THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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

By

ARPAN CHOWDHURY

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DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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Supervisor: **Viktória Jeney PhD, DSc**

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1. Introduction

1.1. Ectopic calcification

Numerous physiological processes, including muscular contraction, blood clotting, nerve impulse transmission, and hormone transduction, depend on calcium [1]. In mammals about 99 percent of the body's calcium is in the bones and teeth [2]. The remaining 1% is present intracellularly within soft tissues and in interstitial fluids and blood. Physiological calcium levels in the blood are between 2.20 and 2.65 mmol/L. One half of the blood calcium ions are in a free form, and the other half is mostly bound to albumin and to a lesser extent is bound to citrate, bicarbonate, and phosphate. With decreasing pH (acidosis), calcium affinity for albumin and other proteins decreases. Hyperventilation-induced respiratory alkalosis (lower bicarbonate concentration in the blood) can rapidly lower the concentration of free ionized calcium in the blood, causing cramping and other neuromuscular symptoms [1].

Calcification is a process in which accumulation of calcium salts, mostly composed by calcium and inorganic phosphate (Pi) occurs [3]. In general, these calcium salts deposit as a crystal known as hydroxyapatite (HA) via an unstable intermediate known as amorphous calcium phosphate. Carbonate and other anions such as HPO_4^{2-}, Cl^-, and F^-, as well as cations such as Mg^{2+}, Na^+, and Fe^{2+}, all contribute to the formation of biological apatite, along with pure hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ [4], [5].

There are several types of calcifications. Physiological calcification occurs in the bones and teeth. Pathologically, the two most common types are the metastatic and the dystrophic calcification. Metastatic calcification is when calcium deposits in a normal tissue due to hypercalcemia, whereas dystrophic calcification occurs in damaged or injured tissue independently of calcium level [6]–[10]. Pathophysiological calcification or ectopic calcification in soft tissues leads to several disease

conditions. Ectopic calcification can occur in almost any soft tissue, including the brain, kidney, skin, tendons, eye, and most importantly, the cardiovascular system [11]–[13].

Under normal conditions various inhibitors, including inorganic pyrophosphate (PPi, a dimer of Pi), actively prevent the precipitation of HA in "soft" tissues. Tissue non-specific alkaline phosphatase (TNAP) regulates PPi levels primarily by cleaving PPi into two molecules of Pi. In this reaction, PPi level decreases whereas Pi level increases, therefore TNAP performs dual functions as an important regulator of physiologic mineralization in "hard" tissues [14].

Extracellular adenosine triphosphate (ATP) is the primary source of PPi and is mainly secreted by hepatocytes via the ATP-binding cassette C subfamily 6 (ABCC6) transporter. There are numerous ABCC6 mutations that have been described as pathogenic, for example pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI) [15]–[17].

Aside from genetic factors, there are several acquired factors that can lead to ectopic calcification. Among them, our study focused on cardiovascular and eye calcification.

1.2. Cardiovascular calcification

Cardiovascular calcification is a type of ectopic calcification that occurs in large elastic arteries, coronary arteries, and heart valves (**Figure 1**). Vascular arteries are composed of three layers: the outermost layer known as tunica adventitia, the middle layer known as tunica media and the innermost layer known as tunica intima. Vascular calcification manifests in two ways depending on where the HA deposition occurs (**Figure 1**). In intima calcification, HA deposition takes place in the intima, whereas media calcification affects the media. These two types of calcification patterns may appear together in some cases. Intimal calcification is focal, and it occurs in the subendothelial space in association with atherosclerotic plaques. The appearance of intimal calcification varies, from microcalcification to large calcification, and affects the plaque's stability in various ways. The media layer of blood vessels is affected by media calcification, which is diffuse and mostly present in the entire cross-section of the blood vessel [18]–[21].

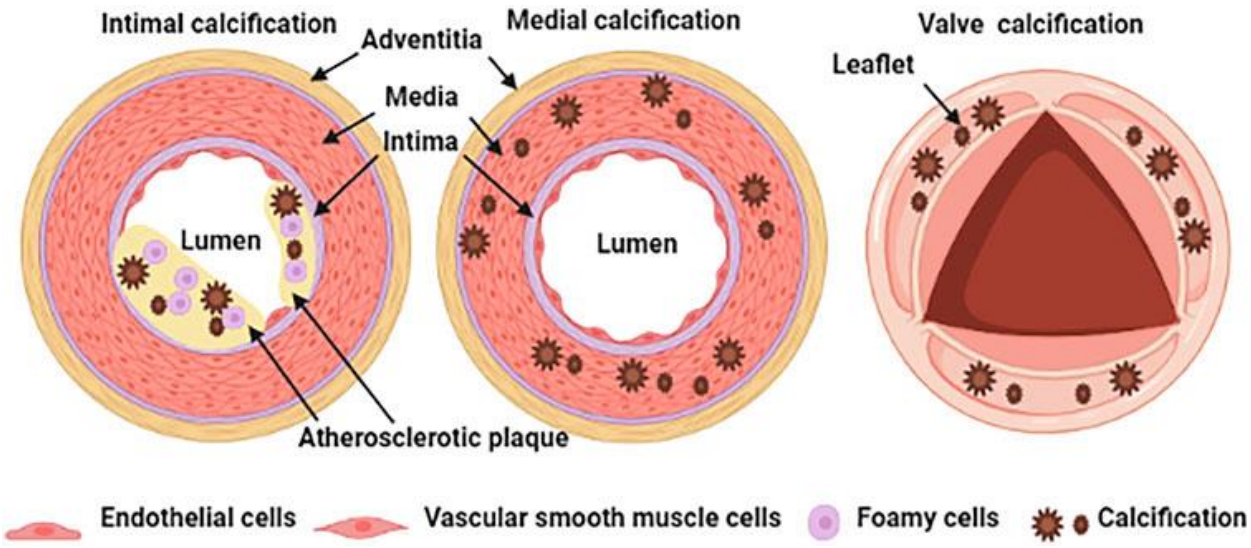

Figure 1. **Types of cardiovascular calcification**. Medial, intimal, and valve calcification are the most common types of cardiovascular calcification. This diagram depicts the disease states of each of these diseases. Medial calcification occurs in the aortic tunica media, around the smooth muscle cells. Intimal calcification develops within an atherosclerotic plaque in the intima. Valve calcification takes place primarily on the leaflet of heart valves. The figure is from reference [21] .

Aside from these two types, valve calcification is a major type of cardiovascular calcification and the focus of our research. Calcific aortic valve disease (CAVD) is the most common valvular heart disease in the aging population of the developed world, with the disease burden expected to rise from 2.5 million in 2000 to 4.5 million in 2030 [22]. 15,000 patients in the United States are facing death for CAVD per year, which forces declaring this disease as a public health issue. It is reported that CAVD affects 13% of the total population above the age of 65 [23]. There is no efficient therapy to prevent or cure CAVD, leaving surgical valve replacement as the only treatment option.

The mitral valve and the 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. While 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, with each having its own set of drawbacks and limitations. As a result, the

implantation of heart valve prostheses has been described as "replacing one disease with another" [24].

An adult normal aortic valve consists of three crescent shaped cusps along with three commissures and three sinuses supported by fibrous annulus. The primitive heart consists of endocardial cells, an overlying layer on the cardiac myosite separated by a hyaluronan-rich matrix namely cardiac jelly. Some of these endocardial cells go through endocardial-to-mesenchymal transformation and swell up in a later stage known as endocardial cushions. These mesenchymal cells work as precursors of the mature valve structures. Primitive heart valves continue to grow and differentiate into two distinct cell lines, valvular interstitial cells (VICs) and valvular endothelial cells (VECs). An aortic valve is made of three layers; the aortic side of the valve known as fibrosa, and the ventricular part known as ventricularis. The layer in between of fibrosa and ventricularis is known as spongiosa that consists of collagen, elastin, and proteoglycans [25], [26] (**Figure 2**). On each side of the cusp, endothelial cells form a monolayer. The body of the cusp is filled mainly with valve interstitial cells (**Figure 2**). They have contractile and secretory properties and oversee the synthesis and repair of the extracellular matrix [27].

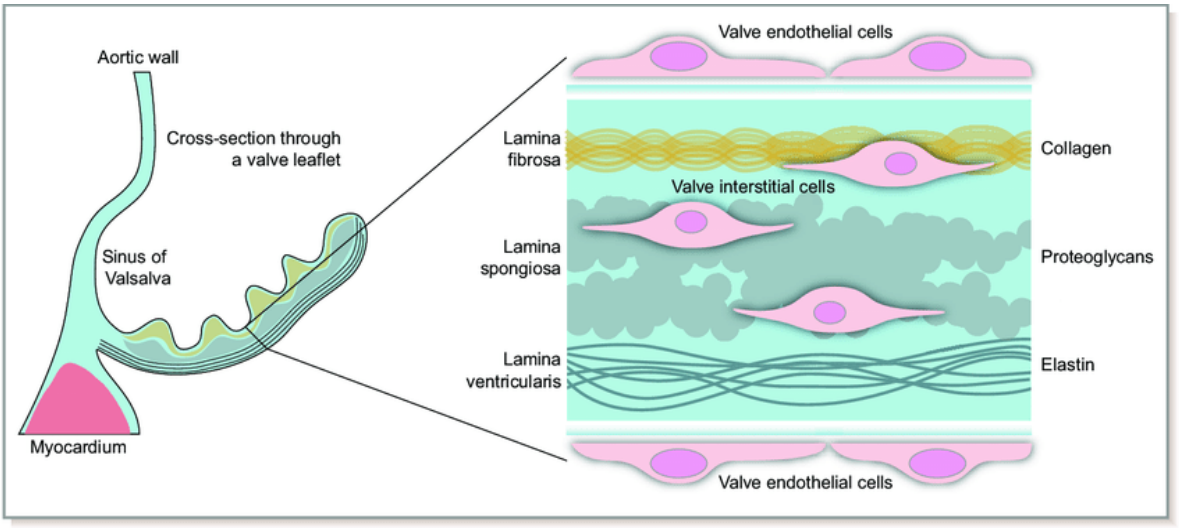

Figure 2. A simplified schematic structure of the human aortic valve leaflets. A schematic cross-section of the aortic valve's leaflet is shown on the left. The right-hand blowup shows the tri layered organization of the extracellular matrix, as well as the localization of aortic valve endothelial cells (referred to as VECs throughout) and interstitial cells (VICs). The figure is from reference [28].

In the absence of disease condition, VICs are present in a quiescent (non-activated) fibroblast-like cell type [29]. On the other hand, due to their mesenchymal origin, VICs have the ability to differentiate into different cell types, including osteoblasts, adipocytes, chondrocytes, and myofibroblasts, depending on the pathogenic stimuli. Osteogenic differentiation of VICs plays an important role in valve calcification. The progression of valve calcification includes different mechanisms such as inflammation, lipid deposition, oxidative and/or mechanical stress, fibrosis, and apoptosis. The phenotypic switch of VICs into osteoblast-like and myofibroblasts-like cells can change the physical and anatomical properties of the valve.

Calcification of the aortic valve is characterized by overall thickening of the valve cusp, the presence of calcium-rich nodules on the aortic valve surface and/or within the annulus region, functional stiffening, and stenosis [29]. Calcification is most noticeable in the center of each cusp [30]. One of the major differences between calcific and normal valves is that in normal conditions, commissures of the valves are fused, whereas in diseased conditions, this fusion of commissure is absent [31].

CAVD is a multifactorial disease, some authors describe it as a 'lifestyle disease'. High fat diet, smoking, gender, and age all have significant impacts on CAVD. Recent research indicates that CAVD is not a degenerative disease, but rather an active process caused by the interaction of molecular pathways, and it is possible to conclude that our daily lifestyle increases the risk factor for this disease [24], [28], [32]–[34].

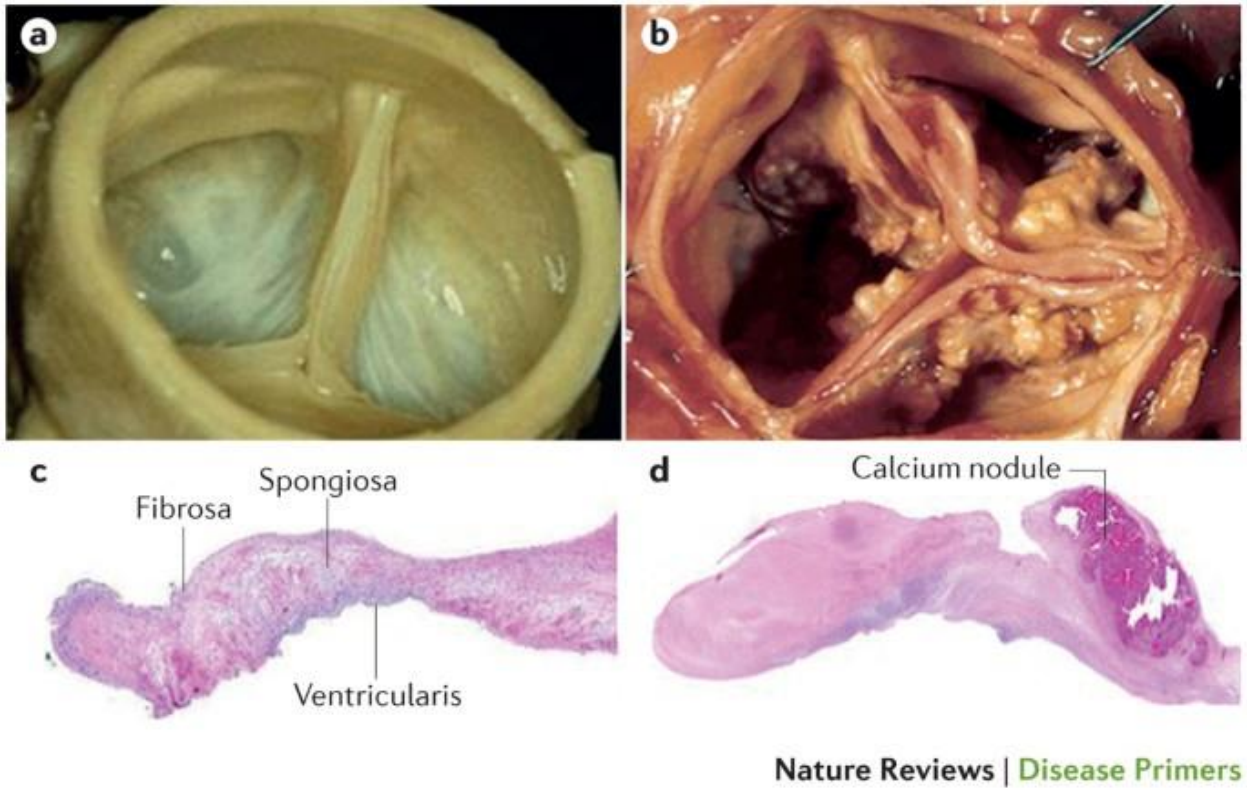

Figure 3. Macroscopic and histopathological appearance of normal and abnormal aortic valves. A normal aortic valve **(a)** and an aortic valve with severe calcific aortic stenosis (AS) are shown in photographs **(b)**. The histopathological section of a normal aortic valve with haematoxylin staining demonstrates the valve's trilaminar structure from top to bottom **(c)**. The histopathological section of a valve with severe calcific AS, stained with haematoxylin, reveals the presence of fibrotic material and a calcified nodule. The excess fibrotic material thickens the tissue, and the calcified nodule in the fibrosa contributes to the alteration of the normal architecture of the leaflet **(d).** The figure is from reference [35]**.**

1.3. Eye calcification

Calcium deposits can be caused by a variety of eye conditions. Depending on their location, they can impair eye function or simply be an asymptomatic finding during a routine eye exam. The eyelids, sclera, cornea, vitreous body, retina, and the lens are the reported locations for calcium deposits in the eye (**Figure 4**) [36]–[41].

