REVIEW ARTICLE

The role of the gasotransmitter hydrogen sulfide in pathological calcification

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Calcification is the deposition of minerals, mainly hydroxyapatite, inside the cell or in the extracellular matrix. Physiological calcification is central for many aspects of development including skeletal and tooth growth; conversely, pathological mineralization occurs in soft tissues and is significantly associated with malfunction and impairment of the tissue where it is located. Various mechanisms have been proposed to explain calcification. However, this research area lacks a more integrative, systemic, and global perspective that could explain both physiological and pathological processes. In this review, we propose such an integrated explanation. Hydrogen sulfide (H₂S) is a newly recognized multifunctional gasotransmitters and its actions have been studied in different physiological and pathological contexts, but little is known about its potential role on calcification. Interestingly, we found that H₂S promotes calcification under physiological conditions and has an inhibitory effect on pathological processes. This makes H₂S a potential therapy for diseases related to pathological calcification.

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1 | INTRODUCTION

Hydrogen sulfide (H₂S) is a naturally occurring gasotransmitter that is involved in a variety of pathophysiological processes, including calcification (Kimura, 2015). The latter is the process by which minerals, mainly calcium-containing crystals, are deposited inside the cell or in the extracellular matrix. Calcium-containing crystals include calcium pyrophosphate dihydrate, basic calcium phosphate (BCP), and calcium oxalate crystals. The main forms of BCP crystals are carbonated-substituted hydroxyapatite (HA), tricalcium phosphate, octacalcium phosphate, and

whitlockite (Ea & Liote, 2009). Calcification occurs physiologically, as part of normal development in skeletal and tooth growth, and may also occur pathologically due to a metabolic imbalance when calcium-containing crystals are formed in tissues such as blood vessels, cartilage, and skin (Orimo, 2010). In this review, we propose an integrative overview of both pathological and physiological calcifications, and we put in context the potential relevance of the gasotransmitter H₂S in the calcification process under physiological and pathological conditions.

2 | H₂S METABOLISM, SIGNALLING, AND BIOLOGICAL EFFECTS

In mammalian cells, the majority of H₂S is synthesized as a by-product of sulfur amino acid (i.e., cysteine) metabolism by three enzymes: **cystathionine β-synthase** (CBS, EC 4.2.1.22), **cystathionine γ-lyase**

Abbreviations: 3-MST, 3-mercaptopropionate sulfurtransferase; BCP, basic calcium phosphate; BMMSCs, bone marrow mesenchymal stem cells; CBS, cystathionine β-synthase; CKD, chronic kidney disease; CPP, calciprotein particles; CPP1, primary calciprotein particles; CPP2, secondary calciprotein particles; CSE, cystathionine γ-lyase; HA, hydroxyapatite; Hcy, homocysteine; MV, matrix vesicles; OA, osteoarthritis; Pi, inorganic phosphate; PPI, pyrophosphate; STS, sodium thiosulfate; TNAP, tissue non-specific alkaline phosphatase; VC, vascular calcification; VSMCs, vascular smooth muscle cells

FIGURE 1 Enzymes involved in cellular H_2S synthesis. H_2S is synthesized in the cells as part of sulfur amino acid metabolism by three enzymes: cystathionine γ -lyase (CSE), which catalyses the conversion from cystathionine to cysteine and produces H_2S from homocysteine and cysteine; cystathionine β -synthase (CBS), which converts homocysteine into cystathionine and produces H_2S from homocysteine and cysteine; and 3-mercaptopyruvate sulfurtransferase (3-MST), which generates H_2S from 3-mercaptopyruvate. The latter is produced by cysteine aminotransferase (CAT) from cysteine

(CTH or CSE, EC 4.4.1.1), and 3-mercaptopyruvate sulfurtransferase (MPST or 3-MST, EC 2.8.1.2). CBS catalyses the condensation of homocysteine (Hcy) and serine to generate cystathionine, and it can also generate H_2S from Hcy and L-cysteine. CSE catalyses the conversion of cystathionine into L-cysteine and generates H_2S from Hcy and L-cysteine. Cysteine aminotransferase catalyses the reaction between L-cysteine and α -ketoglutarate, leading to 3-mercaptopyruvate. 3-MST generates H_2S from 3-mercaptopyruvate (Figure 1; Kimura, 2011).

The levels of free H_2S in circulating blood or cell culture medium at steady state are very low (estimates go from nM to few μM), and the half-life of H_2S is very short as it is serially oxidized to persulfide, sulfite (SO_3^{2-}), thiosulfate ($\text{S}_2\text{O}_3^{2-}$), and sulfate (SO_4^{2-}) by several mitochondrial enzymes. In turn, thiosulfate can be converted into H_2S , thus generating a dynamic equilibrium between H_2S and thiosulfate within the cell. However, the enzymes involved in the latter reaction remain unidentified (Cao et al., 2018).

H_2S exerts many biological functions in the body as it plays important roles in the regulation of the redox state of the cells, inflammation, vasodilation, apoptosis, and metabolism (Cortese-Krott et al., 2017). While H_2S functions have been extensively studied in the context of various conditions, its role in calcification remains incompletely investigated and understood. The biological effects of H_2S can be studied either by providing an exogenous source of H_2S (from H_2S donors) or by interfering with its endogenous production via genetic deletions or overexpression of the enzymes responsible for H_2S synthesis. Under experimental conditions, H_2S donors can be either in the form of inorganic salts (such as NaHS) or as synthetic, organic slow-releasing H_2S donors (such as GYY4137) and plant (garlic)-derived polysulfides. NaHS generates a rapid bolus of H_2S that dissipates within seconds, whereas slow-releasing H_2S donors provide a sustained production of H_2S lasting several hours (Whiteman, Le Trionnaire, Chopra, Fox, & Whatmore, 2011).

