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ORIGINAL RESEARCH

Reactive Oxygen-Forming Nox5 Links Vascular Smooth Muscle Cell Phenotypic Switching and Extracellular Vesicle-Mediated Vascular Calcification

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RATIONALE: Vascular calcification, the formation of calcium phosphate crystals in the vessel wall, is mediated by vascular smooth muscle cells (VSMCs). However, the underlying molecular mechanisms remain elusive, precluding mechanism-based therapies.

OBJECTIVE: Phenotypic switching denotes a loss of contractile proteins and an increase in migration and proliferation, whereby VSMCs are termed synthetic. We examined how VSMC phenotypic switching influences vascular calcification and the possible role of the uniquely calcium-dependent reactive oxygen species (ROS)-forming Nox5 (NADPH oxidase 5).

METHODS AND RESULTS: In vitro cultures of synthetic VSMCs showed decreased expression of contractile markers CNN-1 (calponin 1), α-SMA (α-smooth muscle actin), and SM22-α (smooth muscle protein 22α) and an increase in synthetic marker S100A4 (S100 calcium binding protein A4) compared with contractile VSMCs. This was associated with increased calcification of synthetic cells in response to high extracellular Ca^{2+}. Phenotypic switching was accompanied by increased levels of ROS and Ca^{2+}-dependent Nox5 in synthetic VSMCs. Nox5 itself regulated VSMC phenotype as siRNA knockdown of Nox5 increased contractile marker expression and decreased calcification, while overexpression of Nox5 decreased contractile marker expression. ROS production in synthetic VSMCs was cytosolic Ca^{2+}-dependent, in line with it being mediated by Nox5. Treatment of VSMCs with Ca^{2+} loaded extracellular vesicles (EVs) lead to an increase in cytosolic Ca^{2+}. Inhibiting EV endocytosis with dynasore blocked the increase in cytosolic Ca^{2+} and VSMC calcification. Increased ROS production resulted in increased EV release and decreased phagocytosis by VSMCs.

CONCLUSIONS: We show here that contractile VSMCs are resistant to calcification and identify Nox5 as a key regulator of VSMC phenotypic switching. Additionally, we describe a new mechanism of Ca^{2+} uptake via EVs and show that Ca^{2+} induces ROS production in VSMCs via Nox5. ROS production is required for release of EVs, which promote calcification. Identifying molecular pathways that control Nox5 and VSMC-derived EVs provides potential targets to modulate vascular remodeling and calcification in the context of mineral imbalance.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: calcium ■ extracellular vesicles ■ NADPH oxidase 5 ■ phenotype ■ vascular calcification

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ORIGINAL RESEARCH

Novelty and Significance

What Is Known?

- Vascular calcification causes vascular stiffness that leads to cardiovascular complications. The amount of calcification correlates with atherosclerotic burden and it is prevalent in chronic kidney disease where a serum mineral imbalance occurs.
- Vascular calcification is mediated by vascular smooth muscle cells (VSMCs), which show high phenotypic plasticity.
- Extracellular vesicles (EVs) released by VSMCs contribute to vascular calcification by providing calcification nucleation sites.

What New Information Does This Article Contribute?

- Nox5 (NADPH oxidase 5) contributes to VSMC phenotypic switching. Synthetic (dedifferentiated) VSMCs are more prone to calcification than contractile VSMCs.
- EVs mediate Ca^{2+} entry into VSMCs in the context of elevated extracellular Ca^{2+}, via clathrin- and caveolin-mediated uptake, leading to rises in cytosolic Ca^{2+} levels.
- Ca^{2+}-dependent Nox5 increases oxidative stress, which leads to increased EV release from VSMCs and subsequent calcification.

Understanding vascular calcification is important not only for chronic kidney disease, where VSMCs are directly exposed to elevated Ca^{2+} levels, but other vascular diseases, where vascular remodeling and VSMC phenotypic switching occurs as a result of other mechanisms; for example, increased PDGF signaling in settings such as atherosclerosis, aneurysm, and hypertension. Nox5 is a possible therapeutic target with broad clinical applications. EVs are are key contributors to biomineralization processes, but the mechanisms associated with their release and function are poorly understood. Our study describes a new mechanism of Ca^{2+} entry into the cell via EVs and identifies Nox5-generated oxidative stress as a major stimulus for EV release.

Nonstandard Abbreviations and Acronyms

α-SMA	α-smooth muscle actin
BAPTA-AM	1,2-bis-(o-aminophenoxy)-ethane-N,N,N,N-tetraacetic acid
CNN-1	calponin 1
EV	extracellular vesicle
hVSMC	human VSMC
MLC	myosin heavy and light chain
MYH	myosin heavy chain
MYL	myosin light chain
MYOCD	myocardin
Nox5	NADPH oxidase 5
pVSMC	porcine VSMC
ROS	reactive oxygen species
SM22-α	smooth muscle protein 22α
SMTN	smoothelin
VSMC	vascular smooth muscle cell

Vascular calcification is the formation of calcium phosphate crystals in the blood vessel wall[1] and is associated with a 3- to 4-fold increase in cardiovascular and all-cause mortality.[2] Vascular calcification can be categorized based on its location in the vessel wall into intimal, predominantly associated with atherosclerosis, and medial, associated with aging, chronic kidney disease, and diabetes mellitus.[3] The result of intimal calcification is an increased risk of plaque rupture,[4] which causes myocardial infarction and strokes.[5] Medial calcification in chronic kidney disease is attributed to an imbalance in calcium and phosphate metabolism.[6] The direct result of medial vascular calcification is blood vessel wall stiffening that leads to many cardiovascular complications, such as hypertension and aortic stenosis. These, in turn, give rise to cardiac hypertrophy, myocardial and lower-limb ischemia, congestive heart failure and can eventually result in death.[7] To date, no early detection methods and no proven therapies exist to inhibit or reverse vascular calcification.

Vascular calcification is an active process regulated by vascular smooth muscle cells (VSMCs) via several mechanisms, including apoptosis,[8] osteo/chondrogenic transdifferentiation,[9] extracellular vesicle (EV) release,[10] and cellular senescence.[11] In healthy arteries, most VSMCs of the tunica media maintain a contractile phenotype, which enables them to regulate vascular tone and maintain hemodynamic balance. Physiological or pathological changes may demand adaptation of the involved arteries. Stress signals, such as oxidative or mechanical stress, cause VSMC phenotype to change. These dedifferentiated or synthetic VSMCs are characterized by a decreased expression of contractile proteins (MLC [myosin light chain], calponin [CNN-1], smoothelin, α-SMA [α-smooth muscle actin], SM22α [smooth muscle protein 22α]), increased proliferation and migration.[12,13] The process of dedifferentiation is termed phenotypic switching. Phenotypic switching is thought to precede the

development of vascular disease. In specific pathologies VSMCs have been shown to differentiate even further and give rise to other cell types in the vessel wall including macrophages[14] and osteo/chondrogenic cells.[15,16]

Although phenotypic switching is thought to precede vascular disease, including vascular calcification, molecular events leading to the loss of contractile phenotype and mechanisms driving the synthetic VSMCs towards calcification, are at present not fully understood. Therefore, in this study, we set out to investigate the relationship between VSMC phenotypic switching and vascular calcification and the mechanisms that link these processes.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

An expanded Materials and Methods is available in the Data Supplement. Please see the Major Resources Table in the Data Supplement.

Cell Culture, Treatments, and Transfections

Human and porcine aortic VSMCs (hVSMCs and pVSMCs) were derived from tissue explants and cultured as described previously.[17,18] Collection, storage, and use of tissue and human aortic samples were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue. HASMC66 cell line was cultured as described previously.[19] Each experiment was carried repeated in VSMCs from at least 3 different donors. VSMCs in passages 5 to 12 were used. Heparin, PDGF-BB (platelet derived growth factor subunit B), GKT136901, VAS2870, 1,2-bis-(o-aminophenoxy)-ethane-N,N,N,N-tetraacetic acid (BAPTA-AM), dynasore, and H_2O_2 were used at concentrations stated in the figure legends. For calcification assays VSMCs were treated with control (1.8 mmol/L Ca^{2+}) or high calcium (3.6 mmol/L or 5.4 mmol/L $CaCl_2$) medium. Calcification was measured as previously described.[8] hVSMCs were transduced with lentiviral vectors Nox5 (NADPH oxidase 5)-β and green fluorescent protein (GFP) sequences for 48 hours. SiRNA (S103243856, Qiagen) transfection was performed using a Basic Smooth Muscle Cells Nucleofector Kit (Lonza) for 24 hours.

