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# Hydrogen sulfide inhibits the calcification and osteoblastic differentiation of vascular smooth muscle cells

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Osteoblastic differentiation of vascular smooth muscle cells (VSMCs) is involved in the pathogenesis of vascular calcification. Hydrogen sulfide (H<sub>2</sub>S) is a gas endogenously produced by cystathionine  $\gamma$ -lyase in VSMC. Here we determined whether H<sub>2</sub>S plays a role in phosphate-induced osteoblastic transformation and mineralization of VSMC. Hydrogen sulfide was found to inhibit calcium deposition in the extracellular matrix and to suppress the induction of the genes involved in osteoblastic transformation of VSMC: alkaline phosphatase, osteocalcin, and Cbfa1. Moreover, phosphate uptake and phosphate-triggered upregulation of the sodium-dependent phosphate cotransporter (Pit-1) were also prevented by H<sub>2</sub>S. Reduction of endogenous production of H<sub>2</sub>S by inhibition of cystathionine  $\gamma$ -lyase activity resulted in increased osteoblastic transformation and mineralization. Low plasma levels of H<sub>2</sub>S, associated with decreased cystathionine  $\gamma$ -lyase enzyme activity, were found in patients with chronic kidney disease receiving hemodialysis. Thus, H<sub>2</sub>S is a potent inhibitor of phosphate-induced calcification and osteoblastic differentiation of VSMC. This mechanism might contribute to accelerated vascular calcification in chronic kidney disease.

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Vascular calcification is implicated in the pathogenesis of various vascular diseases and can result in devastating clinical consequences. It is related to an increased risk of cardiovascular morbidity and complications such as atherosclerotic plaque burden,<sup>1–3</sup> myocardial infarction,<sup>4,5</sup> coronary artery disease,<sup>6,7</sup> post-angioplasty dissection,<sup>8</sup> and increased ischemic episodes in peripheral vascular disease.<sup>9</sup> It has also been shown to be a strong marker of cardiovascular events in patients with diabetes and chronic kidney disease (CKD).<sup>6</sup> Studies also indicate that coronary calcification may be predictive of increased propensity for sudden cardiac death.<sup>10,11</sup> Strong associations between arterial calcification and stiffness, pulse pressure, or mortality in dialysis patients have also been shown to contribute to the high rates of cardiac and peripheral ischemic disease and left ventricular hypertrophy in this population.<sup>12–14</sup> Although the precise mechanisms of vascular calcification are not completely understood, abnormalities in mineral metabolism are considered important risk factors. High extracellular phosphate (Pi) has been widely established to induce vascular calcification.<sup>15–19</sup> Pi uptake through a sodium-dependent phosphate cotransporter, Pit-1, is essential for vascular smooth muscle cell (VSMC) calcification and phenotypic modulation in response to elevated Pi.<sup>20</sup> Contrary to previous conception, accumulating evidence now suggests that vascular calcification is a delicate and well-regulated cellular process in which VSMC gain an osteoblastic phenotype. This is indicated by the increase in expression of the core-binding factor alpha-1 (Cbfa1), which is an osteoblast-specific transcription factor required for osteoblast differentiation, bone matrix gene expression, and, consequently, bone mineralization.<sup>21</sup> Upregulation of alkaline phosphatase (ALP; an important enzyme in early osteogenesis) and osteocalcin (OC; a major non-collagenous protein in bone matrix that regulates mineralization) was also shown to occur.<sup>22</sup>

Hydrogen sulfide (H<sub>2</sub>S) has traditionally been considered a toxic gas; however, recently, it has been recognized as the