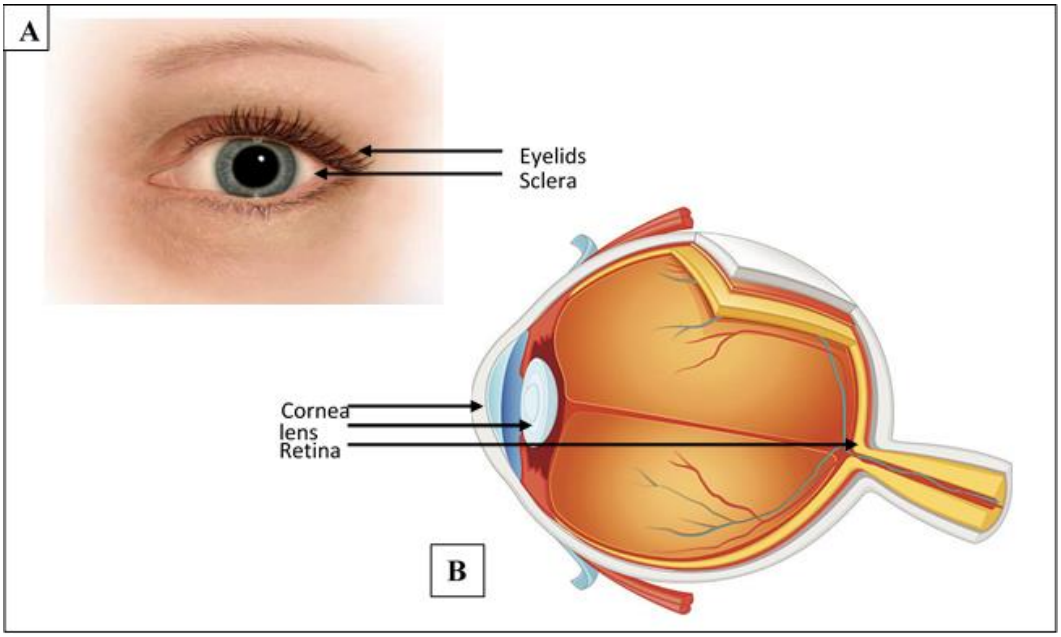

Figure 4. Reported locations of eye calcification. (A) In the outer structure of the eye, eyelids and sclera is susceptible for the calcification. **(B)** The inner structure of the eye. Arrows show cornea, lens and retina which are susceptible for calcification.

Our work focused on lens calcification. The lens is an elastic and transparent biconvex shape organ with epithelial cell origin. It is supported by the vitreous body and is in the posterior chamber (**Figure 5A, B**). The diameter of an adult lens is approximately 9.0-10.0 mm, with an annual growth rate of 0.023 mm and a thickness of approximately 4.0-5.0 mm. The lens is made up of four parts (**Figure 5C**): the lens capsule, epithelial cells, lens fibers, and zonules. The lens zonules, which are attached between the pars plana and the equatorial lens capsule, normally suspend the lens on the ciliary body. Since the epithelial cells in the equatorial part of the lens continue to differentiate and grow throughout life, the shape, weight, and volume of the lens change constantly [42].

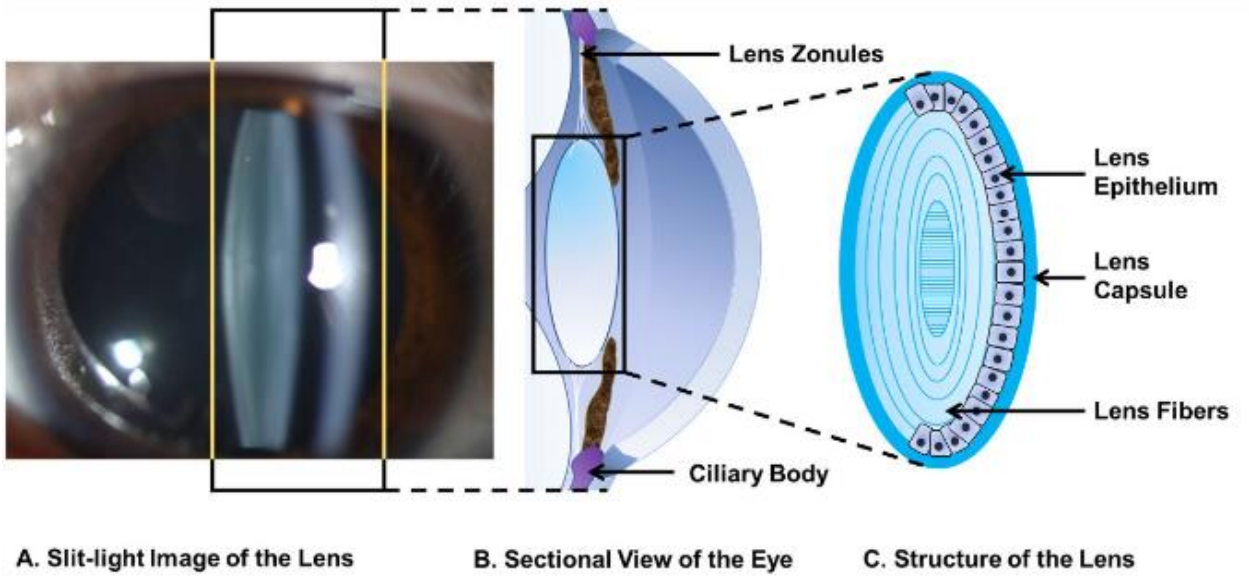

Figure 5. Microphotography with a slit lamp and a lens structure diagram. Slit-lamp microphotography of the eye following mydriasis (A). The lens's biconvex shape is clearly visible. (B) A sectional view of the square part depicted in Panel A. The lens's cellular structure has been shown in panel C. The figure is from reference [42].

The middle layer of the lens known as lens epithelia is made up by a monolayer of cuboidal lens epithelial cells (LECs), a parental cell type, mostly accountable for the development and growth of the transparent ocular lens [43], [44]. LECs exhibit phenotypic plasticity, allowing them to respond to environmental changes. For example, in responding to injury or growth factors (e.g., transforming growth factor beta), LECs can undergo epithelial-to-mesenchymal transition (EMT) [45]. LECs that undergo EMT have altered cell morphology, abnormal proliferation, and migration properties, as well as increased expression of ECM proteins, intermediate filaments and various integrins, in addition to the decreased expression of epithelial cell proteins [45].

Cataract is a commonly encountered disease that affects the lens. It is characterized by greyish-whitish discoloration and opacity of the lens. The causes of cataract vary. Besides a few genetic disorders, *e.g.,* Lowe syndrome and Nance Horan Syndrome, aging is the most common risk factor for cataract formation [46]. This is supported by the fact that approximately 25% of the population over the age of 65 and 50% of the population over the age of 80 are affected [47]. Surgical intervention is currently the only available treatment for cataract [48].

A great deal of effort was made into determining the cause of age-related cataracts, and several relevant mechanisms have been identified.

Progressive lens opacification starts around the age of 45 or 50 as cumulative damage to environmental insults on lens proteins and cells. Lens proteins are essential for maintaining the transparency of the lens. Because mature fiber cells have no protein synthesis or turnover, these proteins are extremely long-lived. As a result, age-related modifications to these proteins accumulate over time, leading to the formation of insoluble protein aggregates, which are found in cataractous lenses [49]–[52]

Besides the protein aggregates, HA is present in the senile cataractous lenses, as revealed by Fourier transform infrared (FTIR) and confocal Raman micro-spectroscopies [53]. A previous study from our research group showed that human LECs (HuLECs) are capable of undergoing through osteochondrogenic trans-differentiation and calcification [54], an active mechanism that might provide an explanation for the presence of HA in the cataractous lens.

1.4. Molecular mechanism of osteogenic trans-differentiation of cells

For a long time, calcification was thought to be a degenerative process in which hydroxyapatite precipitates in the vessel wall due to high levels of inorganic phosphate (Pi) and calcium (Ca) in the plasma. However, it has now been established beyond doubt that calcification is an actively regulated process that shares similarities with physiological bone mineralization [55]–[57].

Cellular plasticity refers to a cell's ability to change its identity or state. Many cells have been shown to change phenotype and begin to express osteochondrogenic markers in response to an osteogenic stimulus. This group includes vascular smooth muscle cells, smooth muscle, and endothelial cell progenitors, pericytes, adventitia cells, circulating mesenchymal and hematopoietic stem cells [58]–[60]. Calcification can also affect the heart valves, which are interstitial in nature. An imbalance of calcification inducers and inhibitors triggers calcification, and the cardiovascular system is prone to calcification through the osteochondrogenic differentiation of its cells [61]–[63]. Many factors, including fetuin-A, matrix gla protein, pyrophosphate, magnesium, and iron/ferritin, have been reported as calcification inhibitors. Pi, Ca, cytokines, lipids, high glucose, vitamin D, and uremic toxins, on the other hand, were identified as circulating inducers of calcification. Local inducers include oxidized LDL, cytokines, and hypoxia (**Figure 6**) [64] [65].

Several calcification mechanisms have been identified like inflammation, apoptosis, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS), matrix vesicles (MVs) release etc. Inflammation not only contributes to the progression of atherosclerosis but also serves as a trigger for vascular calcification. According to recent research, arterial inflammation occurs prior to the development of arterial calcification [66]. The regulation of MV production is poorly understood. MVs are extracellular, membrane-invested particles with a diameter of 100 nm that are exclusively found at sites of early calcification in cartilage, bone, and pre-dentin. The first apatitic bone mineral crystals grow within MVs towards the inner surfaces of their investing membranes [67]. Recent evidence shows that apoptosis regulates human vascular calcification *in vitro*, indicating that apoptotic bodies initiate vascular calcification [68]. ER stress can also promote vascular calcification (VC) through apoptosis, but it can also induce osteogenic differentiation and autophagy of VSMCs, leading to the diseased condition [69].

Additionally, cellular plasticity and subsequent calcification are reported to be regulated by ROS, [70]. Physiological levels of ROS are important in redox signaling, whereas excessive ROS production causes oxidative stress, which has been linked to the onset and progression of a variety of diseases [71], [72]. Accumulating evidence suggests that vascular calcification is associated with increased ROS production, and that excess ROS play a pathophysiological role in the calcification process [73]. Excess ROS activates more than 20 redox-sensitive transcription factors, including NF- E2 p45-related factor 2 (Nrf2). Nrf2, is a key redox-sensitive transcription factor, which maintains cellular redox balance and metabolism, as well as induces an adaptive response upon oxidative stress [74][75].

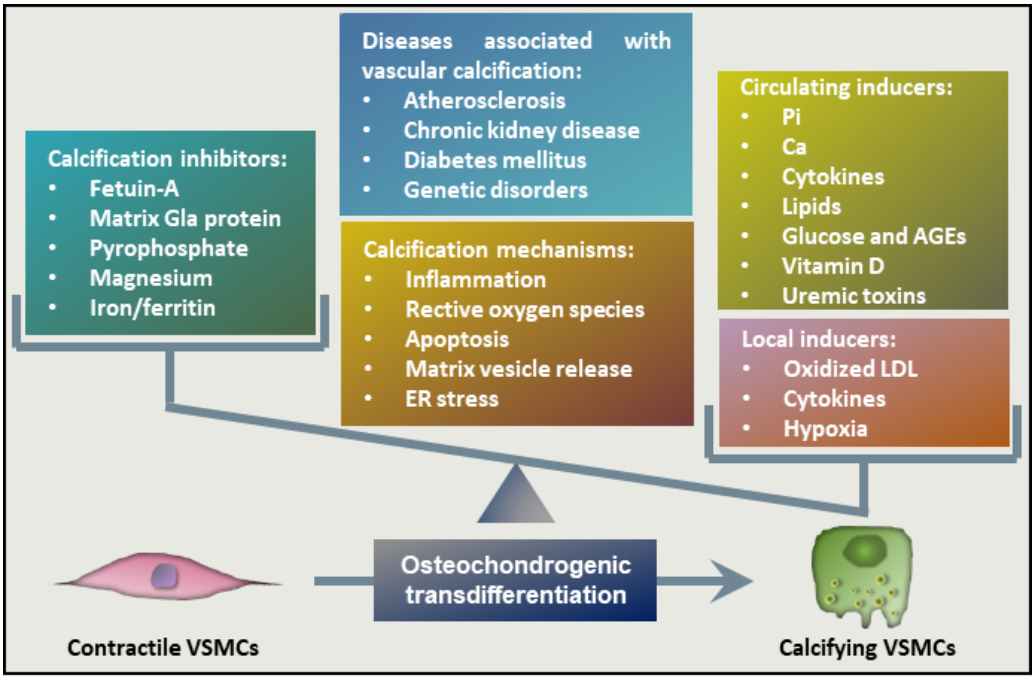

Figure 6. Vascular calcification mechanism. The pathology of vascular calcification is thought to be characterized by an imbalance between calcification inducers and inhibitors. If calcification inhibitors do not suppress the trans-differentiation process, circulating and local triggers cause VSMCs to switch from a contractile to an osteochondrogenic phenotype. Inflammation unchecked reactive oxygen species (ROS) production, apoptosis, matrix vesicle release, and ER stress are the main mechanisms underlying vascular calcification. Abbreviations: AGE stands for advanced glycation end-products. LDL stands for low-density lipoprotein. ER stands for endoplasmic reticulum. The figure is from reference [64].

1.5. The key role of phosphate in calcification

Elevated serum phosphate has emerged as a key risk factor for valve calcification in the general populations and in patients with chronic kidney disease over the last decade [76].

An increase in serum phosphate levels can occur due to an imbalance between phosphate absorption from the intestine, mobilization from bone, and urinary excretion, which is the primary determinant of final serum phosphate levels. Supersaturation of extracellular fluids (such as urine, serum, and synovial fluids) with phosphate and calcium triggers spontaneous calcium phosphate precipitation. Calcification inhibitors such as proteins (Matrix Gla Proteins, Fetuin A, and

osteopontin) and low molecular weight inhibitors such as pyrophosphate (PPi) prevent the passive formation and deposition of calcium phosphate crystals [77].

PPi, the calcification inhibitor is produced during extracellular adenosine-5′ -triphosphate (ATP) hydrolysis via ectonucleotide pyrophosphatase phosphodiesterase 1 (eNPP1) and degraded by tissue non-specific alkaline phosphatase (ALP). ALP and Enpp1 are secreted by the matrix vesicle (MV), which has been identified as an active player in ECM mineralization, particularly in cartilage, bone, and dentin. ALP appears to be a key regulator of the Pi/PPi ratio and promotes calcification by lowering PPi and increasing Pi levels. Pi is recovered from the extracellular space by Pit1 and Pit2, which are type III sodium/phosphate cotransporters (**Figure 7**) [78]. Phosphate transport inhibition prevents phosphate-induced calcification, implying the involvement of these sodium-dependent phosphate cotransporters in this process. Patients with CKD also had high ALP levels, which is a risk factor for cardiovascular disease [79]. ALP upregulation has also been observed in human valve interstitial cells after treatment with osteogenic media [80].

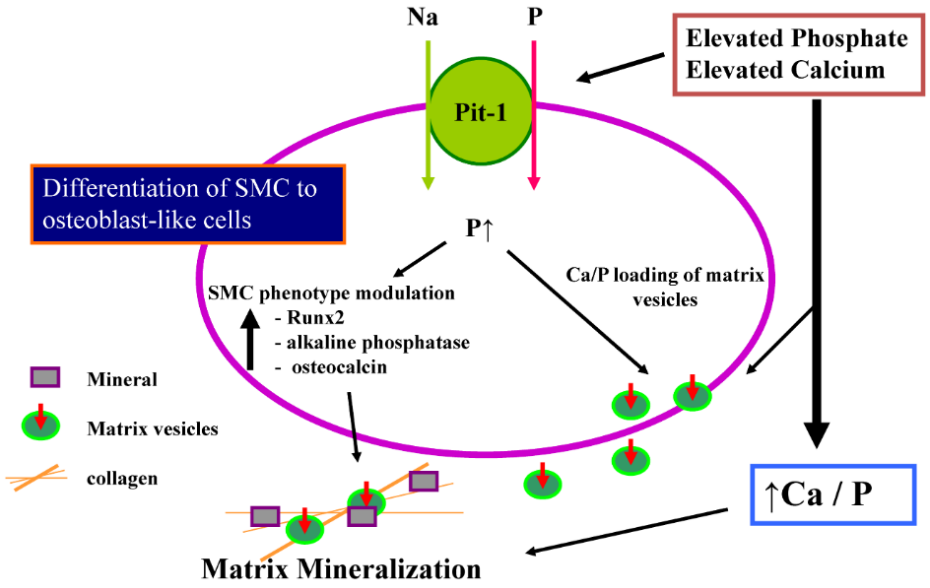

Figure 7. Model for the effects of increased Ca and P on the matrix mineralization of vascular smooth muscle cells. Increased Ca and P can promote vascular matrix mineralization in two different ways. Both Ca and P upregulate P uptake through Pit-1. An increase in intracellular P causes SMC phenotypic modulation, which includes the activation of the osteogenic genes Runx2, osteocalcin, and alkaline phosphatase, as well as the production of an extracellular matrix. Elevated Ca and/or P result in more Ca P ion products, which, through thermodynamic mechanisms, encourages the growth of apatite crystals in the matrix. The figure is from ref [78].