Cell Assays

pVSMC proliferation was measured real-time using the xCELLigence System (ACEA Biosciences). Reactive oxygen species (ROS) were quantified with Amplex red or DCFDA. Cytosolic Ca^{2+} was measured using Fluo-4-AM in Cytation3 (Biotek). Live single-cell imaging of cytosolic Ca^{2+} was performed by monitoring Fura-2 fluorescence using an Olympus Cell^R imaging system as described previously.[20]

Immunoblotting, Quantitative Reverse Transcriptase Polymerase Chain Reaction, and Immunofluorescence

VSMC RNA was reverse transcribed using Mu-MLV reverse transcriptase (Invitrogen) and the SYBR quantitative polymerase chain reaction assay (BioRad) according to manufacturer's protocol. Immunoblotting and immunofluorescence were performed as previously described.[21]

The BiKE Cohort

Patients undergoing surgery for symptomatic (S) or asymptomatic (AS), high-grade (>50% NASCET [North American Symptomatic Carotid Endarterectomy Trial]) carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital, Sweden, were consecutively enrolled in the study and clinical data recorded on admission.[22] All samples were collected with informed consent from patients or organ donors' guardians. All human studies were approved by the regional Ethical Committees. The microarray data set is available from Gene Expression Omnibus (GSE21545).

Immunohistochemistry

Human coronary artery sections were collected during autopsy from 20 patients aged 47 to 86 years, who died from noncardiac causes. Autopsy was performed 6 to 9 hours after death (Department of Pathology, Academic Hospital Maastricht, Maastricht). Tissue collection was approved by the Maastricht Pathology Tissue Collection committee. The Medical Ethics Committee of the Maastricht University approved the study protocol and all subjects gave their informed consent in writing. Immunohistochemical staining was performed as described elsewhere.[23]

EV Isolation and Quantification

EVs were isolated by differential ultracentrifugation as previously described[17] from HASMC66[24] cells, pVSMCs, and hVSMCs. Quantification of EVs secreted in the cell culture media was performed using a bead capture assay, as previously described.[25]

Phagocytosis of EVs and CFSE Loading

EVs (15 μg protein), collected from HASMC66, were labeled with CFSE (carboxyfluorescein succinimidyl ester) for 30 minutes at 37°C and incubated with VSMCs. After incubation, cells were trypsinized, washed, and measured by flow cytometry (Acuri C6, BD biosciences).

Statistical Analysis

Data are shown as mean±SD and were obtained in 3 or more independent experiments. Normality of all data was tested using the Shapiro-Wilk test (for n≥4) or ascertained based on previous reports in literature (for n=3). If data were normally distributed, statistical significance was tested with *t* test, one-sample *t* test, and 1-way ANOVA with Bonferroni post hoc for experiments with 2 and more groups, respectively. If data were not normally distributed, the Mann-Whitney, Wilcoxon signed-rank test, and Kruskal-Wallis with Dunn multiple comparisons tests were used. The exact test used for each data set is mentioned in figure legends. Statistical analysis was performed using using GraphPad Prism 8.2.0. *P<0.05, **P<0.01, and ***P<0.001. No corrections for multiple testing were made across tests. Representative images for figures, which best reflected the data, were selected manually.

Pearson correlations were used to calculate the association between mRNA expression of Nox5 and other markers from the BiKE plaque microarray data set. Significance was considered when $P<0.05$.

RESULTS

Phenotypic Switching of VSMCs In Vitro Is Associated With Calcification Due to Increased ROS Production

Cultured VSMCs display progressive loss of contractile proteins, shifting towards a synthetic phenotype.[26] To study the relationship between VSMC phenotype and calcification in vitro we used 2 model systems: (1) pVSMCs isolated as distinct populations of contractile or synthetic cells[18] and (2) hVSMCs treated with heparin or PDGF-BB to induce the contractile or synthetic phenotype, respectively.[18,27] We confirmed that contractile pVSMCs express higher levels of contractile markers CNN-1 and α-SMA (Figure 1A) than synthetic cells. As previously described,[18] the morphology of the cells differed, with synthetic cells being more rhomboid and contractile cells elongated. Next, we demonstrated that the phenotype switch is reversible, as treatment of contractile pVSMC with PDGF-BB caused a loss of α-SMA (contractile versus synthetic $P=0.0313$, contractile versus contractile + PDGF $P=0.022$), SM22α (contractile versus synthetic $P=0.0006$, contractile versus contractile + PDGF $P=0.0105$) and CNN-1 (contractile versus synthetic $P=0.0001$, contractile versus contractile + PDGF $P=0.0001$) expression and a concomitant gain of S100A4 (S100 calcium binding protein A4) expression (contractile versus synthetic $P=1.7\times10^{-17}$, contractile versus contractile + PDGF $P=0.0001$), a synthetic marker (Figure 1B through 1F).[28] Conversely, treating synthetic pVSMCs with heparin led to upregulated α-SMA (synthetic versus synthetic + heparin $P=0.0013$), SM22-α (synthetic versus synthetic + heparin $P=0.0061$), and CNN-1 (synthetic versus synthetic + heparin $P=0.0070$) and decreased S100A4 expression (synthetic versus synthetic + heparin $P=1.7\times10^{-6}$) indicating a switch towards a contractile phenotype. Similar effects of PDGF-BB and heparin were observed in hVSMCs (Figure IA and IB in the Data Supplement). Additionally, synthetic VSMCs showed higher rates of proliferation (Figure 1G, $P=0.0079$; Figure IC in the Data Supplement, $P=0.0045$) than contractile VSMCs.

To accelerate the process of calcification in vitro, we cultured VSMCs in medium with an increased Ca^{2+} concentration. Contractile pVSMCs showed no significant Ca^{2+} crystal formation, whereas synthetic pVSMCs exposed to calcifying conditions calcified significantly (Figure 2A, $P=1$ for 1.8 mmol/L, $P=1.3\times10^{-5}$ for 5.4 mmol/L; Figure ID in the Data Supplement, $P=1$ for 1.8 mmol/L, $P=1.7\times10^{-10}$ for 5.4 mmol/L). The increased calcification could be partly rescued by reversing pVSMC phenotype with heparin (Figure 2B, $P=2.4\times10^{-5}$ and Figure 2C, $P=1.9\times10^{-5}$). This suggests that the rate of calcification is linked to VSMC phenotype. A similar dependency of calcification on phenotype was observed in hVSMCs (Figure IE in the Data Supplement, $P=1$ for 1.8 mmol/L, $P=9\times10^{-5}$ for 3.6 mmol/L, $P=0.0027$ for 5.4 mmol/L; Figure IF in the Data Supplement, $P=1$ for 1.8 mmol/L, $P=7\times10^{-5}$ for 3.6 mmol/L, $P=8.8\times10^{-5}$ for 5.4 mmol/L).

We next set out to investigate the mechanisms, which could explain the different rates at which contractile and synthetic VSMCs calcify. Expression of osteogenic genes, classically associated with calcification, was not different between the phenotypes at baseline, except a higher expression of matrix Gla protein (MGP) mRNA in contractile pVSMCs compared with synthetic pVSMCs (Figure IG and IH in the Data Supplement $P=2.2\times10^{-7}$ for α-SMA, $P=0.585$ for RUNX2 (RUNX family transcription factor 2), $P=0.609$ for Osterix, $P=0.266$ for BMP2 (bone morphogenetic protein 2), $P=5.6\times10^{-6}$ for MGP). Therefore, we focused on oxidative stress, which is a known mediator of vascular calcification.[29] Contractile and synthetic pVSMCs produced comparable amounts of H_2O_2 under noncalcifying conditions (Figure 2D, $P=0.1407$). However, exposure to high Ca^{2+} levels resulted in increased production of H_2O_2 by synthetic pVSMCs (Figure 2D, $P=0.0020$). This suggested that in these cells the rise in ROS production is Ca^{2+}-dependent. In support of this notion, antioxidant N-acetylcysteine was able to decrease Ca^{2+}-induced calcification of synthetic pVSMCs (Figure 2E, $P=0.0118$).

