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

ATTENUATION OF PHOSPHATE MEDIATED ECTOPIC CALCIFICATION THROUGH THE NRF2/HO-1 AXIS

by

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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 from 1.00 pm.

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INTRODUCTION

Calcium is essential for various physiological processes, including muscular contraction, blood clotting, nerve impulse transmission, and hormone transduction. In mammals, 99% of calcium is found in bones and teeth, while 1% is present in soft tissues and blood. Physiological calcium levels range from 2.20 to 2.65 mmol/L. Calcification is a process involving the accumulation of calcium salts, in the form of hydroxyapatite (HA) crystals. There are various types of calcifications, including metastatic and dystrophic. Ectopic calcification can be present in different soft tissues including brain, kidney, skin, tendons, eye, and the cardiovascular system. Our research focuses on cardiovascular and eye calcification.

Cardiovascular calcification is a type of ectopic calcification that occurs in large elastic arteries, coronary arteries, and the heart valves. Vascular arteries consist of three layers: tunica adventitia, tunica media, and tunica intima. Intima calcification affects the intima, while media calcification affects the media. Intimal calcification is focal and occurs in the subendothelial space, while medial calcification affects the entire cross-section of blood vessels. Valvular calcification is a major type of cardiovascular calcification and the focus of research. Calcific aortic valve disease (CAVD) is the most common valvular heart disease in the aging population of the developed world, with a disease burden expected to rise from 2.5 million in 2000 to 4.5 million in 2030. CAVD affects 13% of the total population above the age of 65. There is no efficient therapy to prevent or cure CAVD, leaving surgical valve replacement as the only treatment option.

The mitral valve and aortic valve are two types of heart valves where calcification occurs. The aortic valve is the most prone to calcification and the most frequently replaced valve. Although minimally invasive catheter-based techniques are becoming more popular, open-heart surgery with aortic valve replacement remains the gold standard. Mechanical or biological heart valve prostheses exist, each with its own drawbacks and limitations. Implantation of heart valve prostheses has been described as "replacing one disease with another."

Valve interstitial cells (VICs) shares similarities with mesenchymal cells, as they can differentiate into various cell types depending on a stimulus. Osteogenic differentiation ofVICs is crucial for valve calcification. The phenotypic switch of VICs can alter valve properties. Calcification of the aortic valve is characterized by thickening, the formation of calcium-rich nodules, functional stiffening, and stenosis. CAVD is a multifactorial disease, with factors like high fat diet, smoking, gender, and age impacting its development.

Calcium deposits in the eye can disrupt eye function or be asymptomatic during routine exams. The lens, an elastic and transparent biconvex organ with epithelial cell origin, is made up of four parts: lens capsule, epithelial cells, lens fibers, and zonules. The lens epithelia, made up of cuboidal lens epithelial cells (LECs), exhibit phenotypic plasticity and can undergo epithelial-to-mesenchymal transition in response to injury or growth factors.

Cataract is a common eye disease characterized by greyish-whitish discoloration and opacity of the lens. Causes vary, but aging is the most common risk factor for cataract formation. Surgical intervention is currently the only available treatment. Progressive lens opacification starts around the age of 45 or 50 due to environmental insults on lens proteins and cells. Age-related modifications to these proteins accumulate over time, leading to the formation of insoluble protein aggregates found in cataractous lenses. Additionally, our group reported before that human LECs (HuLECs) can undergo osteochondrogenic trans-differentiation and calcification, possibly explaining the presence of HA in cataractous lenses.

Calcification, once thought to be a degenerative process, has been found to be an actively regulated process that shares similarities with physiological bone mineralization. Cellular plasticity refers to a cell's ability to change its identity or state, to give an instance, expressing osteochondrogenic markers in VICs in response to an osteogenic stimulus. Calcification can affect heart valves and can be triggered by an imbalance of inducers and inhibitors. Factors such as fetuin-A, matrix gla protein, pyrophosphate, magnesium,

iron/ferritin, and uremic toxins are calcification inhibitors, while circulating inducers include Pi, Ca, cytokines, lipids, high glucose, vitamin D, and uremic toxins.

