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

Regulation of hydrogen sulfide homeostasis in human calcified heart valves: molecular mechanisms and pharmacological implications

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, the 12th of November 2024 1:00 PM

1. INTRODUCTION

According to the World Health Organization, cardiovascular diseases remained the leading cause of death worldwide in 2023. Cardiovascular calcification is often at the root of these conditions. This complex disease affects large arteries, aortas, arterioles, and heart valves, and is frequently accompanied by chronic kidney failure.

Various types of valvular diseases are caused by the differentiation of cells within the tissue matrix of heart valves into bone cells. As this process progresses, the heart valves thicken, stiffen, and ossify, partially or completely losing their native function.

The rationale behind choosing this topic lies in the fact that the major signalling pathways involved in the pathogenesis of calcification are not fully understood. Thus, it is crucial to elucidate the molecular biological mechanisms underlying the disease in greater detail.

This research aims to investigate the relationships between inflammation and mineralization in calcified heart valves, identify new signal transduction pathways, and inhibit the primary pathogenic factors responsible for the pathological milieu using hydrogen sulfide-releasing donor molecules.

The significance of this research topic stems from the high mortality rate in this patient group. Currently, there is no medical treatment available in clinical practice to halt the progression of the disease or reverse calcification. The only solution at present is to replace calcified heart valves with artificial, such as bioprosthetic, valves. Unfortunately, 10-15 years post-intervention, valve failure is often observed again in most patients due to high biomechanical stress.

During my work, I observed regulatory pathways and metabolic changes in heart valves that could potentially serve as future therapeutic targets in cardiovascular drug development.

To support my hypotheses and conduct the experiments, we collected healthy heart valve samples from the Institute of Forensic Medicine at the University of Debrecen. Calcified biological samples from patients who underwent heart valve transplantation were provided by the Institute of Cardiac Surgery at the University of Debrecen.

2. LITERATURE REVIEW

2.1 Valvular Calcification

2.1.1 Epidemiology and Major Risk Factors of Valvular Calcification

According to the World Health Organization (WHO), more than half a billion people worldwide suffer from cardiovascular diseases, accounting for 20.5 million deaths in 2023, which represents nearly one-third of global mortality. The most common reason for heart valve surgeries is calcific aortic valve disease (CAVD). This disease primarily affects the population over the age of 65 (2-7%) and is a frequent condition in patients with chronic kidney disease (CKD) and diabetes. The prevalence of CAVD has increased over the past decade, and its incidence is expected to rise in the future. A simulation model predicts that the number of elderly patients with calcific aortic valve disease will more than double by 2050 in both the United States and Europe. Due to the lack of routinely applicable, effective conservative therapy, surgical intervention remains the only available option for the treatment of CAVD.

There are numerous risk factors for the disease: lifestyle-related risk factors include a sedentary lifestyle, excessive phosphate intake, regular alcohol consumption, and smoking. Physiological causes include high blood pressure, obesity, elevated levels of low-density lipoprotein in the blood serum, diabetes, ageing, and chronic kidney disease. Environmental factors such as air pollution also contribute. Some of these risk factors (e.g., genetics, gender identity, family history) are non-modifiable, while others (e.g., smoking, high blood pressure, reduced physical activity, alcohol consumption) can be prevented through lifestyle changes.

2.1.2 Pathobiology of Heart Valve Calcification

Cardiovascular calcification is one of the most common complex heart diseases in developed countries, affecting large and medium arteries, aortas, smaller arterioles, and heart valves alike. Previously described as a passive, degenerative process associated with ageing, it is now recognized as a dynamic, precisely regulated mechanism at both cellular and molecular levels. It can lead to severe clinical complications such as left ventricular hypertrophy, heart failure, and myocardial infarction. Characteristic features include disruption of the endothelial cell layer integrity, accumulation of plasma lipoproteins, infiltration of proinflammatory cells into the tissue, increased levels of inflammation-inducing cytokines, activation of osteogenic transcription factors, and subsequent appearance of non-

typical bone-specific proteins in the heart valve tissue, along with hydroxyapatite crystal formation in the extracellular matrix, resulting in heterogeneous lesions in the heart valve tissue.

Disruption in systemic phosphate-calcium homeostasis, most commonly due to chronic kidney disease (CKD) or end-stage renal disease (ESRD), often leads to and contributes to the accelerated progression of calcific aortic valve disease (CAVD). CAVD is characterized by high morbidity and mortality rates. Osteoporosis and increased bone resorptive activity have also been associated with CAVD, indicating an active involvement of calcium-phosphate metabolism in its pathogenesis.

In CKD patients, elevated serum phosphate levels are one of the most potent and critical initiators of cardiovascular calcification. It has long been recognized that hyperphosphatemia, as an independent cardiovascular risk factor, is linked to CKD.

In CAVD, interstitial cells comprising heart valve tissue transform into bone-like cells, significantly contributing to tissue mineralization. Specific genes characteristic of vascular smooth muscle cells and VICs, such as α-SMA, show decreased activity. Conversely, the expression of genes involved in osteogenesis and osteo-chondrogenic processes, such as RUNX2 (runt-related transcription factor 2), increases. In bones, RUNX2 is considered a major regulator of osteogenesis, orchestrating the differentiation of pluripotent mesenchymal stem cells into immature osteoblasts, then mature osteoblasts, and finally osteocytes. Its translocation from the cytosol to the nucleus triggers the phenotypic conversion of interstitial cells into osteoblasts in response to pathological factors such as high phosphate levels. Thus, it is a regulatory protein that plays an important role in ectopic mineralization. In the early stage of calcification, nuclear localization of the RUNX2 transcription factor promotes increased alkaline phosphatase enzyme (ALP) activity.

In the later stages of mineralization, the activity of genes characteristic of the bone matrix increases, resulting in the appearance of non-typical bone-specific proteins such as osteocalcin in the heart valve tissue. Surgically excised aortic valves from patients with CAVD often show dystrophic mineralization with disorganized calcification foci. However, in 15% of explanted samples, the well-organized bone matrix is observed, featuring osteogenic metaplasia, including lamellar bone, osteocytes, osteoblast-like cells, bone marrow, and adipocytes.

2.2 Characteristics of Hydrogen Sulfide Donor Molecules

In recent decades, chemical engineers have made significant efforts to synthesize H_2S-containing donors for research and therapeutic purposes. These molecules are designed to release sulfide through hydrolysis or other chemical mechanisms. Under experimental conditions, various inorganic sulfide salts, including sodium sulfide (Na_2S) and sodium hydrosulfide (NaSH), have been used to deliver hydrogen sulfide into biological systems. Upon dissolution in the aqueous medium, they rapidly release sulfide, which dissipates within seconds. However, the use of inorganic sulfide salts in animal experiments can often lead to misleading results. Intravenous or intraperitoneal injections can result in extremely high local concentrations of H_2S at the site of administration, leading to toxic effects. It is also worth noting that commercially available sulfide salts may contain various impurities, which can affect experimental outcomes. Therefore, while suitable for in vitro experiments, inorganic sulfide salts are less advantageous for in vivo studies.

Consequently, attention has shifted towards the design of synthetic, organic molecules that slowly release hydrogen sulfide (e.g., GYY4137), providing sustained H_2S release for hours. Li and colleagues successfully synthesized a new generation sulfide-generating compound (GYY4137) from Lawesson's reagent (2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide) and morpholine. Protonation of GYY4137 leads to the formation of further analogs such as AP67 [(4-methoxyphenyl) pyrrolidin-1-ylphosphinodithioc acid] and AP72 [(4-methoxyphenyl) (piperidin-1-yl) phosphinodithioc acid]. These products likely undergo ionization, forming soluble salts at physiological pH. In biological systems, more stable H_2S-releasing compounds such as AP72 are highly water-soluble and release the active ingredient much slower compared to sulfide salts. These sulfur-containing molecules have been used in both in vitro and in vivo studies, with the latter involving intraperitoneal administration. The therapeutic applicability of sulfur-containing compounds for cardiovascular diseases largely depends on their site-specific activity near lesions.

It is important to note that the concentration of donors does not equate to the total amount of released sulfide, and each slowly-acting donor molecule has a different sulfide-releasing potential. Furthermore, researchers are increasingly recognizing that slowly releasing hydrogen sulfide formulations likely better mimic the effects of endogenous hydrogen sulfide buffering systems, as their dissolution rates do not lead to steep increases in local sulfide concentration as observed with sulfide salts.

One challenge in the clinical application of hydrogen sulfide donors in human medicine is determining the optimal dosing regimen, as most studies administer donors

intraperitoneally. Additionally, human cardiovascular diseases are chronic, while animal experiments are typically short-term compared to human applications. Therefore, long-term studies investigating the effects of hydrogen sulfide application on humans are necessary to identify the most effective local concentration with minimal toxicity. One potential solution may involve the tissue- or organ-specific administration of donor compounds.

