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Original Paper

Role of Cytosolic Serine Hydroxymethyl Transferase 1 (SHMT1) in Phosphate-Induced Vascular Smooth Muscle Cell Calcification

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Key Words
SHMT1 • Phosphate • Oxidative stress • Vascular calcification • Osteo-/chondrogenic signaling • Vascular smooth muscle cells

Abstract
Background/Aims: Hyperphosphatemia promotes medial vascular calcification, at least partly, by induction of osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs). The complex signaling pathways regulating this process are still incompletely understood. The present study investigated the role of cytosolic serine hydroxymethyl transferase 1 (SHMT1) in phosphate-induced vascular calcification. ***Methods:*** Endogenous expression of SHMT1 was suppressed by silencing in primary human aortic smooth muscle cells (HAoSMCs) followed by treatment without and with phosphate or antioxidants. ***Results:*** In HAoSMCs, *SHMT1* mRNA expression was up-regulated by phosphate. Silencing of SHMT1 alone was sufficient to induce osteo-/chondrogenic transdifferentiation of HAoSMCs, as shown by increased tissue-nonspecific alkaline phosphatase (ALPL) activity and osteogenic markers *MSX2*, *CBFA1* and *ALPL* mRNA expression. Furthermore, phosphate-induced *ALPL* mRNA expression and activity as well as calcification were augmented in SHMT1 silenced HAoSMCs as compared to negative control siRNA transfected HAoSMCs. Silencing of SHMT1 decreased total antioxidant capacity and up-regulated NADH/NADPH oxidase system components *NOX4* and *CYBA* mRNA expression in HAoSMCs, effects paralleled by increased mRNA expression

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of matrix metalloproteinase *MMP2* as well as *BAX/BCL2* ratio. More importantly, additional treatment with antioxidants TEMPOL or TIRON blunted the increased osteogenic markers mRNA expression in SHMT1 silenced HAoSMCs. ***Conclusion:*** Silencing of SHMT1 promotes osteo-/chondrogenic signaling in VSMCs, at least in part, by inducing cellular oxidative stress. It thus aggravates phosphate-induced calcification of VSMCs. The present findings support a regulatory role of SHMT1 in vascular calcification during conditions of hyperphosphatemia such as chronic kidney disease.

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Introduction

Medial vascular calcification is strongly associated with cardiovascular morbidity and mortality in chronic kidney disease (CKD) patients [1]. In these patients, impaired renal phosphate excretion leads to hyperphosphatemia, a major pathological factor promoting the initiation and progression of vascular calcification [2, 3]. Accordingly, plasma phosphate concentrations are predictive for an increased risk for cardiovascular events and mortality [4, 5].

The pathological deposition of calcium-phosphate in the media of the arterial wall is an active process, with similarities to physiological bone mineralization [6, 7]. Vascular calcification is promoted mainly by vascular smooth muscle cells (VSMCs) [2, 8, 9]. Pathological factors, especially phosphate, trigger the transdifferentiation of VSMCs into an osteoblast and chondroblast-like phenotype [2, 6, 10] including increased expression and activity of the osteogenic transcription factors msh homeobox 2 (MSX2) and core-binding factor α-1 (CBFA1) [11, 12] as well as osteogenic enzymes such as tissue-nonspecific alkaline phosphatase (ALPL) [9, 13] to initiate mineral deposition in the vascular tissue. The complex intracellular signaling pathways mediating osteo-/chondrogenic transdifferentiation of VSMCs are still incompletely understood.

The cytosolic serine hydroxymethyl transferase 1 (SHMT1) catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5, 10-methylene tetrahydrofolate [14, 15] and is a critical enzyme in one carbon (methyl) metabolism [14-17]. One carbon transfer is a key component in amino acid and nucleotide synthesis as well as methylation of proteins, DNA and RNA [18, 19] and, thus, participating in the regulation of cell function, proliferation and growth [18, 20]. SHMT1 has been associated with apoptotic pathways [21]. SHMT1 differs in targets and effects from the mitochondrial isoform SHMT2 [14, 15, 18]. Polymorphisms in the SHMT1 gene are associated with increased risk of cardiovascular disease [22, 23] including ischemic stroke [24]. Genetic variation in the SHMT1 gene is further associated with carotid intima-media thickness, a marker for atherosclerosis [25]. However, the impact of SHMT1 on VSMC function and vascular calcification remained illdefined.

Therefore, the present study aimed to elucidate the possible involvement of SHMT1 in osteo-/chondrogenic transdifferentiation and calcification of VSMCs during conditions of hyperphosphatemia *in-vitro*.