Several calcification mechanisms have been identified, including inflammation, apoptosis, endoplasmic reticulum stress, reactive oxygen species (ROS), and matrix vesicles (MVs). Inflammation contributes to the progression of atherosclerosis and serves as a trigger for vascular calcification. ER stress can promote vascular calcification through apoptosis but can also induce osteogenic differentiation and autophagy of VSMCs, leading to the diseased condition. Excessive ROS production can cause oxidative stress, which is linked to various diseases. Vascular calcification is associated with increased ROS production, and excess ROS plays a pathophysiological role in the calcification process.

High intracellular Pi induces phenotype modification through osteogenic transcription factors like Runx2, Msh homebox2, Bone morphogenetic protein 2, and Osterix. Runx2 regulates osteoblast development, maturation, and bone formation. Deficiency in Runx2 leads to vascular calcification inhibition. Osteocalcin (OCN), a prominent non-collagenous protein in the bone ECM, is produced by osteoblasts and found in blood after bone resorption. OCN is expressed by differentiated osteoblast-like VSMCs and is used to evaluate ECM calcification extent.

Nrf2, a transcription factor in the human cap'n'collar (CNC) basic-region leucine zipper transcription factor family, is a key regulator of the antioxidant response. It is highly conserved and has a half-life of 20 minutes. In stress, Nrf2 is rapidly degraded by proteasomes, causing low protein levels in cells. It forms complexes with Kelch-like epichlorohydrin-related proteins, which are anchored in the cytoplasm by actin. Stress causes Keap1's cysteine residue to be modified, leading to phosphorylation and nuclear translocation of Nrf2. Nrf2 binds to ARE in the nucleus and initiates transcription in target genes. It is involved in various pathophysiological functions, including autophagy and inflammation inhibition through NFkB. Nrf2's protective mechanism has been shown in various diseases, including cancer, COPD, neurodegenerative diseases, alcohol-induced liver diseases, and acetaminophen hepatotoxicity.

Heme, an iron-containing molecule, is crucial for oxygen transport and aerobic life. It produces pro- and antioxidant compounds, influences cellular oxidant sensitivity, activates the complement system, modulates host defense, induces immune memory, and is required for detoxification and signal transduction. Heme degradation plays a role in cellular homeostasis, with heme oxygenase enzymes catalyzing the first step of heme catabolism. Heme degradation produces biliverdin, ferrous iron, and carbon monoxide possess different protective actions.

Ferritin is an iron storage protein with antioxidant properties. Carbon monoxide is a gasotransmitter, vasomodulator and anti-inflammatory molecule. Bilirubin, that forms in the immediate conversion of biliverdin by biliverdin-reductase is a lipophilic byproduct, which protects lipids from oxidation. There are three HO isoforms (HO-1, HO-2, and HO- 3), with HO-1 is being highly inducible by various stimuli. HO-1 is a stress-induced isoform that is rapidly activated following oxidative stress, acting as a potent endogenousfactor in resolving stress-induced inflammatory injury.

Nrf2, the master regulator of antioxidant pathways. Oxidative stress plays a critical role in the progression of vascular calcification. In human vascular smooth muscle cells (VSMCs), activation of the Nrf2-ARE signaling pathway alleviates vascular calcification caused by hyperphosphatemia. Nrf2 can inhibit Runx2 binding to downstream gene promoters. Labile heme has the potential to trigger Nrf2 signaling, which can regulate its downstream transcription of HO-1. Increasing Nrf2/HO-1 signaling may be a common beneficial strategy for preventing P and Ca-induced osteogenic differentiation and ECM calcification in distinct cell types such as VICs and HuLECs.

AIMS

We aimed to investigate the effect of high phosphate levels on ectopic calcification and see if an antioxidant pathway like the Nrf2/HO-1 axis could reverse the process. We chose the cardiovascular system and the eye as our research interests, focusing on the aortic valve and the lens, respectively.

1. To explore the role of the Nrf2/HO-1 axis in high phosphate-induced calcification of valve interstitial cells and lens epithelial cells.

2. To investigate the impact of Nrf2 and HO-1 inhibition on high-phosphate-induced calcification of valve interstitial cells and lens epithelial cells.

3. To look into the impact of heme breakdown products (iron, bilirubin, and CO) on the calcification of valve interstitial cells and lens epithelial cells in response to high phosphate levels.