However, effective doses at the intracellular level depend on the targets of the donors. Mitochondria-targeted donors, such as AP39, require three orders of magnitude lower concentration (10^{-8} mol/L) for favourable effects compared to cytosol-releasing AP72 (10^{-5} mol/L). It is important to note that each experimental molecule has a specific and narrow therapeutic range, which depends on the administered doses.

Exceeding these concentrations can lead not only to loss of efficacy but also to toxicity.

2.3 The Therapeutic Potential of Hydrogen Sulfide in Cardiovascular Diseases

While high levels of H_2S are toxic in biological systems, it has been demonstrated that low concentrations play important roles in mediating cellular protective processes.

Hydrogen sulfide has numerous potential functions in both physiological and pathological conditions, including calcific aortic valve disease (CAVD). It regulates many physiological processes essential for maintaining vascular homeostasis, such as oxygen sensing, inflammation, immune modulation, and protection against oxidative stress. However, recent studies have shown that reduced H_2S production and tissue concentration contribute to the development of vascular disorders, including atherosclerosis, oxidative stress, and chronic inflammation.

In previous studies, it was demonstrated that H_2S inhibits the transdifferentiation of vascular smooth muscle cells and aortic valve interstitial cells into osteoblast-like cells and eliminates aortic valve calcification in $ApoE^{-/-}$ deficient mice. Furthermore, a decrease in H_2S concentration was observed in valve tissues of patients with CAVD. Three different anti-calcification pathways through which hydrogen sulfide exerts its effects have been identified: a) limiting the uptake of inorganic phosphate by cells, b) preventing the translocation of RUNX2 into the cell nucleus, and c) increasing the level of mineralization-inhibiting pyrophosphate.

In animal models and in vitro experiments, increased calcification of the cardiovascular system was observed with gene silencing of the endogenous hydrogen sulfide

synthesis enzymes (CSE and CBS). In CSE-deficient mice, reduced endogenous H_2S formation promotes atheroma formation, which is compensated by exogenous NaSH treatment. Additionally, CSE regulates flow-dependent vascular remodelling in vascular predilection regions. Human studies have found significantly lower circulating sulfide concentrations in the blood samples of patients with vascular diseases compared to healthy controls. Furthermore, it has been demonstrated that patients with lower H_2S levels have a significantly increased likelihood of death within 36 months following revascularization surgery.

Many diseases are associated with impaired mitochondrial function. The mitochondria-targeted H_2S donor, AP39, is a pharmacological agent primarily developed for studying mitochondrial physiology and has improved mitochondrial bioenergetics in various cell types. It can prevent the formation of reactive oxygen species and preserve mitochondrial membrane integrity. During heart transplantation, AP39 has been shown to protect myocardial cells from ischemia-reperfusion injury and has similar effects on kidney grafts. Thus, this compound appears very promising for the treatment of diseases associated with mitochondrial dysfunction.

3. THE AIMS OF THE STUDY

1. Our research group has previously demonstrated that hydrogen sulfide inhibits calcification of heart valves. Therefore, we investigated whether it also has similar effects on inflammation.
2. Another aim of our study was to uncover the relationships between calcification and inflammation.
3. We observed whether H_2S, through its anti-inflammatory effects, slows the progression of aortic valve calcification.
4. To validate our hypotheses, we planned in vitro, in vivo, and ex vivo experiments using sulfide-releasing donor molecules.
5. Due to the lack of literature data, we designed diagnostics to examine the biologically accessible hydrogen sulfide at physiological concentrations in healthy and calcified heart valves.
6. Our study aimed to assess the response of interstitial cells derived from healthy and calcified aortic valves in a calcifying environment in the presence of proinflammatory cytokines.
7. We aimed to demonstrate the regulation of H_2S levels in the aortic valves of healthy individuals and patients with CAVD.
8. We investigated metabolic changes occurring in healthy and calcified heart valves.
9. We hypothesized that changes in hydrogen sulfide levels in control and calcified heart valves are related to mitochondrial catabolism. Therefore, we analyzed the expression of key enzymes involved in its oxidation.
10. AP39 is a specific mitochondria-targeted sulfide donor that improves mitochondrial bioenergetics in diseased cells. We examined whether it has anti-inflammatory and anti-calcification effects similar to previously studied hydrogen sulfide compounds.
11. Since AP39 has a slow half-life, we aimed to compare its effects with those of a rapidly releasing H_2S donor.

4. MATERIALS AND METHODS

4.1 Chemicals and Reagents Used in Experiments

All chemicals were of analytical reagent grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA), except where otherwise noted. β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The sulfide donor molecules used in our experiments - AP39 [(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl) phenoxy)decyl) triphenylphosphonium bromide] and AP72 [(4 methoxyphenyl) (piperidine-1-yl) phosphinodithioc acid)] - were synthesized for our laboratory by Matthew Whiteman and his team. Sulfide stock solutions were freshly prepared in physiological saline solution before treatments.

4.2 Processing of Human Tissue Samples, Isolation, and Cultivation of Cells

Human calcific aortic valves (CAV) from 58 patients were collected with approval from the Regional Scientific and Research Ethics Committee of the Health Science Council (ETT-TUKEB), under permits 61538-2/2017/EKU and 5643-2021, between August 2018 and May 2021. These were obtained from patients undergoing full aortic valve transplantation due to severe stenosis associated with CAVD. Patients provided written consent for the use of removed tissues for research purposes after verbal information, following the Helsinki Declaration. The samples were obtained in sterile PBS solution after surgery from the Department of Cardiac Surgery at the University of Debrecen. Healthy aortic valves (HAV) were procured from deceased individuals who did not suffer from cardiovascular diseases, through the Department of Forensic Medicine at the University of Debrecen, with the assistance of ETT-TUKEB under permit number 5038-2018. Valvular interstitial cells (VICs) were isolated from valve tissue using type II collagenase (600 U/m^{-1}) (Worthington Biochemical Corporation) enzymatic digestion. Enzymatic digestion of samples was performed at 37 °C for 45 minutes in a cell culture incubator. The cell suspension was then pipetted into cell culture flasks, and the non-adherent cells were washed off the next day, followed by further growth in a fresh medium. All experiments were conducted using cells from five different donors. High-glucose (4.5 g/L D-glucose) growth medium (DMEM: Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Vienna, Austria), 100 U/mL penicillin, 100 U/mL streptomycin, and supplemented with L-glutamine and sodium pyruvate was used for isolation and treatments.

Cells were treated at passage 2 after reaching 90% confluence. It is known from the literature that phenols can absorb large amounts of hydrogen sulfide from liquids; therefore, our sulfide experiments were conducted in a phenol-red-free medium.

4.3 Implementation of Animal Experiments

The methods performed on animals were in accordance with ethical norms set by responsible committees for human experiments (institutional and national) and the revised 1975 Helsinki Declaration. Animal experiments conducted in this study were approved by the Scientific and Research Ethics Committee of the Health Science Council under registration number DE MÁB/157-5/2010, following Institutional and National Guidelines for the care and use of animals (including fishing). C57BL/6 ApoE-/- mice were purchased from Charles River Laboratories International Inc. and housed under specific pathogen-free conditions at the University of Debrecen, at a controlled temperature of 24°C, in accordance with the guidelines of the Institutional Ethics Committee. The mice were randomly divided into four groups. The first group (N=7) received a standard diet. The second group (N=7) had their standard diet replaced with an atherogenic diet (15% fat, 1.25% cholesterol, ssniff Spezialdiäten GmbH, Soest, Germany) from 8 weeks of age onwards, inducing aortic valve inflammation and/or calcification. Concurrently with the atherogenic diet, mice in the third group were intraperitoneally injected with AP72 (266 mmol/kg body weight; N=7), while those in the fourth group were injected with phosphate-buffered saline every other day. Over 8 weeks, every other week, several individuals from each group were sacrificed to model the inflammatory processes in vivo over time. The samples were stored at -70°C. After 8 weeks of treatment, the aortic valves, and other vital organs (heart, liver, kidney, spleen, lung, aorta) were dissected and stored at -70°C after freezing. All animals were euthanized using a slowly rising, controllable, compressed CO_2 inhalation method. No pain or stress was caused to the animals during the interventions. The doses used did not result in mortality among the experimental subjects. The composition of the atherogenic diet was as follows: crude nutrients (%): crude protein 19%; crude fat 15.2%; crude fibre 3.4%; crude ash 6.3%; starch 25.6%; sugar 11.2%; additives (per kilogram): vitamin A 15,000 IU; vitamin D3 1000 IU; vitamin E 110 mg; vitamin K3 5 mg; vitamin C 0 mg; copper 13 mg.