Materials and Methods

Cell culture of primary human aortic smooth muscle cells

Primary human aortic smooth muscle cells (HAoSMCs) were obtained from Thermo Fisher Scientific [26-28]. Cells were grown to confluence in complete medium containing Waymouth's MB 752/1 medium and Ham's F-12 nutrient mixture (1:1 ratio, Thermo Fisher Scientific), 10% FBS (Thermo Fisher Scientific), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific). HAoSMCs were used in all experiments from passages 4 to 11.

HAoSMCs were transfected with 10 nM SHMT1 siRNA (ID no. s12820, Thermo Fisher Scientific) or with 10 nM negative control siRNA (ID no. 4390843, Thermo Fisher Scientific) using siPORT amine transfection

agent (Thermo Fisher Scientific) according to the manufacturer's protocol. The cells were used 48 hours (qRT-PCR and antioxidant capacity), 7 days (ALPL activity) or 9 days (calcium deposition) after transfection. Silencing efficiency was determined by quantitative RT-PCR.

HAoSMCs were treated for 24 hours (qRT-PCR) or 7 days (ALPL activity) with 2 mM β-glycerophosphate (Sigma Aldrich), 10 μM TEMPOL (4-hydroxy-TEMPO, stock in DMSO; Sigma-Aldrich) or 10 μM TIRON (4, 5-dihydroxy-1, 3-benzenedisulfonic acid disodium salt monohydrate; Sigma-Aldrich). Equal amounts of vehicle were used as control. Treatment with calcification medium (10 mM β-glycerophosphate and 1.5 mM $CaCl_2$, Sigma-Aldrich) for 9 days was used for the calcium content analysis and Alizarin Red staining [26, 29, 30]. Fresh media with agents were added every 2-3 days.

Quantitative RT-PCR

Total RNA was isolated from HAoSMCs by using Trizol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions [31, 32]. Reverse transcription of total RNA was performed using oligo$(dT)_{12-18}$ primers (Thermo Fisher Scientific) and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Quantitative RT-PCR was performed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) and iQ™ Sybr Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The following human primers were used (Thermo Fisher Scientific, 5'→3' orientation):

ALPL fw: GGGACTGGTACTCAGACAACG;
ALPL rev: GTAGGCGATGTCCTTACAGCC;
BAX fw: CCCGAGAGGTCTTTTTCCGAG;
BAX rev: CCAGCCCATGATGGTTCTGAT;
BCL2 fw: GGTGGGGTCATGTGTGTGG;
BCL2 rev: CGGTTCAGGTACTCAGTCATCC;
CBFA1 fw: GCCTTCCACTCTCAGTAAGAAGA;
CBFA1 rev: GCCTGGGGTCTGAAAAAGGG;
CYBA fw: CCCAGTGGTACTTTGGTGCC;
CYBA rev: GCGGTCATGTACTTCTGTCCC;
GAPDH fw: GAGTCAACGGATTTGGTCGT;
GAPDH rev: GACAAGCTTCCCGTTCTCAG;
MMP2 fw: TACAGGATCATTGGCTACACACC;
MMP2 rev: GGTCACATCGCTCCAGACT;
MSX2 fw: TGCAGAGCGTGCAGAGTTC;
MSX2 rev: GGCAGCATAGGTTTTGCAGC;
NOX4 fw: TGACGTTGCATGTTTCAGGAG;
NOX4 rev: AGCTGGTTCGGTTAAGACTGAT;
SHMT1 fw: TTGCCTCGGAGAATTTCGCC;
SHMT1 rev: GTCCCGCCATAGTATCTCTGG.

The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate and relative mRNA expression was calculated by using the $2^{-\Delta\Delta Ct}$ method with GAPDH as housekeeping gene. Results were normalized to the negative control silenced group.

Total antioxidant capacity assay

Total antioxidant capacity of HAoSMCs was measured in the cell lysate by using the colorimetric antioxidant assay kit (Cayman Chemical) according to the manufacturer's protocols [26, 33]. Relative antioxidant capacity was compared to that of Trolox as standard. The results were normalized to total protein concentration as assessed by the Bradford assay (Bio-Rad Laboratories) and to the negative control silenced group.

Alkaline phosphatase (ALPL) activity assay

ALPL activity in HAoSMCs was determined by using the ALP colorimetric assay kit (Abcam) according to the manufacturer's protocol [34]. The results are shown normalized to total protein concentration measured by the Bradford assay (Bio-Rad Laboratories).

