein analysis and the measurement of the L-ferritin level of the supernatant, for 8 hours. I showed that, like heme, metHb and ferrylHb also increase the expression of HO-1 and HFT mRNA gene, and the expression of HO-1, HFT, and L-ferritin (LFT) protein. Hb did not increase HO-1 and HFT levels either at the RNA or protein level. Furthermore, as a result of heme, metHb, and ferrylHb treatment, I measured the elevated LFT level in the supernatant, while Hb did not significantly increase LFT. Similar to heme, ferrylHb dose-dependently influenced the expression of HO-1 RNA and the expression of HO-1, HFT, and LFT protein.

5.1.4 OXIDIZED LDL AND ATHEROMA LIPIDS CAUSE HEMOGLOBIN OXIDATION

LOOH produced during the oxidation of LDL and lipids isolated from atheroma cause the oxidation of Hb. The metHb produced during the process can release the heme groups. Oxidized lipids and ferrylHb are present in complicated lesions, but little is known about the role of reactive lipids in the formation of ferrylHb. For this reason, I tested whether native LDL, oxLDL, and PL, which I isolated from human type IV atheroma, result in the formation of ferrylHb. In the tests, I incubated Hb (20 μmol/L) with H_2O_2 (200 μmol/L), native LDL (500 μg/mL), oxLDL (500 μg/mL) and PL (500 μg /mL) for two hours at 37 ^{o}C. After the incubation time, 4 μg of Hb samples were injected into 12% SDS-PAGE and run in an electrophoresis bath, and Hb cross-links were visualized with silver staining. Silver staining

showed that cross-linked Hbs appeared in H_2O_2, oxLDL and PL incubated Hb samples, which proves the formation of ferrylHb. I did not detect cross-linked Hb in the native Hb and the Hb sample with native LDL. I also monitored the changes in the samples using a spectrophotometer. The peaks characteristic of native Hb at 560 nm and 577 nm are reduced during oxidation, and at the same time, a new peak characteristic of oxidized Hb appears at 630 nm. I used metHb (20 μmol/L) as a positive control. I proved that increasing concentrations of oxLDL (50-100-200-400 μg/mL) dose-dependently reduced the peaks of Hb (20 μmol/L) at 560 nm and 577 nm, while it increased dose-dependently at 630 nm the peak characteristic of oxidized hemoglobin. I analyzed the analysis of the samples with the spectrophotometer using Winternbourn's equation. I showed that in Hb (20 μmol/L) samples treated with increasing concentrations of oxLDL (50-100-200-400 μg/mL), the amount of Fe^{3+} characteristic of ferriHb increased in a dose-dependent and significant manner, and simultaneously the Fe^{2+} characteristic of ferroHb its amount decreased significantly and dose-dependently. I also confirmed the result of the spectrophotometric measurement with silver staining. In Hb (20 μmol/L) samples treated with increasing concentrations of oxLDL (50-100-200-400 μg/mL), the amount of cross-linked Hb increased dose-dependently.

5.1.5 HYDROGEN PEROXIDE AND REACTIVE LIPID-INDUCED HEMOGLOBIN OXIDATION ARE INHIBITED BY HAPTOGLOBIN AND GLUTATHIONE/GLUTATHIONE PEROXIDASE

Free Hb released from VVT binds to the acute-phase plasma protein Haptoglobin (Hp), which is then taken up by macrophages via endocytosis via the CD163 Hp receptor. This process helps prevent the accumulation of Hb in the plasma. Glutathione peroxidase (GPx) is an intracellular antioxidant enzyme that enzymatically breaks down reactive oxygen radicals. I previously showed that H_2O_2 and the reactive lipid mediators produced during lipid peroxidation are capable of oxidizing Hb into ferrylHb. As a result, the hypothesis arose that the Hp and glutathione/glutathione peroxidase system can inhibit the oxidation of Hb. The oxidation of Hb (20 μmol/L) was carried out with H_2O_2 (200 μmol/L) and oxLDL (400 μg/mL) alone and with Hp (50 μmol/L) and GPx (200 μmol/L) pretreatment. The oxidation time was 90 minutes, while the Hb and GPx pretreatment time was 10 minutes. Oxidation and possible inhibition were monitored by spectrophotometric measurement and western blot analysis. I showed that the amount of Fe^{3+} increased in the H_2O_2 and oxLDL-treated Hb samples, while I measured significantly lower Fe^{3+} levels in the Hp and GPx pretreated samples. I confirmed the results of the spectrophotometric measurement with Western blot

analysis. Hp inhibited the formation of ferrylHb to a lesser extent, while GPx inhibited it to a greater extent. This result suggests that Hp binds free Hb and prevents its oxidation, while the glutathione (GSH)/GPx system prevents oxidation by disposing of reactive radicals.