H_2S acts via three main mechanisms: (a) At supraphysiological concentrations, H_2S decreases mitochondrial ATP production by inhibition of mitochondrial complex IV (cytochrome c oxidase), whereas at low concentrations, it contributes to energy production and mitochondrial stability by giving electrons to the respiratory chain; (b) H_2S can coordinate and/or reduce metallic centres (i.e., iron), leading either to regulation of protein activity or to H_2S storage; and finally, (c) H_2S signalling can occur via protein sulfhydrylation. Indeed, the action of

H_2S (and of its oxidized forms that are inherently more reactive than H_2S ; Mishanina, Yadav, Ballou, & Banerjee, 2015) on cells involves modification of protein thiol groups to form persulfides (protein persulfhydration/S-sulfhydration; Olson & Straub, 2016). Sulfhydrylation can affect protein function, cellular localization, stability, and resistance to oxidative stress. For instance, H_2S derived either from GYY4137 or from NaHS has been shown to persulfhydrate the p65 subunit of NF- κB at the ^{38}Cys residue, thereby modulating its DNA-binding activity (Sen et al., 2012). H_2S is also able to displace nuclear factor erythroid-2-related factor 2 (Nrf2) from Kelch-like ECH-associated protein-1 (Keap-1) by inducing persulfhydration of ^{151}Cys Keap-1, triggering Nrf2 stabilization and leading to the expression of haem oxygenase (HO-1); Xie et al., 2016). Finally, H_2S persulfhydration can also regulate enzyme activity, as GAPDH activity was enhanced sevenfold after sulfhydrylation of its ^{150}Cys residue (Mustafa et al., 2009).

3 | MECHANISMS OF CALCIFICATION

Biomineralization is a complex process in which interactions between inorganic and organic materials determine the speed of formation and the size of deposited crystals. Crystal formation inside matrix vesicles (MVs) is the main mechanism currently thought to explain physiological mineralization (Bottini et al., 2018). However, MVs are not sufficiently abundant to explain mineralization in bone (Murshed & McKee, 2010), and crystal deposition has also been observed in absence of MVs (Landis, Hodgins, Arena, Song, & McEwen, 1996). Another mechanism that could be involved in mineralization is passive deposition of crystals on collagen fibres, which represent a good scaffold to allow the formation of nucleation centres and subsequent crystal growth (Wiesmann, Meyer, Plate, & Hohling, 2005). In the context of pathological calcification, occurring in inflamed or injured tissues, two additional mechanisms of mineralization have been proposed so far; the first involves transdifferentiation of non-calcifying cells into calcifying-competent cells that secrete MVs in a process similar to physiological mineralization; the second takes place in the liquid phase and depends on the formation of secondary calciprotein particles (CPP2), as those CPP2 are also able to induce transdifferentiation of cells into calcifying-competent cells (Aghagholzadeh et al., 2016). CPP2 contain crystalline calcium phosphate and are formed by spontaneous

FIGURE 2 Mechanisms involved in calcification process. Crystal formation inside matrix vesicles is the main mechanism involved in physiological mineralization. Pathological calcification commonly occurs under hyperphosphataemic and hypercalcaemic conditions. Supersaturation of Ca^{+} and inorganic phosphate (Pi) together with unbalanced regulatory processes leads to the formation of primary and secondary calciprotein particles (CPP). The concurrent release of pro-inflammatory cytokines by macrophages enhances the transdifferentiation of non-calcifying cells into osteogenic or chondrogenic cells (calcifying-competent cells), which express markers such as Runx2 or Sox9, respectively. Transdifferentiated cells have up-regulated pro-calcifying (alkaline phosphatase; ALP) and extracellular matrix (ECM)-degrading enzymes, and they also release matrix vesicles in which crystals are formed. Finally, apoptosis of transdifferentiated cells also contributes to malfunction and tissue degradation. Altogether, these mechanisms ultimately lead to ECM mineralization

transformation of primary calciprotein particles (CPP1), which contain amorphous calcium phosphate (Pasch et al., 2012). Currently, it is unclear if and how the two latter mechanisms are interlinked (Figure 2).

4 | THE ROLE OF H_2S IN PHYSIOLOGICAL CALCIFICATION

In the context of physiological mineralization, osteoblasts facing the osteoid are responsible for calcium-containing crystal formation (Rosenberg, Rosenberg, & Soudry, 2012). In cartilage of children and adolescents, mineralization occurs in the growth plate, where hypertrophic chondrocytes are responsible for mineralization and bone growth (Flores-Silva, Sasso, Sasso-Cerri, Simoes, & Cerri, 2015). In tooth, odontoblasts exert the same function (Bleicher, 2014). Different lines of evidence implicate H_2S in bone and tooth development. Of the three H_2S -producing enzymes, CBS is the most relevant for normal bone formation, as CBS-deficient mice present delayed endochondral ossification (Robert, Maurin, Vayssettes, Siauve, & Janel, 2005) and humans with CBS deficiency (homocystinuria) display skeletal abnormalities (genu valgum, marfanoid features, osteoporosis,

and increased incidence of vertebral fractures; Levasseur, 2009). No skeletal phenotype has been described in either murine or human CSE (Wang & Hegele, 2003) or 3-MST deficiency (Nagahara et al., 2013). CSE is the major enzyme contributing to endogenous H_2S production in primary rat osteoblasts (Zheng et al., 2017). Finally, H_2S is required for the physiological mineralization of periodontal stem cells. Available evidence indicates that endogenous H_2S synthesis has a pro-mineralizing effect not only during physiological bone development and mineralization but also during fracture healing. Indeed, in a fracture model, CSE overexpression promoted healing (Zheng et al., 2017).