MATERIALS AND METHODS

Materials Unless otherwise specified, all reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cell culture Innoprot provided the human VICs (Derio, Spain). Dulbecco's modified Eagle's medium (DMEM) was used as cell culture medium, and it was supplemented with 10% FBS, antibiotic antimycotic solution, sodium pyruvate, and L-glutamine. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. From passages 5 to 8, cells were grown to confluence and used. ATCC supplied immortalized human lens epithelial cells (HuLECs) (Manassas, VA, USA). The cells were grown in DMEM with 10% FBS, penicillin, and streptomycin. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. From passages 3 to 4, cells were grown to confluence and used.

Induction of osteogenesis At confluence, VICs and HuLECs were switched to the osteogenic medium, which was made by combining inorganic phosphate (P) in the form of NaH_2PO_4 and Na_2HPO_4, pH 7.4 (0-2.5 mmol/L), and calcium ($CaCl_2$) (0.3-1.2 mmol/L) to the growth medium. Every three days, both the growth and osteogenic media were changed. We used an osteogenic medium supplemented with 2.5 mmol/L P and 0.3 mmol/L Ca unless otherwise specified.

Cell treatments Ammonium ferric citrate was used to introduce iron, which was dissolved in deionized water. Heme was dissolved in NaOH (20 mmol/L). Tin protoporphyrin IX and zinc protoporphyrin IX were dissolved in DMSO. In all experiments, the final NaOH

concentration was kept below 2 mmol/L, and the DMSO concentration was less than 1%. We used the tricarbonyl-dichloro-ruthenium (II) dimer, also known as CO-releasing molecule 2 (CORM2), $[Ru_2Cl_4(CO)_6]$, to deliver CO. CORM2 was dissolved in DMSO immediately before use and given every 12 hours. DMSO was used to dissolve the Nrf2 inhibitor ML385. Ferritin (FT) was given as holoferritin.

Alizarin red (AR) staining and quantification Following a thorough rinse with deionized water, the cells were fixed in 4% paraformaldehyde and washed with Dulbecco's PBS. For 20 minutes at room temperature, cells were stained with Alizarin Red S solution (2%, pH 4.2). Excess dye was washed away with deionized water several times. To quantify AR staining in 96-well plates, we added 100 µL of hexadecyl-pyridinium chloride solution (100 mmol/L) to the wells and measured the optical density (OD) at 560 nm using hexadecyl-pyridinium chloride solution as a blank.

Quantification of Ca deposition Cells grown in 96-well plates were washed twice with DPBS and decalcified for 30 minutes at room temperature with HCl (0.6 mol/L). The QuantiChrome Calcium Assay Kit was used to determine the Ca content of the HCl supernatants. Following decalcification, cells were washed twice with DPBS, solubilized with a solution of NaOH (0.1 mol/L) and sodium dodecyl sulfate (0.1%), and protein content was determined using the BCA protein assay kit. Cell Ca content was normalized to protein content and expressed as mg/mg protein.

Quantification of OCN ECM of cells, grown in 6-well plates was dissolved in 100 µL of EDTA for OCN detection. An enzyme-linked immunosorbent assay was used to determine the OCN content of the EDTA-solubilized ECM samples, according to the manufacturer's protocol.

Determination of cell viability MTT assay was used to determine cell viability. Following the treatments, the cells in the 96-well plates were washed with PBS before being treated with 100 µL of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). The MTT solution was removed after 4 hours of incubation in the cell culture incubator, the formazan crystals were dissolved in 100 µL of DMSO, and the optical density at 570 nm was measured.

Quantitative RT-PCR TRIzol was used to isolate RNA from cells according to the manufacturer's protocol. Using the High-Capacity cDNA Reverse Transcription Kit, two micrograms of RNA were reverse-transcribed to cDNA. The Real-Time PCR System was used for the PCRs. The ΔΔCt method was used to calculate relative mRNA expressions, with HPRT serving as an internal control.

Intracellular ROS measurement The 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein di-acetate, acetyl ester assay was used to monitor ROS production. Following a 4-hour pre-treatment, cells were washed with DPBS and loaded with CM-H2DCFDA (10 µmol/L, 30 minutes in the dark). Cells were thoroughly washed with DPBS before measuring fluorescence intensity every 30 minutes for 3 hours using 488 nm excitation and 533 nm emission wavelengths. We used the ROS inhibitor N-acetyl cysteine (NAC, 3 mmol/L) in some experiments.