5.1.6 FERRYLHB DISRUPTS THE CONFLUENCE OF ENDOTHELIAL CELLS AND INDUCES THE ADHESION OF MONOCYTES

It has previously been shown that ferrylHb activates EC cells, during which the cell-to-cell connection ceases. During cell activation, adhesion molecules (VCAM-1; ICAM-1; E-selectin) are expressed in EC cells. The expressed adhesion molecules cause the adhesion of monocytes circulating in the vasculature to the surface of EC cells. Based on the mentioned observations, I hypothesized that the confluence of EC cells treated with ferrylHb breaks down, and monocyte adhesion may occur. To prove this, I treated EC cells with increasing concentrations of ferrylHb (1-2.5-5-10-20 μmol/L) and Hb (1-2.5-5-10-20 μmol/L) for 16 hours. At the end of the treatment, I photographed the cells using a light microscope. I found that the holes between the cells increased in a dose-dependent manner as a result of the ferrylHb treatment. I did not detect a significant degree of confluence disruption among the EC cells treated with Hb. This effect is unique to ferrylHb. Neither Hb nor metHb can break cell-cell connections. In another experiment, I cultured EC cells in a hanging cell culture vessel until confluence was reached. The cells were treated with heme (10 μmol/L), Hb (10 μmol/L), metHb (10 μmol/L) and ferrylHb (10 μmol/L) for 12 hours. After the treatment time, I changed the treatment solution to Fluorescein solution (1 μmol/L). After 1 hour of incubation, I removed the hanging cell culture dish. I took a sample from the culture vessel containing HBSS+ located below, in which I measured the fluorescent intensity of Fluorescein using a fluorescent culture vessel reader. The measurements revealed that I was able to measure fluorescence intensity only from the samples taken from the culture vessel treated with ferrylHb. The experiment supports the result of the microscopic experiment, that ferrylHb can break the confluence of EC cells, so Fluorescein was able to penetrate from the apical culture vessel to the lower culture vessel. To further investigate the role of ferrylHb in EC cell activation, I again treated confluent EC cells with heme (10 μmol/L), Hb (10 μmol/L), metHb (10 μmol/L), and ferrylHb (10 μmol/L) for 12 hours. At the end of the treatment, I incubated the EC cells with Calcein-AM labeled monocytes isolated from whole blood for 30 minutes. After the incubation, I washed the cells several times with HBSS+ to remove monocytes that did not adhere to the surface of the cells. After fixing the cells, I painted the cytoskeleton of the cells to show the cell-to-cell connections. The experiment

revealed that only the cells treated with ferrylHb had ruptured confluence, which was supported by cytoskeleton staining. In addition, only in the case of the ferrylHb treatment did I experience a significant adhesion of monocytes to EC cells. The results prove that ferrylHb has an important role in the activation of EC cells and in the initiation of inflammatory processes, which also points to its role in the process of atherosclerosis.

5.2 PART TWO

Hydrogen sulfide is the newest member of endogenous gas transmitters. Extensive literature is not yet available on the physiological effects of hydrogen sulfide in the process of atherosclerosis (at the time of writing this dissertation). In the second part of my research, I examined the effect of hydrogen sulfide on the Hb-lipid interactions observed in complicated lesions.

5.2.1 CSE EXPRESSION IS INCREASED IN HUMAN AND MOUSE ATHEROMA

One of the main enzymes responsible for endogenous hydrogen sulfide production is CSE. To investigate the role of hydrogen sulfide in the process of atherosclerosis, I examined the expression of the CSE enzyme in human healthy, atheroma, and complicated carotid samples, as well as in the aortas of $ApoE^{-/-}$ mice kept on a normal and atherogenic diet. Using Western blot-based protein analysis, I showed that the expression of CSE increases significantly in human atheroma, while in complicated lesions this increase is significant, but significantly less compared to atheroma. A similar phenotypic change was observed in the aortas of $ApoE^{-/-}$ mice maintained on an atherogenic diet. CSE expression was increased in mice fed a high-fat and high-cholesterol diet compared to mice fed a normal diet. Elevated CSE expression is associated with increased hydrogen sulfide production. To prove this, I measured the endogenously produced hydrogen sulfide levels in human healthy, atheroma, and complicated carotid samples. I found that elevated CSE protein expression was associated with elevated endogenous hydrogen sulfide levels. In complicated lesions, the endogenous hydrogen sulfide level did not change significantly. Based on the results obtained in this way, it is not possible to decide whether the H_2S produced by CSE helps to inhibit the formation of atheroma, or perhaps promotes its progression. In addition, the question arises as to how the reduced CSE and endogenous H_2S levels in complicated lesions affect the outcome of atherosclerosis.

5.2.2 H_2S INHIBITS THE PROCESS OF ATHEROSCLEROSIS IN MICE

It was previously shown in animal experiments that hydrogen sulfide inhibits spontaneous atherosclerosis in $ApoE^{-/-}$ mice. As a result, I investigated the anti-atherogenic effect of hydrogen sulfide in ApoE-$^{-/-}$ mice kept on an atherogenic diet. The mice received the atherogenic diet for 8 weeks from the age of 8 weeks. In parallel, I intraperitoneally injected some of the mice (N=9) with freshly dissolved NaSH every two days. The control mice were

injected with PBS every two days on an atherogenic diet (N=21). To determine whether the elevated CSE/H_2S level inhibits or promotes atherosclerosis in atheroma, I injected a group of mice with an atherogenic diet every two days with the CSE inhibitor PPG (N=5). At the end of the experiment, I stained the fatty plaques formed in the aorta of the mice with Oil Red-O staining. I calculated the area of the plaques concerning the area of the entire aorta using ImageJ software. The results showed that mice treated with NaSH had significantly fewer plaques. This result proves that hydrogen sulfide inhibits the process of atherosclerosis. Furthermore, the CSE inhibitor, PPG, significantly increased the extent of plaques compared to mice on a control atherogenic diet, demonstrating that elevated CSE protein levels in human and mouse atheroma are due to reduced atherosclerosis progression. Among lipid peroxidation end products, 4-hyrdoxinone (4-HNE) has been shown to play a key role in the atherogenesis process. It can further stimulate inflammatory processes and initiate cell death. For this reason, I investigated whether the amount of 4-HNE also decreases in mice treated with H_2S. I made sections from the aortic root region of the mice using the frozen fracture method and then stained them for 4-HNE using the fluorescent method. I documented the staining using a fluorescence microscope. I showed that the staining of the lipid peroxidation end product 4-HNE was significantly reduced in mice treated with NaSH compared to mice kept on a control, atherogenic diet. To investigate the role of H_2S in lipid peroxidation, I measured TBARS content in the aorta of NaSH-treated mice and control mice on an atherogenic diet. I showed that the amount of TBARS in the aorta of mice treated with hydrogen sulfide was significantly lower compared to control mice. LOOH, produced during the peroxidation of lipids, increases the level of the oxidative stress marker, HO-1. Since hydrogen sulfide inhibited lipid peroxidation in my experiments, I assumed that the HO-1 level does not increase as a result of NaSH treatment in the mice. RNA was extracted from the aorta of mice fed an atherogenic diet with PBS and treated with NaSH, from which I measured HO-1 mRNA levels using RT-qPCR. The results supported my hypothesis, HO-1 expression was significantly lower in H_2S-treated mice.