To examine the mechanism responsible for increased ROS in synthetic VSMCs, we compared expression of 3 Nox (NADPH oxidase) enzymes between the phenotypes. We found that Nox1 expression was similar in both phenotypes ($P=0.1590$), but both Nox4 and Nox5 were significantly upregulated ($P=0.02399$ and $P=0.0107$, respectively) in synthetic pVSMCs compared with contractile pVSMCs on mRNA level (Figure 2F). However, only Nox5 expression was significantly upregulated when measured by Western blotting ($P=0.0702$ for Nox4, $P=0.0127$ for Nox5; Figure 2G through 2J, Figure II in the Data Supplement).

Nox5 is Highly Expressed in Synthetic VSMCs and Mediates Phenotypic Switching and Calcification

We next set out to investigate whether Nox4 and Nox5 were involved in synthetic phenotype-related calcification in our model. Since Nox4 was expressed at lower levels than Nox5 and we excluded a role for Nox4 in VSMC calcification (Figure IIA in the Data Supplement, $P=0.4848$), we focused on Nox5. First, we confirmed that switching contractile pVSMCs to synthetic cells with PDGF significantly increased expression of Nox5 mRNA (Figure 3A,

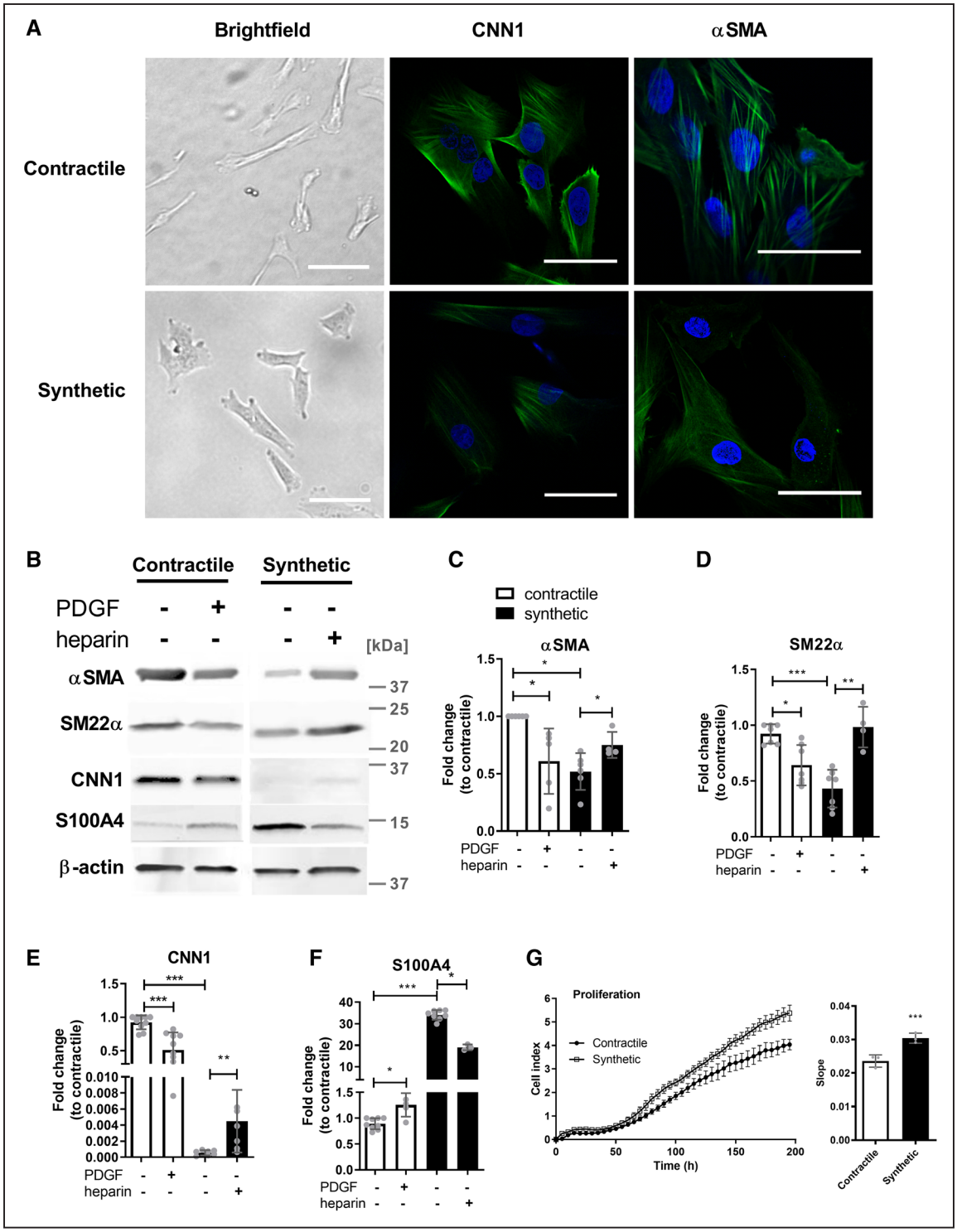

Figure 1. Phenotypic switching of porcine vascular smooth muscle cells (pVSMCs) is associated with changes in contractile gene expression and proliferation.

A, Brightfield images and immunocytochemical staining of contractile proteins in synthetic and contractile pVSMCs showing differences in cell morphology and CNN-1 (calponin 1) and α-SMA (α-smooth muscle actin) expression. Scale bars: 50 μm (immunocytochemistry), 1 mm (brightfield). Representative images from 3 independent experiments. **B**–**F**, Western blotting and quantification of contractile proteins and synthetic marker in contractile cells treated with PDGF and synthetic cells treated with heparin, showing phenotypic switching was partially reversible. Cells were grown in DMEM with 10% FBS with 20 ng/mL PDGF for 2 d or 200 U/mL heparin for 5 d. Molecular weight markers in all Western blots indicated in kDa. Graphs show pooled data from 3 independent experiments, some performed in triplicate. Statistical significance was tested using the Wilcoxon signed-rank test (**C**) or the Mann-Whitney *U* test (**D** and **E**) or *t* test (**F**). **G**, Proliferation of pVSMCs was measured using the xCELLigence system, which measures the impedance of cells adhering to an electrode-covered surface. Synthetic cells show increased proliferation. Data from one representative experiment performed in triplicate. Statistical significance was tested using *t* test.