4.4 Induction of Calcification, Measurement of Calcium, Alizarin Red S Staining

VICs were grown and treated in 12-well plates until reaching 80% confluence, then treated for 5 days in a high-glucose growth medium without phenol red, supplemented with a calcifying

medium containing 2.5 mmol/L inorganic phosphate (Na_2HPO_4/NaH_2PO_4 solution) and 1.8 mmol/L calcium chloride ($CaCl_2$). In some experiments, the calcifying medium was supplemented with 10 μmol/L AP72 or 5 nmol/L AP39 during treatments, along with pro-inflammatory cytokines TNF-α or IL-1β at a concentration of 10 nmol/L. The phosphate and calcium concentrations used in our experiments adequately model the levels measured in the blood samples of CAVD patients. For time-dependent experiments, the first day of culture was considered day 0. After 5 days, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and decalcified with 0.6 mol/L hydrochloric acid solution. Subsequently, cells were solubilized in lysis buffer containing 10 mmol/L Tris-(hydroxymethyl)-aminomethane (Tris-HCl), 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 150 mmol/L sodium chloride (NaCl; pH 7.2), 1% Triton X-100, 0.5% Nonidet P-40, and protease inhibitor (Complete Mini, F. Hoffmann-La Roche Ltd., Basel, Switzerland). The protein content of the samples was measured spectrophotometrically using the Bicinchoninic Acid (BCA) protein assay kit (Pierce, Rockford, IL, United States). This method relies on proteins reducing Cu^{2+} ions to Cu^{+} ions in an alkaline environment. Bicinchoninic acid is a highly specific chromogenic reagent that forms a purple complex with Cu^{+} ions, with an absorption maximum of 562 nm. The concentration of the examined protein is proportional to the absorbance. The calcium content of the collected supernatants was determined quantitatively by colourimetric assay (QuantiChrom Calcium Assay Kit, Gentaur; 65-DICA-500, Brussels, Belgium) and normalized to the protein content of the samples. The kit contains a phenolsulfonphthalein dye that forms a very stable, blue-coloured complex, primarily with free calcium. The complex has a characteristic absorption maximum at 612 nm, and its intensity is directly proportional to the calcium concentration in the samples. The use of the kit minimizes interference from substances such as lipids, proteins, and other nutrients. The results were expressed as μg calcium/mg protein.

Alizarin Red S staining is one of the most widely used methods for the qualitative determination of extracellular calcium deposits. After completion of the experiments, cells were fixed with 3.7% paraformaldehyde solution at room temperature for 10 minutes following washing with PBS. After fixation, cells were washed again with PBS, followed by staining with 2% Alizarin Red S solution for 10 minutes at room temperature to visualize calcium crystals. The excess staining solution was removed by three successive washes with distilled water. The stained cells were visualized using a Leica DMIL LED stereo microscope with a Leica DMC4500 camera, Leica LAS software version 4.9.0, and 10x magnification. Five images were captured from each experimental group. The calcified (red-colored) regions

were evaluated using ImageJ software. Using the program, colours were separated, and only images containing the red shade were retained. Finally, pixel density values related to the red colour were determined and plotted on a graph.

4.5 Detection of Alkaline Phosphatase Enzyme

To visualize alkaline phosphatase enzyme activity, cells were cultured and treated in 12-well plates for 5 days in a high-glucose growth medium, either in calcifying medium alone or supplemented with pro-inflammatory cytokines TNF-α or IL-1β at a concentration of 10 nmol/L. After completion of the experiment, cells were washed twice with PBS. A citrate stock solution was diluted with distilled water in a 1:50 ratio to prepare a citrate solution, which was then mixed with acetone in a 2:3 ratio. Cells were fixed with the citrate-acetone mixture for 1 minute, followed by thorough washing with distilled water at least 3 times. The staining solution was prepared by diluting Naphtanol AS-MX - Fast Violet B in a 1:24 ratio. The mixture is pale yellow and light sensitive. During staining, cells were incubated with the staining solution at room temperature in the dark for 30 minutes, followed by two thorough washes. Visualization of the results was performed using a Leica DMIL LED microscope with a Leica DMC4500 camera and Leica LAS Software version 4.9.0. Increased enzyme expression in calcified regions showed a purplish colouration, which was documented by capturing photographs at 10x magnification. The images were analyzed using ImageJ software as described above, and pixel density values related to the purple colour were plotted.

4.6 Analysis of Proteins by Western Blot Technique

VICs were cultured and treated in 6-well plates for 5 days in a high-glucose growth medium, either in calcifying medium alone or supplemented with 10 μmol/L AP72 or 5 nmol/L AP39 or pro-inflammatory cytokines TNF-α or IL-1β at a concentration of 10 nmol/L. After completion of the experiments, cells were washed in PBS and solubilized to determine protein content as described above. Cytoplasmic and nuclear fractions or total cell lysates were used for the analysis. Lysates were centrifuged at 12,000 rpm for 10 minutes and then denatured in sodium dodecyl sulfate (SDS) solution containing β-mercaptoethanol at 95°C for 10 minutes. Approximately 10 or 20 μg of protein from each sample was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Human healthy and calcified tissue samples were cut into approximately 10-15 mm pieces and ground to powder under liquid nitrogen. In this case, 40 μg of protein sample was used for gel electrophoresis.

Electrophoresis was run at 100 V. Subsequently, proteins in the gel were transferred onto a nitrocellulose membrane with a pore size of 0.45 μm. Blotting was performed at 100 V for 75 minutes. The membranes were then washed at least twice with TBS-T (Tris-buffered saline with 0.1% Tween-20). For blocking, membranes were incubated in 6% milk powder at 4°C for 1 hour on a shaker. After blocking, membranes were washed again with TBS-T. Following the washes, membranes were incubated overnight at 4°C on a shaker with the following primary antibodies: anti-human TNF-α (Thermo Fisher Scientific; PA5-19810; 400 ng/mL), anti-human IL-1β (Invitrogen; 17 h18l16; 400 ng/mL), anti-human ALP (Abcam; ab65834; 1000 ng/mL), anti-human CSE (Proteintech Group; 12217-1-AP; 1000 ng/mL), anti-human SO (Thermo Fisher Scientific; PA5-21705; 1 mg/mL), anti-human ETHE1 (Thermo Fisher Scientific; PA5-56040; 0.30 mg/mL), anti-human SQR (Proteintech Group; 17256-1-AP; 550 μg/mL), anti-human TST (Abcam; ab166625; 0.08 mg/mL), anti-human CBS (Proteintech Group; 14787-1-AP; 700 μg/mL), anti-human RUNX2 (Proteintech; 20700-1-AP; 60 ng/mL), and anti-human NF-κB (Cell Signaling Technology; D14E12; 400 ng/mL) rabbit-produced anti-human NF-κB (Cell Signaling Technology; D14E12; 400 ng/mL). Antibodies were diluted 1:500 in 1% milk powder. After 16 hours, membranes were thoroughly washed with TBS-T. Subsequently, membranes were incubated for 1 hour at 4°C on a shaker with horseradish peroxidase-conjugated anti-rabbit (GE Healthcare Life Sciences, Piscataway, NJ, USA, NA934, diluted 1:15,000) or anti-mouse (GE Healthcare Life Sciences, Piscataway, NJ, USA, NA931, diluted 1:15,000) IgG secondary antibodies. After 1 hour, membranes were washed 3 times for 10 minutes with TBS-T. The formed antigen-antibody complexes were made visible on X-ray film using a horseradish peroxidase chemiluminescence detection system (Amersham Biosciences Corp., Piscataway, NJ, USA; RPN2109) in a dark room. After developing the signals, previously bound antibodies were removed from the membranes and re-blocked in 6% milk powder solution, followed by incubation with anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Cat no. N13300-221, Novus Biologicals, LLC, Littleton, CO, USA) at a dilution of 1:1000. The secondary antibody was also used at a dilution of 1:15,000 in this case. The results were visualized as described above. GAPDH, as a housekeeping protein, was used to confirm that an equal amount of protein was loaded onto the SDS-PAGE gels during the run. The developed X-ray films were densitometrically analyzed using ImageJ Fiji software, and the obtained values were normalized to GAPDH densitometric values. In the case of nuclear fractions, lamin B1 (Proteintech, 12987-I-AP; 55 μg/150 μL) antibody was used as a housekeeping protein, and

normalization was also performed based on this. The obtained relative densitometric results were plotted on a graph.