Whether exogenous H_2S can also play a role in mineralization has been investigated using different H_2S donors. In rabbits, GYY4137 treatment promoted bone formation in a distraction osteogenesis model (Jiang, Chen, Lu, Zhang, & Fan, 2015). Treatment of bone marrow mesenchymal stem cells (BMMSCs) with H_2S increased calcium influx and **Wnt/ β -catenin** signalling and was required to maintain BMMSC self-renewal and osteogenic differentiation (Liu et al., 2014). Wnt also mediated the protective role of H_2S in preventing ovariectomy-induced bone loss in mice (Grassi et al., 2016). Moreover, H_2S enhanced transcription and activity of alkaline phosphatase and osteocalcin (OCN) in the osteoblastic cell line MC3T3-E1 (Xu et al., 2011). In the same cell line, the

H₂S-releasing drug sodium danshensu stimulated the expression of **bone sialoprotein** and Runx2, alkaline phosphatase activity, and bone nodule formation (Yan et al., 2017). As a whole, exogenous H₂S contributes as a pro-calcifying agent in physiological mineralization.

One of the underlying mechanisms by which H₂S exerts its role in mineralization could be via sulfhydration of key proteins. In osteoblasts, H₂S activates Runx2 by sulfhydration, leading to the expression of genes related with differentiation and mineralization (Zheng et al., 2017). In another study, sulfhydration of different Ca²⁺ channels was important for BMMSC self-renewal and osteogenic differentiation (Liu et al., 2014). Other signalling pathways are also significant players in H₂S-induced mineralization. In particular, sodium danshensu attenuates H₂O₂-induced **p38-ERK1/2** and **JNK**-MAPK phosphorylation and stimulates mineralization of an osteoblastic cell line via activation of the **PI3K/Akt** pathway (Yan et al., 2017).

Another mechanism by which H₂S can control physiological mineralization could be via the regulation of microRNAs (miRNA), as H₂S modulates the synthesis of some miRNAs. For instance, NaHS and Na₂S up-regulate the miR-133a levels in cultured cardiomyocytes and elicit a cardioprotective effect via reducing cell hypertrophy (Kesharwani, Nandi, Sharawat, Shahshahan, & Mishra, 2015). Several miRNAs are associated with regulation of bone development and mineralization. Thus, miR-29b promotes osteogenesis and increases the expression of osteogenic markers (Suh, Lee, Choi, Chung, & Park, 2013), while miR-93 blocks osteoblast differentiation by inhibition of Runx2 protein expression (Zhang et al., 2011). It remains to be investigated if those or other miRNAs involved in bone remodelling and development are modulated by H₂S and if this crosstalk could in part explain the mechanisms of H₂S in calcification (Zhai, Tyagi, & Tyagi, 2017).

5 | THE ROLE OF H₂S IN PATHOLOGICAL CALCIFICATION

Pathological calcification has been reported in almost any site of the body, including the liver (Patnana et al., 2018); brain (Jensen et al., 2013); tendon, (Erdogan, Aydingoz, Kesmezacar, & Erginer, 2004; and head and neck (Mori et al., 2012), although the vasculature, articular cartilage, and kidney are three sites particularly prone to undergo pathological mineralization (Kirsch, 2006). Interestingly, virtually, all cell types in the body, except circulating cells, have the potential to calcify (Table 1). In particular, vascular smooth muscle cells (VSMCs), endothelial cells, quiescent articular chondrocytes, and epithelial cells can undergo differentiation into mineralizing-competent cells. We will focus below on the pathological conditions in which ectopic calcifications occur in vasculature, cartilage, and kidney, and then we will review what is known about the role of H₂S on these ectopic calcifications.

5.1 | H₂S in vascular calcification

Vascular calcification (VC) is associated with both hereditary conditions (Nitschke & Rutsch, 2017) and acquired diseases such as atherosclerosis (Doherty et al., 2003), diabetes (Chen & Moe, 2003), and chronic kidney

disease (CKD; Palit & Kendrick, 2014). There are two main types of VC, which are based on the layer of the vessel wall in which BCP crystals accumulate, medial or intimal. Medial VC is most commonly associated with diabetes mellitus and CKD. It is commonly found in the peripheral arteries, along the elastic lamina and in the extracellular matrix, and develops through a process considered similar to bone formation (Lanzer et al., 2014). Intimal VC is associated with atherosclerosis and is triggered by either oxidative stress or pro-inflammatory pathways (Virmani, Joner, & Sakakura, 2014). In addition to blood vessels, myocardium and cardiac valves are also commonly affected (Giachelli, 2004). Crystals in VC are composed of BCP, mainly in the form of HA, and can lead to adverse cardiovascular events with devastating clinical consequences.