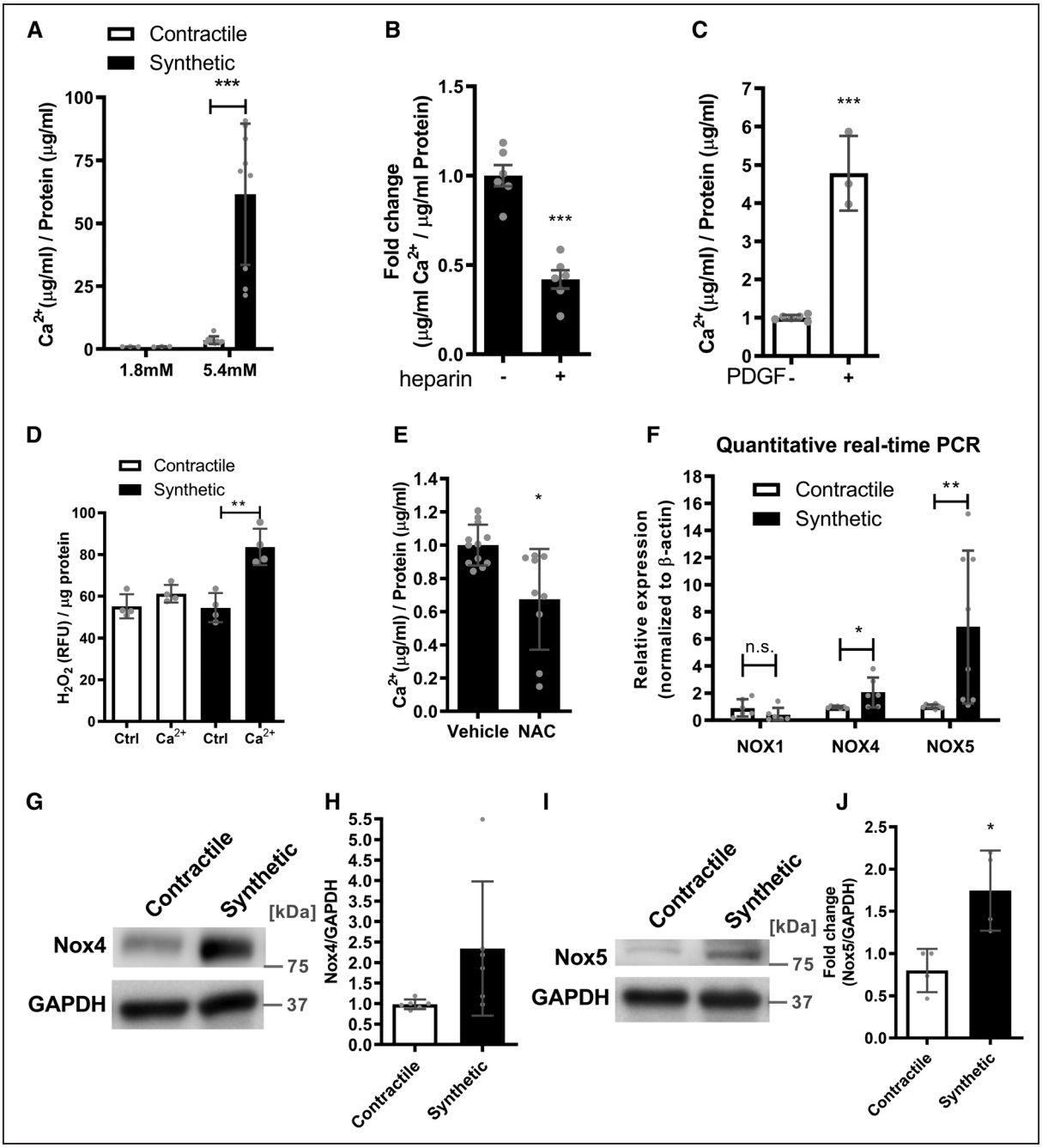

Figure 2. Synthetic phenotype is associated with increased calcification, reactive oxygen species (ROS) production, and Nox (NADPH oxidase) expression.

Calcification was induced by incubation with elevated Ca^{2+} concentrations (5.4 mmol/L) in DMEM with 0.5% FBS and quantified using an o-cresolphthalein colorimetric assay. **A**, Synthetic porcine vascular smooth muscle cells (pVSMCs) showed significant calcification in contrast to contractile cells after 48 h. Statistical significance was tested using the Mann-Whitney *U* test. Pooled data from 3 independent experiments performed in duplicate. **B**, Treating synthetic pVSMCs with heparin decreased calcification after 48 h. Statistical significance was tested with *t* test. Pooled data from 3 independent experiments performed in duplicate. **C**, Treating contractile pVSMCs with PDGF for 48 h increased calcification. Statistical significance was tested with *t* test. Pooled data from 3 independent experiments performed in duplicate. **D**, Synthetic pVSMCs treated with 5.4 mmol/L Ca^{2+} in 0.5% FBS for 22 h produced more H_2O_2 than contractile cells. H_2O_2 production was measured using Amplex red. Statistical significance was tested with *t* test. Data from one representative experiment performed in quadruplicate. **E**, Scavenging H_2O_2 with 1 mmol/L N-acetylcysteine (NAC) decreased calcification of synthetic pVSMCs treated with 5.4 mmol/L Ca^{2+} in 0.5% FBS for 48 h. Statistical significance was tested using the Mann-Whitney *U* test. Pooled data from 3 independent experiments performed in triplicate or quadruplicate. **F**, Quantitative polymerase chain reaction (QPCR) analysis showed increased Nox4 and Nox5 expression in synthetic pVSMCs at baseline. Statistical significance was tested with *t* tests. Pooled data from 3 independent experiments carried out in triplicate or quadruplicate. **G–J**, Western blotting and quantification of Nox4 and Nox5 in contractile and synthetic pVSMCs. Molecular weight markers in all Western blots indicated in kDa. Statistical significance was tested with *t* tests (**H** and **J**). Data from one representative experiment performed in triplicate. Statistical significance was tested using *t* test.

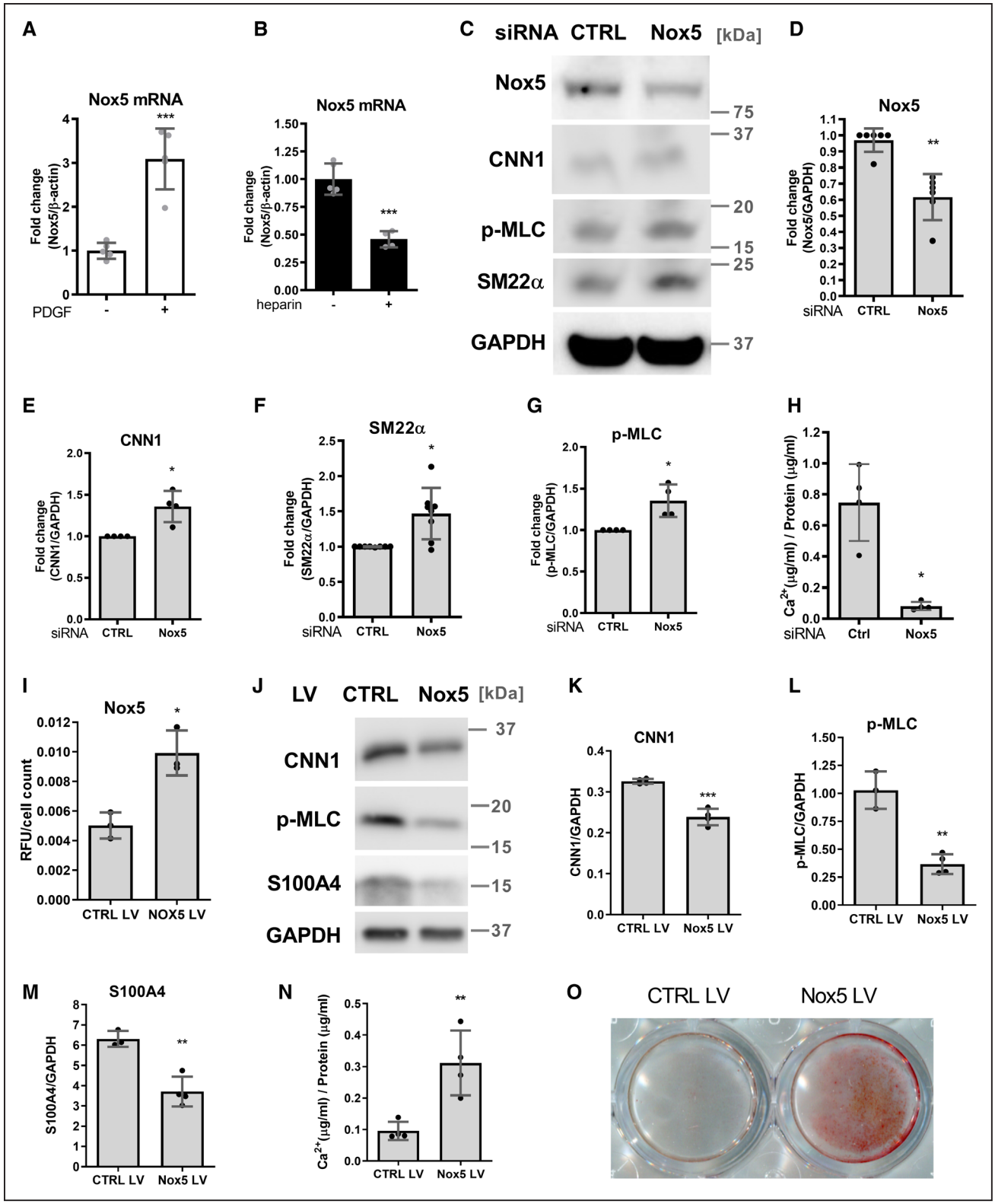

Figure 3. Nox5 (NADPH oxidase 5) mediates phenotypic switching and vascular smooth muscle cell (VSMC) calcification. **A** and **B**, qPCR analysis of Nox5 mRNA expression in synthetic porcine VSMCs (pVSMCs) treated with heparin (200 U/mL, 5 d) and contractile pVSMCs treated with PDGF (20 ng/mL, 2 d). Expression was normalized to β-actin. Statistical significance was tested using *t* test. Pooled data from 2 independent experiments performed in duplicate. **C–G**, SiRNA knockdown (24 h) of Nox5 increased expression of contractile markers in human VSMCs (hVSMCs) (Western blot and quantifications). Molecular weight markers in all Western blots indicated in kDa. Statistical significance was tested using the Mann-Whitney *U* test (**D**), Wilcoxon signed-rank test (**F**), or one-sample *t* test (**E** and **G**). Pooled data from 4 to 8 independent experiments. **H**, SiRNA knockdown (24 h) of Nox5 decreased calcification of hVSMCs (*Continued*)