4.7 Immunofluorescence Staining

VICs were cultured on 24-well plates with 12 mm diameter coverslips for 5 days in a high-glucose growth medium, either in calcifying medium alone or supplemented with 20 μmol/L AP72 or 5 nmol/L AP39. For treatments with hydrogen sulfide-releasing donor molecules, a medium devoid of phenol red was used. Between each step of the staining protocol, coverslips were washed at least three times with PBS. After treatment completion, cells were fixed with 3.7% formalin at room temperature for 15 minutes following three washes with PBS. Subsequently, cells were permeabilized with 0.3% Triton X-100 solution to enhance membrane permeability for antibodies. To block nonspecific binding sites, coverslips were blocked with 10% goat serum at room temperature for 1 hour after permeabilization. After blocking, mouse-produced anti-human TNF-α (Santa Cruz; sc-52746; 100 μg/mL) and rabbit-produced anti-human IL-1β (Invitrogen; 17h18116; 400 ng/mL) antibodies were used as primary antibodies at a dilution of 1:500 to detect TNF-α and IL-1β expressed in VICs. TNF-α antibody was visualized with goat-produced anti-mouse Alexa Fluor 488-conjugated IgG antibody (Thermo Fisher Scientific; A11004), and IL-1β was visualized with goat-produced anti-rabbit Alexa Fluor 488-conjugated IgG antibody (Thermo Fisher Scientific; A11070) in the dark. All secondary antibodies were used at a dilution of 1:500, and coverslips were incubated at room temperature for 1 hour in the dark on a shaker. Hydroxyapatite crystals were stained with IVISense Osteo 680 fluorescent probe (OsteoSense; PerkinElmer; NEV10020EX). Nuclei were stained with Hoechst 33258. To map the nuclear translocation of NF-κB transcription factor, rabbit-produced anti-human NF-κB (Cell Signaling Technology; D14E12; 400 ng/mL) was used as the primary antibody, also at a dilution of 1:500. As a secondary antibody for labelling, goat-produced anti-rabbit Alexa Fluor 488 fluorophore was chosen at a dilution of 1:500. Stainings were captured using Leica TCS SP8 STED-CW (Stimulated Emission Depletion-Continuous Wave) super-resolution fluorescence nanoscopy (Leica Microsystem Mannheim, Germany). The system features three continuous wave excitation lasers (488, 532, 638 nm) and a continuous wave 660 nm depletion laser. Images were acquired using Leica Application Software X, and deconvolution was performed using Huygens Professional software (Scientific Volume Imaging B.V., Hilversum, Netherlands). Image analysis was conducted using ImageJ Fiji software. The colour intensity of individual

proteins and the colocalization of transcription factors in nuclear fractions were determined. The percentage area of colour shades was plotted on a graph.

4.8 Nuclear and Cytoplasmic Protein Extraction

VICs were treated in 6-well plates for 5 days in a high-glucose growth medium, either in calcifying medium alone or supplemented with 10 μmol/L AP72. For treatments with hydrogen sulfide-releasing donor molecules, a medium devoid of phenol red was used. After the experiments concluded, the medium was removed, and cells were washed twice with PBS. Subsequently, cells were collected into 2 mL centrifuge tubes using a cell scraper. To the pellets, ice-cold hypotonic cytoplasmic lysis buffer (20 mmol/L Tris-HCl pH 8.0, 100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L MgCl2, and protease inhibitor solution) was added. The cell suspensions were incubated on ice for 15 minutes. After centrifugation (8000 x g, 4°C for 15 minutes), the supernatant containing the cytoplasmic protein fraction was collected into 1.5 mL centrifuge tubes.

The remaining pellets were washed twice with PBS and then resuspended in ice-cold nuclear extraction buffer (20 mmol/L Tris-HCl pH 8.0, 300 mmol/L NaCl, 2 mmol/L EDTA pH 8.0, and protease inhibitor solution) using a syringe equipped with a 27-gauge needle. Finally, the lysate was centrifuged at 8000 x g, 4°C for 20 minutes. The resulting supernatant contained the nuclear fraction. The protein concentration of the fractions was determined using the method described above.

4.9 Determination of Hydrogen Sulfide Levels from Cell Lysates using Modified Methylene Blue Method

The sulfide levels were measured using a zinc precipitation method, initially developed by Gilboa and Garber, and later modified by A. D. Ang and colleagues. To the cell lysates (50 μL), 350 μl of 1% zinc acetate and 50 μl of 1.5 mol/L sodium hydroxide were added. The samples were then incubated at room temperature on a desktop shaker for 60 minutes to separate sulfide from other chromophores by precipitation.

After the incubation period, the formed zinc sulfide precipitate was centrifuged at 2000 x g for 5 minutes. The supernatant was then removed, and the remaining pellet was washed with 1 mL of distilled water while vigorously vortexing. Subsequently, the samples were centrifuged again at 2000 x g at room temperature for 5 minutes. The supernatant was carefully decanted, and the pellet was dissolved in 160 μl of distilled water. Then, 40 μl of a pre-mixed dye

solution (20 µl of 20 mmol/L NNDP dissolved in 7.2 mol/L hydrochloric acid and 20 µl of 30 mmol/L iron (III) chloride dissolved in 1.2 mol/L HCl) was added, and the samples were incubated.

During incubation in the dark, sulfide reacts with NNDP, catalyzed by $FeCl_3$, producing methylene blue. After 10 minutes, the characteristic absorption wavelength of methylene blue at 667 nm was determined using a spectrophotometer (Beckman DU-800). The exact sulfide concentrations were calculated using a previously constructed calibration curve and the extinction coefficient of methylene blue (30,200 $M^{-1}cm^{-1}$). The results were normalized to the protein content of the samples and expressed as µmol/L total hydrogen sulfide per mg of protein.

4.10 Detection of Biologically Accessible H_2S using LC-MS/MS

The measurements were conducted at the National Institute of Oncology in Budapest and based on the method developed by Akaike and colleagues, as described below:

Healthy and calcified human heart valve tissues were cryogenically powdered and suspended in ice-cold methanol containing 5 mmol/L β-(4-hydroxyphenyl)-ethyl-iodoacetamide (HPE-IAM) for alkylation. The samples were kept continuously on ice between each preparation step. After sonication, the derivatization was carried out at 37 °C for 20 minutes, followed by centrifugation at 14,000 x g for 10 minutes at 4 °C. The supernatants (100 µl) were acidified with 5 µl of 10% formic acid and diluted twofold with 0.1% FA/H2O before injection.

Tissue pellets were dissolved in 1% SDS/PBS, and sonicated, and the protein content was determined by BCA assay. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements were performed using a Thermo Q-Exactive Focus Orbitrap mass spectrometer coupled to a Thermo Vanquish UHPLC system. The derivatized analytes were separated on a Phenomenex Kinetex C18 column (50 × 2.1 mm, 2.6 µm inner diameter) using 0.1% FA/H2O (A) and 0.1% FA/MeOH (B) as mobile phases.

A linear gradient elution was applied, increasing the proportion of eluent B from 5% to 95% over 15 minutes, followed by a gradual decrease to 5% over 2 minutes and holding for 3 minutes until the next injection. The column temperature was maintained at 30 °C, the flow rate at 0.3 mL/min, and the injection volume was 5 µL. The alkylated sulfide products were detected in positive ionization mode by MS/MS. Their quantities were determined by analyzing a specific fragment of a selected precursor ion. Higher Energy Collision Dissociation (HCD) was employed for the detection of the 252 m/z fragment from the 389 m/z precursor ion.

4.11 Experimental Units

"N" indicates the number of tissue samples or experimental animals used in each group. "n" represents the number of independent repetitions of experiments from which the results are derived.

4.12 Statistical Analysis

Data from experiments conducted on interstitial cells were analyzed using GraphPad Prism 5.02 statistical software (GraphPad Software Inc., 7825 Fay Avenue, Suite 230 La Jolla, CA 92037). The statistical results shown in the graphs represent the standard error of the mean (SEM) of data from at least five independent experiments. All statistical data were presented as mean ± SEM. For comparison between the two data groups, a Student's t-test was performed. For comparisons among more than two data groups with equal variances and normally distributed data, a one-way analysis of variance (ANOVA) was conducted, supplemented with Bonferroni's multiple comparison post hoc test. The legends of the figures indicate significant differences according to different p-values: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). A p-value of <0.05 was considered significant. "ns" indicates non-significant results.

5. RESULTS

5.1 Increased Expression of Pro-inflammatory Cytokines in Human Calcified Aortic Valve

In our study, the CAV samples were obtained from patients diagnosed with severe aortic valve insufficiency who underwent complete aortic valve replacement. To assess inflammation, calcified aortic valves were stained for pro-inflammatory cytokines IL-1β and TNF-α. Healthy aortic valve tissues (HAV) obtained from individuals without cardiovascular diseases, suicide, or traumatic events were used as control stains. It was crucial to remove the valves within 20 hours postmortem in such cases. Intense staining of von Kossa in CAV indicated severe valve calcification. In calcified samples, increased expression of mineralization, IL-1β, and TNF-α followed compared to healthy controls. Western blot analysis also demonstrated significantly higher levels of inflammatory cytokines in CAV compared to HAV. These results are consistent with previous findings indicating inflammation and calcification as important characteristics of CAVD.