VC is mainly due to transdifferentiation of VSMCs into osteoblast (Liu, Lin, Ju, Chu, & Zhang, 2015) or chondrocyte-like cells (Speer et al., 2009; mechanism of VSMCs calcification is reviewed in Durham, Speer, Scatena, Giachelli, & Shanahan, 2018; Leopold, 2015). Under physiological conditions, the calcification of VSMCs is prevented by circulating inhibitors such as fetuin-A (Reynolds et al., 2005), by in situ inhibitors such as **osteopontin** (Paloian, Leaf, & Giachelli, 2016), **osteoprotegerin** (Zhou et al., 2013), and matrix gla protein (Schurgers, Cranenburg, & Vermeer, 2008), or by increased pyrophosphate concentration (Persy & McKee, 2011). However, VC occurs when the inhibitory mechanisms are overwhelmed. For example, high inorganic phosphate (Pi) uptake mediated by the **sodium/phosphate cotransporter Pit-1** induces VC (Li, Yang, & Giachelli, 2006). High intracellular Ca²⁺ promotes translocation of annexins to the plasma membrane and the release of MV containing calcium phosphate-phosphatidylserine complexes that serve as nucleation cores (Wang & Kirsch, 2002). In diabetes, sustained elevated glucose levels induce transformation of VSMCs into chondrocyte-like cells and facilitate VC (Bessueille, Fakhry, Hamade, Badran, & Magne, 2015). Furthermore, the involvement of CCP2 in VC has been described, in particular in the context of CKD (Pasch, Jahnke-Dechent, & Smith, 2018).

All three H₂S-producing enzymes are expressed in the cardiovascular system, particularly the endothelium (Papapetropoulos et al., 2009; Saha et al., 2016; Shibuya, Mikami, Kimura, Nagahara, & Kimura, 2009), with CSE being the primary source of H₂S (Yang & Wang, 2015). In a rat model of VC, administration of NaHS decreased VC and was accompanied by inhibition of Ca²⁺ uptake and a reduction of tissue non-specific alkaline phosphatase (TNAP) activity (Wu et al., 2006) or by diminishing endoplasmic reticulum stress via activation of the Akt pathway (Yang et al., 2016). In vitro, the H₂S donor NaHS inhibited calcification of human VSMCs and downmodulated expression of TNAP, osteocalcin, and Runx2 and lowered Pi uptake by Pit-1. Accordingly, inhibition of CSE activity results in increased osteoblast differentiation and mineralization (Zavaczki et al., 2011).

In humans, VC is commonly found in patients with CKD (Shanahan, Crouthamel, Kapustin, & Giachelli, 2011). Calciphylaxis is a rare complication of renal failure characterized by arteriolar and subcutaneous calcification, leading to thrombosis and painful ischaemic tissue necrosis. Treatment with sodium thiosulfate (STS) is effective in this context (Rein et al., 2014). In a rat model of adenine-induced CKD, STS was also able to prevent VC (Pasch et al., 2008).

TABLE 1 Cell types able to calcify and associated mechanisms of calcification

Cell type	Model/disease	Species	Mechanism of calcification	Reference
ECs	PCa metastasis samples and PCa cell line overexpressing BMP4	Human	ECs transition into osteoblasts	Lin et al. (2017)
	Mouse ECs recruited by PCa xenograft	Mouse		
	Tissues from patients with FOP	Human	ECs transition into osteoblasts and chondrocytes	Medici et al. (2010)
	Transgenic mice expressing constitutively active ALK2	Mouse		
	Matrix Gla ^{-/-}	Mouse	ECs transition into osteoblasts due to BMP signalling	Yao et al. (2013)
	Model of diabetes			
	In vitro high-glucose conditions	Human	Aortic ECs transition to chondrocytes	Tang et al. (2012)
Epithelial cells	Healthy and cataractous lenses	Human	Lens epithelial cells transition into osteoblasts	Balogh et al. (2016)
	In vitro pro-calcifying medium	Mouse		
	Calcifying odontogenic tumour	Human	Epithelial cells showing intramatrix calcifications	Bouckaert, Raubenheimer, and Jacobs (2000)
Pericytes	In vivo femur periosteal formation	Rat	Pericytes transition to osteoblasts	Brighton et al. (1992)
	In vitro pro-calcifying medium	Bovine	Pericytes express chondrocyte markers Sox9, aggrecan, and type II collagen	Farrington-Rock et al. (2004)
	In vivo pericytes implanted into athymic mice	Mouse		
Neurons	Electrical stimulation in brain	Cat	HA deposition in neuron cytoplasm	Agnew, Yuen, Bullara, Jacques, and Pudenz (2016)
	Primary familial brain calcification SLC20A2 and PDGFRB mutations	Human	HA deposition in vascular tissue and brain parenchyma	Lemos, Ferreira, Keasey, and Oliveira (2013)
	Clinical cases of different diseases	Human	Non-explained mineralization of brain arteries and parenchyma	McCartney and Squier (2014)
Fibroblast	In vitro standard medium	Human	Vesicles required for mineralization	Yajima, Kumegawa, and Hiramatsu (1984)
	In vitro pro-calcifying medium	Human	Skin-derived fibroblast precursor cells transition into osteoblast	Buranasinsup, Sila-Asna, Bunyaratvej, and Bunyaratvej (2006)
	Pseudoxanthoma elasticum	Human	Fibroblast transition into the osteogenic lineage	Okubo et al. (2017)
VSMCs	Review calcification mechanism	—	VSMC differentiation to osteoblastic-like cells and matrix vesicle formation	Leopold (2015)
Adipocytes	In vitro pro-calcifying medium	Mouse	Adipocyte transition to osteoblast like phenotype	Chen, O'Neill, Akl, and Moe (2014)
Synoviocytes	In vitro pro-calcifying medium	Human	OA and RA fibroblast-like synoviocytes express markers such as Runx2. OA synoviocytes calcify more than the RA cells	Sun, Liu, Chen, and Zhu (2012)
Cardiomyocytes	Review clinical findings of myocardial calcification	Human	Crystal deposition on myocardium	Nance, Crane, Halushka, Fishman, and Zimmerman (2015)
		Human	Calcifications in muscle	Jinnin et al. (2002)