5.2.3 HYDROGEN SULFIDE-RELEASING MOLECULES INHIBIT OXIDATION OF LDL AND ATHEROMA LIPIDS

The NaSH chemical used in animal experiments quickly releases hydrogen sulfide after dissolution. During my research, I was allowed to test new types of potential drug molecules that can release hydrogen sulfide slowly, even for weeks. The sulfide donor molecules used in this research were synthesized for me by Matthew Whiteman and Roberta Torregrossa. In

the lipid peroxidation inhibition experiments, I used the following slowly releasing hydrogen sulfide chemicals: GYY4137, AP67, and AP72. During the experiment, I incubated PL with heme and Hb, thereby modeling the conditions that develop in complicated plaques. I pretreated another group of samples with NaSH, GYY4137, AP67, and AP72. I determined the LOOH and TBARS content of the samples. Since PL is not a transparent pure liquid, spectrophotometric determination of the LOOH concentration cannot be used for these measurements. In this case, I used the iodometric method for LOOH measurements. Incubation of PL with heme and/or Hb increases the LOOH and TBARS content of the samples, the hydrogen sulfide donor molecules were able to inhibit the increase in the level of these lipid peroxidation markers to a different extent. In other experiments, I oxidized human LDL with heme and H_2O_2, and in the presence of hydrogen sulfide donors NaSH, GYY4137, AP67, and AP72. The inhibition of lipid peroxidation by hydrogen sulfide donors was performed by measuring LOOH and TBARS contents. I found that the donors inhibited LDL oxidation to a different extent and in a dose-dependent manner. Among the slow donors, the molecules AP67 and AP72 proved to be the most effective. At this point in the research, the question arose as to whether the inhibition in lipid peroxidation is not caused by the base frame that emits hydrogen sulfide. Furthermore, we know that hydrogen sulfide can transform into polysulfide. As a result, the question arose as to whether the resulting polysulfides are not the cause of the inhibition in lipid peroxidation. As a result, I repeated the experiments with polysulfide and with the ventilated hydrogen sulfide donor molecules, to prove that the observed effect is not caused by the polysulfide produced from hydrogen sulfide or the basic structure of the donor molecules. I found that neither the polysulfide nor the vented H_2S donor molecules were able to inhibit the oxidation of heme-induced LDL and heme- and Hb-induced PL oxidation.

5.2.4 HYDROGEN SULFIDE INHIBITS THE FORMATION OF LIPID PEROXIDATION PRODUCTS IN HUMAN COMPLICATED LESIONS

As a next step, I investigated whether the lipid peroxidation induced by ferrylHb appearing in hemorrhaged plaques can be inhibited by different hydrogen sulfide-releasing molecules. During the experiment, I homogenized human blood-stained carotid plaques from endarterectomy and then incubated the samples (5 mg/mL) at 37 °C with NaSH, GYY4137, AP67, AP72 (200 μmol/L). I measured the LOOH and TBARS contents of the samples on days 0 and 4. I found that the LOOH and TBARS contents measured on the 0th day increased significantly on the 4th day. LOOH and TBARS contents measured from samples containing

hydrogen sulfide donors were significantly lower. Surprisingly, the LOOH content measured on day 4 of samples incubated with AP67 and AP72 donor molecules was lower than the LOOH levels measured on day 0.

5.2.5 HYDROGEN SULFIDE INHIBITS THE FORMATION OF COVALENTLY CROSS-LINKED HEMOGLOBIN MULTIMERS

I previously proved that covalently cross-linked Hb (ferrylHb) can be found in blood-stained atherosclerotic lesions and that hydrogen sulfide can inhibit lipid peroxidation. To model the formation of ferrylHb observed in blood-filled atherosclerotic lesions in vitro, I incubated Hb (5 μmol/L) with H_2O_2 (75 μmol/L) and oxLDL (50 μg/mL) at 37°C for 90 minutes. After that, I investigated whether hydrogen sulfide can inhibit the oxidation of Hb and thus the formation of ferrylHb. To do this, I incubated the above-mentioned samples with NaSH, GYY4137, AP67, and AP72 (200 μmol/L) in the presence of donor molecules. 0.5 μg of the samples were calculated for Hb and run on an SDS-PAGE gel and the formation of Hb multimers was detected with an anti-Hb antibody. I detected Hb dimers, tetramers, and multimers in the samples incubated with H_2O_2 and oxLDL using Western blot. The hydrogen sulfide donors significantly prevented the formation of Hb covalent crosslinks to a different degree. In the experiments, the AP67 and AP72 donor molecules were the most effective.

5.2.6 HYDROGEN SULFIDE INHIBITS HEMOGLOBIN-LIPID INTERACTION AND THEREFORE EFFECT ON ENDOTHELIAL CELLS

My results so far prove that Hb and lipids react with each other in atherosclerotic plaques, resulting in further oxidation of lipids and the formation of pro-oxidative ferrylHb. These oxidized lipids and ferrylHb cause oxidative stress in endothelial cells through their activation. To investigate the protective role of hydrogen sulfide in the dysfunction of endothelial cells, I incubated a lethal dose of oxLDL (200 μg/mL) with NaSH, GYY4137, AP67, AP72 (20 and 200 μmol/L) for 24 hours. After that, I incubated the samples with HAoEC for 4 hours for the cell toxicity tests. 97 % of cells treated with oxLDL died at the end of the treatment. NaSH did not at a concentration of 20 μmol/L, but oxLDL significantly inhibited the induced cell death at a concentration of 20 μmol/L. GYY4137, AP67, and AP72 protected cell survival in a dose-dependent manner. AP67 and AP72 donor molecules at a concentration of 200 μmol/L completely prevented cellular toxicity. In the oxidative stress experiments, I incubated oxLDL (200 μg/mL) with NaSH, GYY4137, AP67, and AP72 (20