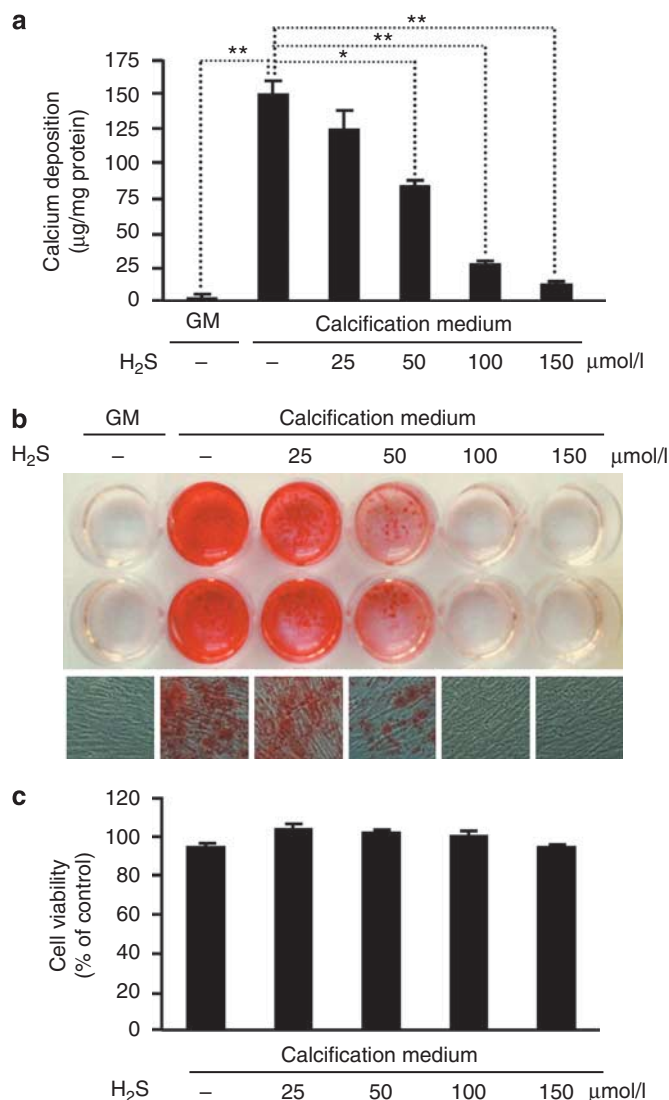
third endogenous gaseous transmitter besides carbon monoxide and nitric oxide.<sup>23</sup> In mammals, H<sub>2</sub>S is produced by two enzymes: cystathionine β-synthase and cystathionine γ-lyase (CSE) via the transsulfuration pathway using homocysteine, cystathionine, and L-cysteine as substrates. On the basis of the literature, the concentration of H<sub>2</sub>S in the peripheral system ranges from 30 to 50 μmol/l,<sup>24</sup> although recently it has been suggested that this is an overestimation (reviewed in ref. 25). It was shown that in the aorta, H<sub>2</sub>S concentration is 20- to 100-fold higher than that in other tissues.<sup>26</sup> In the vasculature, H<sub>2</sub>S is generated mainly by vascular smooth muscle cells by CSE.<sup>27</sup> H<sub>2</sub>S exerts a number of physiological actions in the cardiovascular system: (i) it dilates blood vessels mostly, if not exclusively, by a mechanism that involves opening of adenosine 5' triphosphate-sensitive K<sup>+</sup> channels of smooth muscle cells,<sup>28</sup> (ii) it is cardioprotective against ischemic/reperfusion damage and myocardial inflammation,<sup>29</sup> and (iii) it preserves both mitochondrial structure and function after injury.<sup>30</sup> Accumulating evidence suggests that it has direct inhibitory effects on the development of atherosclerosis. H<sub>2</sub>S induces apoptosis,<sup>31,32</sup> suppresses endothelin-induced proliferation of VSMC,<sup>33</sup> and influences vascular inflammatory reactions.<sup>34</sup> It has also been demonstrated that H<sub>2</sub>S inhibits the oxidation of low-density lipoprotein and lipids from atheromatous plaques.<sup>35</sup> In fact, the progression of atherosclerosis was shown to be significantly slower in patients with Down's syndrome, a state of H<sub>2</sub>S overproduction.<sup>36</sup> In the context of CKD, plasma level of H<sub>2</sub>S was reported to be decreased in stage 5 CKD patients.<sup>37</sup> Calciphylaxis can be successfully treated with intravenous administration of sodium thiosulfate,<sup>38,39</sup> a drug that—as discovered recently—increases H<sub>2</sub>S biogenesis by inducing CSE expression.<sup>40</sup> In addition, H<sub>2</sub>S was recently reported to ameliorate vascular calcification induced by vitamin D<sub>3</sub> plus nicotine in rats.<sup>41</sup> However, the mechanisms underlying the protective effect of H<sub>2</sub>S and the role of CSE in vascular calcification in CKD have not been explored. The purpose of this study was to investigate the role that H<sub>2</sub>S and biogenesis of H<sub>2</sub>S may have in the process of VSMC mineralization and transition of smooth muscle cells into osteoblast-like cells. We observed that H<sub>2</sub>S from both extracellular and intracellular origin suppresses high Pi-induced calcification and osteoblastic differentiation of human aortic smooth muscle cells (HAoSMCs) through suppression of Pit-1, and that the enzyme activity of CSE is decreased in stage 5 CKD.

## RESULTS

### H<sub>2</sub>S decreases HAoSMC mineralization in a dose-responsive manner

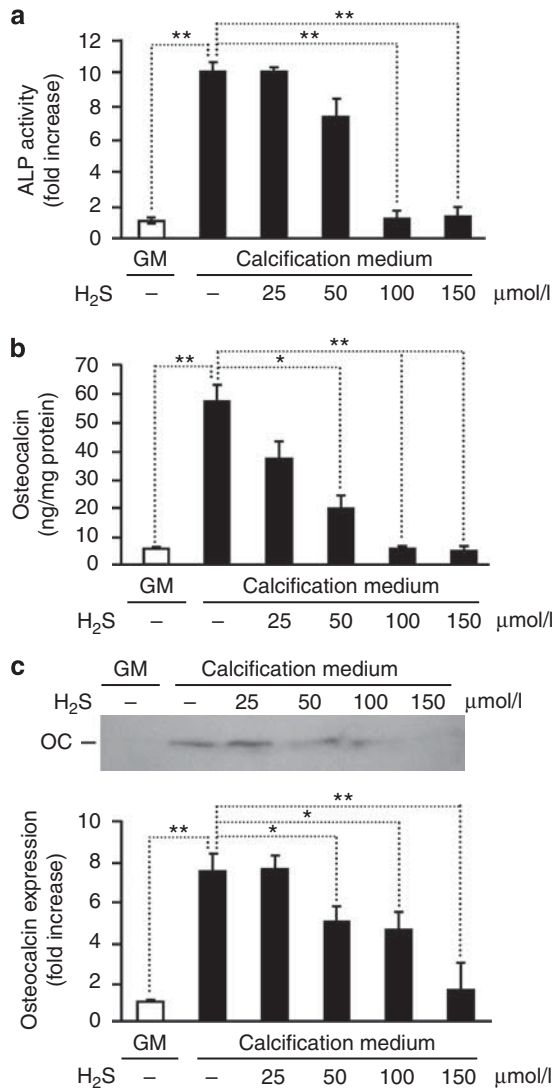
To establish an *in vitro* model of human vascular calcification, we cultured HAoSMC in calcification medium, which was prepared by addition of 3 mmol/l Pi to the growth medium (GM). HAoSMC was cultured in calcification medium in the presence or absence of H<sub>2</sub>S for 7 days, followed by calcium measurement (Figure 1a). As expected, Pi provoked

calcification, whereas in the control culture no deposits were formed during this period. Importantly, H<sub>2</sub>S inhibited calcium deposition in a dose-responsive manner, providing a significant inhibition at concentrations of ≥ 50 μmol/l. To confirm the effect of H<sub>2</sub>S on calcium deposition, we also performed Alizarin red staining of HAoSMC (Figure 1b). HAoSMC maintained in calcification medium showed development of granular calcium deposits throughout the cell culture. Supplementation of the calcification medium



**Figure 1 | H<sub>2</sub>S decreases HAoSMC mineralization in a dose-responsive manner.** (a–c) HAoSMCs were cultured in GM or in calcification medium alone or supplemented with 25, 50, 100, and 150 μmol/l of H<sub>2</sub>S for 7 days. (a) Calcium content is shown as mean ± standard deviation of three independent experiments conducted in duplicates; \**P* < 0.05, \*\**P* < 0.01. (b) Representative images of Alizarin red staining of plates (upper) and microscopic views (×100, lower) from three independent experiments are shown. (c) MTT assay is shown as mean ± standard deviation of two independent experiments conducted in triplicates. GM, growth medium; HAoSMC, human aortic smooth muscle cell; H<sub>2</sub>S, hydrogen sulfide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

with H<sub>2</sub>S prevented the accumulation of calcium in the extracellular matrix. To test the viability of cells exposed to H<sub>2</sub>S, we carried out MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 1c). We did not observe a decline in viability of HAoSMC challenged with H<sub>2</sub>S in the concentration range of 25–150 μmol/l.



