Hydrogen Sulfide (H_2S) Attenuates CPP-induced Calcification of Vascular Smooth Muscle Cells via Activation of the KEAP1/NRF2/NQO1 Signaling Pathway

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Background: Vascular calcification is associated with increased morbidity and mortality in chronic kidney disease (CKD). Secondary calciprotein particles (CPP) and oxidative stress induce calcification of Vascular Smooth Muscle Cells (VSMC) *in vitro*. Hydrogen sulfide (H_2S) is a signaling molecule with antioxidant properties. Here we investigated the anticalcific properties of H_2S in a CPP-induced *in vitro* model of VSMC mineralisation.

Methods: We used next-generation sequencing to investigate differential transcriptomic changes in NaHS- (H_2S-donor) treated versus non-treated calcifying VSMC. The potential role of regulatory pathways in calcification-prevention identified using this approach was investigated using RT-PCR, Western-blotting and silencing with small interfering RNA (siRNA).

Results: To investigate the effect of H_2S on calcification, VSMC were exposed to secondary CPP (50 µg/ml calcium). Furthermore, VSMC were exposed to secondary CPP in the presence of H_2S (300 µmol/L). Secondary CPP induced a pronounced accumulation of calcium within one day, and H_2S significantly ameliorated this accumulation (Figure 1).

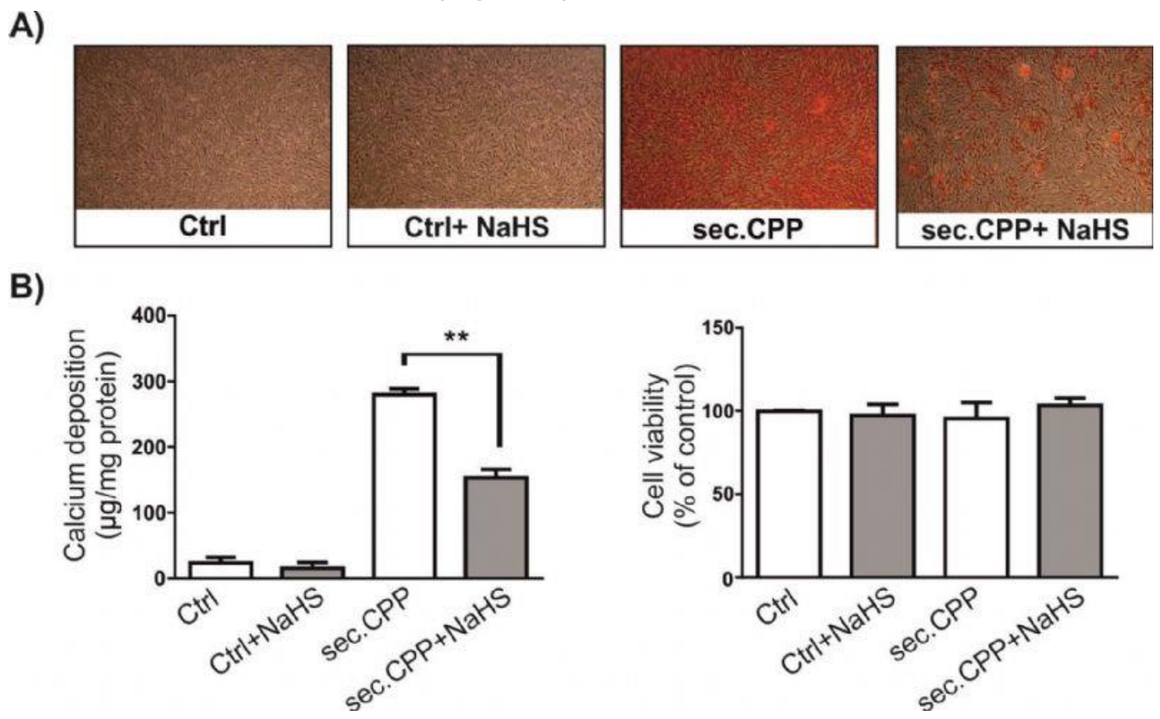

Figure 1: H_2S attenuates the calcification of vascular smooth muscle cells. A, VSMCs were treated with calcification media (including secondary CPP) or with control media (no secondary CPP) in the presence or absence of H_2S. Alizarin red staining was performed after 24 hrs. **B**, Quantification of calcium deposition and cell viability analysis (mean ±SD; N=3; ** *p*< 0.01).