and 200 μmol/L) for 24 hours. After that, I incubated the HAoEC cells with a sub-lethal dose of LDL samples (50 μg/mL) for 8 hours. After lysing, the cells were run using the western blot technique. From the samples, I monitored the expression changes of the HO-1 stress protein, the constitutively expressed HO-2 protein, and the GAPDH protein used for protein normalization. A sublethal dose of oxLDL (50 μg/mL) increased HO-1 expression. GYY4137 did not, while NaSH slightly inhibited the development of oxidative stress in HAoEC. On the other hand, the molecules AP67 and AP72 greatly inhibited the development of oxidative stress in the cells. The expression of HO-2 and GAPDH proteins did not change during the experiments. Donors alone at a concentration of 200 μmol/L did not increase HO-1 expression. FerrylHb is an oxidized form of hemoglobin with unique pro-inflammatory properties that causes the activation of endothelial cells. FerrylHb increases the expression of cell adhesion molecules and breaks the cell-cell connection. Therefore, I investigated whether hydrogen sulfide can prevent the activation of endothelial cells by preventing the formation of ferrylHb. During the experiments, I incubated Hb (10 μmol/L) with H_2O_2 (50 μmol/L) in the presence of NaSH, GYY4137, AP67, AP72 (200 μmol/L) and without the donors at 37°C for 90 minutes. After the incubation period, confluent HAoEC cells were treated with the samples for 8 hours. I detected changes in the expression of the VCAM-1 adhesion molecule from a part of the treated cells using the western blot technique, and from the other part of the cells, I examined the disruption of the cell-cell connection induced by ferrylHb using the immunofluorescence method. I showed that ferrylHb increases the expression of the VCAM-1 protein, which was inhibited by AP67 and AP72, while NaSH, which releases fast hydrogen sulfide, was unable to inhibit it. Using an immunofluorescence technique, I showed that ferrylHb breaks up the confluence of HaoEC cells, this effect was also inhibited by NaSH, GYY4137, and AP67. To support the results of the immunofluorescent staining, I also performed an integrity test of the monolayer of the endothelial cells using an ECIS instrument. I treated confluent HAoEC cells with the solutions used for VCAM-1 protein detection and immunofluorescence testing. I monitored the decrease of monolayer resistance between the cells for 3 hours. I showed that Hb does not change the confluence of HAoEC cells, on the other hand, Hb incubated with H_2O_2 causes a significant decrease in resistance. The hydrogen sulfide donors prevented the rupture of cell confluence, among which AP67 and AP72 significantly improved the cell-to-cell connection compared to the untreated control. The results demonstrate that hydrogen sulfide can inhibit ferrylHb-induced endothelial cell activation by inhibiting the formation of ferrylHb itself. Finally, I also examined the effect of polysulfides and ventilated donor molecules in the activation of

endothelial cells induced by ferrylHb. Surprisingly, I showed that polysulfide was able to inhibit the toxic effect of oxLDL. In the background of this, I believe that through the radical scavenging effect of polysulfide, oxLDL reduced the free radical content, so it was no longer toxic to the endothelial cells. Ventilated donor molecules could not prevent the toxic effect of oxLDL on endothelial cells, which proves that hydrogen sulfide released from donor molecules inhibits lipid peroxidation. Furthermore, neither the polysulfide nor the ventilated donor molecules were able to inhibit the HO-1 and VCAM-1 expression of endothelial cells induced by a sublethal dose of oxLDL.

5.2.7 ATHEROGENIC LIPIDS AND PRO-INFLAMMATORY CYTOKINES INCREASE THE EXPRESSION OF CYSTATION-γ-LYASE IN THE ATHEROSCLEROTIC VESSEL WALL

Macrophages, smooth muscle cells, and endothelial cells also play a central role in the process of atherosclerosis. For this reason, I examined what stimuli the CSE enzyme responsible for hydrogen sulfide production in these cells rises in atherosclerotic plaques. For the experiments, I used stimulants that are found in atherosclerosis plaques. These can be divided into three groups: lipids (LDL, oxLDL, PL, oxPL), found in the hemorrhagic plaque: Hb, ferrylHb, hemes, iron, and cytokines: TNF-α and IL-1β. During the treatments, I used H_2O_2 as a positive control, based on the literature, H_2O_2 increases the expression of the CSE protein. I found that H_2O_2, oxLDL, PL, and oxPL increased CSE expression in smooth muscle cells and macrophages, but not significantly in HAoEC cells. Furthermore, Hb and heme upregulate CSE expression in smooth muscle cells and macrophages. As a result of iron treatment, the level of CSE decreases in smooth muscle cells. In HAoEC cells, Hb, ferrylHb, heme, and iron treatment did not significantly affect the expression of CSE. Finally, I also examined the effect of cytokines TNF-α and IL-1β on CSE. These cytokines increased CSE levels in smooth muscle cells and macrophages, while in HAoEC cells CSE expression increased only under the influence of TNF-α.

6. DISCUSSION

The presence of enzymes and molecules with antioxidant properties in the VVT protects the Hb in it from oxidation. Under normal conditions, damaged VVTs are removed from the circulatory system by hemophagocytic macrophages through the well-regulated reticuloendothelial pathway. There are many pathological conditions in which Hb is released from the VVT, causing oxidative stress in the body. During the oxidation of Hb, various Hb oxidation products are formed, such as heme, globin, and heme radicals containing ferryl iron ($iron^{+4}$), covalently crosslinked heme-globin forms, and covalently crosslinked globin-globin multimers. In the literature, these oxidized forms of Hb containing ferryl iron are called ferrylHb. FerrylHb can be found in human blood under both normal and pathological conditions. Covalently cross-linked ferrylHb has previously been shown to be found in human hemorrhaged atherosclerotic lesions. Although ferrylHb has been detected in atherosclerotic lesions, little is known about its role or its role in the process of atherosclerosis. The first half of my research aimed to investigate the formation and role of ferrylHb in atherosclerosis lesions.