Determination of calcification

HAoSMCs were decalcified in 0.6 M HCl for 24 hours at 4°C. Calcium content in the supernatant was determined by using QuantiChrom Calcium assay kit (BioAssay Systems) according to the manufacturer's protocol. HAoSMCs were lysed with 0.1 M NaOH/ 0.1% SDS and protein concentration was measured by the Bradford assay (Bio-Rad Laboratories). Results are shown normalized to total protein concentration [28, 35]. For Alizarin red staining, HAoSMCs were fixed with 4% paraformaldehyde/PBS and stained with 2% Alizarin Red (pH 4.5). The calcified areas are shown as red staining [26].

Statistics

Data are shown as scatter dot plots and arithmetic means ± SEM. N indicates the number of independent experiments performed at different passages of the cells. Normality was tested with Shapiro-Wilk test. Non-normal datasets were transformed (log, reciprocal or sqrt) prior to statistical testing to provide normality according to Shapiro-Wilk test. Statistical testing was performed by one-way Anova followed by Tukey-test (homoscedastic data) or Games-Howell test (heteroscedastic data). Non-normal data were tested by the Steel-Dwass method. Two groups were compared by unpaired two-tailed t-test. $P<0.05$ was considered statistically significant.

Results

To investigate the role of SHMT1 in vascular calcification, the endogenous expression in primary human aortic smooth muscle cells (HAoSMCs) was suppressed by silencing of the SHMT1 gene followed by additional treatment without and with phosphate. As shown in Fig. 1, S*HMT1* mRNA expression was significantly lower in SHMT1 silenced HAoSMCs than in negative control siRNA transfected HAoSMCs. The negative control transfection conditions alone did not significantly affect *SHMT1* gene expression in HAoSMCs as compared to untransfected HAoSMCs (n=5; 1.004±0.042 a.u. in untransfected HAoSMCs vs. 0.998±0.059 a.u. in negative control siRNA silenced HAoSMCs). Phosphate treatment significantly up-regulated *SHMT1* mRNA expression in negative control silenced HAoSMCs (Fig. 1).

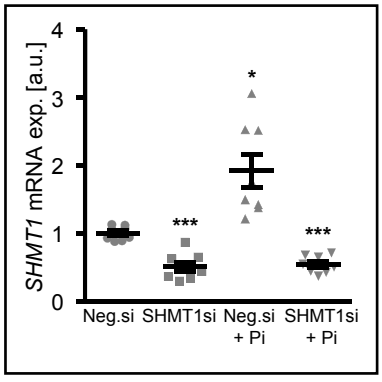

Fig. 1. Phosphate up-regulates cytosolic serine hydroxymethyl transferase 1 (SHMT1) expression in HAoSMCs. Scatter dot plots and arithmetic means ± SEM (n=8; arbitrary units, a.u.) of *SHMT1* relative mRNA expression in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si) and additional treatment for 24 hours with control or with 2 mM β-glycerophosphate (Pi). *($p<0.05$), ***($p<0.001$) statistically significant vs. negative control silenced HAoSMCs.

As illustrated by Alizarin Red staining and quantification of calcium content, treatment with calcification medium increased calcification of negative control silenced HAoSMCs, an effect aggravated in SHMT1 silenced HAoSMCs (Fig. 2A, B). Silencing of SHMT1 alone did not significantly modify calcium deposition in HAoSMCs (Fig. 2A, B). However, SHMT1 knockdown alone was sufficient to significantly increase ALPL activity (Fig. 2C) and mRNA expression of osteogenic transcription factors *MSX2* and *CBFA1* as well as of osteogenic enzyme *ALPL* (Fig. 2D-F) in HAoSMCs to similar high levels as phosphate treatment. Furthermore, the phosphate-induced ALPL activity and mRNA expression were significantly augmented following silencing of SHMT1 in HAoSMCs. Silencing of SHMT1 tended to augment *MSX2* and *CBFA1* mRNA expression in phosphate treated HAoSMCs, an effect, however, not reaching statistical significance. Taken together, silencing of SHMT1 is sufficient to promote osteo-/chondrogenic transdifferentiation and to aggravate the phosphate-induced calcification of VSMCs.