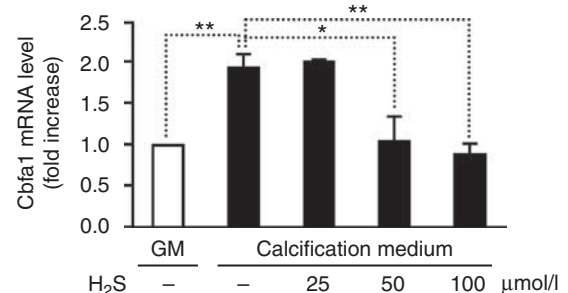
**Figure 2 | H<sub>2</sub>S inhibits Pi-mediated upregulation of osteoblast-specific proteins in HAoSMC.** (a–c) HAoSMCs were cultured in GM or in calcification medium in the absence or presence of different concentrations of H<sub>2</sub>S for 7 days. (a) ALP activity is presented as means ± standard deviation of three independent experiments each conducted in duplicates; \*\*\**P* < 0.01. (b) Osteocalcin level in ethylenediaminetetraacetate (EDTA)-solubilized extracellular matrix was determined by enzyme-linked immunosorbent assay and shown as means ± standard deviation of three independent experiments each conducted in duplicates; \**P* < 0.05, \*\**P* < 0.01. (c) Osteocalcin detected by western blot from EDTA-solubilized extracellular matrix. Blot is representative of three independent experiments. A graph of band intensity means ± standard deviation of three experiments is shown; \**P* < 0.05, \*\**P* < 0.01. ALP, alkaline phosphatase; GM, growth medium; HAoSMC, human aortic smooth muscle cell; H<sub>2</sub>S, hydrogen sulfide.

### H<sub>2</sub>S inhibits osteoblastic differentiation of HAoSMC

It has been shown that vascular calcification *in vivo* resembles bone mineralization; therefore, we examined whether H<sub>2</sub>S suppresses the phenotype transition of HAoSMC into osteoblast-like cells. Because upregulation of ALP, an important enzyme in osteogenesis, and OC, a major non-collagenous protein found in bone matrix, are implicated in the pathogenesis of vascular calcification, we measured the level of their expression in HAoSMC treated with H<sub>2</sub>S. Although HAoSMC maintained in calcification medium for 7 days exhibited around a 10-fold increase in ALP activity compared with controls, addition of H<sub>2</sub>S to the calcification medium resulted in a dose-dependent suppression providing a complete attenuation at a dose of 100 μmol/l (Figure 2a). Similar to ALP activity, the induction of OC was also abolished by H<sub>2</sub>S. Maintaining HAoSMC in calcification medium for 7 days led to a >10-fold increase in OC content in the extracellular matrix compared with control. H<sub>2</sub>S decreased the expression of OC to the basal level, observed in HAoSMC (Figure 2b and c) cultured in GM.

Next, we examined the level of Cbfa1, a transcription factor required for osteoblast differentiation, in our *in vitro* model. Pi increased Cbfa1 mRNA level by ~1.8-fold compared with cells grown in control medium. As shown in Figure 3, H<sub>2</sub>S completely suppressed the induction of Cbfa1 mRNA provoked by elevated Pi.

Evidence suggests that the effects of hyperphosphatemia are mediated via Pit-1, which facilitates entry of Pi into vascular cells. To further explore the mechanism by which H<sub>2</sub>S inhibits vascular calcification, we measured Pi uptake of HAoSMC in the presence or absence of H<sub>2</sub>S. Intriguingly, addition of H<sub>2</sub>S inhibited Pi uptake in a dose-responsive manner, providing a significant and complete suppressions at concentrations of 50 μmol/l and 100 μmol/l, respectively (Figure 4a). To explore the mechanism underlying the inhibition of Pi uptake, we examined the expression of



**Figure 3 | H<sub>2</sub>S prevents Pi-mediated upregulation of osteoblast-specific transcription factor Cbfa1 in HAoSMC.** HAoSMCs were cultured in GM or in calcification medium alone or in the presence of H<sub>2</sub>S (25, 50 and 100 μmol/l) for 24 h. Cbfa1 messenger RNA (mRNA) levels were determined by quantitative reverse transcriptase-polymerase chain reaction. Results are shown as means ± standard deviation of five independent experiments conducted in triplicates; \**P* < 0.05, \*\**P* < 0.01. Cbfa1, core-binding factor alpha-1; GM, growth medium; HAoSMC, human aortic smooth muscle cell; H<sub>2</sub>S, hydrogen sulfide.

Pit-1. Maintaining HAoSMC in calcification medium, we observed a 1.6-fold elevation in Pit-1 mRNA level. Addition of H<sub>2</sub>S into the calcification medium prevented the increase in Pit-1 expression (Figure 4b).

It was recently reported that induction of ferritin prevents Pi-mediated calcification and osteoblastic differentiation of HAoSMC.<sup>42</sup> We therefore tested whether H<sub>2</sub>S modulates its expression in HAoSMC. As shown in Figure 5, the expressions of H- and L-ferritin were not altered, suggesting that the mechanism by which H<sub>2</sub>S prevents calcification is independent of ferritin.

