Atherosclerosis 265 (2017) 78–86

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Hydrogen sulfide attenuates calcification of vascular smooth muscle cells via KEAP1/NRF2/NQO1 activation

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ARTICLE INFO

Article history:
Received 19 December 2016
Received in revised form
19 June 2017
Accepted 17 August 2017
Available online 19 August 2017

Keywords:
Vascular smooth muscle cells
Calciprotein particles
Sulfide
H_2S/HS^-
NQO1

ABSTRACT

Background and aims: Vascular calcification is a common health problem related to oxidative stress, inflammation, and circulating calciprotein particles (CPP). Hydrogen sulfide is an endogenous signaling molecule with antioxidant properties and potential for drug development targeting redox signaling. Yet, its molecular mechanisms of action in vascular smooth muscle cell (VSMC) calcification have not been delineated. We therefore sought to identify key pathways involved in the calcification-inhibitory properties of sulfide employing our recently developed CPP-induced VSMC calcification model.
Methods: Using next-generation sequencing, we investigated the transcriptomic changes of sodium hydrosulfide-treated *versus* non-treated calcifying VSMCs. The potential role of candidate genes and/or regulatory pathways in prevention of calcification was investigated by small interfering RNA (siRNA).
Results: CPP led to a pronounced accumulation of cell-associated calcium, which was decreased by sulfide in a concentration-dependent manner. Both, CPP-induced hydrogen peroxide production and enhanced pro-inflammatory/oxidative stress-related gene expression signatures were attenuated by sulfide-treatment. Gene ontology enrichment and in silico pathway analysis of our transcriptome data suggested NAD(P)H dehydrogenase [quinone] 1 (NQO1) as potential mediator. Corroborating these findings, silencing of Kelch-like ECH-associated protein 1 (KEAP1), an inhibitor of nuclear factor (erythroid-derived 2)-like 2 (NRF2) nuclear activity, enhanced NQO1 expression, whereas NRF2 silencing reduced the expression of NQO1 and abrogated the calcification-suppressing activity of sulfide. Moreover, immunofluorescence microscopy and Western blot analysis confirmed nuclear translocation of NRF2 by sulfide in VSMC.
Conclusions: Sulfide attenuates CPP-induced VSMC calcification *in vitro* via the KEAP1-NRF2 redox sensing/stress response system by enhancing NQO1 expression.

1. Introduction

Arterial medial calcification is the pathological deposition of calcium phosphate in the elastic layer of the blood vessel wall, leading to vascular stiffening and reduced vascular compliance with ageing. An increased propensity for soft tissue calcification is particularly prevalent in chronic kidney disease (CKD) patients, contributing to their markedly higher cardiovascular disease mortality compared to the general population [7,15]. Accumulating

Abbreviations: VSMC, vascular smooth muscle cells; CKD, chronic kidney disease; CPP, calciprotein particles; H_2S, hydrogen sulfide; NaHS, sodium hydrosulfide; NGS, next generation sequencing; NQO1, NAD(P)H dehydrogenase [quinone] 1; NRF2, nuclear factor (erythroid-derived 2)-like 2; KEAP1, Kelch-like ECH-associated protein 1.

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http://dx.doi.org/10.1016/j.atherosclerosis.2017.08.012
0021-9150/

evidence suggests that active cell-mediated processes contribute to the calcification process and that vascular smooth muscle cells (VSMC) are the main cell type affected [14]. Circulating complexes of calcium phosphate and plasma proteins called 'calciprotein particles' (CPP) have been found in the blood of CKD patients [17,35], in the dialysate from patients receiving peritoneal dialysis [8], and in blood of patients with normal renal function suffering from rheumatoid arthritis [35]. CPP exist in the form of primary CPP, which are spherical nano-aggregates containing amorphous calcium phosphate, and secondary CPP, which are spindle-shaped and contain crystalline calcium phosphate. We have previously demonstrated that secondary CPP induce VSMC calcification *in vitro* and trigger the generation of reactive oxygen species (ROS), a process that is further enhanced by endogenous tumor necrosis factor-α [2].

Hydrogen sulfide (H_2S) has recently been identified as a gaseous signaling molecule with a wide range of physiological and pathological functions [39]. It is unclear whether dissolved H_2S, its conjugate hydrosulfide anion (HS^-) or secondary reaction products account for the biological effects observed. Nevertheless, H_2S/HS^- has been shown to reduce the production and/or attenuate the deleterious effects of pro-inflammatory cytokines, chemokines and ROS [6]. Physiologically, H_2S/HS^- is synthesized by the action of three enzymes of the *trans*-sulfuration pathway: cystathionine gamma-lyase (CSE), cystathionine beta-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST). Besides these enzymatic pathways, H_2S/HS^- can also be generated through non-enzymatic processes including the processing of inorganic and organic persulfides and polysulfides as well as elemental sulfur [25]. In fact, reactive sulfur species such as cysteine and protein hydropersulfides themselves may play an important role in redox biology [27]. H_2S/HS^- appears to play important functions in the central nervous and cardiovascular system, and in the gastrointestinal and respiratory tract [38]. Specifically, aberrant endogenous H_2S/HS^- metabolism, with likely consequences for systemic redox status, has been associated with hypertension, atherosclerosis, diabetes and Alzheimer's disease [38].