To explore the mechanisms underlying the pro-calcific role of SHMT1 knockdown, another series of experiments analyzed the effects on oxidative stress in HAoSMCs. Total antioxidant capacity (Fig. 3A) was significantly lower and the mRNA expression of oxidative stress markers *NOX4* and p22phox encoded by the *CYBA* gene (Fig. 3B, C) was significantly higher in SHMT1 silenced HAoSMCs than in negative control silenced HAoSMCs. Similarly, silencing of SHMT1 significantly up-regulated matrix metalloproteinase *MMP2* mRNA expression (Fig. 3D), a downstream target of oxidative stress in VSMCs. These effects were paralleled by increased *BAX/BCL2* mRNA expression ratio as indicator of apoptosis in SHMT1 silenced HAoSMCs (Fig. 3E). Thus, SHMT1 knockdown induces oxidative stress in VSMCs.

Further experiments investigated whether induction of oxidative stress is responsible for the effects of SHMT1 silencing on osteo-/chondrogenic transdifferentiation of VSMCs. As shown in Fig. 4, the increased mRNA expression of osteo-/chondrogenic markers *MSX2*, *CBFA1* and *ALPL* in SHMT1 silenced HAoSMCs was significantly suppressed following additional treatment with antioxidants TEMPOL or TIRON. Thus, the effects of SHMT1 silencing in promoting osteo-/chondrogenic transdifferentiation of VSMCs are due, at least partially, to induction of cellular oxidative stress.

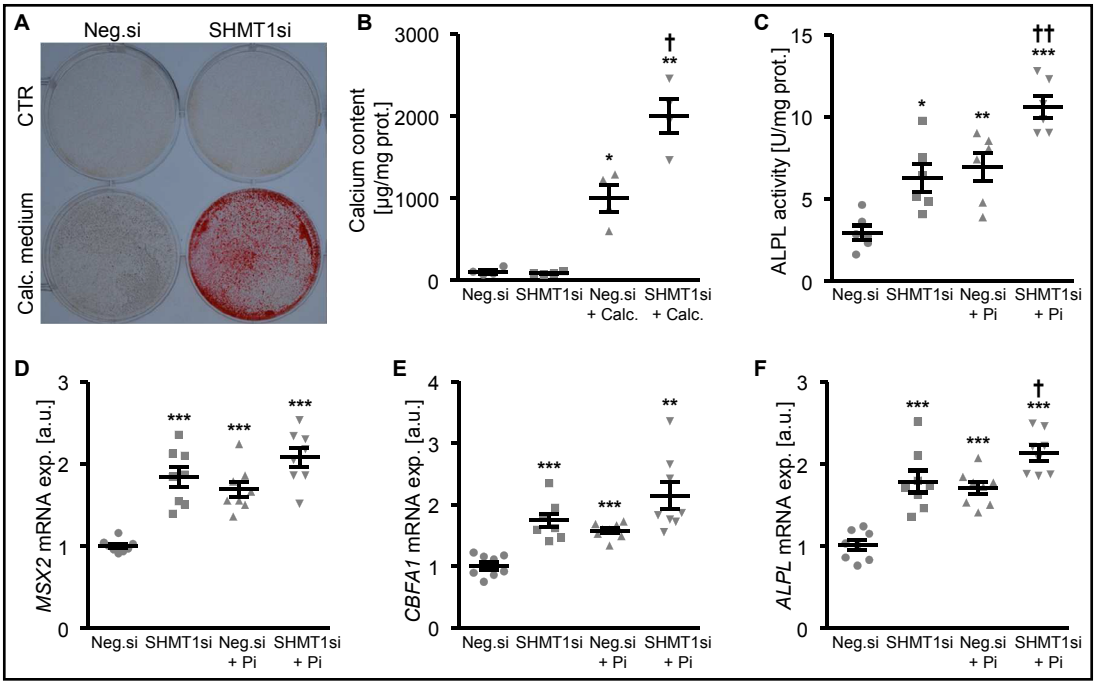

Fig. 2. Silencing of SHMT1 promotes osteoinductive signaling and aggravates phosphate-induced osteo-/chondrogenic transdifferentiation and calcification of HAoSMCs. A. Representative original images showing Alizarin red staining (n=3) in HAoSMCs following silencing for 9 days with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si) and additional treatment with control or with calcification medium (Calc.). The calcified areas are shown as red staining. B. Scatter dot plots and arithmetic means ± SEM (n=4; µg/mg protein) of calcium content in HAoSMCs following silencing for 9 days with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si) and additional treatment with control or with calcification medium (Calc.). C. Scatter dot plots and arithmetic means ± SEM (n=6; U/mg protein) of ALPL activity in HAoSMCs following silencing for 7 days with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si) and additional treatment with control or with 2 mM β-glycerophosphate (Pi). D-F. Scatter dot plots and arithmetic means ± SEM (n=8; arbitrary units, a.u.) of *MSX2* (D), *CBFA1* (E) and *ALPL* (F) relative mRNA expression in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si) and additional treatment for 24 hours with control or with 2 mM β-glycerophosphate (Pi). *(p<0.05), **(p<0.01), ***(p<0.001) statistically significant vs. negative control silenced HAoSMCs; †(p<0.05), ††(p<0.01) statistically significant vs. negative control silenced and Pi treated HAoSMCs.