P=0.0002), whereas switching synthetic pVSMCs with heparin to contractile cells decreased Nox5 levels (Figure 3B, *P*=0.0002). Knockdown of Nox5 (*P*=0.0022) in hVSMCs resulted in increased expression of contractile markers CNN-1 (*P*=0.0319), p-MLC (*P*=0.0008), and SM22-α (*P*=0.0156, Figure 3C through 3G) and decreased calcification (Figure 3H, *P*=0.0018). Conversely, overexpression of Nox5 (*P*=0.0084) resulted in decreased expression of contractile markers p-MLC (*P*=0.0010) and CNN-1 (*P*=0.0002) as well as synthetic marker S100A4 (*P*=0.0028, Figure 3I through 3M, Figure IIB in the Data Supplement) and increased calcification (*P*=0.0068, Figure 3N and 3O). Additionally, H_2O_2 treatment decreased p-MLC (*P*=0.0005) and S100A4 (*P*=0.0024) expression in hVSMCs (Figure IIC in the Data Supplement). These results imply that signals which induce phenotype changes, do so via decreased or increased expression of Nox5 and resulting changes in ROS levels. Interestingly, our results suggest that upregulation of S100A4 in synthetic cells occurs via mechanisms other that Nox5-mediated ROS upregulation.

Ca^{2+}-Dependent Oxidative Stress Mediates pVSMC Calcification

Nox5 is known to be activated by binding of cytosolic Ca^{2+} to its EF hand,[30] therefore, we hypothesized that it is the molecular link between increased extracellular Ca^{2+}, and ROS-dependent calcification of synthetic VSMCs. First, we investigated EV-mediated uptake as a possible mechanism by which increased extracellular Ca^{2+} can increase cytosolic Ca^{2+}. To this end, hVSMCs were treated with dynasore, an inhibitor of clathrin- and caveolin-dependent EV uptake[31] in the presence of high Ca^{2+}. Dynasore blocked the increase in cytosolic Ca^{2+} caused by the high Ca^{2+} treatment (*P*=0.0241 for control versus 3.6 mmol/L Ca^{2+} and *P*=0.0190 for 3.6 mmol/L Ca^{2+} versus 3.6 mmol/L Ca^{2+} + dynasore, *P*=0.059 for control versus ionomycin; Figure 4A), suggesting that Ca^{2+} enters the cells via EVs. Ionomycin, which causes a rapid influx of Ca^{2+} into the cells, was used as a positive control. In line with this, EVs isolated from cells incubated with 3.6 mmol/L Ca^{2+} accumulated significantly higher (1 versus 4.68±1.55, *P*=0.0497) Ca^{2+} compared with EVs isolated from cells treated with normal calcium (1.8 mmol/L Ca^{2+}; Figure 4B). When EVs were isolated from cells treated in normal Ca^{2+}, but later incubated with high Ca^{2+} in the absence of cells, they also accumulated Ca^{2+} (1 versus 3.71±2.32, *P*=0.1812; Figure 4C) suggesting that Ca^{2+} loading into EVs happens outside of the cells. Additionally, we performed a more sensitive cytosolic Ca^{2+} assay in single cells. VSMCs were treated with high Ca^{2+}, which resulted in an insignificant increase in cytosolic Ca^{2+} in 1 hour, compared with control (Figure 4D). Interestingly, when VSMCs cultured in medium with normal Ca^{2+} were treated with EVs isolated from VSMCs treated with high Ca^{2+}, transient increases in cytosolic Ca^{2+} were observed (Figure 4E). In VSMCs treated with EVs isolated from VSMCs incubated with normal Ca^{2+} no such increases were observed. These observations suggest that EVs generated in a high Ca^{2+} environment induce a cytosolic Ca^{2+} rise in VSMCs. Additionally, dynasore inhibited calcification of VSMCs (*P*=0.0213; Figure 4F). This suggests that Ca^{2+} entry via EVs contributes to VSMC calcification.

Next, we set out to investigate whether this rise of cytosolic Ca^{2+} leads to increased ROS production and calcification. First, we confirmed that overexpression of Nox5 in hVSMCs, which induced synthetic differentiation, lead to increased ROS production (*P*=0.0033; Figure 4G) and that siRNA knockdown of Nox5 lead to decreased ROS production (*P*=0.0005; Figure 4H). Then, we demonstrated that cytosolic Ca^{2+} chelator BAPTA-AM blocked the increase in H_2O_2 production by synthetic pVSMCs (*P*=0.0007; Figure 4I). H_2O_2 production was also blocked by the Nox inhibitor GKT136901 (*P*=4.6×10^{-5}; Figure 4I). Moreover, Nox inhibitors GKT136901 and VAS2870 as well as BAPTA-AM decreased VSMC calcification (*P*=0.0007 for GKT, *P*=4.6×10^{-5} for BAPTA, *P*=0.0367, *P*=0.0053, *P*=0.0310, and *P*=0.2894 for 1, 2, 5, and 10 μmol/L VAS, respectively; Figure 4J and 4K, Figure IID in the Data Supplement, Figure IIE in the Data Supplement hVSMCs, *P*=0.0052). Taken together these results show that cytosolic Ca^{2+}-Nox5-dependent ROS production is required for calcification of synthetic VSMCs.

Nox5-Mediated Oxidative Stress Induces EV Release and Inhibits Phagocytosis in Synthetic VSMCs

EVs have recently been shown to play an important role in VSMC calcification.[25] Therefore, we set out to investigate whether EVs could be the mediators of Ca^{2+}-Nox5-ROS-induced calcification of synthetic VSMCs. We first confirmed that externally added EVs (Figure IIIA in the Data Supplement) increased calcification of a collagen matrix (*P*=0.0005; Figure 5A) and of synthetic (*P*=0.0055 for control versus Ca^{2+}, *P*=0.0002 for control versus Ca^{2+} + EVs, *P*=0.0092 for Ca^{2+} versus Ca^{2+} + EVs; Figure 5B) and contractile pVSMCs (*P*=0.981 for control versus Ca^{2+},

Figure 3 Continued. (calcification was induced with 5.4 mmol/L Ca^{2+} in 2.5% FBS for 5 d). Statistical significance was tested using *t* test. Data from a representative experiment carried out in quadruplicate. **I**–**M**, Nox5 overexpression using a lentivirus resulted in decreased contractile protein expression and decreased S100A4 (S100 calcium binding protein A4) expression in hVSMCs (Western blotting and quantification). Nox5 was quantified using immunocytochemistry on the Cytation 3 imaging reader. Statistical significance was tested using *t* tests (**I**, **K**–**M**). Data from a representative experiment performed in quadruplicate. Molecular weight markers in all Western blots indicated in kDa. **N** and **O**, Nox5 overexpression increased calcification of hVSMCs treated with 5.4 mmol/L Ca^{2+} in 2.5% FBS for 3 d, measured using o-cresolphthtalein and visualized with Alizarin Red S. Statistical significance was tested using *t* test. Data from a representative experiment performed in quadruplicate.