Besides matrix vesicle release, high intracellular Pi induces phenotype modification, which is orchestrated by osteogenic transcription factors, namely Runx2 (Runt-related transcription factor 2), Msh homebox2, Bone morphogenetic protein 2 and Osterix. Runx2 binds to specific DNA sequences to regulate osteoblast development from mesenchymal stem cells and osteoblast maturation into osteocytes and bone formation [81]. Articles revealed that the absence of Runx2 prohibits limb formation in mice. Other studies revealed that smooth muscle cell specific Runx2 deficiency leads to inhibition of vascular calcification. Based on these findings, Runx2 has now been established as the most upstream regulator of osteogenic differentiation [82]–[84].

Osteocalcin (OCN) is the most prominent non-collagenous protein found in the bone ECM. It is primarily produced by osteoblasts and can be found in the blood after bone resorption. OCN is classified into three types: carboxylated osteocalcin (cOCN), undercarboxylated osteocalcin (unOCN) and uncarboxylated osteocalcin (ucOCN). cOCN has a high affinity for calcium ions found in HA and is found primarily in the bone matrix [85]–[87]. OCN is also expressed by differentiated osteoblast-like VSMCs. Though reports on circulating OCN concentrations have been conflicting but embedded OCN in calcified vascular regions has been found to be positively correlated with the extent of vascular calcification in humans [88]. OCN is one of the major ECM proteins used to evaluate the extent of ECM calcification [89], [90].

1.6. The redox sensitive Nrf2 transcription factor

A member of the human cap'n'collar (CNC) basic-region leucine zipper transcription factor family described as a transcription factor Nrf2 (encoded by NFE2L2) in 1994. Nrf2 is a key regulator of the antioxidant response [91], [92]. Induction of Nrf2-regulated genes occurs through a specific binding motif known as antioxidant response element (ARE) in the promoter region of target genes [93], [94]. Protein homology scores of 83.4% and 82.5% in human Nrf2 and mouse Nrf2 respectively is a proof of highly evolutionary conserved protein as an antioxidant regulator [95].

Under basal condition, Nrf2 has a half-life of 20 minutes. In the absence of any stress condition, it is rapidly degraded by proteasomes, resulting in a low protein level of Nrf2 in many types of cells. Nrf2 forms complexes with a homodimer of Kelch-like epichlorohydrin-related proteins (Keap1). These complexes are anchored in the cytoplasm by actin and are inactive. Cullin 3 (CUL3) a ubiquitin ligase complex catalyzes the polyubiquitination of Nrf2 to induce its degradation [96]. Under stress, such as when exposed to ROS, or other pathological stimuli, the cysteine residue of

Keap1 is modified, resulting in phosphorylation, release, and nuclear translocation of Nrf2 [97]. In the nucleus, Nrf2 binds to ARE in the promoter region of Nrf2-target genes, and initiates transcription. Nrf2 target genes include cytoprotective genes and a series of antioxidant enzymes and phase II antioxidant enzymes, such as heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase (NQO1), superoxide dismutase (SOD), glutathione peroxidase (GSH) (**Figure 8)** [98]. Aside from Keap1, other protein kinases, such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and PI3K, can phosphorylate Nrf2 and can participate in Nrf2-regulated gene transcription [99], [100].

Nrf2 is involved in several pathophysiological functions. In autophagy, accumulation of p62 activates Nrf2 by suppressing keap1 by binding its KIR motif [101]. Inhibitory effect of Nrf2 in inflammation through NFkB is also reported, though the underlying regulations are still unclear [102], [103]. Different studies revealed the protective mechanism of Nrf2 in several diseased conditions like cancer, COPD, neurodegenerative diseases, alcohol-induced liver diseases, acetaminophen hepatotoxicity [104].

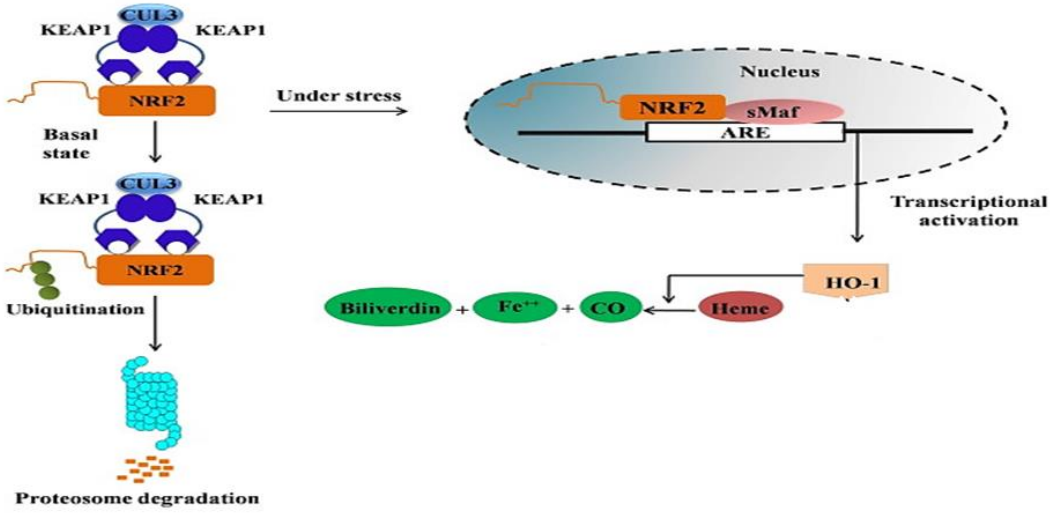

Figure 8. **The ROS-dependent Nrf2 pathway is depicted schematically.** Normally, Nrf2 goes through proteasomal degradation by the Keap1/CUL3 complex. When cells are under stress condition, Nrf2 dissociates from Keap1 and moves into the nucleus, where it binds to ARE and regulates the transcription of various cytoprotective genes and phase II antioxidant enzymes, such as HO-1. HO-1 degrades heme into biliverdin, iron and carbon monoxide. The figure is from reference [75].

1.7. Heme oxygenase-1 (HO-1)

Heme (iron protoporphyrin IX) is a ubiquitous iron-containing molecule which plays an essential role in oxygen transport; therefore, it is vital for aerobic life. The heme biosynthetic and catabolic pathways produce pro- and antioxidant compounds, which influence cellular oxidant sensitivity. From autoxidation and photochemical reactions, heme precursors (δ-aminolevulinic acid, porphyrins) generate reactive oxygen species (ROS). Heme is also a chemoattractant that activates the complement system, modulates host defense mechanisms via innate immune receptor activation and the heme oxygenase-1/ferritin system, and induces innate immune memory. Heme is also required for cellular detoxification, and signal transduction. The potential toxicity of heme and hemoproteins suggests that heme degradation plays an important role in cellular homeostasis [105]–[107].

This function is performed by heme oxygenase enzymes (HO-1-3) that catalyze the first and rate-limiting step of heme catabolism by breaking the porphyrin ring at the α-methene bridge [108]. In the presence of NADPH, cytochrome P450 and three molecules of molecular oxygen (O_2), per heme molecule catalyzes the oxidative cleavage of heme (Fe-protoporphyrin-IX) to yield equimolar amounts of biliverdin, ferrous iron (Fe^{2+}), and carbon monoxide (CO). The cytosolic enzyme biliverdin reductase then converts biliverdin to bilirubin **(Figure 9**) [106].

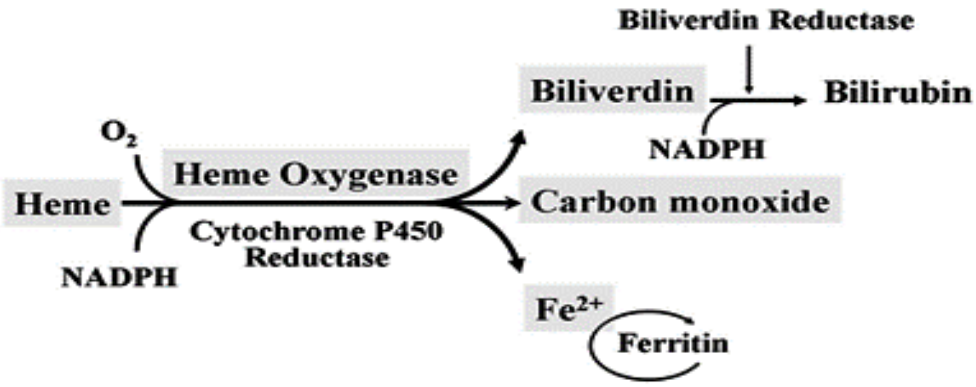

Figure 9. Heme degradation by heme oxygenase. Enzymatic heme degradation results in the formation of biliverdin as well as the release of carbon monoxide (CO) and Fe^{2+}. The biliverdin reductase enzyme converts biliverdin to bilirubin. The iron storage protein ferritin can bind Fe^{2+}. The figure is from reference [106].

The biological effects of HO-1 are largely attributable to its enzymatic activity, which can be conceived as a system with three arms of action, corresponding to its three enzymatic byproducts. Ferritin is yet another molecule that is strongly induced by heme and iron. This is an iron storage protein with antioxidant properties [109]. Ferritin is a large (450 kD) spherical shell that can safely store up to 4500 Fe atoms [110]. It is composed of 24 subunits of two types (heavy [H] chain and light [L] chain), the proportion of which depends on the iron status of the cell, tissue, and organ [111]. The H-chain contains ferroxidase activity, which is important not only for iron incorporation but also for controlling potentially toxic Fe (II) ions and thus reducing oxidative damage [112].

Another end product CO, a gasotransmitter, was discovered in human exhaled breath for the first time in 1949. After two decades, the identification of the HO clarified endogenous CO production. CO was discovered to be a vasomodulator molecule in isolated heart and vascular smooth muscle cells in the 1980s. Endogenous and exogenous CO were widely investigated as a vasomodulator, anti-inflammatory, and anti-apoptotic agent [113].

Bilirubin, a byproduct of heme metabolism, is an important endogenous antioxidant cytoprotectant. Bilirubin, which is lipophilic, protects lipids from oxidation [114].

There are three HO isoforms known (HO-1, HO-2, and HO-3), though HO-3 may be a pseudogene derived from HO-2 transcripts. Unlike HO-2, which is expressed constitutively, HO-1 is normally expressed at low levels in most tissues but is highly inducible by a variety of stimuli. Indeed, HO-1 is a stress-induced isoform of HO that is rapidly activated following oxidative stress to act as a potent endogenous factor in the resolution of stress-induced inflammatory injury [115]. HO-1 mediated vascular protection may be due to a combination of systemic and local effects. There are some evidences that supports the antiatherogenic role of HO-1 as well as the potential pathways and mechanisms mediating cardiovascular protection [116].

It is a Nrf2-regulated gene that is reported to play a critical role in the prevention of vascular inflammation. The activation and transcription of Nrf2 is critical for HO-1 expression. Research indicated that HO-1 has antioxidative potential to protect various cells from oxidative stress-induced injury, which also participates in cellular defense. This hypothesis is based on the observation that HO-1 activation is a common cellular response to oxidative stress. The HO-1 gene's transcriptional regulation by oxidants and the upregulation in mammalian tissues under

stress condition led to the theory that HO-1 served as a cellular defense mechanism during oxidative stress [117], [118].

1.8. Nrf2-HO1 axis in calcification

Nrf2 regulates 3-5% of human cellular proteins and is known as the "master regulator" of antioxidant pathways. [119], [120]. Because of its regulatory function in the antioxidant and anti-inflammatory pathways, the role of Nrf2 in the calcification process is becoming more recognized [121].

Increasing evidence suggests that oxidative stress plays a critical role in the initiation and progression of CAVD [122]. Reduced antioxidant enzyme levels have been shown to increase Runx2 and osteopontin gene expression in isolated human VICs [123]. The calcified valve-derived hVICs were more susceptible to oxidative stress and lacked antioxidant defense mechanisms [124]. According to preliminary research, the Nrf2 antioxidant pathway may play an important role in the progression of vascular calcification and apoptosis in CKD [125]. In renal VSMCs, activation of the Nrf2-ARE signaling pathway alleviates vascular calcification caused by hyperphosphatemia [126]. The expression of the osteogenic markers Runx2 and BMP2 was reduced when Nrf2 was increased [127]. Nrf2 can inhibit Runx2 binding to downstream gene promoters, slowing the process of cellular bone differentiation. On the other hand, experiments in cell culture show that over expression of Nrf2 inhibits cellular bone differentiation by interfering with Runx2. As a result of Nrf2 deletion, Runx2 expression is increased [128]. *In vitro* assays using VSMCs treated with the Nrf2 agonist resveratrol show significantly reduced mineralized matrix deposition, implying that Nrf2 signaling could protect against oxidative stress-induced mitochondrial damage and reduce intracellular calcium deposition. Because resveratrol raises klotho and Nrf2 mRNA expression in VSMCs after calcification [129], it may enhance Nrf2's anti-oxidative effect against hyperphosphatemia-induced calcification [130]. Elevated phosphate levels have been linked to a worse outcome in young healthy adults with coronary atherosclerosis and microvascular dysfunction. Indeed, dimethyl fumarate (DMF), a classic Nrf2 activator, significantly reduced VC in an *in vitro* ring culture system using mouse thoracic aorta and rat carotid artery under hypercalcemic and hyperphosphatemic conditions. DMF hindered VC by activating Nrf2 and decreasing the expression of osteogenic markers in VSMCs [131]–[133]. It has recently been suggested that hydrogen sulfide (H_2S) can effectively protect VSMCs from oxidative stress by

preventing the formation of VC caused by ROS production via the Nrf2-ARE pathway [134], [135]. H_2S treatment resulted in Nrf2 upregulation and subsequent increases in HO-1 and -2 expression in rat aorta [136].

Several factors, including aging, radiation exposure, and trauma, can cause oxidative stress in the lens, particularly LECs, which are prone to produce ROS [137]–[140]. Decreased level of Nrf2 reported as a suppressor of the antioxidant protection against ROS in the lens [141] whereas activation of the Nrf2 mediated antioxidant response genes induced resistance against oxidative stress in eye lens [142].

Labile heme has the potentiality to trigger Nrf2 signaling which can regulate its downstream transcription of HO-1 [143], [144]. There are some indications that HO-1 plays a protective role in atherosclerosis in the ApoE model, such as HO-1 overexpression inhibits AS in ApoE KO mice, whereas HO-1 deficiency accelerates the development of AS lesions [145]–[147]. Further findings showed that HO-1 mutant transgenic mice (HO-1-deficient mice) developed a cataract-like phenotypic character at 12 weeks after birth, and an intensified opaque lens structure into adulthood [148]. Genetic polymorphisms in the human HO-1 gene promoter influence the transcriptional activity and magnitude with which HO-1 responds to a pathophysiological stimulus. These polymorphisms have been linked to a different risk profile for cardiovascular disease. Juan and colleagues demonstrated that selective overexpression of HO-1 reduces lesion size in apolipoprotein E-deficient ($ApoE^{-/-}$) mice using adenovirus-mediated gene transfer. Induction of HO-1 reduces experimental atherosclerosis. In experimental restenosis, HO-1 overexpression reduces intimal thickening. Interestingly, hydrogen peroxide (H_2O_2) induced oxidative stress had been attenuated by HO-1 induction in the lens epithelial cells [149]. However, it is still very much unclear how HO-1 contributes to phosphate-mediated ectopic calcification, especially in valvular calcification and LECs calcification.