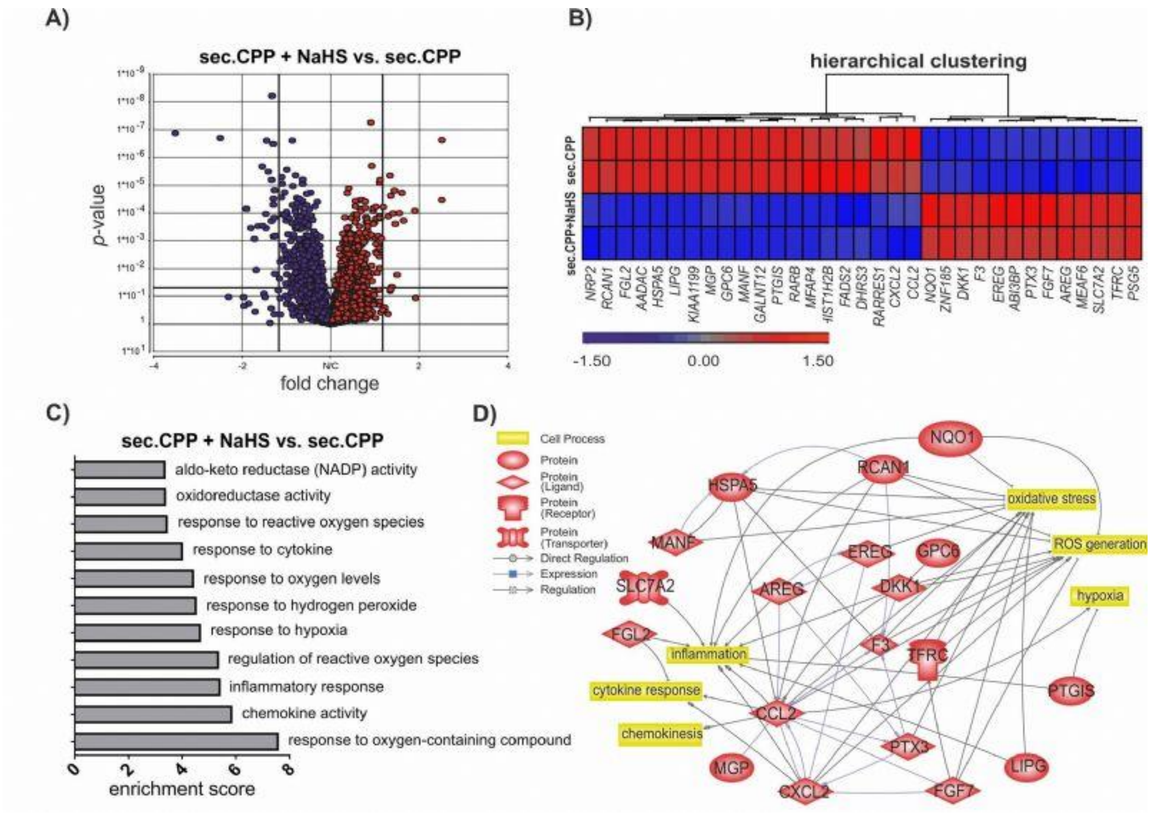

Fig 2. NaHS (H_2S-donor) ameliorates oxidative stress and inflammation in VSMC. **A**, Volcano plot of significant and non-significant differentially expressed genes upon treatment (red or blue colors indicate the up- and down-regulated genes, respectively) **B**, Heatmap representation of significantly expressed genes of sec.CPP + NaHS vs. sec.CPP alone. **C**, Significantly enriched gene ontology (GO) terms associated with dysregulated genes upon NaHS treatment (enrichment scores equal or higher than three are considered statistically significant). **D**, Gene network analysis to explore the possible interactions among the significantly expressed genes and selected cellular processes (cellular responses to oxidative stress or inflammation).