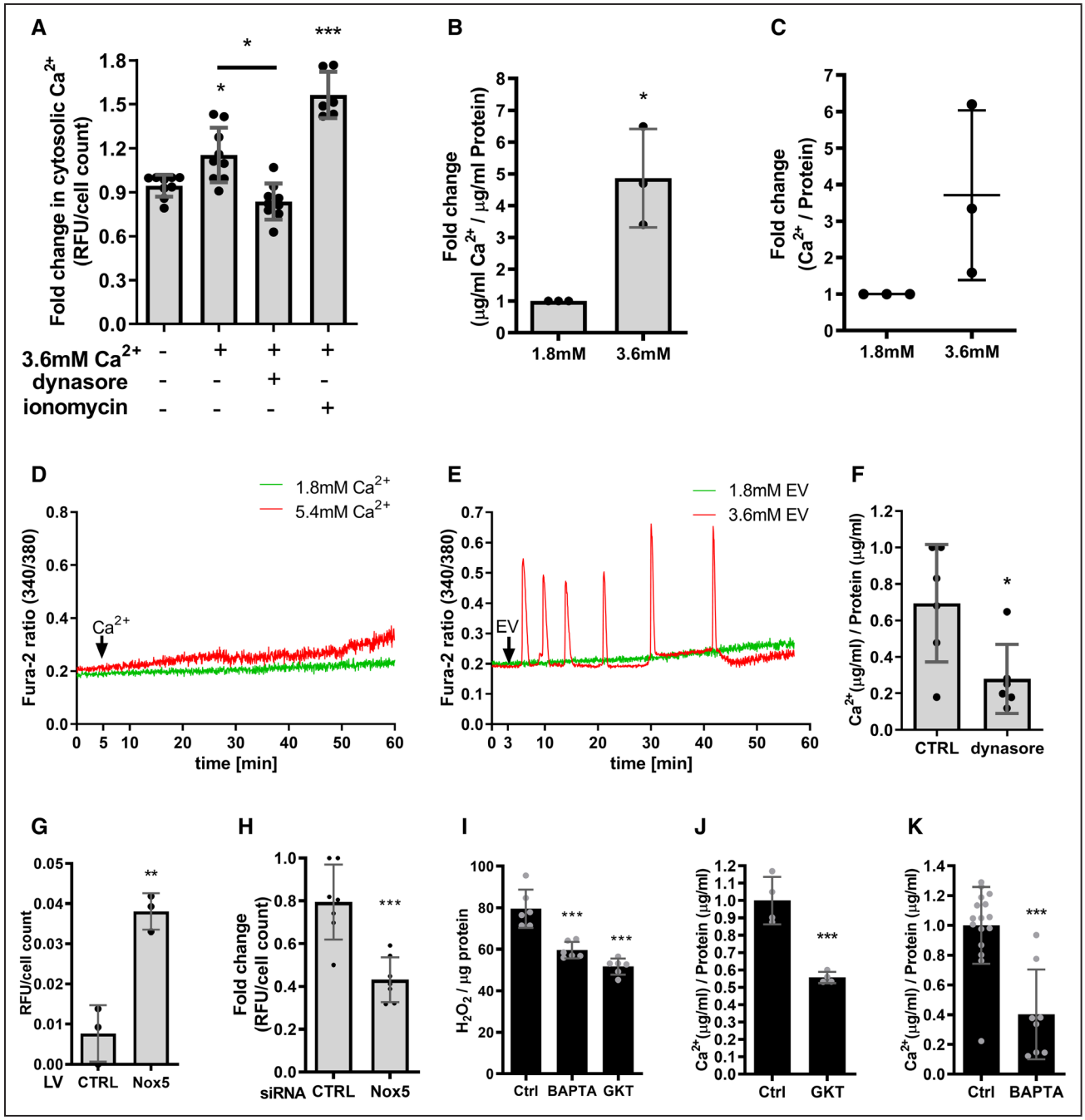

Figure 4. Ca2+-dependent oxidative stress mediates calcification of synthetic vascular smooth muscle cells (VSMCs). **A**, Human VSMCs (hVSMCs) were treated with 3.6 mmol/L Ca^{2+}, 40 µmol/L dynasore, or 1 µmol/L ionomycin for 4 h in M199 with 0.5% FBS. Cytosolic Ca^{2+} was measured using Fluo-4-AM in Cytation 3 imager. Graph shows pooled data from 3 independent experiments. Statistical significance was tested using the Kruskal-Wallis test. Pooled data from 3 experiments performed in triplicate. **B**, hVSMCs were treated with M199 with 2.5% FBS with normal (1.8 mmol/L) or high (3.6 mmol/L) Ca^{2+} for 48 h. No calcification was observed. Extracellular vesicles (EVs) were isolated by ultracentrifugation, Ca^{2+} content in EVs was quantified using an o-cresolphthalein colorimetric assay and normalized to protein content. Pooled data from 3 experiments, significance tested using one-sample *t* test. Pooled data from 3 experiments. **C**, Medium was collected from 10^6 hVSMCs grown in M199 with 20% FBS and incubated with CD63 (CD63 molecule)-coupled beads overnight to capture EVs. Next, the EV bead pool was aliquoted and aliquots were treated with 3.6 mmol/L Ca^{2+} or normal Ca^{2+}, washed and Ca^{2+} content was quantified using an o-cresolphthalein colorimetric assay. Graph shows pooled data from 3 experiments. Statistical significance was tested using one-sample *t* test. Pooled data from 3 experiments. **D**, hVSMCs were loaded with Fura-2-AM which was alternately excited at 340 and 380 nm and the ratiometric emission, corresponding to cytosolic Ca^{2+} concentration, is presented. Cells were treated with normal (1.8 mmol/L) or high (5.4 mmol/L) extracellular Ca^{2+} after 5 min of live monitoring and continued monitoring up to 60 min. Results shown are responses for 2 individual cells, representing ≈20 cells from 2 independent experiments. **E**, As for **D**, but hVSMCs were treated with EVs isolated by ultracentrifugation from hVSMCs treated for 48 h with medium containing 1.8 or 3.6 mmol/L Ca^{2+}. Some cells showed clear oscillatory cytosolic Ca^{2+} responses to EVs isolated from 3.6 mmol/L Ca^{2+} conditions but no cells showed responses to EV from 1.8 mmol/L Ca^{2+} conditions. (*Continued*)

P=0.0030 for control versus Ca^{2+} + EVs, P=0.0086 for Ca^{2+} versus Ca^{2+} + EVs; Figure IIIB in the Data Supplement). This was further confirmed in a Boyden chamber experiment with pVSMC in the upper chamber and a collagen coating in the lower chamber. Synthetic pVSMCs in the upper chamber caused significantly more calcification in the lower chamber as compared with contractile VSMCs (P=0.0056; Figure IIIC in the Data Supplement). Additionally, dynasore attenuated calcification induced by externally added EVs (P=4.4×10^{-7} for control versus Ca^{2+} + EVs, P=0.0001 for Ca^{2+} + EVs versus Ca^{2+} + EVs + dynasore; Figure 5C). Quantification of EVs using a bead capture assay showed that synthetic hVSMCs released significantly more EVs than contractile hVSMCs (P=0.0008 for control versus heparin, P=0.0074 for control versus PDGF; Figure 5D). These results suggest that synthetic VSMCs secrete more EVs, which leads to increased calcification.

We next showed that high extracellular Ca^{2+} levels (which lead to increased ROS production in synthetic cells, Figure 2D) induced EV release (P=0.0109; Figure 5E). Additionally, H_2O_2 dose-dependently enhanced EV release (P=0.9669 for 25 µmol/L H_2O_2, P=2.1×10^{-5} for 50 µmol/L H_2O_2; Figure 5F). To investigate whether EV release is mediated by Nox5, we treated hVSMCs with pan-Nox inhibitor GKT136901 and observed decreased EV release in the presence of Ca^{2+} (P=0.0013; Figure 5G, Figure IIID in the Data Supplement). Conversely, overexpression of Nox5 in hVSMCs, which leads to increased ROS production (Figure 4G), induced a 5-fold increase in EV release (P=0.0001; Figure 5H).