5.2 Exogenously Administered H_2S Inhibits Inflammation in Apolipoprotein E-deficient ($ApoE^{-/-}$) Aortic Valves

Our research group previously demonstrated that hydrogen sulfide prevented aortic valve calcification in $ApoE^{-/-}$ mice fed a high-fat diet. We investigated whether H_2S influences inflammation in $ApoE^{-/-}$ mouse aortic valves under similar conditions. In our experiments, we used AP72, a slow-release hydrogen sulfide donor compound previously shown to strongly inhibit human heart valve mineralization. AP72 sulfide-containing compound was administered to the mice every other day via intraperitoneal injection at a dose of 266 mmol/kg. Our results showed that the tested hydrogen sulfide compound significantly inhibited aortic valve thickening and the expression of inflammatory cytokines IL-1β and TNF-α. Similar to human CAV, the number of interstitial cells staining positive for IL-1β and TNF-α was much more pronounced in the aortic valves of $ApoE^{-/-}$ mice fed a high-fat diet compared to control mice. However, AP72 treatment significantly reduced both IL-1β and TNF-α expression in the aortic valve tissue of $ApoE^{-/-}$ mice fed a high-fat diet. Similar results were observed under ex vivo experimental conditions. Tissue sections of $ApoE^{-/-}$ mouse valves were cultured, and high phosphate exposure was used to induce calcification. It was

evident that Alizarin Red S staining significantly reduced the calcification of interstitial cells in AP72-treated valves.

5.3 Decreased Endogenous H_2S Production Enhances Inflammation Induced by High Phosphate in Human Aortic Valve Interstitial Cells

As expected, significant calcification occurred in human aortic valve interstitial cells exposed to high phosphate treatment. Furthermore, we observed strong induction of pro-inflammatory cytokines IL-1β and TNF-α at both translational and transcriptional levels in human VICs exposed to high phosphate. Following treatment with AP72, there was a significant reduction in calcium deposition in the extracellular matrix, which was accompanied by a decrease in the induction of inflammatory cytokines. Our research group previously observed that decreased expression of CSE and CBS, the major enzymes responsible for physiological hydrogen sulfide production, leads to decreased endogenous H_2S production, promoting VIC calcification. Thus, to test the anti-inflammatory effects of endogenous H_2S, experiments were conducted by inhibiting the function of the CSE and CBS genes. Our results showed that double knockdown of CSE and CBS genes using specific small interfering RNAs significantly increased the expression of IL-1β and TNF-α, the inflammation-inducing cytokines, in VICs exposed to high phosphate. Based on our experiments, we concluded that under calcification conditions, hydrogen sulfide regulates the progression of inflammation.

5.4 H_2S Inhibits the Progression of Inflammatory Processes via Inhibition of NF-κB Activity

It is well known that the NF-κB transcription factor regulates the signalling pathway that initiates inflammation, and its activation is based on nuclear translocation, followed by the expression of inflammatory cytokines such as IL-1β and TNF-α. Previous studies by other research groups have demonstrated that H_2S is capable of sulfhydrating NF-κB, resulting in inhibition of its nuclear translocation. Therefore, we investigated whether AP72 affects NF-κB activation in cells maintained in calcification-inducing medium. Confocal microscopic evaluation of immunofluorescent stains showed that NF-κB was present in the cytoplasm of aortic valve interstitial cells cultured in growth medium. However, phosphate exposure induced the translocation of NF-κB from the cytoplasm to the nucleus. Treatment with AP72 prevented the appearance of NF-κB in the nuclei of VICs maintained in the calcification-inducing medium. To confirm the results of immunofluorescent staining, the cytoplasmic and

nuclear fractions of VICs were analyzed for NF-κB by Western blot analysis. Our results showed that NF-κB appeared in the nucleus in response to phosphate exposure, while its level decreased in the cytoplasmic fraction. Importantly, at the translational level, we were able to confirm that AP72 treatment significantly inhibited the translocation of NF-κB into the nucleus in VICs exposed to high phosphate.

5.5 Az NF-κB activation promotes the nuclear translocation of RUNX2, followed by mineralization of aortic valve interstitial cells (VICs).

RUNX2 is a key transcription factor in osteogenic processes, regulating the early osteoblastic differentiation of vascular smooth muscle cells and aortic valve interstitial cells. We were interested in exploring the relationship between NF-κB and RUNX2 transcription factors in human VICs under calcification-inducing conditions. In the experimental design, human VICs were cultured in calcification medium, where the NF-κB gene activity was reduced by gene silencing. As expected, exposure to high phosphate significantly increased the level of RUNX2 in the cell nucleus. Following gene silencing with NF-κB-specific siRNA, we observed a significant reduction in the nuclear translocation of RUNX2. To confirm this observation, we also inhibited NF-κB activity using a pharmacological inhibitor (SC75741) via another pathway. The results clearly show that treatment of cells with the synthetic NF-κB inhibitor SC75741 significantly reduced both the appearance of NF-κB, the transcription factor regulating inflammation, and RUNX2, the transcription factor regulating calcification, in the nucleus.

To further reinforce the idea that inflammation regulates mineralization through NF-κB in VICs, we cultured the cells again in calcification-inducing medium, where we silenced the NF-κB gene and monitored the accumulation of calcium in the extracellular matrix. Importantly, reducing NF-κB gene activity inhibited the accumulation of calcium deposits in the extracellular matrix of cells cultured in calcification-inducing conditions.

5.6 In ApoE$^{-/-}$ mice, the expression and nuclear colocalization of NF-κB and RUNX2 increase during valve interstitial cell calcification – both processes inhibited by hydrogen sulfide.

High-fat diets induce valve calcification in ApoE$^{-/-}$ mice. Previous findings in calcific aortic valve disease (CAVD) demonstrated severe inflammation accompanying valve calcification. Given our in vitro experiments showing hydrogen sulfide (H_2S) inhibiting both NF-κB and

RUNX2 nuclear translocation, with RUNX2 nuclear localization being NF-κB-dependent, we investigated their localization in the aortic valves of high-fat diet-fed ApoE$^{-/-}$ mice. The regulatory effect of hydrogen sulfide was modelled by intraperitoneal administration of the AP72 donor molecule. Dual immunohistochemical staining showed peak expression of NF-κB and RUNX2 in the aortic valves of ApoE$^{-/-}$ mice on the atherogenic diet at week 8. Treatment of experimental animals with AP72 resulted in significantly weaker staining for both NF-κB and RUNX2 in the valve tissue. Confocal microscopy revealed NF-κB and RUNX2 transcription factors mainly localized to the perinuclear region of cells at week 4, transitioning to the nucleus by week 8. STED nanoscopy of valve samples demonstrated colocalization of NF-κB and RUNX2 in both the perinuclear regions and the nuclei of cells (colocalization ratio: 60.75% ± 7%). Treatment of high-fat diet-fed ApoE$^{-/-}$ mice with AP72 substantially reduced the nuclear presence of both NF-κB and RUNX2.

5.7 In CAVD, elevated CSE levels and low biologically accessible H_2S levels are associated with increased expression of proinflammatory cytokines.

It is a known fact that CAVD is an inflammatory disease, and H_2S regulates vascular calcification. Therefore, we investigated the expression of proinflammatory cytokines and CSE in human aortic valves. Aortic valves from patients diagnosed with severe CAVD who underwent valve replacement surgery were stained for proinflammatory cytokines IL-1β and TNF-α, as well as for CSE. Control samples were obtained from healthy aortic valves of individuals who died from suicide or traumatic events, and according to available information, did not suffer from cardiovascular diseases. Calcification was accompanied by increased expression of proinflammatory cytokines IL-1β and TNF-α in the calcified aortic valves (CAV). We observed significantly elevated CSE expression in the aortic valves of patients diagnosed with CAVD compared to healthy individuals. Since elevated CSE levels were found in the aortic valves of CAVD patients, we measured the biologically accessible H_2S levels in tissues from CAVD patients and healthy individuals using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Surprisingly, the biologically accessible H_2S levels were significantly lower in the aortic valve tissues of CAVD patients compared to HAV tissues.

In addition to CSE, CBS is another enzyme responsible for catalyzing endogenous hydrogen sulfide biosynthesis. Therefore, we examined the expression of CBS in healthy and calcified

aortic valves. Immunohistochemical and Western blot analyses clearly showed that CBS levels did not change in CAV compared to HAV.

5.8 Assessment of VIC cell mineralization and inflammatory response to high phosphate concentration.

To assess the mineralization potential of VIC cells and their inflammatory response to calcification-inducing stimuli, interstitial cells isolated from HAV and CAV tissues were exposed to a high phosphate concentration environment, which triggered the cells' phenotypic shift towards osteoblastic differentiation. VIC cells derived from patients with CAVD exhibited a robust response to excessive phosphate exposure, reflected in the accumulation of calcium in the extracellular matrix and elevated levels of the proinflammatory cytokines IL-1β and TNF-α. In VIC cells from healthy donors, significantly less calcium deposition and lower expression of IL-1β and TNF-α were observed compared to cells from CAVD patients. We examined cellular responses in VIC cells from healthy individuals cultured in calcification-inducing medium, compared to cells maintained in growth medium. It is important to note that the values on the y-axis in the right panels are substantially lower than those in the left panels. As seen in the right panels, increased phosphate concentration resulted in enhanced mineralization and elevated levels of IL-1β and TNF-α in VIC cells derived from HAV tissues, while cells maintained in growth medium did not exhibit activation of calcification and inflammatory responses.