As a result, activating Nrf2/HO-1 signaling could be a common beneficial mechanism to prevent P and Ca-induced osteogenic differentiation and ECM calcification in various cell types like VICs and HuLECs. Moreover, we scrutinized the effect of the end-products like carbon monoxide (CO), bilirubin and iron in terms of the ectopic calcification as well.

2. Objective

Our primary aim was to investigate the effect of high phosphate on ectopic calcification and to observe if an antioxidant pathway like the Nrf2/ HO-1 axis can reverse the procedure. As we discussed earlier about the several occurrences of ectopic calcification in different organs, we chose the cardiovascular system and the eye as our research interest, focusing on the aortic valve and the lens respectively.

Specific aims:

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

2. To address the effects of inhibition of Nrf2 and HO-1 activities in high phosphate-induced calcification of valve interstitial cells and lens epithelial cells.

3. To explore the individual effects of heme degradation products (iron, bilirubin, and CO) in high phosphate-induced calcification of valve interstitial cells and lens epithelial cells.

4. To investigate the role of reactive oxygen species (ROS) in high phosphate-induced calcification of lens epithelial cells.

3. Materials and methods

3.1. Materials

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

3.2. Cell culture

Innoprot provided the human VICs (Derio, Spain). Dulbecco's modified Eagle's medium (DMEM, D6171, Sigma, St. Louis, MO, USA) was used as cell culture medium, and it was supplemented with 10% FBS (10270-106, Gibco, Grand Island, NY, USA), antibiotic antimycotic solution (A5955, Sigma, St. Louis, MO, USA), sodium pyruvate (S8636, Sigma, St. Louis, MO, USA), and L-glutamine (G7513, Sigma, St. Louis, MO, USA). 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 (ScienceCell Research Laboratories, Carlsbad, CA, USA). 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.

3.3. 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.

3.4. Cell treatments

Ammonium ferric citrate (F5879, Sigma, St. Louis, MO, USA) was used to introduce iron, which was dissolved in deionized water. Heme (H9039, Sigma, St. Louis, MO, USA) was dissolved in NaOH (20 mmol/L). Tin protoporphyrin IX (Cayman Chemical, Ann Arbor, MI, USA) and zinc protoporphyrin IX (EMD Millipore Corp., Burlington, MA, USA) 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 (288144, Sigma, St. Louis, MO, USA). CORM2 was dissolved in DMSO immediately before use and given every 12 hours. DMSO was used to dissolve the Nrf2 inhibitor ML385 (SML1833, Sigma, St. Louis, MO, USA). Ferritin (FT) was given as holoferritin (F4503, Sigma, St. Louis, MO, USA).

3.5. Alizarin red (AR) staining and quantification

Following a thorough rinse with deionized water, the cells were fixed in 4% paraformaldehyde (16005, Sigma, St. Louis, MO, USA) and washed with Dulbecco's PBS (DPBS; D8537, Sigma, St. Louis, MO, USA). For 20 minutes at room temperature, cells were stained with Alizarin Red S (A5533, Sigma, St. Louis, MO, USA) 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 (C9002, Sigma, St. Louis, MO, USA) solution (100 mmol/L) to the wells and measured the optical density (OD) at 560 nm using hexadecyl-pyridinium chloride solution as a blank.

3.6. 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 (30721, Sigma, St. Louis, MO, USA, 0.6 mol/L). The QuantiChrome Calcium Assay Kit was used to determine the Ca content of the HCl supernatants (DICA-500, Gentaur, Kampenhout, Belgium). Following decalcification, cells were washed twice with DPBS, solubilized with a solution of NaOH (S8045, Sigma, St. Louis, MO, USA, 0.1 mol/L) and sodium dodecyl sulfate (11667289001, Sigma, St. Louis, MO, USA, 0.1%), and protein content was determined using the BCA protein assay kit (23225, Pierce Biotechnology, Rockford, IL, USA). Cell Ca content was normalized to protein content and expressed as mg/mg protein.

3.7. Quantification of OCN

The ECM of cells, grown in 6-well plates was dissolved in 100 µL of EDTA (E6758, Sigma, St. Louis, MO, USA, 0.5 mol/L, pH 6.9) for OCN detection. An enzyme-linked immunosorbent assay (DY1419-05, DuoSet ELISA, R&D, Minneapolis, MN, USA) was used to determine the OCN content of the EDTA-solubilized ECM samples, according to the manufacturer's protocol. The observer who took all the ELISA measurements was unaware of the group assignment.

3.8. Determination of cell viability.

The MTT assay was used to determine cell viability, as previously described [150]. 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, ML2128, Sigma, St. Louis, MO, USA, 0.5 mg/mL). 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.

3.9. Quantitative RT-PCR

TRIzol (CS502, RNA-STAT60, Tel-Test Inc., Friendswood, TX, USA) 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 (4368813, Applied Biosystems, Waltman, MA, USA). The reaction mixture contained 0.1 µg reverse-transcribed sample, 10 µmol/L of forward (5'-GAGACAGGTGAATTTCCCAAT-3') and reverse (5'-GGGAGTAGTTGGCAGA TCCA-3') primers for Nrf2, forward (5'-TTCAGAAGGGCCAGGTGA-3') and reverse (5'-TGTTGCTCAATCTCCTC-3') primers for HO-1, forward (5'- (04887352001, Bio-Rad Laboratories, Hercules, CA, USA). The Real-Time PCR System was used for the PCRs (Bio-Rad Laboratories, Hercules, CA, USA). The ΔΔCt method was used to calculate relative mRNA expressions, with HPRT serving as an internal control.

3.10. Measurement of intracellular ROS

The 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein di-acetate, acetyl ester (CM-H2DCFDA) assay (C6827, Life Technologies, Carlsbad, CA, USA) 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 (A9165, Sigma).

3.11. 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 (1060003, Amersham Proton, GE Healthcare, Chicago, IL, USA). Western blotting was performed using an anti-ALP antibody (sc30203, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at a 1:1000 dilution, an anti-HO-1 antibody (70081, Cell Signaling Technology, Leiden, The Netherlands) at a 1:1000 dilution, an anti-Nrf2 antibody (16396-1-AP, Proteintech, Rosemont, IL, USA) at a 1:1000 dilution, an anti-NQO1 antibody (#3187, Cell Signaling Technology, Leiden, The Netherlands), at a 1:1000 dilution, an anti-FTH antibody (4393, Cell Signaling Technology, Leiden, Netherlands) at a 1:1000 dilution, an anti-FTL antibody (ab69090, Abcam, Cambridge, United Kingdom) at a 1:500 dilution and an anti-RUNX2 antibody (GTX81326, GeneTex International Corporation, Irvine, CA, USA) at a 1:1000 dilution followed by HRP-labeled anti-rabbit or anti-mouse IgG secondary antibodies (NA-934 and NA-931, Amersham Biosciences Corp., Piscataway, NJ, USA). ClarityTM Western ECL Substrate (170-5060, Bio-Rad Laboratories, Hercules, CA, USA) was used to detect antigen-antibody complexes. Chemiluminescent signals were detected using a C-Digit Blot Scanner or conventionally on an X-ray film (LI-COR Biosciences, Lincoln, NE, USA). After detection, the membranes were stripped and reprobed for ß-actin with a 1:2000 dilution of anti ß-actin antibody (sc-47778, Santa Cruz Biotechnology Inc., Dallas, TX, USA). Blots were quantified using the C-Digit Blot Scanner's built-in software (LI-COR Biosciences, Lincoln, NE, USA).

3.12. Statistics

The results are presented as mean ± SD. For all *in vitro* studies, at least three independent experiments were carried out. GraphPad Prism software was used for statistical analysis (v.8.01, San Diego, CA, USA). 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 the two groups. One-way ANOVA was used to compare more than two groups, followed by Tukey's multiple comparisons test. We used a one-

way ANOVA followed by Dunnett's post hoc test to compare each of the treatment groups to a single control group. A $p \leq 0.05$ value was considered significant.

4. Results

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

4.1.1. Phosphate and calcium induce extracellular matrix mineralization and cell death in valve interstitial cells synergistically

We used human primary VICs with morphological characteristics of both fibroblasts and smooth muscle cells to create an *in vitro* model of aortic valve calcification (**Figure 10a**). To induce calcification, we cultured human VICs in calcification medium containing varying concentrations of Pi (0-2.5 mmol/L) and Ca (0-1.2 mmol/L). AR staining was used to assess ECM calcification after 7 days of treatment (**Figure 10b, c**). We discovered that Pi and Ca induced ECM calcification in VICs in a synergistic and dose-dependent manner. Then, we measured Ca levels in HCl-solubilized ECM samples, which confirmed the AR staining results (**Figure 10d**). The combination of 2.5 mmol/L Pi and 0.3 mmol/L Ca resulted in an approximately 5-fold increase in ECM Ca content over the control (0.20 ± 0.03 vs. 1.10 ± 0.13 mg Ca/mg protein) **(Figure 10d**), whereas the 2.0 mmol/L Pi and 0.6 mmol/L Ca in media resulted in an approximately 8.5-fold increase. The maximum calcification of VIC cells occurred in the presence of 0.6 mmol/L Ca and 2.5 mmol/L Pi media (**Figure 10d**). A further increase in Ca resulted in the loss of Pi-sensitivity and maximum calcification regardless of Pi concentration (**Figure 10d**). Because calcification of VICs has previously been linked to apoptotic cell death [33], we next investigated the effect of Pi- and Ca-driven osteogenic stimulation on cell viability (**Figure 10e**). These findings imply that excess Pi and Ca cause cell death and Ca deposition in VIC ECMs. Based on these results, we set the Pi and Ca concentrations to 2.5 mmol/L and 0.3 mmol/L, respectively, to induce VIC calcification without causing massive cell death, and used this supplementation to the osteogenic medium (OM) in all subsequent experiments **(Figure 10e**). The master transcription factor of osteogenic differentiation is Runx2, therefore next we investigated whether OM increased Runx2 expression. We discovered that OM increased Runx2 expression approximately 4-fold (**Figure 10f**).

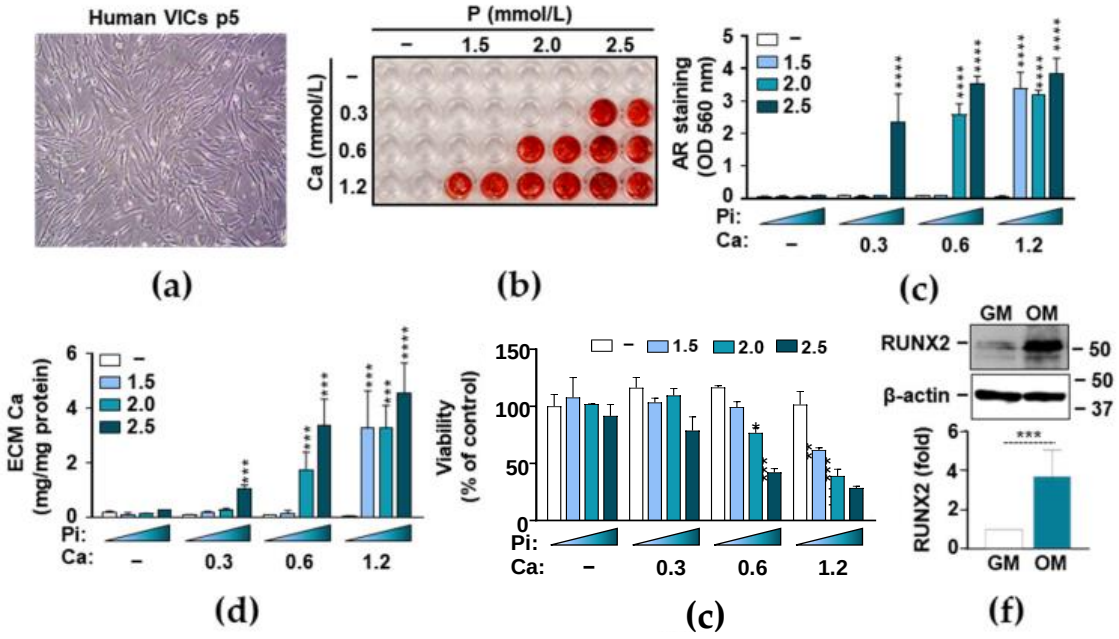

Figure 10. Extracellular matrix mineralization and Ca deposition of valve interstitial cells (VICs) are induced by osteogenic stimuli. Osteogenic medium was made by adding phosphate (P) (0–2.5 mmol/L) and calcium (0–1.2 mmol/L) to growth medium (GM). **(a)** Bright-field microscope image of human VICs at 100x magnification in passage number 5. **(b, c)** Alizarin Red (AR) staining was used to assess Ca deposition in the extracellular matrix (ECM) following 7 days of treatment. Shown are representative stained plate images and quantification from three separate experiments. **(d)** The HCl-solubilized ECM's Ca content is displayed. **(e)** The MTT assay was used to assess cell viability seven days after treatment. **(f)** Whole cell lysates were used to determine RUNX2 protein expression (24 h). Reprobing membranes for β-actin. Three independent experiments' representative Western blots and relative expression of RUNX2 normalized to β-actin are displayed. Results are shown as the mean ± SD of three separate experiments that were carried out in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

4.1.2. Phosphate and calcium-induced extracellular matrix calcification and osteogenic trans-differentiation in valve interstitial cells can be prevented by heme

We treated VICs with OM in the presence of various concentrations of heme (1-50 μmol/L) to investigate the effect of heme on VIC calcification. First, we used AR staining to examine ECM calcification and discovered that heme strongly inhibited VIC calcification in a dose-dependent manner (**Figure 11a**). Based on AR staining quantification, the effect of heme reaches its maximal value at 10 μM since the differences between the OD values for 10, 25 and 50 μM are not significant (**Figure 11a**). Then we looked at how heme affected ECM Ca levels. Heme inhibited OM-induced calcification significantly at 1 μmol/L (2.02 ± 0.10 vs. 1.21 ± 0.31 mg Ca/mg protein), and increasing concentrations produced even more significant effects (**Figure 11b**). Osteogenic stimulation causes VICs to transdifferentiate into osteoblast-like cells. This process can be tracked by looking for osteoblast-specific proteins like ALP and OCN. As a result, we investigated the impact of heme on the expression of these markers next. When compared to control VICs (GM), osteogenic stimulation with OM resulted in a 1.5-fold increase in ALP expression (**Figure 11c**). This increase was mitigated by heme at concentrations of 5, 10, and 25 μmol/L (**Figure 11c**). The level of OCN was then determined in the ECM of the VICs. The OCN level in OM-treated VICs was approximately 10-fold higher than in controls (GM) (**Figure 11d**). This increase was significantly reduced in the presence of heme at concentrations of 10 and 25 μmol/L (**Figure 11d**).