Extracellular levels of EVs are the result of a balance between secretion and uptake and we showed that ROS induced secretion of EVs. Therefore, we investigated whether uptake of EVs by VSMCs is influenced by ROS. First, we confirmed that EVs secreted by VSMCs present phosphatidylserine, a phagocytosis signal[19] (Figure IIIE in the Data Supplement) and that CFSE-labeled EVs were phagocytosed when added to synthetic hVSMCs in EV-free medium. Significantly more uptake occurred at 37°C than at 4°C indicative of an active phagocytic process (Figure IIIF in the Data Supplement). Next, we showed that H2O2 inhibited phagocytosis of EVs (P=0.0152; Figure 5I). Taken together these results suggest that Ca^{2+}-Nox5- induced ROS lead to increased release and inhibited uptake of EVs, which causes their net accumulation and increased calcification in the extracellular environment.

Nox5 Is Expressed in Synthetic VSMCs in Human Vascular Disease Tissues

To examine the relationship between Nox5 expression and VSMC markers in human vascular disease context, we analyzed global transcriptomic data from 127 advanced human atherosclerotic plaques (Table, Figure IV in the Data Supplement). Nox5 mRNA expression showed a positive correlation with contractile markers α-SMA, SMTN (smoothelin), and MYL (myosin light chain). There was no correlation with CNN-1, SM22-α, or MYOCD (myocardin) and a negative correlation with MYH11 (myosin heavy chain), one of the first markers lost in dedifferentiating VSMCs.[32] Unexpectedly, there was a negative correlation with S100A4 and KLF-4, which promote synthetic differentiation. We also examined EV-related gene expression and found a negative correlation with EV/multivesicular body markers CD63 (CD63 molecule), CD81 (CD81 molecule), and TSG101 (tumor susceptibility 101), but strong positive correlations with CD9 (CD9 molecule) and enzyme regulating EV release SMPD3 (sphingomyelin phosphodiesterase 3). Additionally, there was a positive correlation with PDGF expression. Since atherosclerotic plaques are comprised many other cell types, we also examined Nox5 expression in correlation with markers of other cell types (Table I in the Data Supplement). Nox5 expression negatively correlated with endothelial cell, platelet, lipid-laden foam cell, macrophage, and some lymphocyte markers (CD8B [CD8b molecule], CD4 [CD4 molecule], and FOXP3 [forkhead box P3]). Positive correlation was observed with other lymphocyte markers (ITGAE [integrin subunit α E], CD80 [CD80 molecule] , and CD86 [CD86 molecule]).

To further assess whether Nox5-dependent phenotype switching occurred in human vasculature we localized synthetic and contractile markers and Nox5 in human arteries on protein level (Figure 6). Immunohistochemical staining of human coronary artery samples showed extensive Nox5 staining in regions with α-SMA expression, indicating the presence of VSMCs, but with low expression of contractile marker CNN-1 and high expression of synthetic marker S100A4. Some Nox5 staining was present

Figure 4 Continued. Results shown are responses for 2 individual cells, representing ≈15 cells from 2 independent experiments. **F**, Inhibiting EV uptake with dynasore decreased calcification. hVSMCs were treated in DMEM with 0.5% FBS and 3.6 mmol/L Ca^{2+} with or without 8 µmol/L dynasore for 48 h. Representative data from 3 experiments. Statistical significance was tested using *t* test. Pooled data from 2 experiments performed in triplicate. **G**, Overexpression of Nox5 (NADPH oxidase 5) induced an increase in cytosolic reactive oxygen species (ROS) measured using the DCFDA probe. Statistical significance was tested using *t* test. Data from a representative experiment performed in triplicate. **H**, Nox5 knockdown decreased ROS production in hVSMCs. Pooled data from 2 experiments. Statistical significance was tested using *t* test. Pooled data from 2 experiments (in triplicate and inquadruplicate). **I**, Both Nox (NADPH oxidase) inhibitor GKT136901 (10 µmol/L) and cytosolic Ca^{2+} chelator 1,2-bis-(o-aminophenoxy)-ethane-N,N,N,N-tetraacetic acid (BAPTA; 2 µmol/L) decreased H_2O_2 production measured with Amplex red in synthetic cells treated with 5.4 mmol/L Ca^{2+} in 0.5% FBS. Statistical significance was tested using *t* tests. Pooled data from 3 experiments performed in triplicate. **J** and **K**, GKT136901 (10 µmol/L) and BAPTA (2 µmol/L) decreased calcification of synthetic porcine vascular smooth muscle cells (pVSMCs) treated with 5.4 mmol/L Ca^{2+} in 0.5% FBS for 48 and 36 h, respectively. Statistical significance was tested using *t* tests. Data from representative experiment in quadruplicate (**J**) and pooled data from 3 experiments in triplicate (**K**).

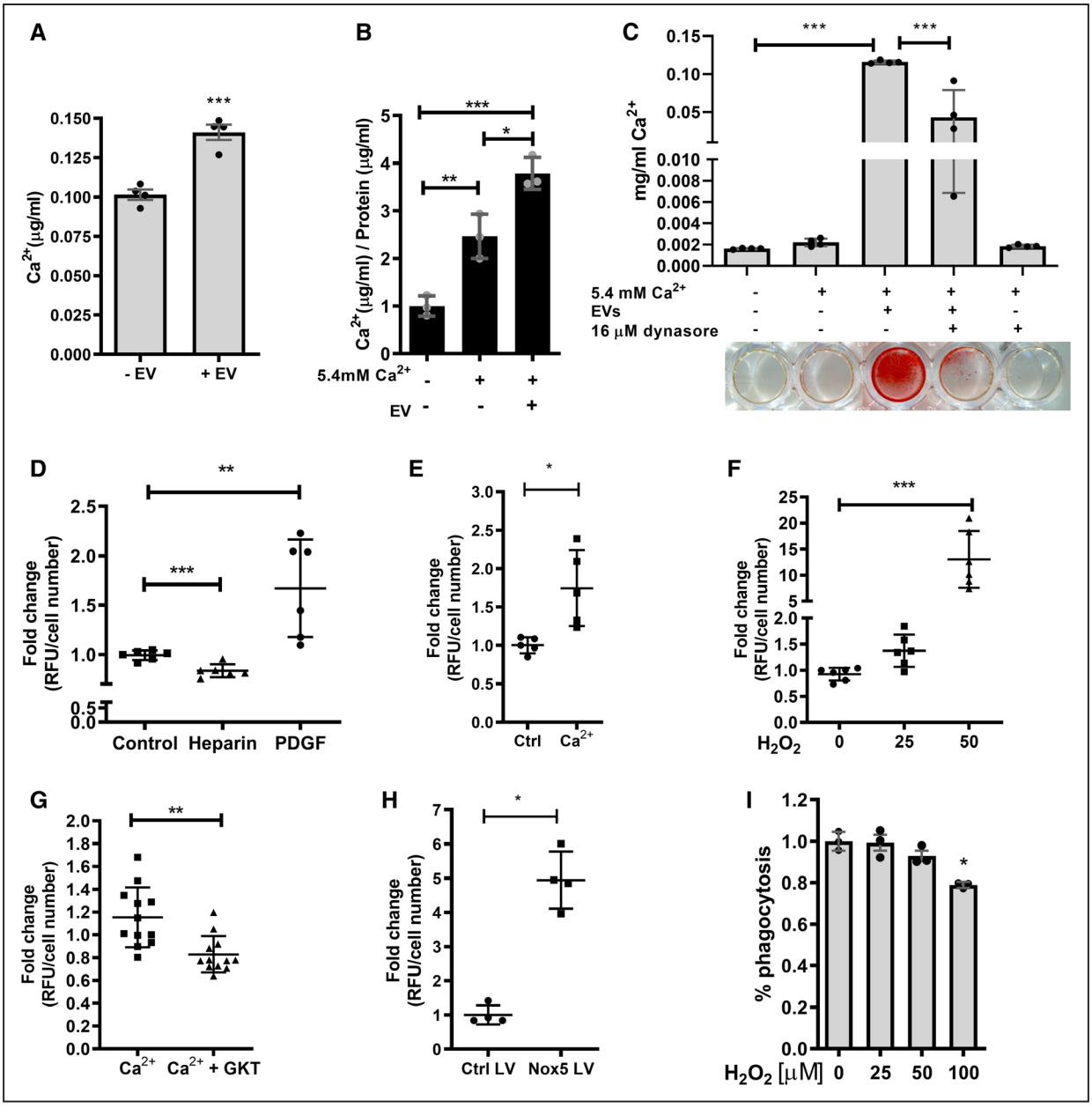

Figure 5. Nox5 (NADPH oxidase 5)-dependent oxidative stress increases extracellular vesicle (EV) release and inhibits phagocytosis.