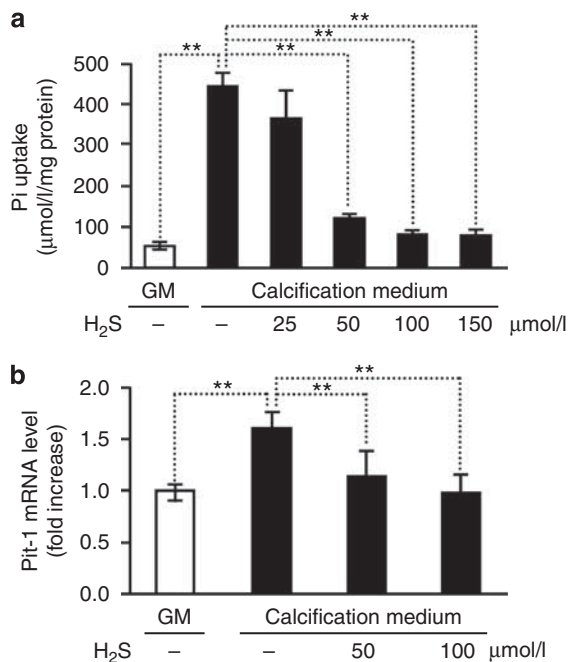
#### Endogenous production of H<sub>2</sub>S inhibits calcification and osteoblastic differentiation of HAoSMC

In the vasculature, H<sub>2</sub>S is produced by VSMC expressing the pyridoxal-5'-phosphate-dependent enzyme CSE. On the basis of our observations, we thereby hypothesized that inhibition of CSE enzyme activity would lead to increased mineralization. Therefore, first we inhibited CSE using dl-propargylglycine (PPG), a well-known inhibitor of CSE activity. Cells treated with PPG showed a gradual decrease in CSE enzyme activity (Figure 6a). Suppression of CSE by PPG almost doubled the deposition of calcium in the extracellular

matrix of HAoSMC maintained in calcification medium for 7 days (Figure 6b), as compared with cells cultured in calcification medium without PPG. Accordingly, PPG provoked a significant additional increase in the activity of ALP (Figure 6c) and expression of OC (Figure 6d and e) by 73% and 120%, respectively. As a second approach to decrease CSE activity, we transfected HAoSMC with CSE-specific small interfering RNA (siRNA). Transfection provided an ~70% reduction in CSE enzyme activity, which was accompanied by increased calcium deposition in HAoSMC (Figure 7).

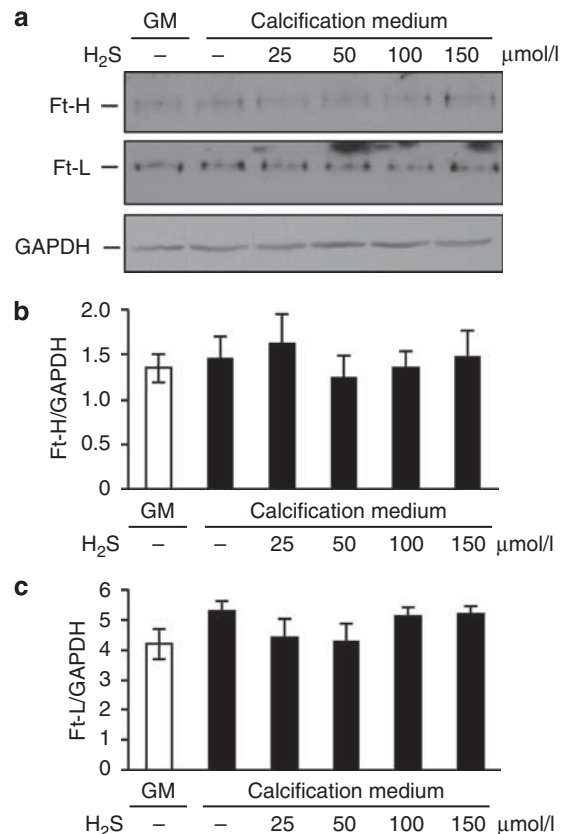
#### Decreased plasma H<sub>2</sub>S levels are associated with reduced CSE activity in stage 5 CKD patients

In agreement with previous observations,<sup>37</sup> we found that plasma concentration of H<sub>2</sub>S was decreased in stage 5 CKD patients, and that H<sub>2</sub>S level was further lowered by hemodialysis (Figure 8a). As the main enzyme responsible for H<sub>2</sub>S biogenesis in the vasculature is CSE, we compared its expression and activity in mononuclear cells derived from stage 5 CKD patients treated with hemodialysis and



**Figure 4 | H<sub>2</sub>S inhibits phosphate uptake by HAoSMCs.**

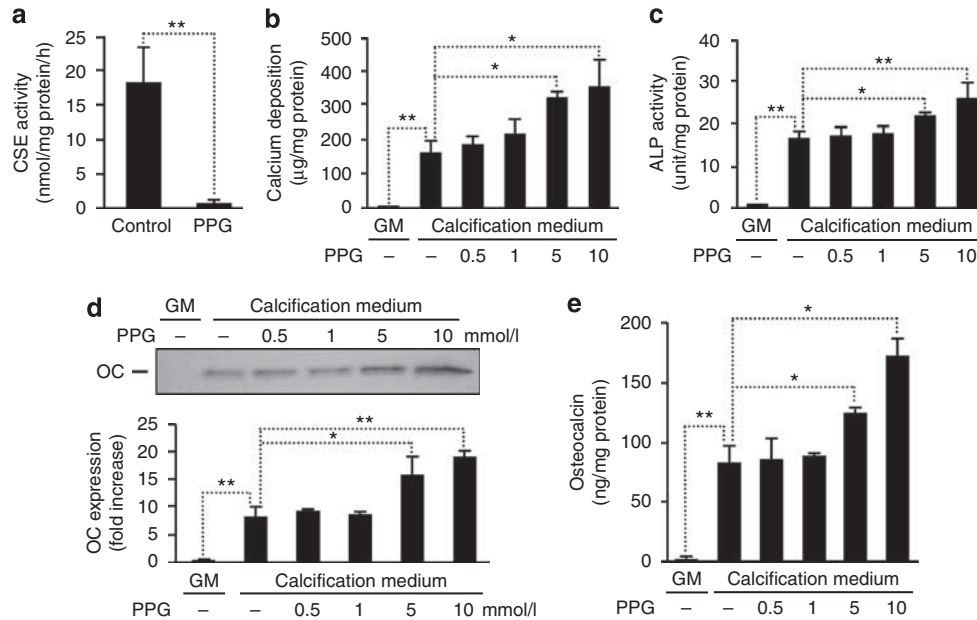
(a) HAoSMCs were cultured in GM and calcification medium alone, or supplemented with H<sub>2</sub>S (25, 50, 100, 150 µmol/l) for 4 days. Pi levels were determined from cell lysates and shown as means ± standard deviation of three independent experiments conducted in duplicates; \*\**P* < 0.01. (b) HAoSMCs were cultured in GM and calcification medium alone, or supplemented with H<sub>2</sub>S (25, 50, 100, 150 µmol/l) for 48 h and Pit-1 messenger RNA level was determined by quantitative reverse transcriptase-polymerase chain reaction. Results are presented as means ± standard deviation of five independent experiments conducted in triplicates; \*\**P* < 0.01. GM, growth medium; HAoSMC, human aortic smooth muscle cell; H<sub>2</sub>S, hydrogen sulfide.