Western blot Cells were lysed in Laemmli lysis buffer to assess the expression of ALP, Nrf2, HO-1, NQO1, ferritin H-chain (FtH), and ferritin L-chain (FtL) and RUNX2 proteins. Whole-cell lysates were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. We detected antigen-antibody complexes with Clarity Western ECL Substrate. Chemiluminescent signals were detected using a C-Digit Blot Scanner or conventionally on an X-ray film. After detection, the membranes were stripped and reprobed for ß-actin. Blots were quantified using the C-Digit Blot Scanner's built-in software.

Statistics The results are presented as mean ± SD. For all studies, at least three independent experiments were carried out. GraphPad Prism software was used for statistical analysis. The Shapiro-Wilk test was used to determine the distribution's normality. Because all the data passed the normality and equal variance tests, parametric tests were used to calculate p values. A two-tailed Student's t-test was used to determine whether there were statistically significant differences between two groups. One-way ANOVA was used to compare more than two groups, followed by Tukey's multiple comparisons test. A $p \leq 0.05$ value was considered significant.

RESULTS

1. Heme-mediated activation of the Nrf2/HO-1 axis attenuates calcification of valve interstitial cells

In an in vitro model of aortic valve calcification, human VICs were grown in calcification medium with different amounts of Pi (0-2.5 mmol/L) and Ca (0-1.2 mmol/L). AR staining was performed to assess ECM calcification after 7 days of treatment. Pi and Ca promoted ECM calcification in VICs in a synergistic and dose-dependent manner. The combination of 2.5 mmol/L Pi and 0.3 mmol/L Ca resulted in a 5-fold increase in ECM Ca content, while 2.0 mmol/L Pi and 0.6 mmol/L Ca in medium resulted in an 8.5-fold increase. The addition of 0.6 mmol/L Ca and 2.5 mmol/L Pi medium led to the greatest calcification of VIC cells. The study examined the effect of Pi and Ca-driven osteogenic stimulation on cell survival, as calcification of VICs has been linked to apoptotic cell death. To induce VIC calcification without substantial cell death, Pi and Ca concentrations were adjusted to 2.5 mmol/L and 0.3 mmol/L, respectively. The study also examined whether OM increased Runx2 expression, which was found to be fourfold higher in OM.

The study investigated the effect of heme on VIC calcification in vitro. AR staining showed that heme strongly inhibited VIC calcification in a dose-dependent manner, with its maximum effect at 10 μmol/L. Heme also significantly inhibited Ca accumulation in ECM, with increasing concentrations producing even more significant effects. Osteogenic stimulation caused VICs to transdifferentiate into osteoblast-like cells, which were tracked by osteoblast-specific proteins like ALP and OCN. The study found that heme at concentrations of 5, 10, and 25 μmol/L mitigated the increase in ALP expression. The OCN level in OM-treated VICs was approximately 10-fold higher than in controls, but was significantly reduced in the presence of heme at concentrations of 10 and 25 μmol/L.

The Nrf2 transcription factor, which regulates antioxidant gene expression, is linked to vascular disease. Upregulating the Nrf2 system reduces high Pi-induced calcification of vascular smooth muscle cells (VSMCs). Heme, a known inducer of Nrf2 expression, was found to induce Nrf2 expression in vitro in VICs. Heme-mediated Nrf2 upregulation led

to a 4-fold increase in Nrf2 mRNA, which controls HO-1, a protein with antioxidant and anti-inflammatory properties. Heme-treated VICs showed a 2.5-fold increase in Nrf2 expression compared to the control, and a significant and dose-dependent increase in HO-1 expression.

The study investigated the role of the Nrf2/HO-1 system in heme-mediated inhibition of VIC calcification. We inhibited Nrf2 transcriptional activity with ML385. The presence of ML385 also led to heme losing its ability to inhibit OM-induced calcification. The study also examined the role of HO-1 in heme's anti-calcification effect, finding that heme completely inhibited OM-induced ECM calcification in the absence of HO-1 inhibitors. The Ca content of ECM was found to be significantly lower in heme-treated VIC cells, which was reversed in the presence of HO-1 inhibitors.

Inhibiting HO-1 activity reduces heme's anti-calcification effect, suggesting that heme degradation products may be responsible for its inhibitory effects. We investigated the effect of bilirubin, iron, and CO on VIC calcification. All the heme degradation products inhibited completely or partially the calcification of VICs. Heme and bilirubin inhibited OM-induced increases in SOX9 and OPN mRNA expression, while Fe and CO had no effect.