(Continues)

TABLE 1 (Continued)

Cell type	Model/disease	Species	Mechanism of calcification	Reference
Skeletal muscle cells	Clinical case of scleroderma with muscle calcification			
Chondrocytes	In vitro calcification medium and HA crystals	Human and mouse	Chondrocyte calcification and human explants response to crystals	Nasi, So, Combes, Daudon, and Busso (2016)
Osteoblasts	In vitro culture of osteoblast on biomaterials	Rat	Matrix vesicle-dependent and independent process	Declercq, Verbeeck, De Ridder, Schacht, and Cornelissen (2005)

Abbreviations: ECs, endothelial cells; HA, hydroxyapatite; OA, osteoarthritis; PCa, prostate cancer; RA, rheumatoid arthritis; VSMCs, vascular smooth muscle cells.

Coexistence of osteoporosis and VC has been frequently observed (Hofbauer, Brueck, Shanahan, Schoppet, & Dobnig, 2007), suggesting a shift of calcium from the skeleton to the arterial vascular wall (Hayden, Tyagi, Kolb, Sowers, & Khanna, 2005). In a murine model of postmenopausal bone loss, CBS and CSE expression in bone marrow was decreased. Serum H₂S level was lower compared with control mice, and treatment with H₂S donors increased bone formation and prevented trabecular bone loss (Grassi et al., 2016). In this case, the pro-mineralizing effect of H₂S in restoring the physiological process, under the conditions of osteoporosis, potentially leads to inhibition of the pathological VC associated to osteoporosis.

VC is also associated with increased local ROS production. H₂O₂ produced by vascular cells promotes the phenotypic switch of VSMCs into the osteogenic lineage (Byon et al., 2008). H₂S is a powerful ROS scavenger (Xie, Liu, & Bian, 2016) suggesting that inhibition of ROS production or direct reaction of H₂S with ROS could be a possible explanation for the anti-mineralizing effect. Furthermore, persulfhydration of crucial proteins by H₂S inhibited mineralization. Sulfhydration of Src homologous-collagen homologue (Shc) adaptor protein (P66Shc) by H₂S inhibited mitochondrial ROS production. P66Shc sulfhydration also impeded its phosphorylation and subsequent activation of mitochondrial redox signalling (Xie et al., 2014). CPP2-induced calcification of VSMCs was largely ameliorated by the H₂S-donor NaHS via activation of the KEAP1/NRF2/NQO1 pathway (Aghagolzadeh et al., 2017).

Overall, even though the mechanisms are not completely understood, H₂S appears to have a protective, anti-calcifying role in the cardiovascular system.

5.2 | H₂S in cartilage calcification

Calcification of joint structures (cartilage, synovium, tendon, and joint capsule) is common and primarily associated with aging and **osteoarthritis** (OA) and leads to cartilage destruction and joint stiffness. Calcium pyrophosphate dihydrate deposits are found primarily in cartilage and synovial fluid, and BCP has been found in cartilage, synovial membrane, and synovial fluid. The prevalence of BCP deposition is related to the severity of OA, and crystals have been identified in 50% of synovial fluids from OA patients (Nalbant et al., 2003) and in 100%

of cartilage samples obtained during joint replacement surgery (Fuerst et al., 2009; Hawellek et al., 2016).

Altered expression of genes involved in the mineralization process, including pyrophosphate channel progressive ankylosis (ANK), the ectonucleotide pyrophosphatase/phosphodiesterase1 (ENPP1), and the TNAP have been observed on cartilage from OA patients (Nguyen et al., 2013). We have demonstrated that injection of BCP crystals in mouse knee joints leads to a chronic arthropathy that reproduces human OA, with low-grade synovial inflammation and cartilage degradation (Ea et al., 2013). Cartilage calcification may modify its biomechanical properties and trigger crystal-induced cell stress, chondrocyte apoptosis, secretion of metalloproteinases, and acute or chronic synovitis via pro-inflammatory cytokine production (Liote & Ea, 2014). Factors that induce cartilage calcification include inflammation, ROS production, and chondrocyte apoptosis. We have shown that in vitro exogenous IL-6 stimulation of chondrocytes promotes calcium crystal formation and up-regulates gene expression of ANK, annexin V, and Pit-1 (Nasi, So, et al., 2016). Increased ROS production and stimulation trigger chondrocyte hypertrophy and apoptosis by activation of **ERK** and **p38 MAPK** pathways in chondrogenic cells (Morita et al., 2007); in turn, chondrocyte hypertrophy induces mineralization (Dreier, 2010). Some authors showed that apoptosis is required for mineralization with annexin V release by hypertrophic chondrocytes (Johnson, Pritzker, Goding, & Terkeltaub, 2001), but this is a topic under debate (Pourmand, Binderman, Doty, Kudryashov, & Boskey, 2007). All these findings suggest that calcium-containing crystals are of etiological and pathogenic importance in OA. However, the involvement of CPP2 in cartilage pathological calcification has not been directly described.