The accumulation of LDL in the subendothelial space and the immune response to oxLDL formed during its oxidation is the initial step in the initiation of atherosclerosis. It has been shown that oxLDL, apoptotic cells, and modified proteins accumulating in the vessel wall lead to the development of myocardial infarction and stroke. Considering that there is a close relationship between the development of cardiovascular diseases and the presence of oxidative changes in the atherosclerotic vessel wall, it is therefore important to study potential oxidative substances. Hb can cause strong oxidative damage in atherosclerotic lesions. Two mechanisms have been described by which Hb can appear in atherosclerotic plaques. One is due to the rupture on the intima side of the plaque, and the other is due to the formation of hemorrhage within the plaque. Hemorrhage within the plaque primarily results from the rupture of small neovascularized blood vessels, which grow from the adventitia of the blood vessels to the interior of the plaque due to the hypoxia occurring during the oxidation processes occurring in the plaque and the vascular endothelial growth factor (VEGF) secreted as a result of the hypoxia. Furthermore, it has been shown that hemorrhage within the plaque contributes to the weakening of the plaque and its rupture, which thus directly contributes to the appearance of acute clinical symptoms associated with atherosclerosis. After hemorrhaging within a plaque, VVT-derived membrane proteins and iron accumulation can be observed, which indicates that the VVTs appearing in the lesions

are lysed, the Hb contained in them is released, which is quickly oxidized by the oxidative substances in the plaque, such as oxLDL. During the oxidation of Hb, due to the splitting of the globin, the heme contained in it is also released and degraded. It was previously shown that both lipids extracted from human atheromatous lesions and oxLDL lyse VVTs, and Hb can oxidize them. In theory, oxidized Hb forms can also initiate LDL modification in two different ways. One such mechanism is LDL oxidation induced by globin radicals, and the other is heme-iron-induced LDL oxidation. In my experiments, I proved that these mechanisms cause the oxidation of LDL. During the in vitro experiments, heme (5 μmol/L), H_2O_2 (75 μmol/L), and LDL (200 μg/mL) were incubated, during which the lipid peroxidation products (conjugated diene, LOOH, TBARS). During the incubation of Hb and H_2O_2, the resulting globin radicals initiate the oxidation of LDL. On the other hand, during the oxidation of Hb, heme is released from the globin ring, which also results in the oxidation of LDL. The metHb is the oxidized form of Hb that does not contain globin radicals but can release heme, thus causing the oxidation of LDL. This observation supports the fact that heme itself is a strong inducer of LDL oxidation. Furthermore, if heme or metHb is incubated on Hpx, which can bind heme, the oxidation of LDL is inhibited. It was previously shown and we confirmed that the heme released from ferrylHb can associate with the LDL in the plasma, during which the slow oxidation of LDL takes place. During this period, the heme released from ferrylHb bound to LDL is degraded. These results show that ferrylHb is capable of oxidizing LDL in two different ways, one is due to the globin radicals found in ferrylHb, and the other mechanism is the association of heme from ferrylHb with LDL. H_2O_2 is one of the best-studied reactive oxygen metabolites, capable of oxidizing Hb in a cell-free medium and intact VVTs. In these complex reactions, Hb forms such as metHb and ferrylHb are formed. $Iron^{4+}$ in ferrylHb is not stable, it can react with specific amino acids on the globin chains, during which globin radicals are formed. The resulting globin radicals can covalently connect, thus forming Hb dimers, tetramers, and multimers. Furthermore, it was shown that the classical reactive oxygen metabolites, organic peroxides, are also capable of rupturing the membrane of VVTs, thereby generating metHb after the release of Hb. It was previously shown that ferrylHb can be formed after the lysis of VVTs during the reaction with LDL. In parallel, we showed that oxLDL, but not native LDL, leads to the oxidation of Hb and the formation of covalently cross-linked ferrylHb. We proved that the formation of ferrylHb cross-links occurs in a way that depends on the concentration of oxLDL. Furthermore, we showed that pretreatment of oxLDL with the GSH/GPx system, which converts LOOH into lipid alcohol, inhibits Hb oxidation and the formation of covalently

cross-linked ferrylHb. This result shows that the content of the intermediate lipid peroxidation product LOOH in oxLDL is responsible for the oxidation of Hb.

During intravascular and extravascular hemolysis and hemorrhage, Hb released from VVTs can associate with Hp with high affinity. Hb bound to Hp is removed by monocytes and resident macrophages through the reticuloendothelial system via the scavenger receptor CD163. Endocytosis of the Hb-Hp complex mediated by the CD163 receptor is followed by rapid HO-1 induction, during which heme is catabolized into carbon monoxide, biliverdin, and iron. At the same time, the expression of ferritin increases, and the iron released from heme is stored. Colocalization between the CD163 receptor and HO-1 has previously been demonstrated in subpopulations of macrophages observed in neovascularized atherosclerotic lesions and hemorrhaged lesions. This subset of macrophages can rapidly degrade Hb, producing fewer reactive radicals and producing higher amounts of the anti-inflammatory cytokine IL-10. These results show that the macrophages associated with hemorrhage are polarized in an anti-inflammatory direction, thus having an atheroprotective effect. They also showed that the affinity of ferrylHb for Hp is lower than that of non-oxidized Hb, suggesting that the Hp-CD163 receptor pathway is less efficient in the presence of ferrylHb. Furthermore, since the Hp-CD163 receptor-mediated uptake of ferrylHb is low, it is associated with no increase in macrophage HO-1 protein expression, which is associated with a reduced anti-inflammatory and lower athero-protective effect in macrophages in infarcted plaques. Absence of ferrylHb uptake results in the presence of free heme, leading to programmed necrosis and apoptosis of apoptotic macrophages and other plaque-resident cells. Overall, it can be said that Hp can prevent the oxidation of Hb, thereby inhibiting the release of heme from Hb. Cooper et al showed the mechanism behind the protective effect of Hp, by which it can prevent the oxidation of Hb. They found that Hp binds to ferrylHb iron^{4+} ion, thus increasing the amount of ferryl ion, simultaneously inhibiting lipid peroxidation. It was shown that Hp stabilizes ferryl iron and binds to the tyrosine globin radical 145 of the beta chain of Hb. We also supported this observation in our experiments. We showed that Hb oxidation mediated by oxLDL and the formation of covalently cross-linked Hb multimers were inhibited by Hp.