Both, endogenous H_2S synthesis and plasma concentrations of sulfide metabolites are reduced in CKD [5,32]. Of note, pharmacological administration of H_2S/HS^- was shown to ameliorate vascular calcification load induced by vitamin D3 and nicotine feeding in rats [40], and also prevents calcification of VSMC *in vitro* [43]. Moreover, the endogenous H_2S/HS^--precursor sodium thiosulfate (STS, $Na_2S_2O_3$), which is also available as a drug [28], attenuates the progression of vascular calcifications in CKD, both in animals and humans [1,31].

To date, the molecular mechanisms underlying the protective effects of sulfide in VSMC calcification have not been delineated. We therefore sought to shed light on the mechanism(s) of H_2S/HS^--mediated inhibition of CPP-induced calcification in cultured VSMC using a combination of high-throughput whole transcriptome analysis and RNA silencing. Here, we find that the sulfide donor NaHS ameliorates calcification in this system and that these beneficial effects are mediated by activation of the KEAP1/NRF2/NQO1 pathway.

2. Materials and methods

Primary Human Aortic Vascular Smooth Muscle Cells (VSMC) were exposed to control growth medium without CPP, growth medium supplemented with secondary CPP (final concentration equivalent to 50 μg/mL calcium) or growth medium supplemented concurrently with secondary CPP and sodium hydrosulfide for 24 h. Mineralization was assessed qualitatively by staining with Alizarin Red S, and quantitatively by measurement of cell-associated calcium using the QuantiChrome calcium assay kit (Sococchim) and normalized to cellular protein content. Cell viability was determined using MTT [3-(4,5-dime-thylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay kit (BioVision).

Total RNA was extracted from VSMC lysates using the RNeasy Micro Kit (QIAGEN AG). For performing high-throughput transcriptome analysis using next generation sequencing (NGS), the library preparation was performed with 650 ng of total RNA using the TruSeq Stranded mRNA Library Prep Kit (Illumina). The enriched RNA-seq libraries were sequenced using the NextSeq 500 High Output Kit 75-cycles (Illumina). The RNA-seq data was analyzed using the ArrayStar software v.12.2 (DNASTAR). Gene networks and canonical pathways representing differentially expressed genes were identified using the Ariadne Genomics Pathway Studio® software (Elsevier). The potential role of candidate genes and/or regulatory pathways in prevention of calcification was investigated by quantitative real-time PCR, Western blot, small interfering RNA (siRNA), nuclear and cytoplasmic extraction and immunofluorescence microscopy. The complete details of methods are available in Supplementary data.

3. Results

3.1. Sulfide inhibits the calcification of VSMC

To investigate the effects of sulfide on calcification, VSMC were exposed to secondary CPP (50 μg/mL calcium), in the absence and presence of increasing NaHS concentrations. Calcium deposits at baseline were low in the absence of secondary CPP, but following incubation with secondary CPP, Alizarin staining was vastly increased, demonstrating enhanced mineralization of VSMC (Fig. 1A). This increase in calcification was significantly ameliorated by concomitant exposure of VSMC to NaHS, starting at a concentration of 100 μmol/L. CPP and NaHS, alone or in combination, did not impair cell viability up to a concentration of 300 μmol/L (Fig. 1C). The effective NaHS concentration for the ensuing experiments was selected as the one with maximum calcification prevention potency and concurrent minimum cytotoxicity (300 μmol/L, Fig. 1B and C). Concentrations between 300 μmol/L and 1000 μmol/L will exhibit increasing cell toxicity while further reducing calcification.

In summary, secondary CPP induced a pronounced accumulation of VSMC-associated calcium, whereas sulfide reduced this accrual in a concentration dependent manner.

3.2. Sulfide ameliorates oxidative stress and cytokine expression in VSMC

Using next-generation sequencing, we investigated the whole-transcriptome changes in NaHS-treated versus non-treated calcifying VSMC. The associated volcano plot illustrates the differential expression of more than 8000 genes (Fig. 2A). Further data analysis using a threshold fold change of 1.5 revealed that only 33 transcripts were significantly differentially up- or down-regulated between both groups (Fig. 2B and Suppl. Table 2). Gene Ontology enriched those genes into processes such as e.g. regulation of reactive oxygen species, response to hydrogen peroxide and inflammatory responses (Fig. 2C). Next, we used gene network analysis to explore the possible interactions among the 33 differentially expressed genes and selected cellular processes. This analysis revealed that 19 out of 33 genes are directly or indirectly involved in cellular responses to oxidative stress or inflammation (Fig. 2D). The expression of *CCL2* and *CXCL2* as representative inflammatory response genes; and *DKK1* and *RCAN1* genes as representative regulators for oxidative stress, were chosen for the