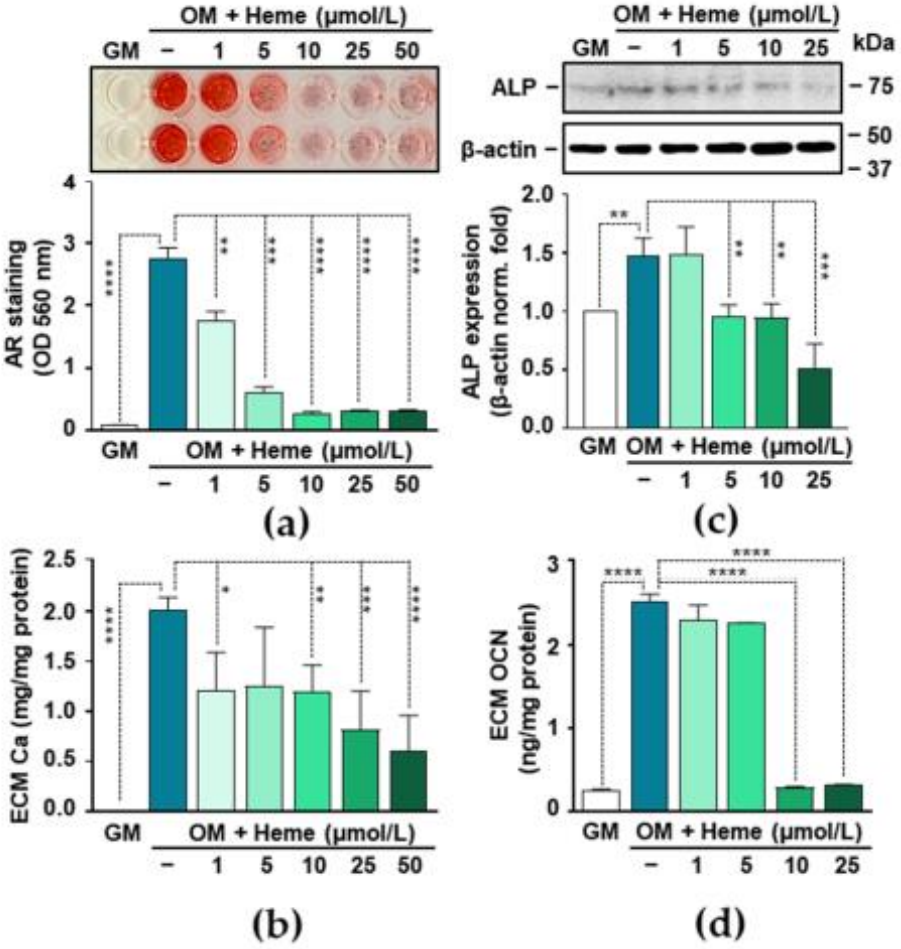

Figure 11. Heme inhibits VIC calcification brought on by OM in a dose-dependent way. (a–d) In the presence or absence of heme (1–50 μmol/L), confluent VICs (passage numbers 5-8) were maintained in GM or OM (2.5 mmol/L P and 0.3 mmol/L Ca). Three separate experiments' representative AR staining and quantification (day 5) results are shown in **(a)**. **(b)** The HCl-solubilized ECM's Ca content is displayed (day 5). The results of three independent experiments carried out in triplicate are expressed as the mean ± SD. **(c)** Whole cell lysates were used to determine the protein expression of ALP (72 h). Reprobing membranes for β-actin. Three independent experiments' representative Western blots and relative expression of ALP normalized to β-actin are displayed. The data is presented as mean ± SD. **(d)** By using an ELISA, the levels of OCN in EDTA-solubilized ECM samples were determined (day 5). Results are shown as the mean ± SD of three separate experiments that were carried out in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

4.1.3 Heme induces Nrf2/HO-1 axis in valve interstitial cells

Nrf2, a transcription factor that regulates the expression of numerous antioxidant genes, has been linked to vascular disease. Previous research has shown that upregulating the Nrf2 system reduces high Pi-induced calcification of VSMCs. Because heme is known to induce Nrf2 expression in a variety of cell types, we first investigated whether heme induces Nrf2 expression in VICs. Heme at a concentration of 50 µmol/L resulted in a 4-fold increase in Nrf2 mRNA (4 h) (**Figure 12a**). Nrf2 controls the expression of HO-1, a protein with antioxidant and anti-inflammatory properties. Therefore, we investigated whether heme-mediated Nrf2 upregulation was accompanied by increased HO-1 expression. Heme, as expected, caused a strong and dose-dependent increase in HO-1 mRNA (4 h) expression in VICs (**Figure 12b**). After that, we looked at the protein expression of Nrf2 and HO-1 in heme-treated VICs (12 hours). Every heme concentration (5-50 µmol/L) used resulted in a 2.5-fold increase in Nrf2 expression when compared to the control (GM) (**Figure 12c**). Furthermore, we discovered a significant and dose-dependent increase in HO-1 expression in heme-treated VICs (**Figure 12c**).

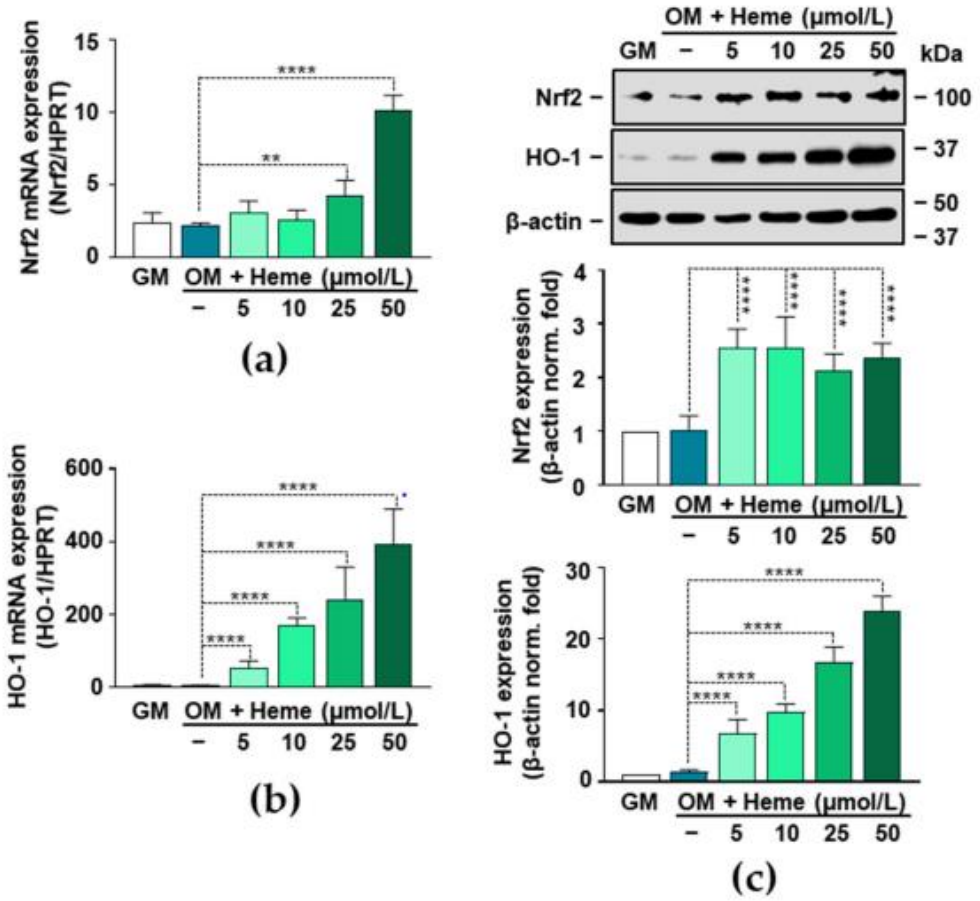

Figure 12. In VICs, heme induces Nrf2 and HO-1. VICs (passage numbers 5-8) were cultured in GM or OM with or without heme (5-50 μmol/L) in the figures in (a–c). **(a, b)** Real-time RT-PCR was used to measure the levels of Nrf2 and HO-1 mRNA (4 h). **(c)** From whole cell lysates, Nrf2 and HO-1 protein expression was assessed (12 h). Reprobing membranes for β-actin. Three separate experiments' representative Western blots and relative expression of Nrf2 and HO-1 normalized to β-actin are displayed. Results are shown as the mean ± SD of three separate experiments that were carried out in triplicate. ** $p < 0.01$, **** $p < 0.001$.

4.1.4. The anti-calcification effect of heme requires Nrf2/HO-1 axis integrity

Next, we wanted to see if the induction of the Nrf2/HO-1 system was important in the heme-mediated inhibition of VIC calcification. Our first strategy was to inhibit Nrf2 transcriptional activity with the well-known inhibitor ML385. We induced calcification of the VICs with OM in the presence or absence of heme (10 µmol/L) and ML385 (10 µmol/L). On day 5, we stained the cells with AR to assess calcification (**Figure 13a**). In the absence of ML385, heme completely inhibited OM-induced VIC calcification (**Figure 13a**). In the presence of ML385, however, heme lost its ability to inhibit OM-induced calcification of VICs (**Figure 13a**), implying that Nrf2 transcriptional activity was required for heme's anti-calcification effect. Furthermore, we discovered that OM caused more calcification in the presence of ML385, highlighting Nrf2's protective role in Pi and Ca-induced VIC calcification (**Figure 13a**). The assessment of ECM Ca levels confirmed these findings (**Figure 13b**). Then we looked into the role of HO-1 in heme's anti-calcification effect. We used OM to cause calcification of the VICs in the presence of heme and the HO-1 enzyme inhibitors SnPP and ZnPP. In the absence of HO-1 inhibitors, heme (10 µmol/L) completely inhibited OM-induced ECM calcification (**Figure 13c**). In contrast, heme lost its ability to inhibit OM-induced VIC calcification in the presence of either SnPP (10 µmol/L) or ZnPP (10 µmol/L) (**Figure 13c**). We measured the Ca content of HCl-solubilized ECM to confirm these findings. The Ca level in heme-treated VIC was significantly lower than in OM-treated cells, and this decrease was completely reversed in the presence of HO-1 inhibitors (**Figure 13d**).

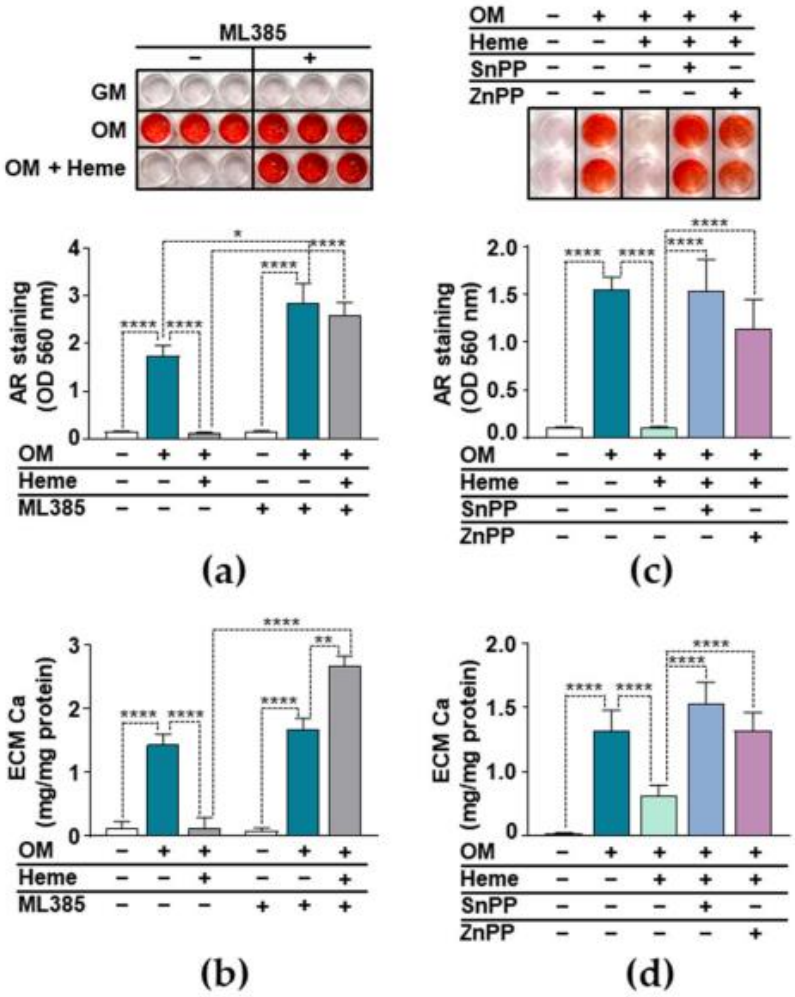

Figure 13. Inhibition of Nrf2 or HO-1 diminishes the anti-calcification effect of heme. **(a,b)** VICs (passage number 5–8) were pretreated with vehicle or ML385 (10 µmol/L) for 3 h. **(c,d)** VICs were pretreated with vehicle, SnPP (10 µmol/L), or ZnPP (10 µmol/L) for 3 h. **(a–d)** After the pretreatment, cells were maintained in GM, OM, or OM supplemented with heme (10 µmol/L). **(a,c)** Representative AR staining from three experiments and quantification (day 5) are shown. **(b,d)** The Ca content of the HCl-solubilized ECM is presented (day 5). Data are expressed as mean ± SD of three independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$.

4.1.5. Products of heme degradation engage in anti-calcification activities

Inhibiting HO-1 activity reduces heme's anti-calcification effect, raising the possibility that heme degradation products are responsible for heme's inhibitory effects. By means of HO-1-catalized heme degradation, biliverdin, iron (Fe), and carbon monoxide (CO) are produced. Biliverdin is then swiftly converted to bilirubin (BR) by biliverdin reductase (BVR) (**Figure 14a**). As a result, we investigated the effect of BR, Fe, and CO on VIC calcification next. We used OM to stimulate ECM calcification in VICs in the presence or absence of BR (5-50 µmol/L), Fe (5-50 µmol/L), and CO administered as CORM2 (5-50 µmol/L). AR staining on day 5 revealed that all three heme degradation products had dose-dependent anti-calcification potential (**Figure 14b–d**). We validated these findings by determining the Ca content of solubilized ECM. Calcium accumulation in the ECM of VICs was inhibited by BR, Fe, and CO in response to osteogenic stimulation. At a concentration of 50 µmol/L, BR completely inhibited OM-induced calcification, whereas Fe and CO provided only partial protection (**Figure 14e–g**). Then, we looked at how heme degradation products affected the levels of OCN in the ECMs of OM-stimulated VICs (**Figure 14h**). When compared to controls (GM), osteogenic stimulation (OM) caused an approximately 80-fold increase in OCN in the ECM of VICs (0.037 ± 0.016 vs. 3.025 ± 0.304 ng/mg protein) (**Figure 14h**). This increase was completely inhibited in the presence of heme and BR, and it was partially inhibited in the presence of Fe and CO at a concentration of 50 µmol/L. (**Figure 14h**). The mRNA levels of SOX9, a transcription factor involved in the osteochondrogenic trans-differentiation of VICs, and OPN, a bone-related glycoprotein, were then measured (**Figure 14i, j**). Heme and BR inhibited OM-induced increases in both SOX9 and OPN mRNA expression, whereas Fe and CO had no such effect (**Figure 14i, j**).