Heme degradation by HO-1 releases iron, which upregulates ferritin (FT) expression. The study investigated the role of FT in vascular calcification. At a concentration of 5 μmol/L, heme increased FTH and FTL expression. Holo-FT completely inhibited OM-induced VIC calcification at 100 μg/mL.

2. Activation of Nrf2/HO-1 antioxidant pathway by heme attenuates calcification of human lens epithelial cells

The study investigated whether activation of the Nrf2/HO-1 system inhibits calcification in HuLECs. We used heme at a concentration of 1 to 50 μmol/L to activate the Nrf2/HO-1 antioxidant pathway. We found that heme upregulated Nrf2 expression in HuLECs in a dose-dependent manner, with modest upregulation of NQO1 and significant increase of

HO-1. The study also found that heme-induced Nrf2 upregulation in HuLECs was dependent on ROS production.

Prevention of osteochondrogenic differentiation and calcification of vascular smooth muscle cells by heme has already been reported. In a dose-dependent manner, heme reduced OM-induced HuLEC calcification in ECM. Heme also reduced the expression of key indicators of osteogenic differentiation, such as Runx2, ALP and OCN expression. Heme completely abolished the robust increase in OCN expression induced by osteogenic stimuli in HuLECs.

The study investigated the role of Nrf2/HO-1 pathway activation in heme-mediated inhibition of HuLEC calcification. The results showed that heme protects HuLECs from calcification and its anti-calcification effect relies on Nrf2 upregulation. The study also investigated whether HO-1 is responsible for the anti-calcification effect of heme in HuLECs. Heme (10 μmol/L) completely prevented OM-induced calcification in the absence of HO-1 inhibitors, but in the presence of HO-1 inhibitors, heme-mediated calcification inhibition was lost. These findings suggest that the anti-calcification properties of heme require an intact and active Nrf2/HO-1 system.

Among the heme degradation products, iron was found to have a potent, while bilirubin had a minimal anti-calcification potential in HuLECs.

DISCUSSION

A study using an osteogenic medium with high levels of P and Ca revealed that it can cause calcification in valve interstitial cells (VICs). Elevated levels of P and Ca caused ECM calcification in VICs, which was consistent with earlier reports. Heme, a common iron compound was used to activate the Nrf2/HO-1 pathway, which is a potent antioxidant and anti-inflammatory pathway. Heme can catalyze the Haber-Weiss reaction, which produces reactive hydroxyl radicals and is a major cause of oxidant-mediated cell death in endothelial cells (ECs). Heme also stimulates the nuclear factor kappa B (NF-κB) signaling pathway and toll-like receptor 4 in ECs, resulting in endothelial activation and

dysfunction marked by the production of vascular adhesion molecules, pro-inflammatory cytokines, and ROS.

We found that heme is a powerful inhibitor of VIC calcification, inhibiting osteoblast markers ALP and OCN expression and ECM calcification in OM-stimulated VICs in a dose-dependent manner. This finding is in agreement with Zarjou *et al*.'s previous study on heme's effect on P-induced calcification of VSMCs in vitro. The study also found that the heme-degrading inducible enzyme HO-1, which is regulated by Nrf2, is significantly upregulated in VICs exposed to heme. The inhibitory effect of heme required HO-1 enzyme activity due to the protective nature of heme degradation products. The most potent inhibitor of VIC calcification among them was bilirubin, which also had a similar inhibitory effect on the OM-induced upregulation of SOX9 and OPN. Iron and FTH were previously identified as the primary regulators of this process by Zarjou *et al*., who also demonstrated that heme inhibits VSMC calcification. Since FT mimics the inhibitory effects of heme and iron, it was found to be a potent anti-calcification agent in VICs. Further research is needed to determine whether VICs take up extracellular FT and whether the iron dissociated from it is responsible for the inhibitory effect of FT. The cellular mechanism underlying vascular and valve calcification is the phenotype switch of VSMCs and VICs into osteoblast-like cells, respectively. The differentiation of mesenchymal stem cells (MSCs) into osteoblasts and this process are strikingly similar. There is mounting evidence that the Nrf2/HO-1 axis regulates osteogenesis. Yoon *et al*. discovered that blocking the nuclear translocation of Nrf2 reduced the ability of MSCs to differentiate into osteogenic cells and that proper Nrf2 signaling is necessary for maintaining the stemness and self-renewal capacity of MSCs. High iron has been shown to prevent MSCs from differentiating into osteoblasts by inducing the expression of FT, while Zarjou *et al.* demonstrated that high iron reduces osteoblast activity and ECM calcification by upregulating the expression of FTH and ferroxidase activity. High iron also affects the formation of hydroxyapatite accumulation in soft tissues outside the cardiovascular system. This phenomenon was supported by our group's demonstration that HuLECs can differentiate into osteoblast-like cells and calcify the ECM in response to osteogenic stimulation. Previous research has found high levels of calcium and OCN,