Activation and damage of endothelial cells can be linked to the initiation and progression of atherosclerosis. Oxidation of Hb can damage endothelial cells in different ways. One such way is that the heme released during the oxidation of Hb can sensitize endothelial cells through oxidant-mediated cell death, and heme can initiate the oxidative modification of

LDL, during which cytotoxic LOOH and 4-HNE are produced. During my work, I showed that ferrylHb has a cytotoxic effect on endothelial cells by initiating the oxidation of LDL and sensitizing endothelial cells in an H_2O_2-mediated manner. The release of heme from metHb triggers a cellular response. If endothelial cells are treated with metHb, the HO-1 expression of the cells increases, followed by heme catabolism and iron storage in the upregulated HFT. HFT contains iron in the form of iron^{3+} ions, which is an inactive form of iron for redox processes. By treating the cells with ferrylHb, I found that similarly to metHb, ferrylHb also induces the expression of HO-1 and HFT protein, which suggests that heme can escape from the covalently cross-linked globin chain of ferrylHb.

It has been shown that ferrylHb produced during the oxidation of Hb has proinflammatory properties for endothelial cells. As a result of ferrylHb, the actin cytoskeleton of the endothelial cells is rearranged, during which the confluence of the cells is broken and holes are formed between them. The induction of pro-inflammatory genes such as ICAM-1, E-selectin, and VCAM-1 is increased in the treated cells. In the course of my work, I showed that in endothelial cells treated with ferrylHb, cell permeability also increases parallel to the increased adhesion molecules, which results in monocytes adhering to the surface of the endothelial cells. I did not see this effect on either heme or metHb, which suggests that the release of heme from oxidized Hb forms does not affect the inflammatory processes in the cells. Furthermore, I also showed that non-oxidized Hb cannot induce HO-1 and HFT in endothelial cells at the protein level, which proves that ferrylHb is a covalently cross-linked oxidized Hb form with unique pro-inflammatory properties.

In summary, in the first half of my work, I showed that the lipids in human atheroma interact with Hb not associated with VVT appearing during hemorrhage, thus provoking a vicious circle, where the oxidation of lipids in the plaque and Hb interact to cause the activation of endothelial cells, leading to endothelial cell toxicity.

In various biological systems, hydrogen sulfide not only has an antioxidant capacity (binding reactive oxygen intermediates), but also plays a role in cellular adaptation, metabolic regulation, cell death signaling, oxidative stress regulation, enzyme processes, and changes in the intramolecular structure of proteins. During my experiments, I demonstrated the protective effects of the hydrogen sulfide cell, these data are consistent with the increasingly large number of publications appearing in the literature. Being oxidative stress, ferrylHb formed during hemorrhage within an atherosclerotic plaque contributes to the

pathophysiological changes of the complicated lesions that have developed. FerrylHb has previously been shown to have pro-inflammatory effects on EC cells. During my experiments, I inhibited the oxidation of Hb with hydrogen sulfide donors. This inhibition in oxidation prevented the disintegration of the cohesive cell layer of EC cells induced by ferrylHb, the formation of holes between the cells, the increase in the expression of the proinflammatory protein VCAM-1, and the adhesion of monocytes to the surface of EC cells.

Oxidized Hb forms can release their heme group, thereby inducing lipid peroxidation. During my research, I showed that hydrogen sulfide donor molecules inhibit the oxidation of Hb and plaque lipids isolated from heme-induced human atheroma. Furthermore, I also showed that the lipid peroxidation processes taking place in human complicated lesions can also be inhibited by hydrogen sulfide-emitting molecules. Chemically, H_2S is a reducing agent. Previous research has shown that hydrogen sulfide converts LOOH to lipid alcohol *in vitro*. I was able to support this observation with an ex vivo experiment. Hydrogen sulfide was able to reduce the LOOH content of the human complicated lesion below the LOOH level measured at baseline.

Our main enzyme responsible for endogenous hydrogen sulfide production in cardiovascular tissues is CSE. Examining the human carotid artery and aorta from $ApoE^{-/-}$ mice, I observed that CSE protein expression increases in both human and mouse atheroma, as well as in human complicated lesions. Interestingly, in complicated lesions, CSE protein expression is lower compared to the expression level observed in atheroma. Furthermore, the level of hydrogen sulfide produced in human carotid arteries was not significantly higher compared to the healthy blood vessel section.

Atherosclerotic plaque contains large amounts of reactive oxygen mediators, reactive lipid mediators, proinflammatory cytokines, and Hb oxidation products produced during plaque bleeding. The main resident cells of the arteriosclerotic vessel wall are EC cells, smooth muscle cells, and macrophages. As a stress response, the CSE protein expression of these cells increases. During our studies, we established that proinflammatory cytokines such as IL-1β and TNF-α, oxLDL and PL, and ferrylHb increase the expression of CSE in EC cells, smooth muscle cells, and macrophages found in the vessel wall. Since these mediators are found in human atheroma and complicated lesions, they contribute to the elevated CSE expressions that I also observed.

Previously, Wang, Mani et al showed that hydrogen sulfide inhibited the development of atherosclerotic lesions in ApoE$^{-/-}$ mice. They performed the experiments without an atherogenic diet. During the experiments, I induced the formation of atherosclerotic lesions in ApoE$^{-/-}$ mice with a diet high in fat and cholesterol. Even under these conditions, I managed to demonstrate the atheroprotective effect of hydrogen sulfide. Wang, Mani et al. also showed that pharmacological inhibition of the CSE enzyme with PPG accelerates the formation of atherosclerotic plaques, thus proving that the hydrogen sulfide produced by CSE has an atheroprotective effect. In an in vivo experiment, ApoE$^{-/-}$ mice maintained on a high-fat and cholesterol-containing diet were injected intraperitoneally with PPG. My results supported Wang and Mani's observations that CSE inhibition increases the progression of atherosclerosis.