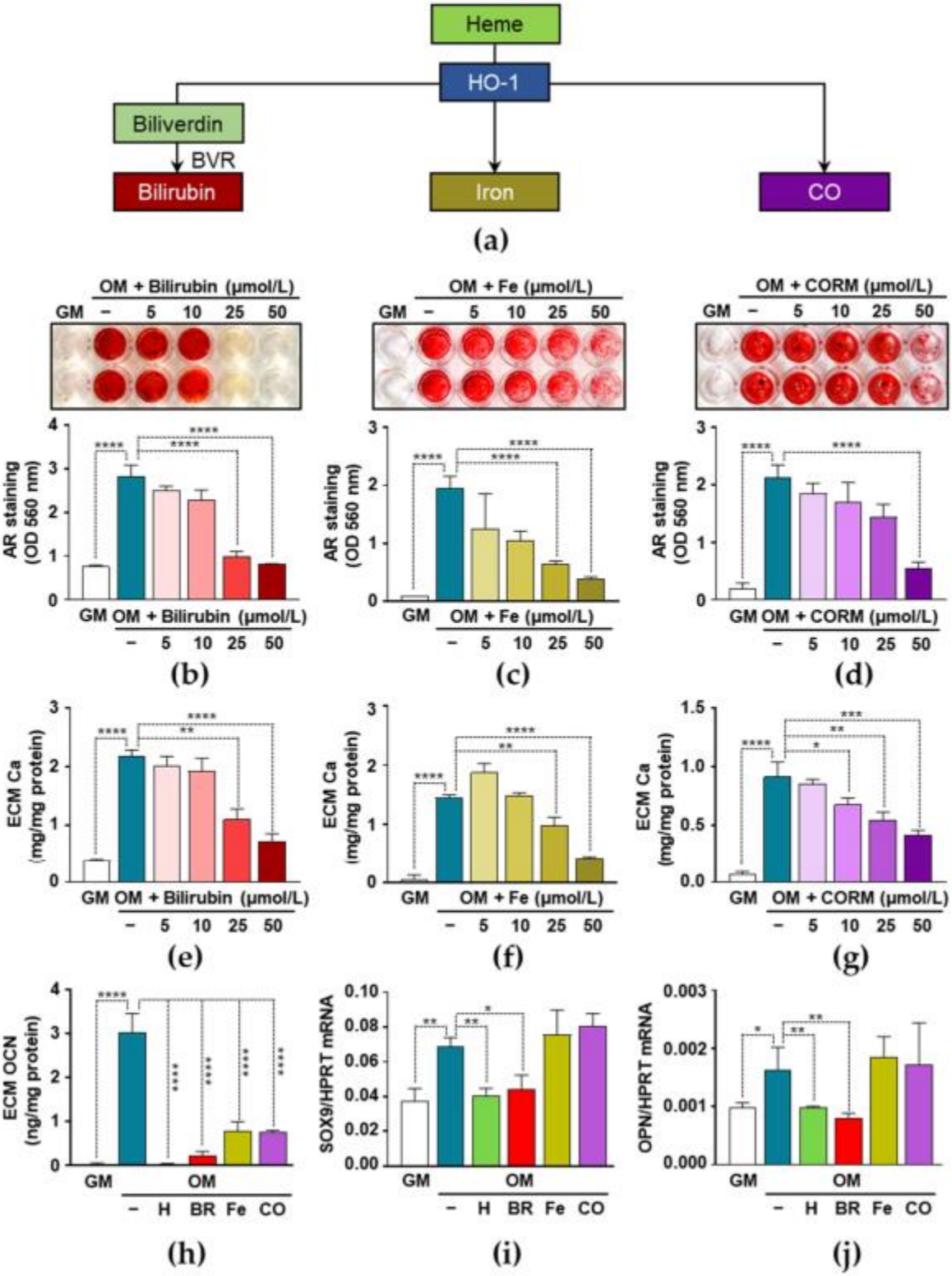
Heme
HO-1
Biliverdin
BVR
Bilirubin
Iron
CO
(a)
OM + Bilirubin (μmol/L)
GM – 5 10 25 50
OM + Fe (μmol/L)
OM + CORM (μmol/L)
AR staining (OD 560 nm)
GM OM + Bilirubin (μmol/L)
– 5 10 25 50
(b)
GM OM + Fe (μmol/L)
(c)
GM OM + CORM (μmol/L)
(d)
ECM Ca (mg/mg protein)
(e)
(f)
(g)
ECM OCN (ng/mg protein)
GM OM
– H BR Fe CO
(h)
SOX9/HPRT mRNA
(i)
OPN/HPRT mRNA
(j)

Figure 14. The impact of iron, CO, and bilirubin on the calcification of VICs. Diagram of HO-1-catalyzed heme degradation in **(a)**. GM, OM, or OM supplemented with **(b, e)** bilirubin (BR; 5-50 µmol/L), **(c, f)** iron (Fe); **(d, g)** CO donor (CORM; 5-50 µmol/L) were used to treat **(b-g)** VICs (passage numbers 5-8) for 5 days. **(b-d)** AR staining was used to assess the amount of Ca deposited in the ECM after 5 days. Shown are representative stained plate images and quantification from three separate experiments. Ca content of the HCl-solubilized ECM is displayed in **(e–g)**. (H, 25 µmol/L), BR (50 µmol/L), Fe (50 mol/L), or CORM (50 µmol/L) were added to GM, OM, or OM to maintain VICs. **(h)** Using an ELISA, the levels of OCN in EDTA-solubilized ECM samples were determined (day 5). **(i,j)** Real time RT-PCR was used to measure the levels of SOX9 and OPN mRNA (72 h). The results of three independent experiments carried out in triplicate are expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

4.1.6. Ferritin resembles heme's inhibitory activity on valve interstitial cells calcification

Heme degradation by HO-1 results in the release of free iron from the heme moiety and, as a result, an increase in ferritin (FT), the key molecule in iron storage. We investigated the role of FT in VIC calcification because it has many beneficial effects in vascular pathology. We started by looking at the protein expression of both ferritin subunits, FTH (ferritin heavy/heart) and FTL (ferritin light/liver). At a concentration of 5 µmol/L, heme increased the expression of FTH and FTL by about 15 and 20-fold, respectively (**Figure 15a**). The next question was whether ferritin could prevent OM-induced VIC calcification. Ferritin was given as a holoprotein, and calcification was assessed using AR staining and ECM Ca measurements. At a concentration of 100 µg/mL, both methods demonstrated that holo-FT completely inhibits OM-induced calcification (**Figure 15b, c**).

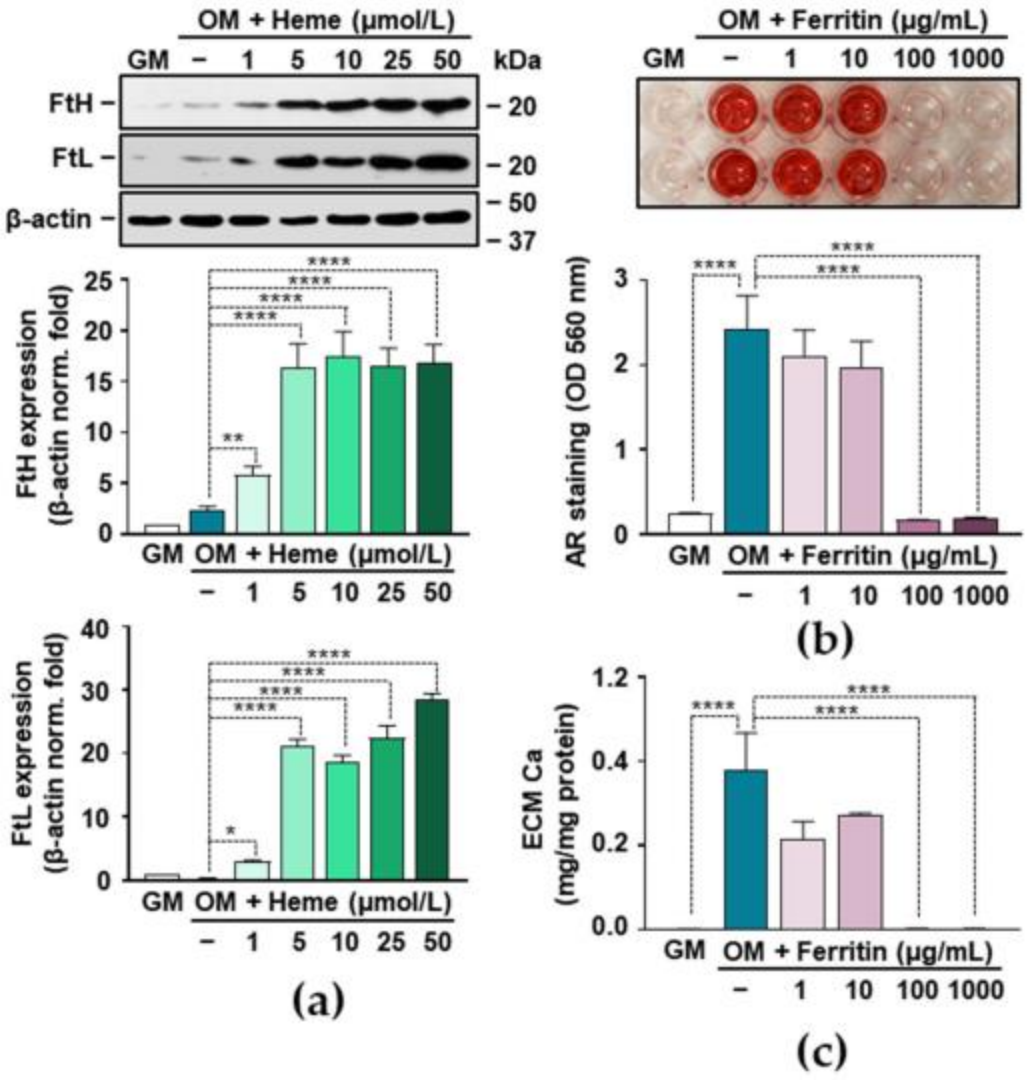

Figure 15. Ferritin acts similarly to heme in preventing VICs from mineralizing. (a) For 12 hours, VICs (passage numbers 5-8) were given GM or OM in either the absence or presence of heme (1-50 μmol/L). Western blot was used to assess the expression of the H and L chains of ferritin (FTH and FTL). The immunoblots represent three separate experiments and were reprobed with β-actin. (b,c) For 5 days, ferritin (1-1000 μg/mL) was either present or absent when GM and OM were administered to VICs. **(b)** Three independent experiments' representative AR staining and quantification (day 5) are displayed. **(c)** The HCl-solubilized ECM's Ca content is displayed. Results from three independent experiments carried out in triplicate are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$.

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

4.2.1. Heme-induced activation of the Nrf2/HO-1 axis is reactive oxygen species dependent in human lens epithelial cells

It has been demonstrated in the past that activating the Nrf2/HO-1 axis stops the calcification of vascular smooth muscle cells induced by high Pi. Based on this, we set out to investigate whether HuLEC calcification is inhibited by the activation of the Nrf2/HO-1 system. We used heme at a supraphysiological concentration of 1 to 50 µmol/L to activate the Nrf2/HO-1 antioxidant pathway based on prior publications. We demonstrated that heme upregulated Nrf2 expression in HuLECs in a dose-dependent manner (**Figure 16a, b**). We looked into the expression of two Nrf2 target genes, NQO1 and HO-1, in response to heme. By the highest concentration of heme (50 µmol/L), we discovered a modest upregulation of NQO1 (2-fold) and a significant increase of HO-1 (200-fold) **(Figure 16c, d**).

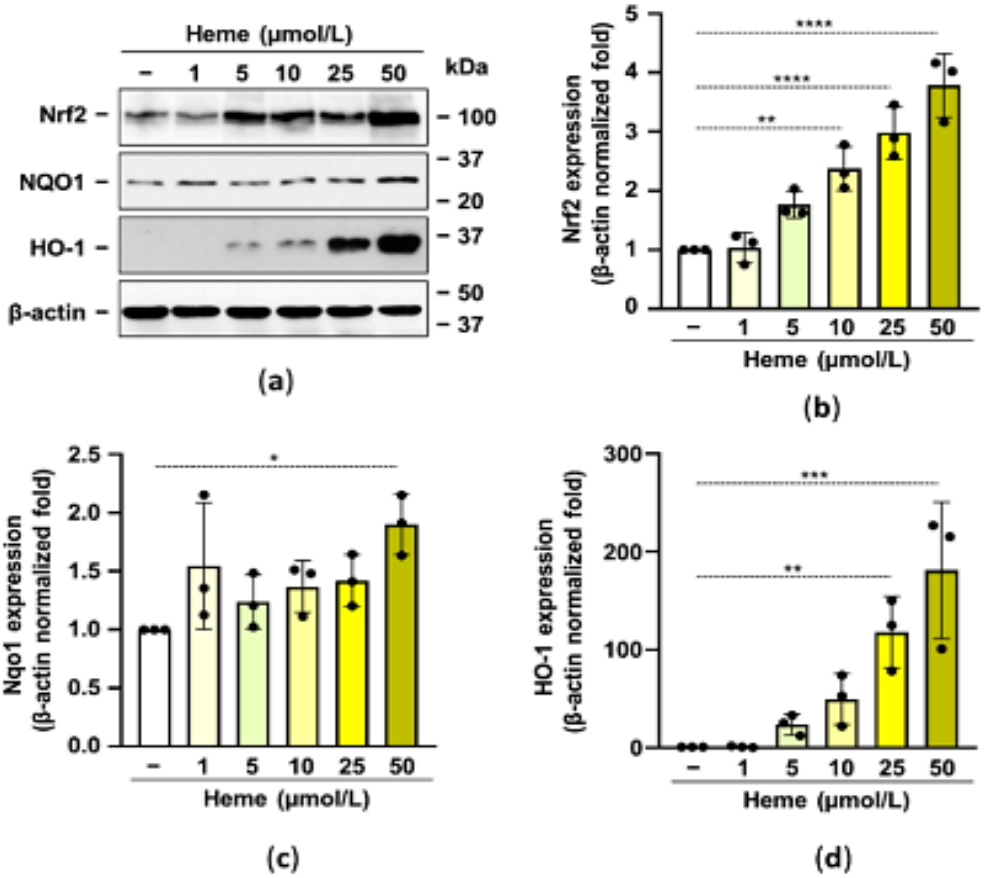

Figure 16. Heme activates the Nrf2/HO-1 axis in HuLECs. Heme was applied to confluent HuLECs (passage 5-8) in the following images: (a–d). From whole-cell lysates, the protein expressions of Nrf2, NQO1, HO-1, and β-actin were determined (12 h). **(a)** Three representative Western blots. Nrf2, NQO1, and HO-1 relative expression, normalized to actin **(b–d)**. The mean and standard deviation are represented by bars. To determine p values, a standard one-way ANOVA was used, then Dunett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

The heme iron participates in the Fenton reaction-driven ROS production in many cell types, acting as a pro-oxidant. Oxidative stress and Nrf2 interact in a complex way, whereby ROS activate Nrf2 and Nrf2 target genes regulate oxidative stress. Based on these findings, we next looked into how heme-induced Nrf2 upregulation in HuLECs was dependent on ROS. First, we demonstrated that heme causes HuLECs to produce ROS (**Figure 17a**). Heme-induced ROS formation in HuLECs was completely eliminated by the glutathione precursor N-acetylcysteine (NAC) (**Figure 17b**). NAC also reduced the upregulation of Nrf2 caused by heme in HuLECs (**Figure 17c, d**)

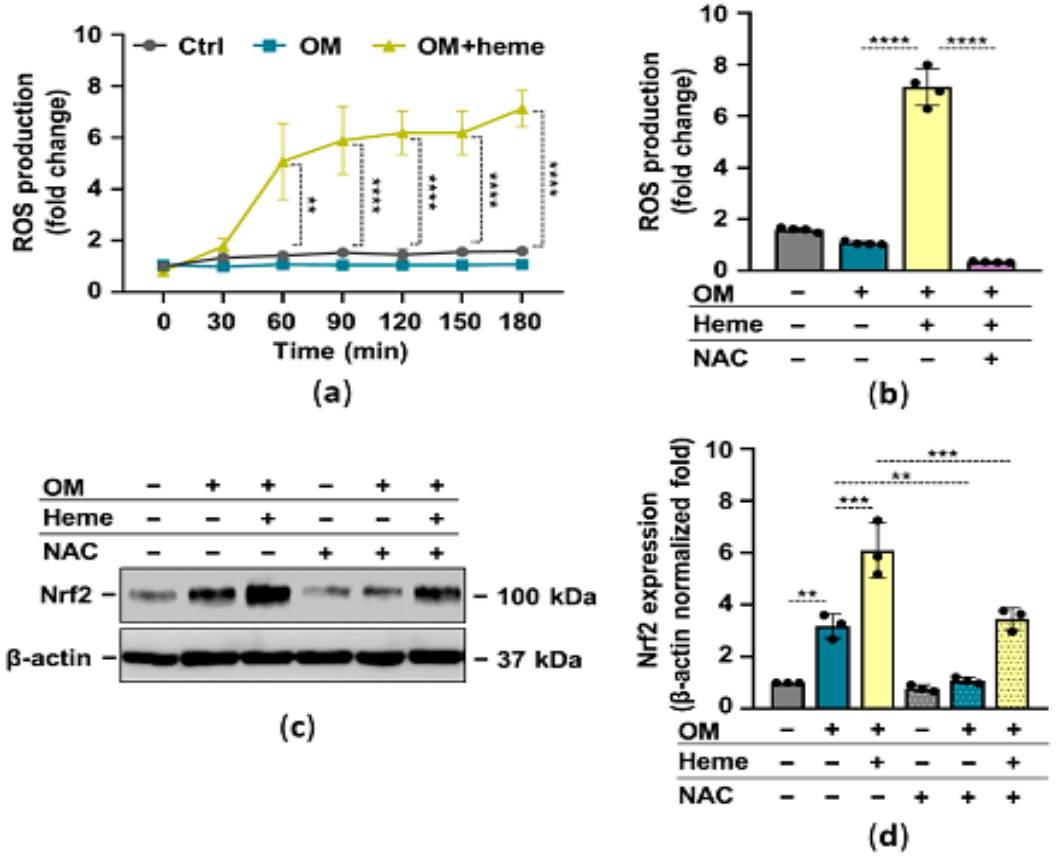

Figure 17. Heme-mediated ROS production plays a role in Nrf2 activation. Confluent HuLECs (passage number 5–8) were treated with OM and OM + heme (25 μmol/L) in the presence or absence of NAC (5 mmol/L). (**a**) Kinetics of intracellular ROS production was monitored for 3 h. (**a**, **b**) Data points are derived from 4 independent experiments. (**b**) ROS production (3 h), $n = 3$. (**c**, **d**) Protein expressions of Nrf2 and β-actin in whole-cell lysates (12h). (**c**) Representative Western blots ($n = 3$). (**d**) β-actin-normalized Nrf2 expression. Data are expressed as mean ± SD. Ordinary one-way ANOVA followed by Tukey's multiple comparison test was used to obtain *p* values. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

4.2.2. Heme prevents human lens epithelial cells extracellular matrix calcification caused by osteogenic stimuli

Heme has been demonstrated to prevent osteochondrogenic differentiation and calcification of vascular smooth muscle cells. Therefore, we next looked into whether heme reduces HuLEC calcification. Heme reduced HuLECs' OM-induced ECM calcification in a dose-dependent manner, as shown by AR staining and the determination of calcium in HCl-solubilized ECM (**Figure 18a–c**). Next, we looked at how heme affected key indicators of the osteogenic trans-differentiation process. Runx2, the master transcription factor of osteogenesis, was increased by OM, but this was abolished by heme (25 μmol/L), which also decreased its expression below the control level (**Figure 18d, e**). ALP expression was reduced by heme (25 μmol/L) compared to control and OM-induced HuLECs (**Figure 18d, f**). Heme completely eliminated the robust increase in OCN expression that osteogenic stimuli induced in HuLECs' ECM (**Figure 18g**).