CSE, CBS, and 3-MST are all expressed in joint tissues (bone, cartilage, and synovial membrane; Vela-Anero et al., 2015). The role of H₂S in joint diseases such as OA has been investigated in very few studies but anti-catabolic, anti-inflammatory, and antioxidant properties were demonstrated. Diallyl sulfide, an H₂S donor found in garlic and other alliums, showed to down-regulate **MMP1**, **MMP3**, and **MMP13** expression in chondrosarcoma cells (Williams et al., 2010) and rabbit chondrocytes (Chen et al., 2011) and reduce cartilage degradation in rabbit experimental OA (Chen et al., 2011).

In addition to its anti-catabolic effects, H₂S also has anti-inflammatory properties (Kloesch, Liszt, Steiner, & Broll, 2012; Sieghart

et al., 2015). Short-term treatment with NaHS leads to decreased basal and IL-1 β -induced secretion of IL-6 and IL-8 via modulation of the MAPK and the PI3K/Akt pathways. Moreover, H₂S donors significantly down-regulated IL-1 β -induced expression of NOS2, COX2, PGE synthase, and IL-6 and downstream synthesis of NO, PGE₂, and IL-6. NF- κ B nuclear translocation was also reduced in articular chondrocytes (Burguera, Vela-Anero, Magalhaes, Meijide-Failde, & Blanco, 2014; Ha et al., 2015). Finally, H₂S antioxidant properties were demonstrated in human chondrocytes and mesenchymal progenitor cells (Fox et al., 2012). GYY4137 treatment inhibited oxidative stress-induced cell death, while pharmacological inhibition of H₂S synthesis or knockdown of CBS/CSE with siRNA had the opposite effect. An additional observation, though it is in apparent contrast with the previous findings, showed that IL-1 β , TNF- α , IL-6, or LPS treatment of human chondrogenically differentiated mesenchymal cells up-regulated CSE expression (Fox et al., 2012). As a plausible explanation, H₂S synthesis could be locally induced by pro-inflammatory cytokines by increasing H₂S-producing enzymes, in order to limit joint diseases (Fox et al., 2012).

There is also indirect evidence for the beneficial effect of H₂S from studies in OA. Patients with knee and hip OA who received the allosteric CBS activator S-adenosyl-L-methionine showed a small positive effect in pain and functional scores (Bradley et al., 1994), but the quality of the studies and reports was poor (Rutjes, Nuesch, Reichenbach, & Juni, 2009). Sulfur balneotherapy was found to have positive clinical effects in terms of pain and stiffness when used to treat patients with hand OA (Kovács et al., 2012). Finally, dietary garlic supplementation protected against hip OA (Williams et al., 2010), possibly through the H₂S-releasing activity of diallyl sulfide in garlic (Nakata et al., 2018).

In line with the protective effect of H₂S in chondrocytes and in the context of OA, our group found that the H₂S donor STS reduces the severity of calcification and cartilage damage in an animal model of OA, by decreasing chondrocyte mineralization and crystal-induced inflammation, as well as down-regulating synthesis of catabolic enzymes and ROS generation (Nasi, Ea, Liote, So, & Busso, 2016).

Taken together, the findings suggest that increased exogenous or endogenous levels of H₂S have an anti-mineralizing and potentially clinically relevant effect in OA.

5.3 | H₂S in kidney calcification

Nephrocalcinosis is the generalized deposition of calcium oxalate or calcium phosphate in the kidney (Shavit, Jaeger, & Unwin, 2015). Nevertheless, nephrocalcinosis does not necessarily give rise to renal stone formation, which is commonly an independent event. Medullary nephrocalcinosis is the most frequent form of kidney calcification where characteristic crystal clusters are present in each renal pyramid (Priante et al., 2017).

Kidney calcification is caused by either increased crystal retention or increased crystal formation. Crystals should be continuously formed in urine due to Pi and Ca²⁺ supersaturation, but the presence of citrate, magnesium, and small amounts of inhibitory proteins creates an anti-aggregation environment (Kumar, Pena de la Vega, Farrell, & Lieske, 2005). Moreover, healthy kidney has a non-adherent epithelium, which facilitates tubular passage of crystals (Verhulst, 2003). Damage to renal epithelium by inflammation or other injury can decrease its anti-adherent properties, thus facilitating crystal

FIGURE 3 Effects of H₂S on physiological and pathological calcification. H₂S exhibits selective roles in physiological or pathological contexts. (a) Under physiological conditions, H₂S (1) promotes differentiation of mesenchymal stem cells into calcifying-competent cells (osteoblast, odontoblast, and chondrocytes at the growth plate), (2) promotes the expression of calcifying genes such as alkaline phosphatase (ALP), and (3) inhibits mesenchymal-induced osteoclast precursor differentiation. (b) In the pathological context, H₂S (1) acts as ROS scavenger decreasing oxidative stress and inhibiting cytokine production by macrophages, (2) inhibits transdifferentiation of non-calcifying cells to a pro-calcifying phenotype, and (3) acts as ROS scavenger for ROS produced by transdifferentiated cells and also inhibits cytokine and calcification genes from transdifferentiated cells. H₂S could also act on the transition of primary calciprotein particles (CPP) to secondary CPP, although this has not yet been investigated