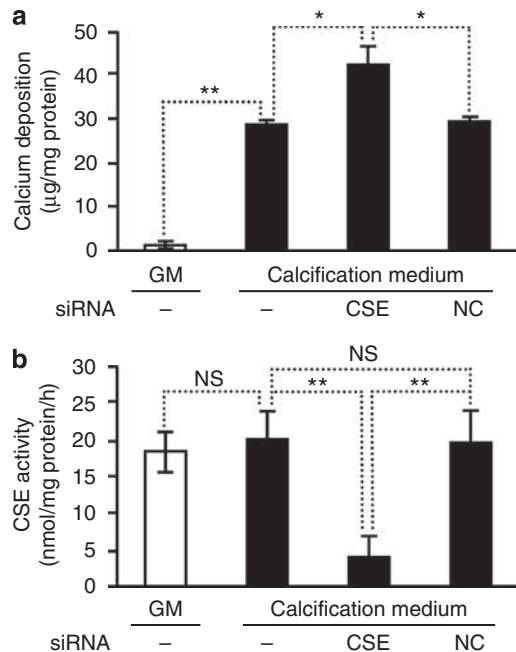
**Figure 5 | H<sub>2</sub>S does not alter the expression of H- and L-ferritin in HAoSMCs.**

(a-c) HAoSMCs were cultured in growth medium or in calcification medium in the absence or presence of H<sub>2</sub>S (25, 50, 100, 150 µmol/l) for 24 h. (a) H- and L-ferritin detected by western blot from cell lysates. Membranes were reprobbed with GAPDH for normalization. Blots are representatives of three experiments. (b, c) Bar graphs show densitometric measurement of band intensities as means ± standard deviation of three experiments. Ft, ferritin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM, growth medium; HAoSMC, human aortic smooth muscle cell; H<sub>2</sub>S, hydrogen sulfide.





**Figure 6 | Inhibition of endogenous production of H<sub>2</sub>S by dl-propargylglycine (PPG) promotes calcification and osteoblastic differentiation of HAoSMCs.** (a) CSE activity determined from HAoSMC in the absence or presence of PPG (10 mmol/l) is shown as means ± standard deviation of three independent experiments; \*\**P* < 0.01. (b–e) HAoSMCs were cultured in GM or in calcification medium alone or in the presence of PPG (0.5, 1, 5 and 10 mmol/l) for 7 days. Extracellular calcium deposition (b), ALP activity (c), and osteocalcin (OC) expression (d, e) are shown. Data are expressed as means ± standard deviation of three independent experiments each conducted in duplicates; \**P* < 0.05, \*\**P* < 0.01. ALP, alkaline phosphatase; CSE, cystathionine γ-lyase; GM, growth medium; HAoSMC, human aortic smooth muscle cell; H<sub>2</sub>S, hydrogen sulfide.



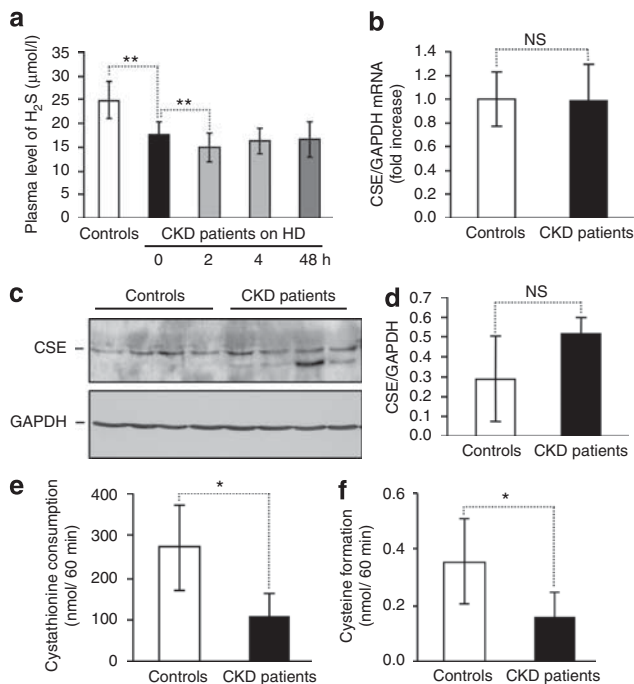
**Figure 7 | Silencing of cystathionine γ-lyase (CSE) provokes mineralization of HAoSMCs.** (a) Calcium deposition of HAoSMC transfected with small interfering RNA (siRNA) for CSE or negative control siRNA (NC) after culturing cells in calcification medium for 4 days is shown as means ± standard deviation of three independent experiments; \**P* < 0.05, \*\**P* < 0.01. (b) CSE activity of transfected HAoSMC 48 h post transfection is shown as means ± standard deviation of three independent experiments; \*\**P* < 0.01. GM, growth medium; HAoSMC, human aortic smooth muscle cell; H<sub>2</sub>S, hydrogen sulfide; NS, non-significant.

healthy controls. Levels of CSE mRNA and protein expression were similar in CKD patients and controls (Figure 8b–d). Importantly, CSE enzyme activity—which was measured by cystathionine consumption and cysteine production—was markedly decreased in mononuclear cells derived from stage 5 CKD patients treated with hemodialysis compared with healthy individuals (Figure 8e and f).