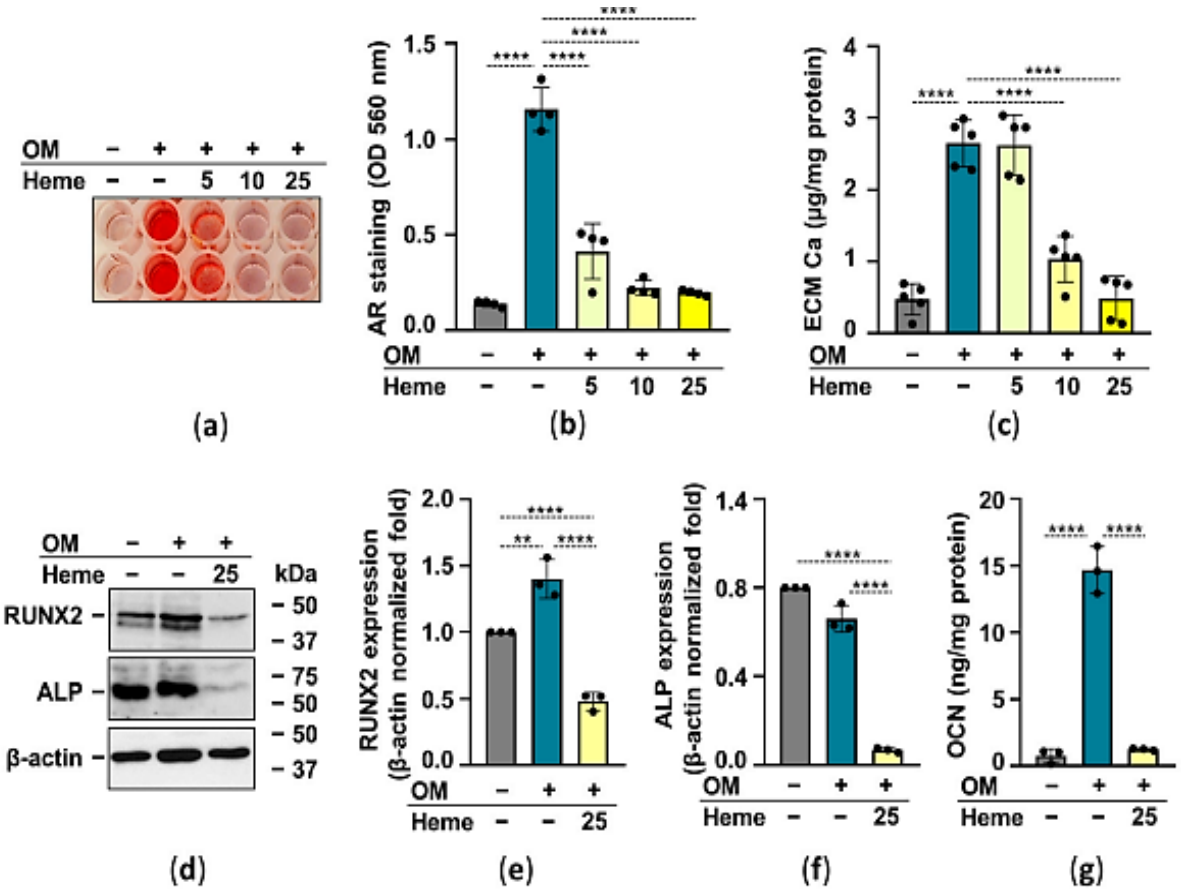

Figure 18. OM-induced ECM calcification is inhibited by heme in HuLECs. Confluent HuLECs (passage numbers 5-8) were handled under three culture conditions, Ctrl, OM, and OM + heme. **(a, b)** Heme's dose-dependent effect on OM-induced Ca deposition in the ECM as measured by AR staining (day6). Four independent experiments' representative images and quantification are displayed. **(c)** The HCl-solubilized ECM's Ca content (day6). Independent experiments are represented by data points. **(d–f)** From whole-cell lysates, Runx2 and ALP protein expressions were discovered (day6). β-actin probes were applied to the membranes. (d) Typical Western blots, n = 3. Relative Runx2 and ALP expressions normalized to β-actin are displayed in **(e,f)**. OCN levels in EDTA-solubilized ECM (day 6) from 3 separate experiments are shown in **(g)**. The data is presented as mean ± SD. To obtain p values, a standard one-way ANOVA was used, followed by a Tukey's multiple comparison test. ** $p < 0.01$, **** $p < 0.001$.

4.2.3. Heme-mediated calcification inhibition requires the Nrf2/HO-1 antioxidant pathway

The next set of experiments examined the role of Nrf2/HO-1 pathway activation in heme-mediated inhibition of HuLEC calcification. We examined the impact of Nrf2 modulation on HuLEC calcification using heme (10 µmol/L) to induce, and ML385 (10 µmol/L) to inhibit Nrf2. HuLECs treated with ML385 calcified more quickly than cells treated with vehicle, that is 10 µmol/L DMSO (**Figure 19a, b**). Additionally, heme was unable to stop HuLEC calcification brought on by OM in the presence of ML385 (**Figure 19a, b**). These findings indicate that Nrf2 protects HuLECs from calcification and that heme's anti-calcification effect relies on Nrf2 upregulation (**Figure 19a, b**).

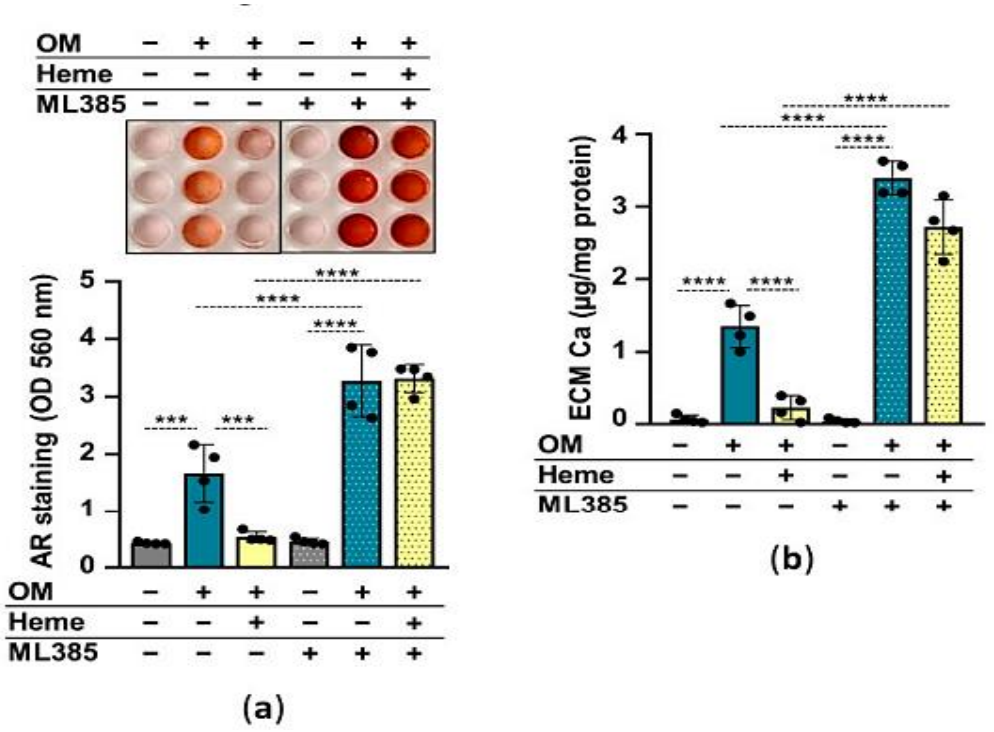

Figure 19. The ability of heme to prevent calcification is reduced by Nrf2 inhibition. Confluent HuLECs (passages 5-8) were pretreated for 3 hours with either the vehicle or ML385 (10 µmol/L). Cells were kept in Ctrl, OM, or OM + heme (10 µmol/L) conditions following the pretreatments. **(a)** Four independent experiments' representative AR staining and quantification are displayed (day5). **(b)** The HCl-solubilized ECM's Ca content is displayed (day5). Four separate experiments were used to generate the data points. The data is presented as mean ± SD. To obtain p values, a standard one-way ANOVA was used, followed by a Tukey's multiple comparison test. *** $p < 0.005$, **** $p < 0.001$.

We then investigated whether HO-1 is responsible for the anti-calcification effect of heme in HuLECs. We used heme to induce HO-1 activity and SnPPIX and ZnPPIX to inhibit it. Heme (10 µmol/L) completely prevented OM-induced calcification of HuLECs as determined by AR staining and calcium measurement in the absence of HO-1 inhibitors (**Figure 20a, b**). However, in the presence of HO-1 inhibitors, heme-mediated calcification inhibition was lost (**Figure 20a, b**). These findings imply that the anti-calcification properties of heme require an intact and active Nrf2/HO-1 system.

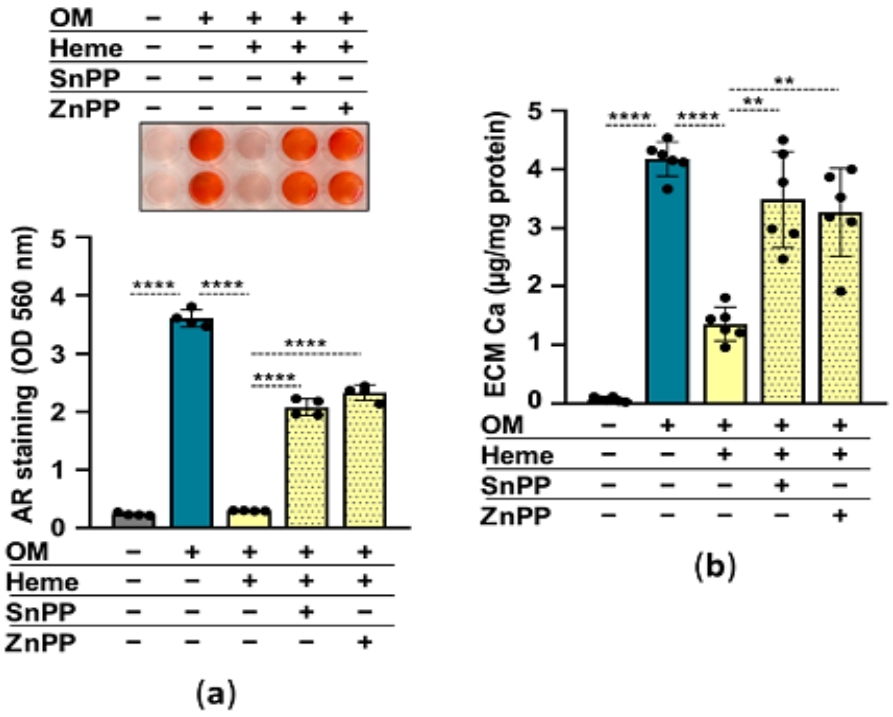

Figure 20. Heme's ability to prevent calcification is reduced by the activity of the HO-1 enzyme being inhibited. (a, b) For a 3-hour pretreatment, confluent HuLECs (passages 5-8) were exposed to vehicle, SnPP (10 µmol/L), or ZnPP (10 µmol/L). Cells were kept in Ctrl, OM, or OM + heme (10 µmol/L) conditions following the pretreatments. **(a)** Quantitation and representative AR staining (day5). **(b)** The HCl-solubilized ECM's Ca content is displayed (day5). Four separate experiments were used to generate the data points. The data is presented as mean ± SD. To obtain p values, a standard one-way ANOVA was used, followed by a Tukey's multiple comparison test. ** $p < 0.01$, **** $p < 0.001$.

4.2.4. Products of heme degradation have anti-calcification effect

Heme-induced inhibition of calcification is abolished, when HO-1 activity is eliminated, indicating that heme degradation products may be involved. Iron (Fe), carbon monoxide (CO), and biliverdin are the three byproducts of heme degradation by HO-1. Biliverdin is quickly converted to bilirubin (BR) by biliverdin reductase. So, in our subsequent experiment, we looked into how these heme degradation products affected HuLEC calcification. In either the presence or absence of BR (5-50 µmol/L), Fe (5-50 µmol/L), and CORM2 (25-200 µmol/L), we administered OM to HuLECs. Iron is the final heme degradation product that exhibits a potent anti-calcification potential, according to AR staining and ECM calcium measurements (**Figure 21**). In HuLECs treated with OM, BR had minimal anti-calcification potential, while CORM had none (**Figure 21**).

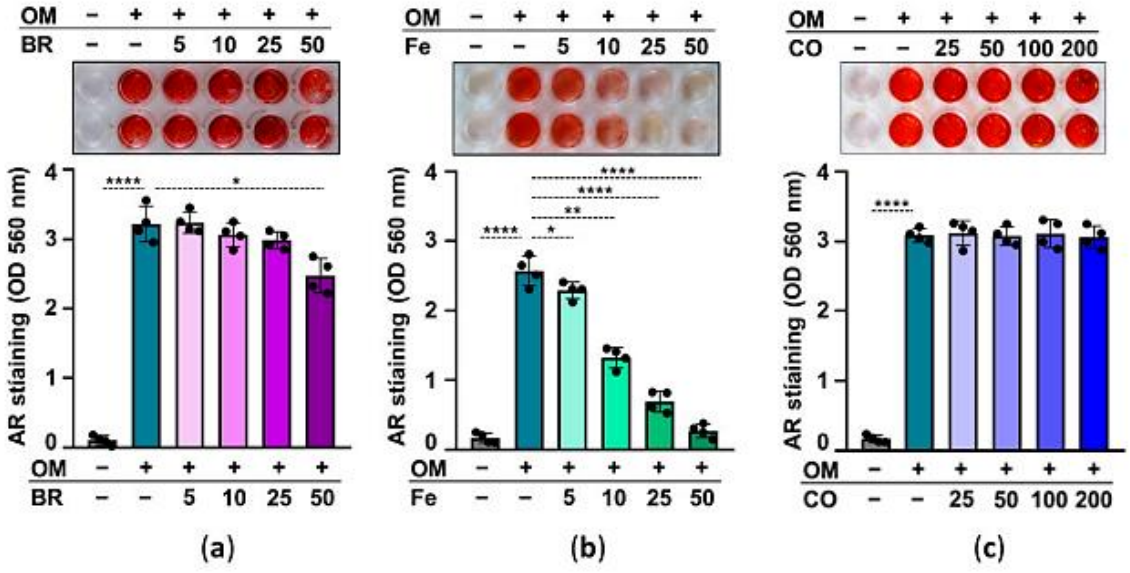

Figure 21. Effect of heme degradation products on HuLECs calcification brought on by OM. (a–c) In the presence of bilirubin (BR; 5–50 µmol/L), iron ($FeSO_4$; 5–50 µmol/L), or CO (CORM; 25–200 µmol/L), confluent HuLECs (passage 5-8) were treated with OM. AR staining was used to measure calcification (day5). Shown are representative images and quantification from four independent experiments. The data is presented as mean ± SD. To obtain p values, a standard one-way ANOVA was used, followed by a Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$.