5.9 Proinflammatory cytokines enhance mineralization of VIC cells derived from CAVD patients.

Proinflammatory cytokines play a central role in the development and progression of CAVD, so we examined whether IL-1β and TNF-α cytokines exacerbate mineralization induced by elevated phosphate levels in VIC cells. Treatment of VIC cells derived from CAVD patients with phosphate triggered the deposition of calcium deposits in the extracellular matrix. Following treatment with IL-1β or TNF-α proinflammatory cytokines, we observed increased calcium accumulation in these cells compared to cells treated with phosphate alone. Accordingly, treatment with IL-1β or TNF-α in VIC cells from CAVD patients further increased alkaline phosphatase enzyme expression in high phosphate concentration medium, supported by Western blot analysis and confirmed by increased ALP staining. These results

are consistent with our hypothesis that the transdifferentiation of VIC cells into an osteoblastic phenotype is associated with inflammation.

5.10 Proinflammatory cytokines cause transient inhibition of mineralization in VIC cells derived from healthy aortic valves

Surprisingly, when VIC cells derived from HAV tissues were treated with IL-1β or TNF-α proinflammatory cytokines, mineralization induced by high phosphate concentration did not occur. Calcium accumulation in the extracellular matrix of VIC cells derived from healthy aortic valves increased in high phosphate medium. An important observation was that in cells exposed to IL-1β or TNF-α treatment for 5 days, calcium deposition significantly decreased; however, this anti-calcific effect was no longer detectable by day 14. Accordingly, both proinflammatory cytokines inhibited the expression of the osteoblast-specific gene, ALP, as confirmed by Western blot analysis and ALP staining. These data indicate that in VIC cells derived from healthy aortic valves, treatment with IL-1β and TNF-α transiently inhibits the osteoblastic phenotypic transition induced by high phosphate levels.

5.11 CSE/H_2S regulates the transient inhibition of IL-1β- and TNF-α-induced mineralization in VIC cells derived from healthy aortic valves

Based on the previous findings, proinflammatory cytokines are capable of transiently inhibiting the progression of phosphate-induced mineralization in HAV-VIC cells. However, the molecular mechanisms underlying this process are unknown. Therefore, to explore them, we examined the levels of endogenously produced H_2S in the cells using a modified methylene blue method. We observed a gradual decrease in total H_2S levels in VIC cells isolated from healthy aortic valves cultured in high-phosphate medium. In contrast, in cells treated with the proinflammatory cytokines IL-1β or TNF-α, hydrogen sulfide levels increased until day 5 and then gradually decreased by day 14. Previously, we identified several activators, including IL-1β and TNF-α, capable of inducing CSE enzyme expression in vascular cells. Therefore, we examined changes in CSE enzyme expression in HAV-VIC cells. Both IL-1β and TNF-α significantly increased CSE enzyme protein levels in HAV-VIC cells, indicating the regulation of mineralization through endogenous H_2S production mediated by CSE.

We previously observed that reducing endogenous hydrogen sulfide production by decreasing the expression of CSE and CBS hydrogen sulfide-producing enzymes promotes VIC calcification. Therefore, we investigated whether the transient, anti-mineralization effect of proinflammatory cytokines depends on CSE levels. We found that in a calcifying environment when the expression of hydrogen sulfide-producing enzymes CSE and CBS was reduced for 5 days using gene-specific small interfering RNAs (siRNAs), both IL-1β and TNF-α proinflammatory cytokines lost their inhibitory effect on calcification. This was reflected in a significant increase in calcium deposition in the extracellular matrix of HAV cells.
We examined whether the level of CSE increased in interstitial cells isolated from aortic valves of CAVD patients upon treatment with proinflammatory cytokines. While inorganic phosphate alone significantly increased CSE protein expression, neither IL-1β nor TNF-α altered its level. In contrast, in interstitial cells cultured in high-phosphate medium, the total H_2S level measured by the modified methylene blue method was significantly lower than in control medium-cultured cells. Moreover, when the calcification medium was supplemented with IL-1β or TNF-α, cytokine treatment further decreased the total endogenous H_2S level.

5.12 The mitochondrial metabolism of H_2S is increased in aortic valve interstitial cells (VICs) from CAVD patients.

Increased expression of CSE accompanied by decreased H_2S levels was observed in aortic valve tissues of CAVD patients compared to healthy controls. This contradictory observation may indicate that CSE activity is reduced in the aortic valve tissues of CAVD patients, leading to decreased H_2S production. To gain deeper insights into the enzymatic activities of the transsulfuration process, sulfur metabolome analyses were performed. The levels of cysteine and homocysteine were similar, while cystathionine levels were lower in aortic valve tissues of CAVD patients compared to healthy aortic valves. These analytes can be substrates and products of various enzymatic pathways involving CSE and other transsulfuration enzymes, thereby being interconnected in enzymatic pathways. Lanthionine is mainly a byproduct of CSE enzyme H_2S production, which is formed when the enzyme utilizes two cysteine molecules for hydrogen sulfide synthesis. Therefore, we measured the level of lanthionine in valve tissues. Importantly, the level of lanthionine was higher in the aortic valve tissues of CAVD patients than in healthy aortic valves.

Taken together, these results indicate that the CSE enzyme is functional in the aortic valve tissues of CAVD patients. Based on this observation, we hypothesize that in calcified aortic

valves, the lower level of biologically available hydrogen sulfide, despite increased CSE expression, is a consequence of the increased metabolic flux of H_2S. Therefore, we examined the expression of SQR, ETHE1, SO, and TST proteins, which are key enzymes involved in the mitochondrial catabolism of H_2S. The expression of these enzymes was significantly higher in the calcified aortic valves of CAVD patients than in healthy aortic valves, indicating indeed an increased rate of hydrogen sulfide catabolism. This may explain the lower biologically available hydrogen sulfide levels observed alongside increased CSE expression.
These enzymes are also involved in cysteine-persulfide metabolism; therefore, considering transpersulfidation reactions, we measured the level of protein persulfidation (CysSH/Cys%) in healthy and calcified aortic valves; however, no significant difference was found.
Since elevated plasma phosphate levels are one of the strongest known inducers of vascular calcification, we investigated whether excessive phosphate exposure to VICs alters the expression of mitochondrial enzymes involved in H_2S oxidation. Importantly, healthy VICs cultured in phosphate-rich medium showed increased expression of SQR, ETHE1, SO, and TST. These results, along with the observation that HAV-VIC cells cultured in high-phosphate medium for 14 days showed increased CSE expression and lower H_2S levels compared to control conditions, suggest that high phosphate exposure indeed induces higher sulfide flux.

5.13 Mitochondrial hydrogen sulfide donor inhibits high phosphate-induced calcification in human aortic valve interstitial cells.

Mitochondria-targeted H_2S donors offer a new approach to test whether restoring hydrogen sulfide levels in mitochondria can prevent VIC cells from transdifferentiating into an osteoblastic phenotype. Interstitial cells isolated from the aortic valves of CAVD patients were treated with AP39 compound, a specific mitochondria-targeted hydrogen sulfide-releasing donor molecule, in a high-phosphate medium, and its effect on calcification was examined. As expected, under high phosphate concentration, VIC cells initiated transdifferentiation into an osteoblastic phenotype, reflected by the accumulation of calcium in the extracellular matrix of the cells. Treatment of VIC cells with mitochondria-targeted hydrogen sulfide using the AP39 donor significantly reduced extracellular calcium accumulation, as demonstrated by Alizarin Red S staining and direct calcium measurements, thus confirming the key role of mitochondrial H_2S metabolism in maintaining aortic valve integrity.

5.14 Mitochondrial hydrogen sulfide donor inhibits high phosphate-induced inflammation in human aortic valve interstitial cells.

Since the processes of calcification and inflammation are interconnected in the pathogenesis of CAVD, we investigated whether the mitochondrial H_2S donor molecule AP39 affects inflammation in VIC cells. As expected, the expression of inflammatory cytokines such as IL-1β and TNF-α significantly increased at both transcriptional and translational levels in human interstitial cells cultured in calcification medium. However, treatment of the cells with AP39 markedly reduced the levels of proinflammatory cytokines elevated due to high phosphate levels. To further validate the anti-inflammatory effect of the specific mitochondrial-targeted H_2S donor, immunofluorescence staining was performed for calcification (Osteosense) as well as IL-1β and TNF-α inflammatory cytokines. Immunofluorescence staining was visualized using STED-CW nanoscopy technology. The cells were cultured in high phosphate medium, and the accumulation of calcium in the extracellular matrix was significantly increased, accompanied by strong staining for IL-1β and TNF-α. The evaluation revealed that AP39 treatment significantly inhibited calcium accumulation in the extracellular matrix and prevented the expression of IL-1β and TNF-α.