**DISCUSSION**

Elevated Pi level has long been recognized as a significant predictor of soft tissue mineralization. Such elevated Pi levels are commonly seen in patients with end-stage kidney disease in which the Pi homeostasis is deranged because of inability of the kidneys to excrete phosphate. Evidence shows that vascular calcification results in increased cardiovascular mortality in hemodialysis patients.<sup>43–45</sup> Vascular calcification follows two distinct patterns: (i) intimal calcification, which occurs with atherosclerotic plaques, and (ii) medial calcification, which is characterized by diffuse calcification of the media, particularly at the level of the internal elastic lamina that is commonly seen in hemodialysis patients and is not always accompanied by atherosclerosis. Furthermore, development of calciphylaxis, which is a syndrome of diffuse arteriolar calcification and skin necrosis, is almost exclusively seen in patients with stage 5 CKD and correlates with extremely high fatal rates.

Previous studies indicated that elevated phosphate could induce VSMC calcification as well as an osteochondrogenic phenotypic change.<sup>46,47</sup> Evidence suggests that this transition



**Figure 8 | Decreased plasma level of H<sub>2</sub>S and reduced CSE activity in stage 5 CKD patients.** (a) Plasma levels of hydrogen sulfide in healthy individuals ( $n = 23$ ) and in stage 5 CKD patients ( $n = 21$ ) on hemodialysis (HD) treatment at different time points is shown as means  $\pm$  standard deviation;  $**P < 0.01$ . (b) CSE gene expression was measured by quantitative reverse transcriptase-polymerase chain reaction from PBMC messenger RNA (mRNA) isolated from controls ( $n = 16$ ) and stage 5 CKD patients ( $n = 14$ ). (c, d) CSE protein expression determined by western blot in PBMC isolated from controls ( $n = 4$ ) and stage 5 CKD patients ( $n = 4$ ). Levels of CSE were normalized to GAPDH. (e, f) CSE enzyme activities assessed by cystathionine consumption (e) and cysteine production (f) in PBMC isolated from controls ( $n = 10$ ) and stage 5 CKD patients ( $n = 10$ ) are shown as means  $\pm$  standard deviation;  $*P < 0.05$ . CKD, chronic kidney disease; CSE, cystathionine  $\gamma$ -lyase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H<sub>2</sub>S, hydrogen sulfide; NS, non-significant; PBMC, peripheral blood mononuclear cell.

is a highly regulated cellular process<sup>18,20–22</sup> involving upregulation of Cbfa1, a key regulatory transcription factor critical for the differentiation of osteoblasts, and its downstream transcript proteins such as ALP, a crucial enzyme in the context of bone and teeth formation, and OC, which is a very specific protein indicative of osteoblast activity.

H<sub>2</sub>S is now recognized as a gas with important functions in the cardiovascular system.<sup>23</sup> In the vasculature, it is produced by VSMC by CSE enzyme,<sup>27</sup> and is involved in the regulation of vascular tone<sup>28</sup> and myocardial contractility.<sup>48</sup> H<sub>2</sub>S deficiency was observed in various animal models of arterial<sup>49</sup> and pulmonary hypertension.<sup>50</sup> Exogenous H<sub>2</sub>S ameliorates myocardial dysfunction associated with the ischemia/reperfusion injury.<sup>51,52</sup> More recently, it was reported that H<sub>2</sub>S reduces vascular calcification induced by vitamin D<sub>3</sub> plus nicotine in rats.<sup>41</sup> The potential inhibitory effect of H<sub>2</sub>S on vascular calcification has recently emerged in calcific uremic

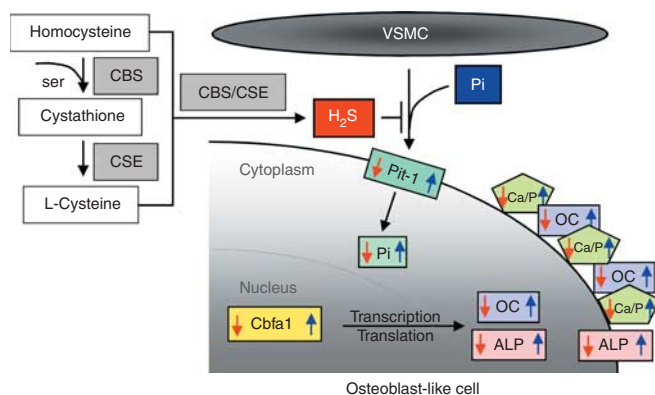
arteriopathy/calciphylaxis, a life-threatening complication of renal failure. Calciphylaxis can be successfully treated with intravenous administration of sodium thiosulfate.<sup>38,39</sup> Sodium thiosulfate was shown to enhance H<sub>2</sub>S generation by inducing CSE expression.<sup>40</sup> However, the effector function(s) of H<sub>2</sub>S in the treatment of calciphylaxis remained to be elucidated.

Drawing upon these previous observations, we examined the role that H<sub>2</sub>S may have in VSMC calcification and transition of VSMC into osteoblast-like cells. We observed that H<sub>2</sub>S suppressed deposition of calcium in the extracellular matrix of HAoSMC induced by Pi in a dose-responsive manner. More importantly, the inhibitory effect of H<sub>2</sub>S was not limited to calcium deposition. In fact, H<sub>2</sub>S suppressed the induction of genes involved in osteoblastic transformation of HAoSMC. H<sub>2</sub>S inhibited Pi-mediated upregulation of ALP and OC in a dose-dependent manner. Cbfa1 is required for osteoblast differentiation, bone matrix gene expression, and, consequently, mineralization;<sup>21</sup> therefore, we examined the effect of H<sub>2</sub>S on Cbfa1 expression in HAoSMC. Similar to ALP and OC, the Pi-provoked upregulation of Cbfa1 was attenuated by H<sub>2</sub>S.