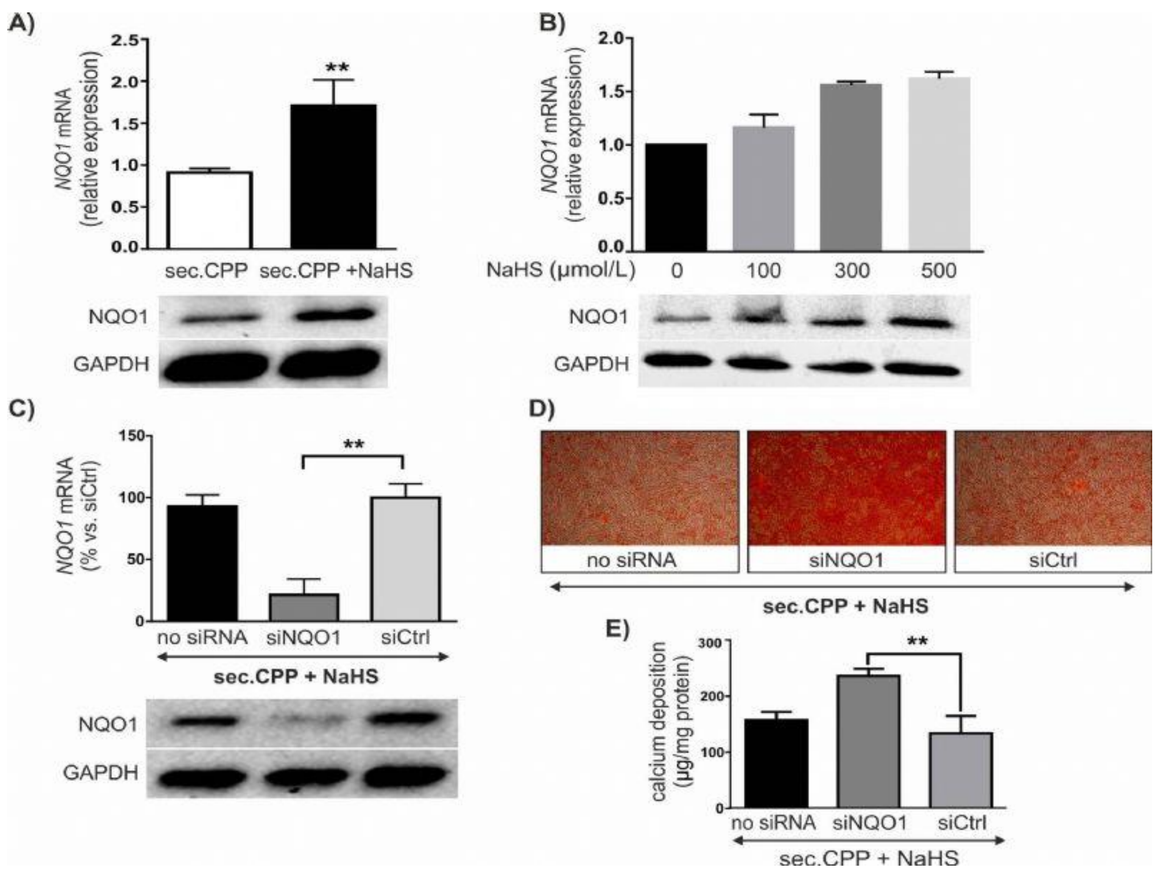

Fig 3. NQO1 mediates the inhibitory effects of NaHS on VSMC calcification. **A**, VSMC and sec.CPP were incubated in the presence or absence of 300 µmol/L NaHS for 24 hrs. NQO1 was determined by RT-PCR and Western blot analysis. **B**, NaHS stimulates NQO1 mRNA and protein expression in a concentration-dependent manner in the absence of sec.CPP. **C**, VSMC were treated with siNQO1 or siCtrl and incubated with secondary CPP + NaHS for 24 hrs. Significant down-regulation of NQO1 was confirmed by RT-PCR and Western blot analysis. **D**, The inhibitory effect of NaHS on calcification is blunted by NQO1 gene knockdown. Calcification was visualized by Alizarin Red staining, and confirmed by **E**, Quantification of calcium deposition (mean±SD; N=3; ** p< 0.01).