In humans, the development of complicated lesions with macroscopically visible hemorrhage is the main event of atherosclerosis that causes mortality. We have shown that the CSE protein expression is lower in complicated lesions than in atheroma. These results show the beneficial effect of the CSE/H_2S system in the progression of atherosclerosis and not that elevated CSE protein expression accelerates the process of atherosclerosis. This is also supported by PPG-treated animal experiments and ex vivo experiments on complicated lesions. Since the complicated lesion observed in humans does not develop in the ApoE$^{-/-}$ animal model, we cannot obtain information about the effect of hydrogen sulfide on the development of complicated lesions. An animal model with a complicated lesion would provide an opportunity to investigate the effect of hydrogen sulfide on the formation of ferrylHb, which has pro-oxidant and pro-inflammatory properties. The prevention of plaque rupture and the infiltration of VVTs in the atheroma could provide a new approach to the inhibition of the progression of human atherosclerotic lesions.

In conclusion, in the second half of my work, I proved that the increased expression of CSE in the vascular cells of human and mouse atheroma, as well as in the human complicated lesion, affects the outcome of atherosclerosis. Based on the in vitro experiments, it can be said that the increased CSE expression is a compensatory atheroprotective response of the cells, where the generated hydrogen sulfide inhibits the formation of prooxidant and proinflammatory lipid mediators and ferrylHb.

7. SUMMARY

The accumulation and oxidation of lipids in the vessel wall are the main inducers of atherosclerosis. As a result of lipid peroxidation, small blood vessels grow inside the fatty plaques from the vasa vasorum in the atherosclerotic vessel section. The endothelial cell layer of these small blood vessels ruptures in contact with the cytotoxic lipid peroxidation products, and the VVTs in the blood vessels get inside the plaque, thus creating complicated arteriosclerotic lesions. Red blood cells lyse in contact with oxidized lipids. The released Hb is oxidized to ferrylHb. FerrylHb contains radicals on both α and β globin chains, which form globin dimers, tetramers, and multimers by covalently cooperating. In addition to the pro-inflammatory effect of ferrylHb, it also causes the rupture of the monolayer of endothelial cells.

H_2S is the most recently discovered member of endogenous gas transmitters. It is involved in the regulation of many physiologically significant processes. From a medical point of view, drug molecules that release hydrogen sulfide stably, for a long time, and at a low physiological concentration under controlled conditions seem promising in the prevention and treatment of cardiovascular diseases.

In the first half of the study that forms the basis of the doctoral dissertation, we modeled the conditions for the formation of ferrylHb. We found that ferrylHb can induce oxidative stress and cell death in EC cells through the oxidation of LDL. Furthermore, we also demonstrated that ferrylHb ruptures the confluent layer of EC cells.

In the second part of my research, we demonstrated that the expression of the CSE enzyme responsible for hydrogen sulfide production increases in human atheroma and the aorta of $ApoE^{-/-}$ mice kept on an atherogenic diet. We have shown that hydrogen sulfide inhibits lipid peroxidation both in vitro and in vivo. We demonstrated that hydrogen sulfide inhibits atherosclerosis in $ApoE^{-/-}$ mice maintained on an atherogenic diet, while pharmacological inhibition of the CSE enzyme increases the progression of plaque formation. We proved that the formation of ferrylHb is inhibited by H_2S. Furthermore, we showed that H_2S prevented cell layer disruption of ferrylHb-induced EC cells.

In summary, it can be said that ferrylHb accelerates the progression of arteriosclerosis, while hydrogen sulfide-releasing molecules have an atheroprotective effect by inhibiting the action and formation of ferrylHb.

8. NEW RESULTS AND FINDINGS OF THE DISSERTATION

1. Oxidation of LDL is caused by oxidized forms of hemoglobin.

2. Oxidized forms of hemoglobin cause the death of endothelial cells through oxidative stress.

3. OxLDL and oxPLs can oxidize Hb into ferrylHb.

4. Haptoglobin and Glutathione/glutathione peroxidase can inhibit Hb oxidation induced by hydrogen peroxide and reactive lipids.

5. FerrylHb disrupts the confluence of endothelial cells and induces the adhesion of monocytes.

6. CSE enzyme expression increases in human and mouse atheroma.

7. Hydrogen sulfide inhibits the formation of atheroma in an animal model with atherosclerosis.

8. Molecules releasing hydrogen sulfide can inhibit the oxidation of LDL and PLs to a different degree.

9. Hydrogen sulfide inhibits the formation of lipid peroxidation products in human complicated lesions.

10. The formation of ferrylHb is inhibited by hydrogen sulfide.

11. Hydrogen sulfide inhibits hemoglobin-lipid interactions and thus their harmful effect on endothelial cells in atherosclerosis plaques.

12. Atherogenic lipids and pro-inflammatory cytokines increase the expression of CSE in the resident cells of the atherosclerotic vessel wall.