TABLE 2 Summary of H₂S role on pathological and physiological calcification

Calcification	Organ	Model	Specie	Source of H ₂ S	Effect/role	Reference
Physiological	Bone and teeth	CBS mutations	Human	Endo (CBS)	Pro-mineralizing	Levasseur (2009)
		CBS ^{-/-}	Mouse	Endo (CBS)	Pro-mineralizing	Robert et al. (2005)
		CBS ^{-/-}	Mouse	Endo (CBS)	Pro-mineralizing	Liu et al. (2014)
		BMSCs		Exo (NaHS and GYY)		
		Overexpression or silencing of CSE in osteoblasts	Rat	Endo (CSE)	Pro-mineralizing	Zheng et al. (2017)
		Femur fracture		Exo (NaHS and GYY)		
		Periodontal stem cells	Human	Endo (CBS and CSE)	Pro-mineralizing	Cen et al. (2016)
		Distraction osteogenesis	Rabbit	Exo (GYY)	Pro-mineralizing	Jiang et al. (2015)
Pathological	Vasculature	Osteoblastic cell line MC3T3-E1	Mouse	Exo (NaHS or SDSS)	Pro-mineralizing	Xu et al. (2011) and Yan et al. (2017)
		Vitamin D ₃ plus nicotine	Rats	Exo (NaHS)	Anti-mineralizing	Wu et al. (2006) and Yang et al. (2016)
		VSMCs in calcification media	Human	Endo (CSE) and exo (NaHS)	Anti-mineralizing	Zavaczki et al. (2011)
		CKD patients				
	Cartilage	VC by adenine-induced CKD	Rats	Exo (STS)	Anti-mineralizing	Pasch et al. (2008)
		Calciophylaxis	Human	Exo (STS)	Anti-mineralizing	Bourgeois and De Haes (2016)
		OA Mnx model and primary chondrocytes	Mouse	Exo (STS)	Anti-mineralizing	Nasi, So, et al. (2016)
		Genetic hypercalciuric	Rat	Exo (STS)	Anti-mineralizing	Asplin et al. (2009)
	Kidney	Passive precipitation of crystals in urine	—	NaHS and STS	Anti-mineralizing	Vaitheeswari et al. (2015)

Abbreviations: BMSCs, bone marrow stem cells; CBS, cystathionine β-synthase; CKD, chronic kidney disease; CSE, cystathionine γ-lyase; OA, osteoarthritis; SDSS, sodium danshensu; STS, sodium thiosulfate; VSMCs, vascular smooth muscle cells.

accumulation in the kidney pelvis (Priante et al., 2017). Transdifferentiation of human kidney medullary cells into osteoblastic-like cells has been described (Gambaro et al., 2004; Mezzabotta et al., 2015) and, in addition, a canine kidney epithelial cell line, cultured in high phosphate medium, was able to calcify in vitro (Kageyama et al., 1996). Osteogenic genes such as Runx1 and 2, osterix, **BMP2** and BMP7, **bone morphogenetic protein receptor 2** (BMPR2), and collagen I are up-regulated in the kidney of hyperoxaluric rats (Joshi, Clapp, Wang, & Khan, 2015). Altogether, these results suggest that both a passive process and a cell-mediated mechanism could contribute to nephrocalcinosis.

Expression of the three H₂S-producing enzymes has been reported in the kidney. While CSE is mainly expressed by endothelial cells, podocytes, mesangial cells, proximal and distal tubular epithelial cells, and peritubular capillaries (Bos et al., 2013; Lee et al., 2012), CBS is expressed by the proximal tubules (Yamamoto et al., 2013). However, it has recently been described that H₂S in the kidney is generated from D-cysteine rather than L-cysteine, involving D-amino acid oxidase and 3-MST (Shibuya et al., 2013).

There are only a few reports of the effects of H₂S on kidney calcification. Genetic hypercalciuric, stone-forming, rats treated with thiosulfate showed significantly decreased stones compared with non-treated animals, in part due to lower urine pH and thus lower calcium phosphate supersaturation (Asplin et al., 2009). In another in vitro study, H₂S and thiosulfate inhibited passive calcium oxalate

agglomeration in both urine and phosphate buffer (Vaitheeswari, Sriram, Brindha, & Kurian, 2015). Finally, in a case report, oral supplementation with potassium gluconate, STS and alkalinization in a patient with erythrocytosis associated with renal tubular acidosis type I, halted the progression of nephrocalcinosis (Agroyannis, Tzanatos, Vlahakos, & Mallas, 2001). Clinical nephrolithiasis in humans was also prevented by daily intake of thiosulfate (Yatzidis, 2004a; Yatzidis, 2004b).

These observations suggest that H₂S could have a protective effect in both passive and cell-mediated mechanisms of kidney calcification.

6 | DISCUSSION AND CONCLUSION

In both physiological and pathological calcifications, the formation and deposition of HA crystals in tissues is a regulated process that takes place in calcification-competent cells, mediated by MV formation that leads to crystal deposition on collagen and other connective tissue structures. The formation of MV containing HA crystals has been extensively studied and reviewed (Anderson, 2007; Golub, 2009).