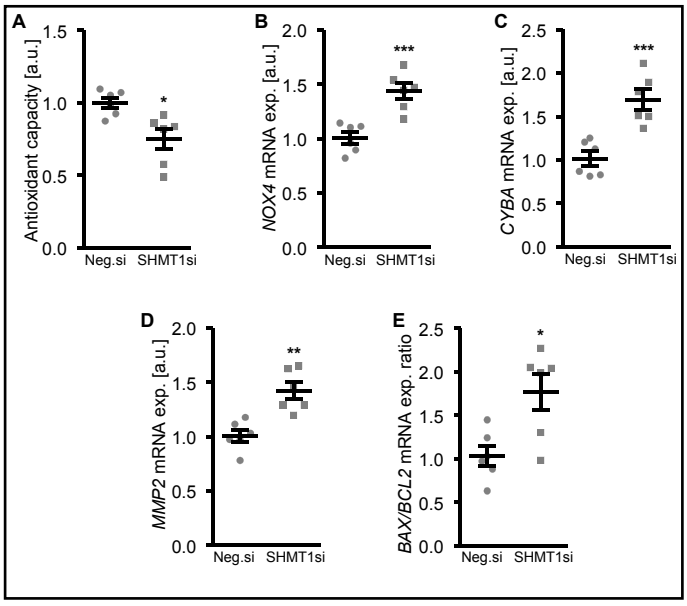

Fig. 3. Silencing of SHMT1 increases oxidative stress and apoptosis in HAoSMCs. A. Scatter dot plots and arithmetic means ± SEM (n=6; arbitrary units, a.u.) of total antioxidant capacity in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si). B-E. Scatter dot plots and arithmetic means ± SEM (n=6; a.u.) of *NOX4* (B), *CYBA* (C) and MMP2 (D) relative mRNA expression as well as *BAX/BCL2* (E) relative mRNA expression ratio in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si). *(p<0.05), **(p<0.01), ***(p<0.001) statistically significant vs. negative control silenced HAoSMCs.

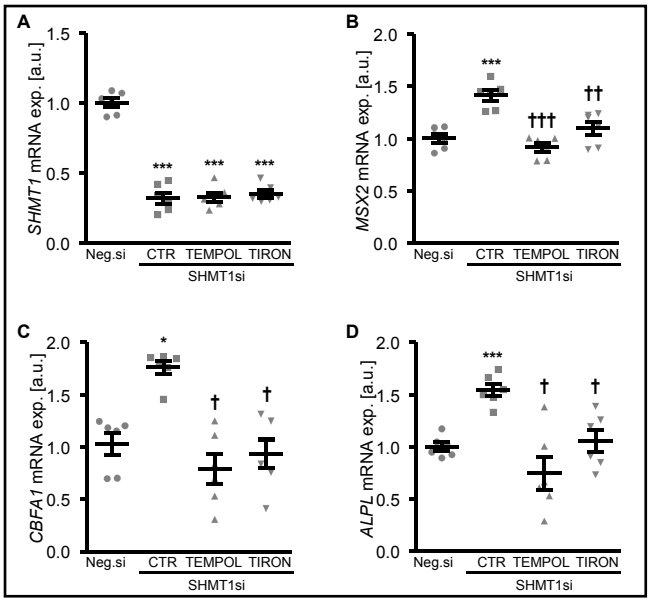

Fig. 4. The osteoinductive effects of SHMT1 silencing in HAoSMCs are blunted in the presence of antioxidants. Scatter dot plots and arithmetic means ± SEM (n=6; arbitrary units, a.u.) of *SHMT1* (A), *MSX2* (B), *CBFA1* (C) and *ALPL* (D) relative mRNA expression in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or SHMT1 siRNA (SHMT1si) and additional treatment with control (CTR), 10 μM TEMPOL or 10 μM TIRON. *(p<0.05), ***(p<0.001) statistically significant vs. negative control silenced HAoSMCs; †(p<0.05), ††(p<0.01), †††(p<0.001) statistically significant vs. control treated and SHMT1 silenced HAoSMCs.