LIST OF ANNOUNCEMENTS

UNIVERSITY of DEBRECEN

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

Registry number: DEENK/455/2022.PL
Subject: PhD Publication List

Candidate: László Potor
Doctoral School: Kálmán Laki Doctoral School
MTMT ID: 10043377

List of publications related to the dissertation

1. **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., 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

2. **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

List of other publications

3. Balla, S., Zöld, E., **Potor, L.**, Lukucz, B., Vajas, A., Ujhelyi, B., Nagy, V.: Analysis of intravitreal bevacizumab treatment for macular oedema due to retinal vein occlusion.
   Eur. J. Ophthalmol. 31 (5), 2528-2534, 2021.
   DOI: http://dx.doi.org/10.1177/1120672120962051
   IF: 1.922

4. 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

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

5. Fige, É., Szendrei, J., Sós, L., Kraszewska, I., **Potor, L.**, Balla, J., Szondy, Z.: Heme Oxygenase-1 Contributes to Both the Engulfment and the Anti-Inflammatory Program of Macrophages during Efferocytosis.
Cells. 10 (3), 652-669, 2021.
DOI: http://dx.doi.org/10.3390/cells10030652
IF: 7.666

6. Sikura, K. É., Combi, Z., **Potor, L.**, Szerafin, T., Hendrik, Z., Méhes, G., Gergely, P., Whiteman, M., Beke, L., Fürtös, I., Balla, G., Balla, J.: Hydrogen sulfide inhibits aortic valve calcification in heart via regulating RUNX2 by NF-[kappa]B, a link between inflammation and mineralization.
J. Adv. Res. 27, 165-176, 2021.
DOI: http://dx.doi.org/10.1016/j.jare.2020.07.005
IF: 12.822

7. **Potor, L.**, Hendrik, Z., Patsalos, A., Katona, É., Méhes, G., Póliska, S., Csősz, É., Kalló, G., Komáromi, I., Combi, Z., Posta, N., Sikura, K. É., Pethő, D., Oros, M., Vereb, G., Tóth, C., Gergely, P., Nagy, L., Balla, G., Balla, J.: Oxidation of hemoglobin drives a pro-atherogenic polarization of macrophages in human atherosclerosis.
Antioxid. Redox Signal. 35 (12), 917-950, 2021.
DOI: http://dx.doi.org/10.1089/ars.2020.8234
IF: 7.468

8. Zavaczki, E., Gáll, T., Zarjou, A., Hendrik, Z., **Potor, L.**, Tóth, C., Méhes, G., Gyetvai, Á., Agarwal, A., Balla, G., Balla, J.: Ferryl Hemoglobin Inhibits Osteoclastic Differentiation of Macrophages in Hemorrhaged Atherosclerotic Plaques.
Oxidative Medicine and Cellular Longevity. 2020, 1-17, 2020.
DOI: http://dx.doi.org/10.1155/2020/3721383
IF: 6.543

9. Posta, N., Csősz, É., Oros, M., Pethő, D., **Potor, L.**, Kalló, G., Hendrik, Z., Sikura, K. É., Méhes, G., Tóth, C., Posta, J., Balla, G., Balla, J.: Hemoglobin oxidation generates globin-derived peptides in atherosclerotic lesions and intraventricular hemorrhage of the brain, provoking endothelial dysfunction.
Lab. Invest. 100 (7), 986-1002, 2020.
DOI: http://dx.doi.org/10.1038/s41374-020-0403-x
IF: 5.662

10. Sikura, K. É., **Potor, L.**, Szerafin, T., Oros, M., Nagy, P., Méhes, G., Hendrik, Z., Zarjou, A., Agarwal, A., Posta, N., Torregrossa, R., Whiteman, M., Fürtös, I., Balla, G., Balla, J.: Hydrogen sulfide inhibits calcification of heart valves; implications for calcific aortic valve disease.
Br. J. Pharmacol. 177 (4), 793-809, 2020.
DOI: http://dx.doi.org/10.1111/bph.14691
IF: 8.739

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

11. **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.
DOI: http://dx.doi.org/10.3390/ijms21134746
IF: 5.924

12. Sikura, K. É., **Potor, L.**, Szerafin, T., Zarjou, A., Agarwal, A., Arosio, P., Poli, M., Hendrik, Z., Méhes, G., Oros, M., Posta, N., Beke, L., Fürtös, I., Balla, G., Balla, J.: Potential Role of H-Ferritin in Mitigating Valvular Mineralization.
Arterioscler. Thromb. Vasc. Biol. 39 (3), 413-431, 2019.
DOI: http://dx.doi.org/10.1161/ATVBAHA.118.312191
IF: 6.604

13. Gáll, T., Pethő, D., Nagy, A., Hendrik, Z., Méhes, G., **Potor, L.**, Gram, M., Akerström, B., Smith, A., Nagy, P., 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

14. Jeney, V., Silva, G., **Potor, L.**, Balla, G., Soares, M. P., Balla, J.: Ferryl hemoglobin is a proinflammatory agonist that activates endothelial cells and induces mouse peritonitis.
Am. J. Hematol. 88 (5), E234-E235, 2013.
IF: 3.477

Total IF of journals (all publications): 83,255
Total IF of journals (publications related to the dissertation): 8,231

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.

20 October, 2022

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

ACKNOWLEDGMENT

I would like to thank my supervisor, Prof. Dr. József Balla, with whom it has been a pleasure and an honor to work with him over the past years. I am grateful to him for countless consultations and opinions, and especially for supporting my work both personally and professionally.

I would like to thank Prof. Dr. György Balla, who constantly monitored my work. His advice and guidance helped me throughout my research.

I would like to thank all my colleagues who helped me during my research work.

I would especially like to thank Zoltán Hendrik, Ibolya Fürtös, Lívia Beke, Tamás Czirják, Katalin Sikura, Zsolt Combi, Xéniánka Nemesdy, Edit Dajka Szabóné, and Judit Tóth, who greatly contributed to the completion of this dissertation with their selfless support. I thank Dr. Anita Nánási-Molnár and Antal Nánási for the language proofreading.

Last but not least, I would like to thank my beloved children: Ádám, Ákos, and Anna for their continued interest. Without their advice and childlike attention, this scientific work would have turned out differently. Finally, I would like to thank my wonderful wife for her constant encouragement and support, since without her this thesis would not have been possible. I dedicate this dissertation with much love to them, to my family: my wife and children.

Competitions supporting research work::

ELKH (11003), OTKA-K 132828, GINOP-2.3.2-15-2016-00043 (IRONHEARTH), EFOP-3.6.2-16-2017-00006 (LIVE LONGER), TKP2020-NKA-04, TKP2021-EGA -18, Laki Kálmán Doctoral School.