In physiological calcification, chondrocytes form MV in a polarized manner that is regulated by factors that stimulate chondrocyte maturation and differentiation. Osteoblasts also form MV at the

calcification front, though the detailed understanding of how this process is regulated is still lacking. In pathological calcification, cells that normally are not known to calcify become calcification-competent through a process of transdifferentiation. Recent studies have shown that both inflammation (Lencel, Hardouin, & Magne, 2010) and oxidative stress (You et al., 2009) can induce this transition. In addition, the role of CPP has been highlighted as a factor that potentiates transdifferentiation. Higher circulating levels of CPP2 are associated with increased pathological calcification in kidney disease (Figure 3). To date, there are no data to show that CPP participates in physiological calcification nor is it established whether CPP can directly induce MV formation in pathological calcification.

In the context of physiological calcification (in precursor cells or naturally calcifying-competent cells), H₂S has a pro-mineralizing effect (Table 2, physiological). In contrast, H₂S has an anti-mineralizing effect on cells that calcify only under pathological conditions due to transdifferentiation (Table 2, pathological; Figure 3). A general mechanism that could explain the dual role of H₂S is protein sulfhydrylation, which is one of the main H₂S signalling mechanisms. H₂S could sulfhydrylate selective proteins in naturally calcifying cells, such as Runx2, activating calcification proteins, whereas under pathological situation, protein sulfhydrylation of targets such as Keap-1, which activates expression of HO-1 via Nrf2 nuclear translocation (Guo, Liang, Shah Masood, & Yan, 2014), could lead to negative regulation of mineralization (Lin et al., 2010). One interesting approach to explore the role of H₂S in the context of both pathological and physiological mineralization could be to obtain and compare the profile of sulfhydrated proteins in both conditions. Our hypothesis would be that H₂S differentially sulfhydrates proteins in physiological and pathological process. Nevertheless, the techniques to detect sulfhydrated proteins are relatively new and still under development, and currently, there are no data on the proteomic differences in these two calcification pathways.

The anti-calcification effects of H₂S may also be via its activities on pathways such as oxidative stress, inflammation, and endoplasmic reticulum stress that have all been shown to drive transdifferentiation during pathological mineralization. H₂S acts as a ROS scavenger (Xie, Liu, & Bian, 2016) and has an anti-inflammatory effect in most studies (Wallace et al., 2015). It can also block the transition of cells from endothelial to mesenchymal phenotype (a process that precedes calcification) via inhibition of endoplasmic reticulum stress (Ying et al., 2016). Altogether, the data demonstrate that H₂S can impair mineralization process at multiple steps.

The positive role of H₂S in physiological calcification is based in part on genetic studies that examined the phenotypes of CSE and CBS deficiency (Table 2). However, CSE- and CBS-deficient mice are characterized by elevated plasma levels of Hcy (Robert et al., 2005), which also has effects on mineralization. Thus, it is difficult to decide whether the main mechanism is due to CSE/CBS loss or Hcy accumulation and controversial results have been obtained. In both mice (Ishii et al., 2010) and humana (Wang, 2003) with CSE-deficiency, there is cystathioninuria and hyperhomocysteinaemia but no evidence of skeletal abnormalities. Hcy decreased mineralization in differentiating chick limb-bud mesenchymal cells (Khan et al., 2001), whereas it

potentiated calcification in cultured rat aortic smooth muscle cells via induction of Ca²⁺ accumulation and TNAP activity (Li, Chai, Tang, & Du, 2003). Hcy is also known to be toxic for vascular cells, while other cell types that contain the full complement of metabolic enzymes to process Hcy through remethylation and transsulfuration catabolic pathways are less affected. As the metabolism of Hcy in vascular cells is dependent on B₁₂-folate-dependent remethylation, they are especially vulnerable to higher levels of Hcy (Hayden et al., 2005). In man, CSE deficiency results in homocysteinuria that is associated with early osteoporosis (Lampropoulos et al., 2016) and bone fracture in aged persons (McLean et al., 2004). Interestingly, treatment of CBS-deficient mice with H₂S donors rescued the osteopenia phenotype, without changing Hcy levels, indicating that the phenotype could be more related with lower levels of H₂S than the hyperhomocysteinaemia (Liu et al., 2014). Nonetheless, it has been reported that H₂S is a free radical scavenger in rat VSMCs cultured in vitro (Yan et al., 2006). Further research is required to distinguish between direct effects of H₂S levels and Hcy, as an explanation of its effect on mineralization in the context of CSE/CBS deficiency.

In conclusion, we have highlighted how endogenous and exogenous H₂S production can affect the calcification process. In all examples of pathological calcification, H₂S exerted an anti-mineralizing effect. Therefore, strategies able to increase H₂S by synthetic, slow-releasing, controllable H₂S donors could be valuable. Interestingly, an **H₂S-releasing derivative of naproxen (ATB346)** is being developed for the treatment of osteoarthritis (Wallace & Wang, 2015). ATB-346 was able to prevent bone loss in a model of ligature-induced periodontitis (Herrera et al., 2015), but its effect on joint calcification has to be evaluated. Finally, new CBS/CSE selective stimulating compounds, yet to be discovered, could be promising therapeutic drugs to fight ectopic calcifications.

6.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Fabbro et al., 2017a, 2017b; Alexander, Kelly et al., 2017).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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