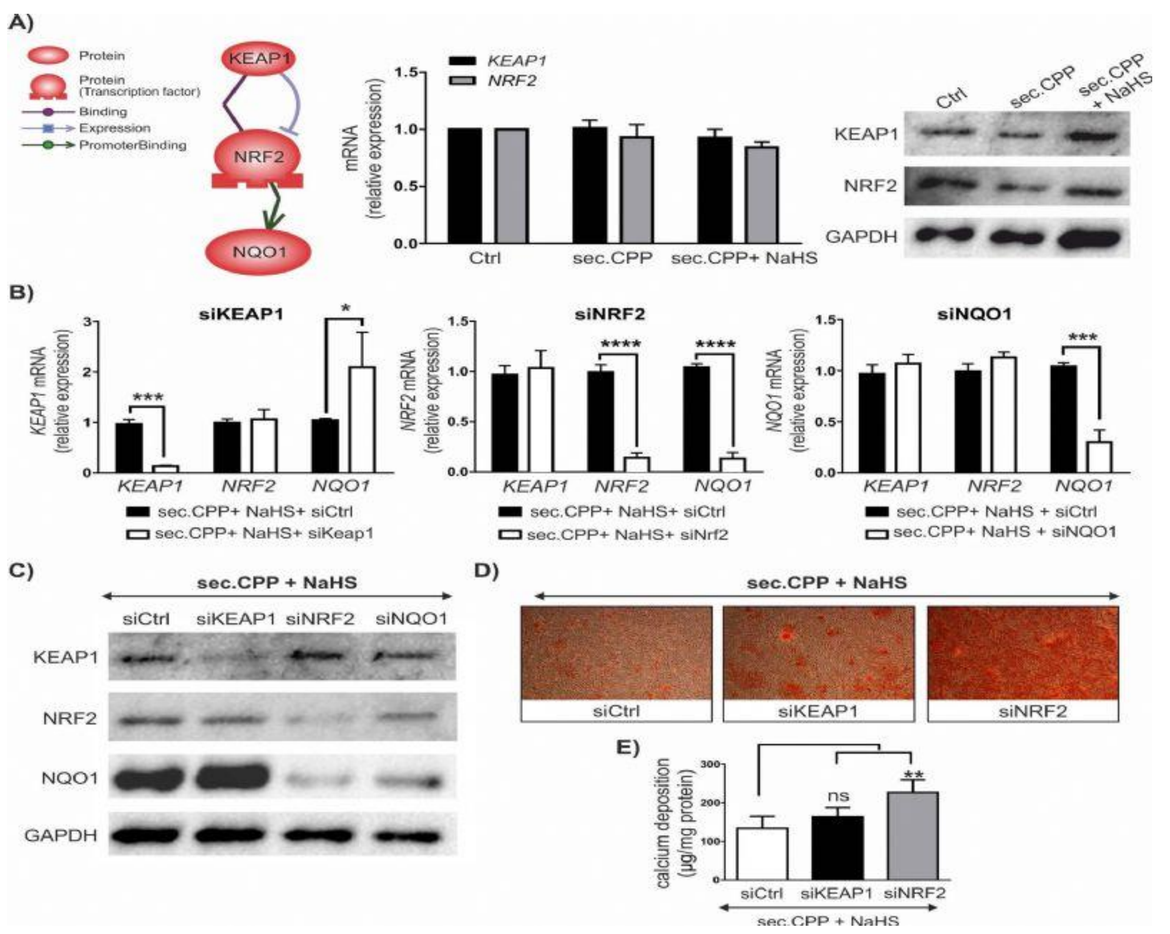

Fig 4. NaHS acts via modulating KEAP1- and NRF2-regulated NQO1 gene expression. A, Effects of NaHS on KEAP1 and NRF2 expression, determined by RT-PCR and Western blot analysis. GAPDH was used as internal control. **B**, Gene silencing with specific siRNA. After 24 hrs, cells were treated with a combination of sec.CPP and 300 µmol/L NaHS, and the resulting mRNA levels were determined by RT-PCR. **C**, Western blot analysis of KEAP1, NRF2 and NQO1 protein levels. **D**, Effect of KEAP1 and NRF2 silencing on calcification inhibition by sulfide. Calcification was visualized by Alizarin Red staining and **E**, Quantification of calcium deposition (mean±SD; N=3; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

Conclusions:

Our data provide evidence that secondary CPP induce genes related to oxidative stress and inflammation, an effect ameliorated by the anti-oxidant activity of H2S/HS-. The latter is mediated by NQO1 and under regulatory control of the KEAP1/NRF2 pathway. These results may open potential novel strategies to prevent vascular calcification.

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