5. Discussion

The most accurate parameter for predicting the likelihood of valve calcification in CKD patients is the Ca x P product [151]. Using a cellular model of valve calcification, we used an osteogenic medium with high levels of P and Ca to cause calcification in VICs (**Figure 10**). We discovered that elevated levels of P and Ca caused ECM calcification in VICs, which is consistent with earlier reports [152]. P and Ca had a dose-dependent and synergistic effect, and excessive Ca accumulation in VICs' ECM was linked to cell death (**Figure 10**).

We used heme, a common iron compound with well-known harmful effects in vascular pathology, to activate the Nrf2/HO-1 pathway. Heme can catalyze the Haber-Weiss reaction, which produces highly reactive hydroxyl radicals and is a major cause of oxidant-mediated cell death in endothelial cells (ECs) [153], [154]. Heme can indirectly harm ECs by causing low-density lipoprotein to undergo oxidative modification [155]. Heme also stimulates the nuclear factor kappa B (NF-κB) signaling pathway and toll-like receptor 4 in ECs, which results in endothelial activation and dysfunction marked by the production of vascular adhesion molecules, pro-inflammatory cytokines, and ROS [156]–[158]. The leucine-rich repeat-containing family, the pyrin domain-containing 3 (NLRP3) inflammasome, and the nucleotide-binding domain are also activated by heme [159].

Recent research has shown that excessive ROS production and NF-κB activation are key mediators of vascular calcification [160]–[165]. These facts allow one to infer that heme would cause vascular calcification. Contrary to our expectations, we discovered that heme is a powerful inhibitor of VIC calcification (**Figure 11**). Our findings demonstrated that heme inhibits osteoblast markers ALP and OCN expression as well as ECM calcification in OM-stimulated VICs in a dose-dependent manner (**Figure 11**). This finding is in agreement with Zarjou *et al*. who described a similar effect of heme on P-induced calcification of VSMCs *in vitro* previously [166]. The impact of heme on vascular and valvular calcification *in vivo* requires additional research.

We proposed that induction of the Nrf2/HO-1 pathway, a potent antioxidant and anti-inflammatory pathway, could account for the anti-calcification effect of heme. We first established that heme induces the Nrf2/HO-1 system in VICs to inhibit Pi mediated ectopic calcification. Additionally, we discovered that when Nrf2 was pharmacologically inhibited in VICs, heme lost its ability to

prevent calcification (**Figure 13**). Our findings are consistent with earlier studies that highlighted Nrf2's critical role in the calcification of VSMCs. Numerous endogenous, organic, and pharmaceutical agents can activate Nrf2. Numerous Nrf2 inducers, such as hydrogen sulfide, rosmarinic acid, dimethyl fumarate, and resveratrol, have been shown to inhibit high P-induced VSMC calcification in studies [167]–[171]. By encouraging autophagy and reducing the production of ROS, activation of the Nrf2 signaling pathway prevents high P-induced calcification [172], [173]. Furthermore, by reducing oxidative stress and VSMC apoptosis through Nrf2 activation, the mitochondria-targeted antioxidant mitoquinone reduces vascular calcification [174].

Here, we show that the heme-degrading inducible enzyme HO-1, which is regulated by Nrf2, is significantly upregulated in VICs that have been exposed to heme. We investigated whether the inhibitory effect of heme required HO-1 enzyme activity due to the protective nature of heme degradation products. We noticed that the HO-1 inhibitors ZnPP and SnPP prevented the anti-calcification effects of heme (**Figure 13**). Similar results were seen for osteoblast mineralization, where heme inhibited osteoblast maturation and mineralization while ZnPP's blockade of HO-1 activity counteracted heme's inhibitory effect [175].

Biliverdin is quickly converted to bilirubin by biliverdin reductase after being produced as a result of heme degradation by HO-1. Primarily due to its propensity to bind to Fe^{2+} centers in heme proteins, CO is a crucial signaling molecule [176], [177]. The pro-oxidant iron released during heme catabolism is controlled by the FT, and particularly the FTH, which is co-expressed with HO-1 [178]. A slight increase in bilirubin, the third byproduct of heme catabolism, is linked to cardiac and vascular protection as well as a reduction in adult all-cause mortality [179]–[181].

Here, we show that calcium accumulation in the ECM of OM-induced VICs was reduced by all three heme catabolic products. The most potent inhibitor of VIC calcification among them was bilirubin. Additionally, bilirubin and heme both 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 [166]. Since FT mimics the inhibitory effects of heme and iron, we also looked at its role in VIC calcification and discovered that it was a potent anti-calcification agent in VICs (**Figure 15**). In healthy adults, the extracellular

FT concentration in serum is less than 500 ng/mL, which is significantly lower than the FT concentration we used in this study (1–1000 µg/mL). Extracellular FT is taken up by particular receptors that are present on human lymphocytes, erythroblasts, adipocytes, liver cells, and other cell lines [182], but we are unaware of how FT is taken up by VICs. Additional research is required to ascertain 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. It is interesting to note that *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 [183]. It was discovered that the increased osteogenic and decreased adipogenic differentiation potential of MSCs were related to the overexpression of HO-1 [184]. Regarding iron's impact, Balogh *et al*. demonstrated that high iron prevents MSCs from differentiating into osteoblasts by inducing the expression of FT [185], and Zarjou *et al.* demonstrated that high iron reduces osteoblast activity and ECM calcification by upregulating the expression of FTH and ferroxidase activity [186].

Hydroxiapatite accumulation also occurs in soft tissues outside the cardiovascular system. One example is the eye, in which Fourier transform infrared and Raman microspectroscopies revealed the presence of hydroxyapatite in the lens affected by senile cataract [187]–[189]. Current understanding of cataract formation has not provided an explanation for this phenomenon, until our group demonstrated that HuLECs can differentiate into osteoblast-like cells and calcify the ECM in response to osteogenic stimulation [190]. Previously we found high levels of calcium and OCN, the main non-collagenous bone protein, in human cataractous lenses [190]. This finding 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. Numerous age-related eye diseases, such as glaucoma, macular degeneration, diabetic retinopathy, and cataract, have been linked to Nrf2-driven cytoprotective mechanisms [191], [192]. Gao *et al*.

looked into the expression of Nrf2 and Keap1 genes and proteins in human lenses. In comparison to lenses of younger population, they discovered that Nrf2 expression was significantly lower and Keap1 expression was higher in lenses made from elderly people (65–80 years old) [193]. This pattern of expression causes the oxidative stress defense in the aging eye to deteriorate, which can aid in the emergence of age-related eye diseases like cataracts. The evidence is mounting that Nrf2 is a therapeutic target with promise for preventing age-related cataracts [191], [194].

The rate-limiting enzyme of heme degradation with a variety of antioxidant and anti-inflammatory properties is called HO-1 [195]. It has been demonstrated that HO-1 shields HuLECs from hydrogen peroxide-induced oxidative stress and apoptosis when it comes to lens epithelial cells [196]. Through the induction of oxidative and endoplasmic reticulum stress, HO-1 gene loss of function causes early-onset nuclear cataracts [105].

Here, we looked into how HuLECs' osteogenic differentiation was affected by the Nrf2/HO-1 pathway being activated. Heme was used to activate the Nrf2/HO-1 axis. In order to control the Keap1/Nrf2 system, ROS is essential. Under unstressed circumstances, Keap1, a protein with a high concentration of thiols, binds Nrf2 and encourages its degradation. Overproduction of ROS changes Keap1's conformation directly, enabling Nrf2 stabilization and nuclear translocation [197], [198].

Here, we demonstrate that, as anticipated, the ROS scavenger NAC reduced heme-induced upregulation of Nrf2 expression (**Figure 17**). HuLECs' ECM calcification caused by OM was reduced by heme in a dose-dependent manner (**Figure 18**). Additionally, heme reduced the expression of ALP in HuLECs and inhibited OM-induced upregulation of RUNX2, the master transcription factor of osteogenesis **(Figure 18**). These findings support earlier studies that found heme inhibited the phosphate-induced calcification of VSMCs [199].

We used ML385, a Nrf2 inhibitor that interacts with Nrf2 and blocks the DNA binding activity of the Nrf2-containing heterodimeric transcription factors, to better understand the role of Nrf2 in the anti-calcification effect of heme. Heme no longer has the ability to prevent HuLECs from calcifying in the presence of ML385 (**Figure 19**).

It has been demonstrated that VSMC calcification is reduced when the Nrf2 system is activated by a variety of substances, including dimethyl fumarate, hydrogen sulfide, tert-butylhydroquinone,

rosmarinic acid, or mitoquinone. Dimethyl fumarate, tert-butylhydroquinone, rosmarinic acid, and mitoquinone were among them that reduced excessive ROS production in VSMCs under osteogenic conditions and inhibited calcification, whereas NQO1 upregulation was crucial in hydrogen sulfide-mediated inhibition [91], [200]–[204].

We demonstrated here that heme significantly increases ROS production while only slightly increasing NQO1 expression, leading us to infer that heme-mediated inhibition of calcification in HuLECs was NQO1 independent. Since HO-1 is strongly induced by heme, we demonstrated that heme's anti-calcification effect is eliminated when HO-1 activity is inhibited with ZnPP or SnPP (**Figure 20**). Equimolar amounts of biliverdin, iron, and CO are produced as a result of heme degradation mediated by HO-1. Rapid biliverdin to bilirubin conversion occurs. We investigated whether these molecules have an impact on OM-induced calcification in HuLECs because these heme degradation products have distinct biological effects. We demonstrated that CO has no inhibitory effect on OM-induced HuLECs calcification, while iron has a very strong and bilirubin has a mild effect (**Figure 21**). This outcome is consistent with the iron's strong inhibitory effects on the osteogenic differentiation of VSMCs, osteoblasts, and mesenchymal stem cells that have previously been reported [205]–[207]. Research into and comprehension of iron metabolism in the lens and its role in cataractogenesis is lacking [208]. However, the Lens Opacities Case-Control Study, which included 1380 participants, 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 [209].

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 [92], [210]–[214]. Numerous clinical trials are focusing on the Nrf2 system in a variety of clinical conditions, such as diabetes, cancer, chronic kidney disease, and aging issues [215], [216].

Here, we found that HuLEC calcification is inhibited by heme-mediated Nrf2/HO-1 system activation, a mechanism that may help prevent cataract-related lens calcification. Our findings showed that the most effective anti-calcification agent is intracellular iron, which is liberated from heme during HO-1 catalyzed heme degradation.

For a better understanding of the calcification phenomenon of LECs, additional research is required. In particular, we have no knowledge of the metabolism of pyrophosphate (PPi) in LECs. A highly effective endogenous inhibitor of soft tissue calcification is extracellular PPi. Since PPi is cleaved by ALP, low PPi levels and calcification are typically linked to high ALP expression [35], [217]. We observed elevated ALP levels in calcifying HuLECs; consequently, additional research is required to measure PPi levels and the contribution of this pathway to LEC calcification. Additionally, we must develop *in vivo* animal models of lens calcification and *ex vivo* organ culture. The development of this field would be impossible without these experimental tools, which could also prove to be very helpful when researching and testing potential treatments for lens calcification.

6. Summary

We found that heme-mediated activation of the Nrf2/HO-1 axis is able to protect against high phosphate-induced calcification of valve interstitial cells and lens epithelial cells. We also discovered that inhibition of Nrf2 and HO-1 activities altered the protective effect of heme towards the high phosphate-induced calcification of valve interstitial cells and lens epithelial cells. Since iron is indeed the most important among the degradation products of heme in lens calcification (Fig. 21), the effect of bilirubin, CO, and iron on valvular interstitial cells is more variegated. The generation of reactive oxygen species (ROS) by heme is important to activate the Nrf2/HO-1 axis in high phosphate-induced calcification of lens epithelial cells.

This research, I feel, can contribute to a better understanding of the relationship between CKD, cataracts, and CAVD. Although we show here a beneficial effect of heme against VIC and HuLEC calcification, before we would administer heme to patients to prevent calcification (as a form of heme arginate, a drug in use to treat acute porphyrias), we should consider the well-known pro-oxidant and pro-inflammatory nature of heme. We showed here that the beneficial effect of heme relies on the activation of the Nrf2 antioxidant system. Therefore, using other dietary Nrf2 inducers such as curcumin, resveratrol, or sulphoraphane could be a safer approach.

7. Keywords

- Ectopic calcification
- Hyperphosphatemia
- Osteogenic differentiation
- Aortic valve calcification
- Valve interstitial cells (VICs)
- Lens calcification
- Lens epithelial cells (LECs)
- Nuclear factor erythroid 2-related factor 2 (Nrf2)
- Heme oxygenase-1 (HO-1)
- Nrf2/HO-1 axis
- Heme degradation products

8. List of abbreviations

ABCC6: ATP-binding cassette C subfamily 6

ALP: Alkaline phosphatase

ARE: Antioxidant response element

ATP: Adenosine triphosphate

ApoE: Apolipoprotein E

CAVD: Calcific aortic valve disease

CKD: Chronic kidney disease

CNC: Cap'n'collar

CO: Carbon monoxide

CORM2: CO-releasing molecule 2

CUL3: Cullin 3

Ca: Calcium

DMF: Dimethyl fumarate

ECM: Extra cellular matrix

EMT: Epithelial-to-mesenchymal transition

ER: Endoplasmic reticulum

FT: Ferritin

FTIR: Fourier transform infrared

GACI: Generalized arterial calcification of infancy

GM: Gross-media

GSH: Glutathione peroxidase

H_2O_2: Hydrogen peroxide

H_2S: Hydrogen sulfide

HA: Hydroxyapatite

HO-1: Heme oxygenase-1

HO: Hydroxyl radical

HuLECs: Human Lens epithelial cells

Keap1: Kelch-like epichlorohydrin-related proteins

LECs: Lens epithelial cells

MAPKs: Mitogen-activated protein kinases

MV: Matrix vesicle

NAC: N-acetyl cysteine

NQO1: NAD(P)H:quinone oxidoreductase

Nrf2: NF- E2 p45-related factor 2

O_2: Oxygen

OCN: Osteocalcin

OM: Osteogenic media

PKC: Protein kinase C

PPi: inorganic pyrophosphate

PXE: Pseudoxanthoma elasticum

Pi: Inorganic phosphate

ROS: Reactive oxygen species

Runx2: Runt-related transcription factor 2

SOD: Superoxide dismutase

SnPPIX: Tin-protoporphyrin

TNAP: Tissue non-specific alkaline phosphatase

VC: Vascular calcification

VECs: Valvular endothelial cells

VICs: Valvular interstitial cells

VSMCs: Vascular smooth muscle cells

ZnPPIX: Zinc-protoporphyrin

cOCN: Carboxylated osteocalcin

eNPP1: Ectonucleotide pyrophosphatase phosphodiesterase 1

ucOCN: Uncarboxylated osteocalcin

unOCN: Undercarboxylated osteocalcin

9. Acknowledgement

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11. Appendix

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Subject: PhD Publication List

Candidate: Arpan Chowdhury
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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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