6. DISCUSSION

This study sheds light on the relationship between NF-κB and RUNX2 as mediators between inflammation and vascular mineralization and demonstrates how hydrogen sulfide (H_2S) regulates the expression of proinflammatory cytokines, subsequently halting the further progression of aortic valve calcification. In calcific aortic valve disease (CAVD), inflammation is a significant pathological process alongside mineralization, resulting in tissue heterogeneity within the affected aortic valve. Distinguishing the mineralization status of the tissue relies on the ratio of calcified to non-calcified areas. CAVD is an actively regulated inflammatory disease at both cellular and molecular levels. As demonstrated by the National Heart Lung and Blood Institute and other research groups, inflammation is a critical initiating factor in the development of heart valve diseases. It is worth noting, however, that the specific molecular and biochemical mechanisms are not fully understood to date.

There is increasing evidence suggesting that RUNX2, an osteogenic transcription factor, plays a crucial role in cardiovascular mineralization, including CAVD. For instance, the elevation of intracellular phosphate levels promotes the nuclear translocation of RUNX2, leading to osteoblastic phenotype transition in cells. The upregulation of RUNX2 represents a maladaptive response to vascular injury, uremic environments, and hyperglycemic conditions, presenting a potential therapeutic target in vascular mineralization. In our and others' previous studies, RUNX2 has been detected in the nuclei of interstitial cells composing heart valve tissue, serving as an early indicator of osteoblastic phenotype transition.

We observed that hydrogen sulfide produced by the enzymes CSE and CBS, as well as the application of H_2S-releasing donor molecules like AP72, inhibits aortic valve calcification in $ApoE^{-/-}$ mice and prevents the transdifferentiation of human aortic valve interstitial cells into osteoblast-like cells. We identified three distinct anti-calcification pathways through which hydrogen sulfide may exert its effects: I. inhibition of RUNX2 nuclear translocation, II. reduction of inorganic phosphate uptake into cells, and III. promotion of pyrophosphate formation, an anti-calcification compound. These previous studies and our findings prompt us to investigate whether the anti-inflammatory effect of H_2S contributes to the inhibition of aortic valve mineralization and whether this mechanism occurs through the regulation of RUNX2 in interstitial cells.

AP72 is a donor molecule with excellent water solubility and slow H_2S release compared to compounds that rapidly release sulfide, such as Na_2S and NaSH. It is increasingly recognized that donors releasing hydrogen sulfide slowly may better mimic the effects of the endogenous

H_2S buffering system, as they generate low sulfide levels slowly. Therefore, for our experiments, we chose to use the AP72 compound. An important result is that exogenous administration of H_2S abolished the inflammation induced by a high-fat diet in the aortic valves of $ApoE^{-/-}$ mice, as reflected by the decrease in TNF-α and IL-1β levels. Accordingly, the elevation of TNF-α and IL-1β levels induced by high phosphate exposure decreased in human aortic valve interstitial cells after treatment with AP72. To investigate whether endogenous hydrogen sulfide production has anti-inflammatory effects, we silenced the activity of the CSE and CBS genes. As the interaction between CSE/CBS expression was revealed by Nandi and Mishra, proving that the absence of CBS upregulates CSE protein levels, we employed double gene silencing. Reducing endogenous H_2S production exacerbated the phosphate-induced increase in TNF-α and IL-1β levels in the interstitial cells of heart valve tissues. This result indicates the regulation of inflammation by endogenous hydrogen sulfide production.

Proinflammatory cytokines, IL-1β and TNF-α expression are regulated by nuclear translocation of NF-κB. It has recently been found that hydrogen sulfide inhibits NF-κB activation. This study prompted us to examine whether inflammation and mineralization are related at the level of major regulatory transcription factors, NF-κB and RUNX2. Reinforcing the above findings, AP72 prevented NF-κB translocation into the cell nucleus in human aortic valve interstitial cells exposed to high phosphate concentration. We also observed that H_2S inhibits the activation of RUNX2. Therefore, we limited NF-κB activity by gene silencing and pharmacological inhibitor application and then monitored RUNX2 activation in human aortic valve interstitial cells exposed to phosphate treatment. An important finding is that phosphate did not induce RUNX2 translocation into the cell nucleus in cells that did not express NF-κB, indicating a connection between inflammation-promoting and osteogenic signalling pathways. To confirm our findings, we conducted in vivo experiments and demonstrated that the expression of NF-κB and RUNX2 increased during aortic valve calcification in $ApoE^{-/-}$ mice, but hydrogen sulfide therapy significantly reduced the levels of both transcription factors. During the progression of aortic valve calcification, we observed strong colocalization of NF-κB and RUNX2 in the perinuclear region and cell nuclei of interstitial cells. Therefore, our study suggests that the regulation of RUNX2 by hydrogen sulfide (CSE/CBS) occurs through NF-κB, resulting in anti-calcification and thus establishing a link between inflammation and mineralization in CAVD.

We have demonstrated for the first time the metabolic regulation of hydrogen sulfide levels in interstitial cells isolated from aortic valves of CAVD patients and their relationship with the

process of mineralization. Our hypothesis was based on the significantly lower hydrogen sulfide levels detected in the aortic valves of CAVD patients compared to healthy aortic valves, which were associated with higher expression of the hydrogen sulfide-generating CSE enzyme and mitochondrial enzymes involved in its oxidation. Accordingly, VIC cells isolated from healthy human aortic valves mimicked the expression profile of transsulfuration enzymes characteristic of the pathological condition in the calcification milieu, namely, increased expression of CSE and elevated levels of key enzymes involved in mitochondrial hydrogen sulfide oxidation, such as SQR, ETHE1, SO, and TST. We speculate that the lower biologically accessible H_2S levels observed in CAVD aortic valves and VIC cells isolated from them are consequences of the increased mitochondrial catabolism of hydrogen sulfide induced by the calcific environment.

The increased mitochondrial catabolism of H_2S may explain the lower biologically accessible H_2S levels we observed, although it is known that other mechanisms can also cause this phenomenon. Elevated expression of CSE does not necessarily correlate with an increase in the rate of H_2S production in calcified aortic valves. Based on literature data, we know that the CSE enzyme is capable of phosphorylation at the Ser377 amino acid, which can lead to a decrease in enzyme activity. However, the elevated level of lanthionine observed in the calcified aortic valves of CAVD patients compared to healthy tissues suggests that the CSE enzyme is active, and the rate of H_2S formation may even increase in CAVD.

The altered expression of CBS, another important endogenous hydrogen sulfide-producing enzyme, would provide further explanation for the low biologically accessible H_2S levels observed in CAVD. However, immunohistochemical and Western blot analyses showed that CBS expression in calcified aortic valves was similar to that observed in healthy valves. This result suggests that the lower biologically accessible H_2S levels observed in CAVD are not related to the function of CBS.

Hydrogen sulfide is an important signalling molecule that has received increasing attention in recent years due to its versatile functions in the cardiovascular system. Our laboratory previously demonstrated the role of hydrogen sulfide in inhibiting aortic valve calcification in $ApoE^{-/-}$ mice through endogenous production by CSE and exogenous administration of hydrogen sulfide-releasing donor molecules, as well as inhibiting osteoblastic transdifferentiation of human valvular interstitial cells and vascular smooth muscle cells.

Due to the hypothesized interactions between cysteine persulfidation and the mitochondrial electron transport chain, enzymes activated during hydrogen sulfide catabolism, including SQR, ETHE1, SO, and TST, may also increase their expression during persulfide/polysulfide

metabolism. However, hydrogen sulfide formation may also induce increased levels or metabolism of persulfides/polysulfides. It should also be noted that the most advanced detection methods currently available for reactive sulfur species (including H_2S) can artificially modify their specificity. Therefore, it is very difficult to distinguish whether the observed biological effect is mediated by hydrogen sulfide or persulfides/polysulfides. Nevertheless, we measured the total protein persulfidation levels in healthy and calcified aortic valves. During the study, we observed unchanged protein CysSH/Cys% persulfide levels, which, due to the detection issues, do not exclude the role of persulfides but also do not support the direct role of hydrogen sulfide in our experimental systems.

The increased expression of proinflammatory cytokines such as IL-1β and TNF-α is associated with CAVD, making inflammation one of the disease's key features. CAVD is an active, complex, osteogenic process in which inflammation plays a central role in both development and progression. These findings prompted us to study the effects of IL-1β and TNF-α on human VIC cells under calcification conditions. As we have previously uncovered, both IL-1β and TNF-α promoted calcification of human VIC cells, regardless of the cells' origin, specifically whether they originated from healthy individuals or CAVD patients' aortic valves. Although significant differences in the magnitude and duration of cellular responses exist depending on the origin of the cells, as reflected in the intensity of calcification and production of inflammatory cytokines, the ultimate direction of these responses was the same in both conditions. The progression of calcification was faster, and the synthesis of IL-1β and TNF-α was more pronounced in VIC cells from CAVD patients, attributable to the calcification environment of diseased aortic valves and their inherent commitment to an inherited osteoblastic phenotype.