Discussion

This study reveals a role of SHMT1 in the regulation of VSMC osteo/chondrogenic transdifferentiation and calcification. Phosphate up-regulates *SHMT1* expression in VSMCs, while silencing of SHMT1 aggravates phosphate-induced osteo/chondrogenic transdifferentiation and calcification of VSMCs. Silencing of SHMT1 in VSMCs induces oxidative stress and additional treatment with antioxidants blocks the pro-calcific effects of SHMT1 silencing. The present observations, therefore, suggest that vascular SHMT1 is a powerful regulator of the cellular response in pathological conditions such as hyperphosphatemia in CKD [2].

Hyperphosphatemia and dysregulation of mineral homeostasis are critical pathological factors promoting vascular calcification [2, 6]. *In-vitro*, elevated extracellular phosphate levels trigger osteo-/chondrogenic transdifferentiation and calcification of VSMCs [36]. We show

here that silencing of SHMT1 augments phosphate-induced osteo-/chondrogenic signaling and calcification of VSMCs. Increased SHMT1 levels in phosphate-treated VSMCs are, thus, apparently necessary to limit the extent of calcification. VSMC calcification models *in-vitro* require calcium and phosphate supplementation in the cell culture media as substrate for calcification, to permit maximal mineralization [29]. Accordingly, SHMT1 knockdown alone induces osteo-/chondrogenic transdifferentiation, but does not affect mineralization of HAoSMCs, as the substrate for calcification is insufficient. These interpretations are however limited, as the *in-vitro* findings may not directly reflect vascular calcification *in-vivo* [37, 38]. Nonetheless, the present observations indicate that vascular SHMT1 interferes with the osteo-/chondrogenic signaling in phosphate-induced VSMC calcification *in-vitro*.

Oxidative stress is a critical mediator of vascular calcification [39-41] by inducing osteo-/chondrogenic transdifferentiation of VSMCs [4, 26, 33, 39]. Silencing of SHMT1 in VSMCs induces an imbalance between antioxidant systems and oxidative products, as shown by reduced total antioxidant capacity as well as up-regulation of *NOX4* and *CYBA* mRNA expression, important components of the superoxide-generating NADH/NADPH oxidase system [42].

Oxidative stress may induce osteo-/chondrogenic transdifferentiation of VSMCs via various mechanisms [33, 39, 43-45]. Silencing of SHMT1 is able to increase the expression of downstream effectors of oxidative stress such as matrix gelatinase *MMP2* [43], an essential factor in phenotypical transformation of VSMCs and matrix remodeling to initiate mineralization [44, 45]. Oxidative stress may further promote vascular calcification by inducing apoptosis of VSMCs [6, 46]. Also, the *BAX/BCL2* expression ratio was higher in SHMT1 silenced VSMCs, indicative of increased apoptosis.

Nonetheless, other mechanisms may contribute to the osteoinductive effects of SHMT1 knockdown in VSMCs. The reaction catalyzed by SHMT1 is an important source of glycine [14, 15] and hypoglycinemia was shown to contribute to occurrence of vascular calcification and elevated cardiovascular risk in diabetes and CKD [47]. Furthermore, direct targets of SHMT1-mediated methylation [18-21] may be involved in osteoinductive signaling in VSMCs. Conversely, SHMT1 may mediate anti-calcific effects of folate-dependent pathways to protect against vascular calcification [48].

Excessive oxidative stress is prevalent in CKD patients [49, 50] and associated with vascular calcification [42] and with increased cardiovascular and all-cause mortality [51]. NADPH oxidase activation is associated with coronary artery calcification [52]. Accordingly, antioxidants may prevent the progression of vascular calcification in CKD [53]. Beyond its role in vascular calcification, SHMT1 may further play an important role in vascular disease in other conditions associated with oxidative stress and apoptosis [49, 50]. Thus, SHMT1 may potentially have a role in systemic changes during disease progression. Further studies are necessary to confirm the potential role of SHMT1 in these conditions.

Conclusion

SHMT1 knockdown promotes osteo-/chondrogenic transdifferentiation of VSMCs, at least in part, by increasing cellular oxidative stress and oxidative stress-dependent osteoinductive signaling. It aggravates vascular calcification during conditions of hyperphosphatemia *in-vitro*. The present observations, thus, reveal SHMT1 as novel player in the signaling of vascular calcification.

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Disclosure Statement

All authors disclose that they have no potential conflict of interest.

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