Emerging evidence suggests that the effect of hyperphosphatemia on VSMC calcification is mediated through Pit-1, which facilitates entry of Pi into vascular cells.<sup>20</sup> We therefore tested whether H<sub>2</sub>S alters intracellular Pi level in HAoSMC. Importantly, the marked increase in the level of intracellular Pi due to phosphate exposure was substantially reversed by H<sub>2</sub>S. We demonstrate that inhibition of entry of Pi into vascular cells provided by H<sub>2</sub>S occurs through suppression of Pit-1 expression, thus decreasing the intracellular level of Pi that is fundamental to osteoblastic transformation of HAoSMC. Apoptosis of VSMC is also implicated in the pathogenesis of calcification in vessels, which is seen both in the intima in advanced plaques and in the media in CKD. Apoptotic smooth muscle cells may function as both a nidus for calcification and actively concentrate both calcium and phosphate to generate hydroxyapatite.<sup>53–55</sup> H<sub>2</sub>S was shown to induce apoptosis of HAoSMC. The concentration at which H<sub>2</sub>S exhibits proapoptotic effect is  $\geq 200$   $\mu$ mol/l. In our study, we did not observe alterations in viability of HAoSMC challenged with Pi or H<sub>2</sub>S at concentrations studied.

H<sub>2</sub>S is generated as an alternative product of the transsulfuration pathway (Figure 9), and in the vasculature it is produced mainly by VSMC via CSE-catalyzed reaction.<sup>27</sup> Recently, it has been shown that deletion of CSE in mice results in hypertension.<sup>56</sup> Our data confirmed that the plasma concentration of H<sub>2</sub>S was decreased in stage 5 CKD patients. Moreover, the level of H<sub>2</sub>S was further lowered by hemodialysis. We revealed that enzyme activity of CSE in monocytes derived from stage 5 CKD patients treated with hemodialysis was markedly reduced compared with healthy individuals, without changes in mRNA or protein expression. These results suggest potential post-translational modifications in CSE in CKD that remain to be determined.

To prove the role of CSE in inhibiting Pi-induced HAoSMC mineralization, we used strategies to decrease



**Figure 9 | Scheme of H<sub>2</sub>S biogenesis and its involvement in Pi-induced osteoblastic transformation of VSMC.** H<sub>2</sub>S is generated as an alternative product of the transsulfuration pathway. H<sub>2</sub>S inhibits all the steps of osteoblast transition of VSMC. Pi-induced phosphate uptake, Pit-1 upregulation, Cbfa1, ALP, osetocalcin (OC) expression, and Ca deposition are all inhibited by H<sub>2</sub>S. Blue arrows represent responses to elevated Pi, whereas red arrows represent the effect of H<sub>2</sub>S. ALP, alkaline phosphatase; Cbfa1, core-binding factor alpha-1; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; H<sub>2</sub>S, hydrogen sulfide; VSMC, vascular smooth muscle cell.

endogenous production of H<sub>2</sub>S via both pharmacological inhibition of CSE enzyme activity and silencing of CSE gene expression. By reducing CSE-mediated endogenous H<sub>2</sub>S biogenesis, we observed a significant increase in both HAoSMC calcification and the expression of ALP and OC.

Our findings further corroborate the imperative role of H<sub>2</sub>S and CSE in the vasculature and suggest that reduced activity of CSE and subsequent decrease in the level of H<sub>2</sub>S in stage 5 CKD patients could exacerbate the cardiovascular complications that commonly accompany this particular group of patients.

In conclusion, our study demonstrates a novel role of H<sub>2</sub>S in the process of Pi-provoked mineralization and transition of HAoSMC into osteoblast-like cells (Figure 9). We provide evidence that H<sub>2</sub>S, regardless of its exogenous or endogenous origin, inhibits the upregulation of osteoblast-specific genes such as ALP, OC, and Cbfa1. The inhibition of Pi uptake through Pit-1 is essential for providing beneficial effects against calcification and phenotypic modulation of HAoSMC by H<sub>2</sub>S. Reduced CSE activity leading to decreased H<sub>2</sub>S levels in stage 5 CKD patients might facilitate calcification of vasculature. These results offer a new strategy to prevent vascular calcification.

## MATERIALS AND METHODS

### Cell culture and reagents

HAoSMCs were obtained from Cambrex Bioscience (Wokingham, UK) and fetal bovine serum from Invitrogen/GIBCO (Carlsbad, CA). Unless otherwise mentioned, all other reagents were obtained from Sigma (St Louis, MO). Cell cultures were maintained in GM DMEM (high glucose) containing 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and neomycin, and 1 mmol/l sodium pyruvate. Cells were grown to confluence and used

from passages 3–7. H<sub>2</sub>S was introduced as sodium hydrosulfide dissolved in deionized water.

### Patients

Controls were healthy subjects recruited among hospital staff without any known diseases including hypertension, dyslipidemia, and liver and kidney malfunctions ( $n = 23$ , mean age 53 years, F/M 9/14). Stage 5 CKD patients treated with hemodialysis were selected ( $n = 21$ , mean age 61 years, F/M 13/8) from our dialysis unit. The patients were on hemodiafiltration therapy of three 4-h sessions weekly, using High-Flux Dialysers FX 60 and FX 600 (Fresenius, Fresenius AG, Homburg, Germany). The Kt/V ratio was  $1.25 \pm 0.3$ . Folate (3 mg/day) and intravenous iron (Ferrlecit, Aventis Pharma, Dagenham, UK) (62.5 mg every other week) were given to patients from the beginning of hemodialysis therapy. They also received rHu-EPO depending on their hemoglobin value. Blood was drawn in Vacutainers (BD, Franklin Lakes, NJ) using ethylenediaminetetraacetate immediately before the hemodialysis session from CKD patients by venipuncture. Participants gave their informed consent to the study, which was approved by the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Nr. DEOEC RKEB/IKEB 3287A-2010).