Lagoutte and colleagues have demonstrated that hydrogen sulfide plays a significant role in the bioenergetics of breast cells by stimulating the mitochondrial electron transport chain. Studies with the mitochondrial-targeted H_2S donor AP39 have shown a beneficial effect on the bioenergetic parameters of cells. Therefore, we examined whether restoring hydrogen sulfide levels in mitochondria with a specific mitochondrial-targeted sulfide donor regulates the calcification of VIC cells. The observed inhibitory effect of the AP39 molecule on calcification in VIC cells supports the fundamental role of mitochondrial hydrogen sulfide metabolism in maintaining aortic valve integrity.

During our investigations, hydrogen sulfide measurements were not specific to mitochondria, so we conducted experiments with NaSH (a general H_2S donor) and compared it with AP39 to exclude the effect of cytoplasmic hydrogen sulfide on the inhibition of VIC cell calcification.

NaSH did not affect VIC cell mineralization at a dose of 5 nmol/L, which was the optimal concentration for AP39. When comparing the effectiveness of AP39 and NaSH, we found that the mitochondria-targeted AP39 hydrogen donor was more effective at a concentration 5000 times lower. This observation indicates that mitochondrial hydrogen sulfide plays a critical role in inhibiting VIC cell calcification. The reduction in the progression of mineralization and the decrease in the expression of proinflammatory cytokines with low-concentration AP39 treatment are consistent with previous observations showing the pathophysiological relationship between calcification and inflammation in CAVD.

An important finding was the transient inhibition of calcification and osteoblast phenotype switching in interstitial cells derived from healthy aortic valves upon treatment with IL-1β and TNF-α, compared to cells isolated from CAVD patients. This advantageous property of proinflammatory cytokines can be associated with H_2S biogenesis since both IL-1β and TNF-α increased CSE expression, resulting in increased hydrogen sulfide levels. To test whether hydrogen sulfide produced by the CSE enzyme is responsible for the inhibition of VIC cell calcification and osteoblast phenotype switching, we reduced the expression of CSE and CBS enzymes using gene silencing. Studies conducted in various biological systems have shown that considering sulfide/persulfide-producing activity, CSE and CBS can compensate for each other to some extent, so we applied double gene silencing of these enzymes. The decrease in endogenous hydrogen sulfide production led to the loss of anti-calcification effects in the calcifying environment. This means that hydrogen sulfide produced by CSE participated in the transient inhibition of VIC cell calcification induced by TNF-α or IL-1β.

Similar to valvular interstitial cells, CSE expression can be enhanced by TNF-α and IL-1β in other cells of the vascular system, including smooth muscle cells, endothelium, and macrophages. CSE can also be induced by biologically active molecules or pathological metabolic products involved in vascular pathologies, such as peroxides associated with oxidized LDL, lipid components of atherosclerotic plaques, and hem or oxidized haemoglobin. These components are closely related to the development of CAVD and may contribute to increased CSE expression in aortic valves.

We hypothesize that in a calcifying environment, the significantly decreased level of endogenous hydrogen sulfide due to increased mitochondrial catabolism promotes VIC cell calcification in CAVD. However, upregulation of CSE enzyme expression is accompanied by increased hydrogen sulfide generation, providing an adaptive, anti-calcification protective mechanism for the cells.

7. SUMMARY

It is well known that inflammation plays a key role in the pathogenesis of CAVD, and the NF-κB transcription factor is a central regulator of inflammation. The expression of proinflammatory cytokines such as IL-1β and TNF-α is significantly elevated in the calcifying aortic valves of patients with CAVD and in $ApoE^{-/-}$ mice on a high-fat diet. Our research group previously demonstrated that hydrogen sulfide (H_2S) inhibits calcification in human aortic valves. This was confirmed by our experimental results, as dual gene silencing of endogenous H_2S-producing enzymes, CSE and CBS, enhanced mineralization and inflammation in VIC cells isolated from human calcified aortic valves. We investigated whether H_2S exerts its anti-calcification effects through the reduction of inflammation. Based on our results, silencing or pharmacological inhibition of the NF-κB transcription factor prevented the nuclear translocation of RUNX2, a transcription factor that regulates bone formation, thereby inhibiting calcification in VIC cells.

We observed that treatment with the H_2S-releasing donor molecule AP72 prevented the nuclear translocation of NF-κB and RUNX2 in the aortic valves of $ApoE^{-/-}$ mice and human VIC cells, resulting in a reduced cytokine response. For the first time, we demonstrated that the regulation of RUNX2 by hydrogen sulfide (CSE/CBS) occurs via NF-κB, establishing a link between inflammation and valvular mineralization.

We explored the metabolic regulation of H_2S levels in human aortic valves. Despite the higher expression of the CSE enzyme, we identified lower biologically available H_2S and higher levels of IL-1β and TNF-α in the aortic valves of CAVD patients compared to healthy donors. We were the first to analyze the role of enzymes involved in the mitochondrial oxidation of H_2S, including SQR, ETHE1, SO, and TST in valvular mineralization.

We demonstrated the anti-calcification and anti-inflammatory effects of AP39, a specific mitochondria-targeting H_2S-releasing donor molecule.

The clinical relevance of the dissertation lies in the fact that the H_2S-releasing donor molecules we studied have a long half-life, thus providing the active substance in a slow and controlled manner, ensuring physiologically tolerable H_2S concentrations. Therefore, in the future, they could become potential drug candidates for the treatment of cardiovascular diseases.

8. NEW RESULTS OF THE DISSERTATION

1. The H_2S produced by CSE/CBS inhibits inflammation and calcification of aortic valve-derived interstitial cells.
2. H_2S-releasing donor molecules reduce inflammation and calcification of aortic valve-derived interstitial cells.
3. H_2S prevents the development of inflammation and calcification in the aortic valve of apolipoprotein E-deficient mice.
4. H_2S inhibits the nuclear translocation of NF-κB and the expression of IL-1β and TNF-α.
5. RUNX2 activation and nuclear translocation in the milieu triggering calcification regulated by NF-κB.
6. Inflammation and mineralization processes are linked by the two main transcription factors, NF-κB and RUNX2.
7. Despite higher expression of the CSE enzyme, there is lower biologically accessible H_2S and higher IL-1β and TNF-α levels in the aortic valve tissue of CAVD patients compared to healthy individuals.
8. VICs isolated from healthy human aortic valves mimic human pathology under conditions that induce calcification, as elevated CSE expression is associated with low H_2S levels here as well.
9. Mitochondrial enzymes involved in H_2S catabolism, including SQR, ETHE1, SO, and TST, are more tightly regulated in calcified aortic valve tissues, leading to decreased endogenous H_2S levels.
10. Similar protein expression patterns were observed in VICs in response to high phosphate exposure in in vitro experiments.
11. A specific H_2S donor improving mitochondrial bioenergetics reduced inflammation and calcification in VICs.
12. Both proinflammatory cytokines (IL-1β and TNF-α) increased calcification in VICs derived from calcified aortic valves. In contrast, in cells isolated from healthy valves, they transiently inhibited calcification at the initiation stage, an effect mediated through CSE induction and endogenous H_2S generation.

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LIST OF PUBLICATIONS

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List of publications related to the dissertation

1. **Combi, Z.**, Potor, L., Nagy, P., Sikura, K. É., Ditrói, T., Jurányi, E. P., Galambos, K., Szerafin, T., Gergely, P., Whiteman, M., Torregrossa, R., Ding, Y., Beke, L., Hendrik, Z., Méhes, G., Balla, G., Balla, J.: Hydrogen sulfide as an anti-calcification stratagem in human aortic valve: altered biogenesis and mitochondrial metabolism of H2S lead to H2S deficiency in calcific aortic valve disease.
   Redox Biol. 60, 1-19, 2023.
   DOI: http://dx.doi.org/10.1016/j.redox.2023.102629
   IF: 11.4 (2022)
2. 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

List of other publications

3. 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 proatherogenic 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

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4. 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

5. Kristóf, E., Klusóczki, Á., Veress, R., Shaw, A., **Combi, Z.**, Varga, K., Győry, F., Balajthy, Z., Bai, P., Bacsó, Z., Fésüs, L.: Interleukin-6 released from differentiating human beige adipocytes improves browning.
Exp. Cell Res. 377 (1-2), 47-55, 2019.
DOI: http://dx.doi.org/10.1016/j.yexcr.2019.02.015
IF: 3.383

6. Oláh, V., **Combi, Z.**, Szőllősi, E., Kanalas, P., Mészáros, I.: Anthocyanins: possible antioxidants against Cr(VI) induced oxidative stress in Spirodela polyrrhiza.
Cereal Res. Commun. 37 (Suppl.), 533-536, 2009.
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