the main non-collagenous bone protein in human cataractous lenses, which supports our theory that HuLECs may calcify *in vivo*, and that this active mechanism may be involved in the formation of hydroxyapatite in cataractous lenses. Age-related eye diseases, such as cataract development, are greatly influenced by oxidative stress. Gao *et al*. investigated the expression of Nrf2 and Keap1 genes and proteins in human lenses, finding that Nrf2 expression was significantly lower and Keap1 expression was higher in lenses made from elderly people (65-80 years old). The study used heme to activate the Nrf2/HO-1 pathway, which is essential for controlling the Keap1/Nrf2 system. Overproduction of ROS changes Keap1's conformation directly, enabling Nrf2 stabilization and nuclear translocation. The results showed that heme significantly increased ROS production while only slightly increasing NQO1 expression, leading to the inferred that heme-mediated inhibition of calcification in HuLECs was NQO1 independent. The Lens Opacities Case-Control Study assessed risk factors for age-related nuclear, cortical, posterior subcapsular, and mixed cataracts and discovered that dietary iron intake reduced the risk of cortical, nuclear, and mixed cataracts. Numerous studies have discussed how the Nrf2 antioxidant system contributes to the prevention or reduction of age-related diseases like atherosclerosis, vascular calcification, cataract development, and macular degeneration. Clinical trials are focusing on the Nrf2 system in various clinical conditions, such as diabetes, cancer, chronic kidney disease, and aging issues.

Additional research is needed to understand the calcification phenomenon in lens epithelium (LECs) and the metabolism of pyrophosphate (PPi). Extracellular PPi is an effective endogenous inhibitor of soft tissue calcification, and low PPi levels and calcification are typically linked to high ALP expression. Elevated ALP levels in calcifying HuLECs require further investigation to measure PPi levels and its contribution to LEC calcification. Additionally, developing *in vivo* animal models and ex vivo organ culture is crucial for the development of this field and potential treatments for lens calcification.

SUMMARY

We discovered that heme-mediated activation of the Nrf2/HO-1 axis can protect valve interstitial cells and lens epithelial cells against high phosphate-induced calcification. Inhibiting Nrf2 and HO-1 activity also affected heme's protective action against high phosphate-induced calcification of valve interstitial cells and lens epithelial cells. Although iron is the most important of the heme breakdown products in lens calcification, the effects of bilirubin, CO, and iron on valvular interstitial cells are more varied. Heme-generated reactive oxygen species (ROS) are required to activate the Nrf2/HO-1 axis in high phosphate-induced calcification of lens epithelial cells.

This study, I believe, will help us better understand the link between CKD, cataracts, and CAVD. Although we demonstrate a protective effect of heme against VIC and HuLEC calcification in this study, before administering heme to patients to prevent calcification (as a form of Heme arginate, a drug used to treat acute porphyrias), we should consider heme's well-known prooxidant and pro-inflammatory properties. We demonstrated that heme's positive effect is dependent on the activation of the Nrf2 antioxidant system. As a result, using other dietary Nrf2 inducers like curcumin, resveratrol, or sulphoraphane may be a safer option.

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List of publications related to the dissertation

1. **Chowdhury, A.**, Balogh, E., Ababneh, H., Tóth, A., Jeney, V.: Activation of Nrf2/HO-1 Antioxidant Pathway by Heme Attenuates Calcification of Human Lens Epithelial Cells.
   Pharmaceuticals (Basel). 15 (5), 1-13, 2022.
   DOI: http://dx.doi.org/10.3390/ph15050493
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2. Balogh, E., **Chowdhury, A.**, Ababneh, H., Csiki, D. M., Tóth, A., Jeney, V.: Heme-Mediated Activation of the Nrf2/HO-1 Axis Attenuates Calcification of Valve Interstitial Cells.
   Biomedicines. 9 (4), 1-17, 2021.
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