### Separation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from blood by density gradient centrifugation using Histopaque 1077 (Sigma). Cells were disrupted by ultrasonication in 100 mmol/l phosphate buffer, pH 7.4, for immediate determination of CSE activity or in 100 mmol/l phosphate buffer containing 1% Triton X-100 and a mixture of protease inhibitors (Sigmafast Protease Inhibitor Tablets, Sigma). Cell lysate was maintained at  $-70^{\circ}\text{C}$  until use.

### Measurement of plasma H<sub>2</sub>S concentration

Immediately after drawing, blood was centrifuged (3 min, 3000 g) and 40 μl of plasma was rapidly added to the assay mixture that contained 50 μl of 1% zinc acetate, 40 μl of FeCl<sub>3</sub> (30 mmol/l in 1.2 mol/l HCl), and 50 μl of *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (20 mmol/l in 7.2 mol/l HCl). To deprotonize samples, 70 μl of 10% trichloroacetic acid was added, and then the mixture was centrifuged at 3000 g for 30 min at room temperature. The absorbance of the supernatant was read at 670 nm, and concentration was calculated using a calibration curve.

### Induction of calcification

At confluence, HAoSMCs were switched to calcification medium, which was prepared by adding 3 mmol/l of inorganic phosphate to the GM. Both GM and calcification medium were changed every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0.

### Quantification of calcium deposition

Cells grown on 48-well plates were washed twice with phosphate buffered saline and decalcified with 0.6 mol/l HCl for 24 h. Calcium content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur, Hayward, CA). After decalcification, cells were solubilized with a solution of 0.1 mol/l NaOH and 0.1% sodium dodecyl sulfate, and protein content of the samples were measured with the BCA protein assay kit (Thermo Scientific, Rockford, IL). Calcium content of the cells was normalized to protein content and expressed as μg/mg protein. Mineralization was also determined by Alizarin red staining.

### Alkaline phosphatase activity assay

HAoSMCs grown on six-well plates were washed with phosphate-buffered saline twice, solubilized with 1% Triton X-100 in 0.9% NaCl, and assayed for ALP activity. Briefly, 130 µl of Alkaline Phosphatase Yellow Liquid Substrate (Sigma) was combined with 50 µg of protein samples, and then the kinetics of *p*-nitrophenol formation was followed for 30 min at 405 nm at 37 °C. Maximum slope of the kinetic curves was used for calculation.

### Phosphate measurement

Pi content of the cell lysate was determined by the QuantiChrome Phosphate Assay Kit (Gentaur). After 4 days of culture, cells were washed twice with phosphate-buffered saline and solubilized with 1% Triton, and the cell lysates were assayed for Pi. Phosphate content of the cells was normalized to protein content and expressed as µmol/l/mg cell protein.

### Quantification of OC

Osteocalcin was quantified as described previously.<sup>42</sup>

### Detection of OC, CSE, ferritin H- and L-chain by western blot

To determine the protein expression level of OC, extracellular matrix was dissolved in ethylenediaminetetraacetate (0.5 mol/l, pH 6.9) and was electrophoresed in 16.5% Tris-Tricin/Peptide-PAGE (Bio-Rad Laboratories, Hercules, CA). Ferritin western blot was performed as described previously.<sup>42</sup> For CSE determination, cell lysate of PBMC was electrophoresed in 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting was performed with a polyclonal anti-OC antibody at 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) and with anti-CSE antibody (Sigma) at 1:100 dilution, followed by horseradish peroxidase-labeled anti-mouse immunoglobulin-G antibody. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK). Chemiluminescence was quantified by using Alpha DigiDoc RT quantification software (Alpha Innotech, San Leandro, CA).

### Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated, reverse transcribed, and polymerase chain reactions were performed using iQ SYBR Green Supermix (Bio-Rad) and the following primers: Cbfa1 forward 5'-ATGGCGGGTAACG ATGAAAAT-3' and reverse 5'-ACGGCGGGGAAGACTGTG-3' Pit-1 forward 5'-GCCAAAGTGAGCGAAACCATCC-3' and reverse 5'-CCACACAGCAGAACCAAACATAGC-3'. To measure CSE mRNA levels in CKD patients and controls, blood mononuclear cell mRNA was isolated from whole blood with the QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers for CSE were the following: forward: 5'-GCCTTCATAATAGACTTCGTTTC-3' and reverse: 5'-GCAGCCC AGGATAAATAACC-3'. Polymerase chain reactions were carried out using the iCycler iQ Real-Time PCR System (Bio-Rad). Results were normalized by glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

### Cystathionine γ-lyase siRNA transfection

Small interfering RNA specific to CSE and negative control siRNA were obtained from Ambion (Austin, TX). Transfection of siRNA into HAoSMC was achieved using the Oligofectamine Reagent (Invitrogen). Briefly, the cells were plated overnight to form 60–70% confluent monolayers. CSE siRNA at 30 nmol/l and transfection

reagent complex were added to the cells in serum-free medium for 4 h. Fresh normal GM was added further and the cells were incubated for another 20 h.

### Cystathionine γ-lyase activity measurement

CSE activity was measured according to the Stipanuk method.<sup>57</sup> CSE activity from PBMC was assessed by cystathionine consumption and cysteine production. Lysed PBMC was incubated with cystathionine (2 mmol/l), pyridoxal 5'-phosphate (0.25 mmol/l) in Tris-HCl buffer (100 mmol/l, pH 8.3) for 60 min at 37 °C. Thereafter, acid ninhydrine reagent was added, and samples were boiled for 5 min. After cooling the samples, optical densities at 455 and 560 nm were determined and used for calculation of cystathionine and cysteine concentrations, respectively.

### MTT assay

MTT assay was performed as described previously.<sup>42</sup>

### Statistical analysis

Data are shown as mean ± standard deviation. Statistical analysis was performed by analysis of variance test, followed by *post hoc*, Newmann-Keuls test for multiple comparisons. A value of  $P < 0.05$  was considered significant and marked with \*, and  $P < 0.01$  was considered highly significant and marked with \*\*.

### DISCLOSURE

All the authors declared no competing interests.

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