A, EVs isolated by ultracentrifugation from HASMC66 cultured in normal medium (7.5 µg/mL protein concentration) increased calcification of a collagen matrix in the absence of cells (calcification was induced with 5.4 mmol/L Ca^{2+} in 0.5% of EV-free FBS for 24 h). Statistical significance was tested using *t* test. Data from representative experiment in quadruplicate. **B**, EVs isolated from HASMC66 cells (15 µg/mL) increased calcification of synthetic porcine vascular smooth muscle cells (pVSMCs). Statistical significance was tested using ANOVA with Bonferroni post hoc test. Data from representative experiment in triplicate. **C**, EV uptake inhibitor dynasore attenuated calcification induced by externally added EVs. Equal numbers of hVSMCs were incubated with 5.4 mmol/L Ca^{2+} and EVs for 48 h in medium with 0.5% FBS. Calcification was quantified using o-cresolphthalein assay and visualized using Alizarin Red S staining. Statistical significance was tested using ANOVA with Bonferroni post hoc test. Data from representative experiment in quadruplicate. **D**, Contractile hVSMCs (treated with 200 U/mL heparin for 5 d) secreted fewer EVs than synthetic hVSMCs (treated with 20 ng/mL PDGF for 2 d). EVs were captured with anti-CD63 (CD63 molecule)-coupled beads, detected with a fluorescently labeled anti-CD81 (CD81 molecule) antibody and quantified using flow cytometry. Statistical significance was tested using *t* tests. Pooled data from 2 experiments in triplicate. **E**, 5.4 mmol/L Ca^{2+} increased EV release in hVSMCs (in 2.5% of EV-free FBS, 48 h). Statistical significance was tested using *t* test. Data from representative experiment in quadruplicate. **F**, H_2O_2 increased EV release in hVSMCs (2.5% FBS, 24 h). Statistical significance was tested using ANOVA with Bonferroni post hoc test. Pooled data from 3 experiments in duplicate. **G**, Nox (NADPH oxidase) inhibitor GKT136901 (10 µmol/L) decreased EV release in the presence of 5.4 mmol/L Ca^{2+} in hVSMCs (2.5% FBS, 24 h). Statistical significance was tested using *t* test. Pooled data from 3 experiments in quadruplicate. **H**, Nox5 overexpression in hVSMCs using a lentiviral vector increased EV release. Statistical significance was tested using *t* test. Data from representative experiment in quadruplicate. **I**, CFSE (carboxyfluorescein succinimidyl ester)-labeled EVs (15 µg/mL) from HASMC66 were added to hVSMCs pretreated with H_2O_2 for 20 h in 2.5% FBS. Uptake was quantified after 4 h by flow cytometry. H_2O_2 decreased EV uptake. Statistical significance was tested using the Kruskal-Wallis test. Data from representative experiment in triplicate.

Table. Nox5 Expression Correlates With SMC Markers in Atherosclerotic Plaques

Cell	Marker	Pearson *r*	95% CI	*P* (2-Tailed)	*P* Value Summary
Smooth muscle	MYH11	−0.2691	−0.4240 to −0.09884	0.0023	**
	CNN-1	−0.0161	−0.1898 to 0.1586	0.8574	ns
	MYOCD	0.1171	−0.05898 to 0.2862	0.1915	ns
	SM22-α	0.1255	−0.05048 to 0.2940	0.1613	ns
	α-SMA	0.3943	0.2356 to 0.5325	4.9×10^{-6}	****
	MYL10	0.4328	0.2796 to 0.5644	3.8×10^{-7}	****
	SMTN	0.6218	0.5014 to 0.7186	8×10^{-15}	****
	MYL1	0.6692	0.5603 to 0.7554	7.7×10^{-18}	****
Synthetic	S100A4	−0.313	−0.4620 to −0.1467	0.0003	***
	KLF-4	−0.3701	−0.5113 to −0.2093	1.9×10^{-5}	****
Extracellular vesicles	CD63	−0.1774	−0.3411 to −0.0033	0.046	*
	CD81	−0.3507	−0.4947 to −0.1880	5.7×10^{-5}	****
	TSG101	−0.3624	−0.5047 to −0.2008	3.1×10^{-5}	****
	SMPD3	0.6594	0.5482 to 0.7478	3.6×10^{-17}	****
	CD9	0.7246	0.6299 to 0.7981	6×10^{-22}	****
Growth factor	PDGFB	0.417	0.2611 to 0.5517	1.2×10^{-5}	****

Pearson correlations were performed to investigate the association between the transcriptomic expression levels of Nox5 and SMC markers, based on the microarray data from human plaques in the BiKE cohort, n=127. CNN-1 indicates calponin 1; MYH, myosin heavy chain; MYL, myosin light chain; MYOCD, myocardin; Nox5, NADPH oxidase 5; SM22-α, smooth muscle protein 22α; SMTN, smoothelin; and α-SMA, α-smooth muscle actin.

in the adventitia, suggesting possible expression of Nox5 in other cell types. Taken together, these results support the notion that Nox5 is expressed in synthetic VSMCs in human vessels and that EV release is involved.

DISCUSSION

In the present study, we identify Nox5 as a key regulator of VSMC phenotype and calcification. We demonstrate that contractile VSMCs have low calcification capacity and that switching towards a synthetic phenotype promotes calcification. We show that extracellular factors known to induce phenotype switching cause increased Nox5 expression. Subsequently, increased Nox5 expression then induces phenotypic switching to synthetic. We elucidate the mechanism by which synthetic VSMCs are prone to calcification, showing that extracellular Ca^{2+} enters cells via EVs, which results in a cytosolic Ca^{2+} rise. This activates Nox5, which is responsible for increased ROS production. Increased ROS leads to decreased contractile marker expression, enhanced EV release, and decreased uptake, which promotes calcification (Figure 7).

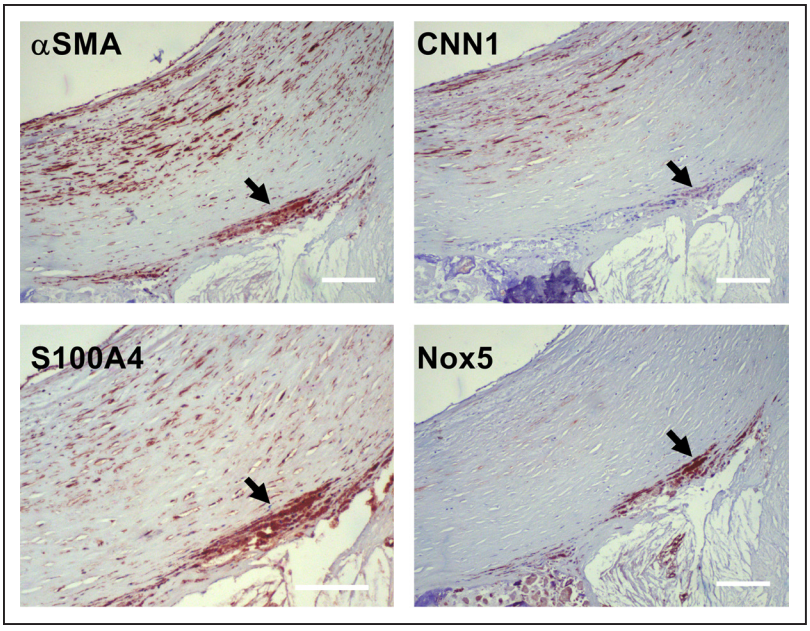

Figure 6. Nox5 (NADPH oxidase 5) is expressed in synthetic cells in human arteries.
Immunohistochemical staining of human coronary artery with atherosclerotic plaque. Vascular smooth muscle cells (VSMCs) were α-SMA (α-smooth muscle actin)-positive. Nox5 was present in VSMCs expressing S100A4 (S100 calcium binding protein A4), but not contractile marker CNN-1 (calponin 1; black arrows). Figure shows representative images from a total of 20 coronary artery and aortic samples that were stained. Scale bars are 250 μm.

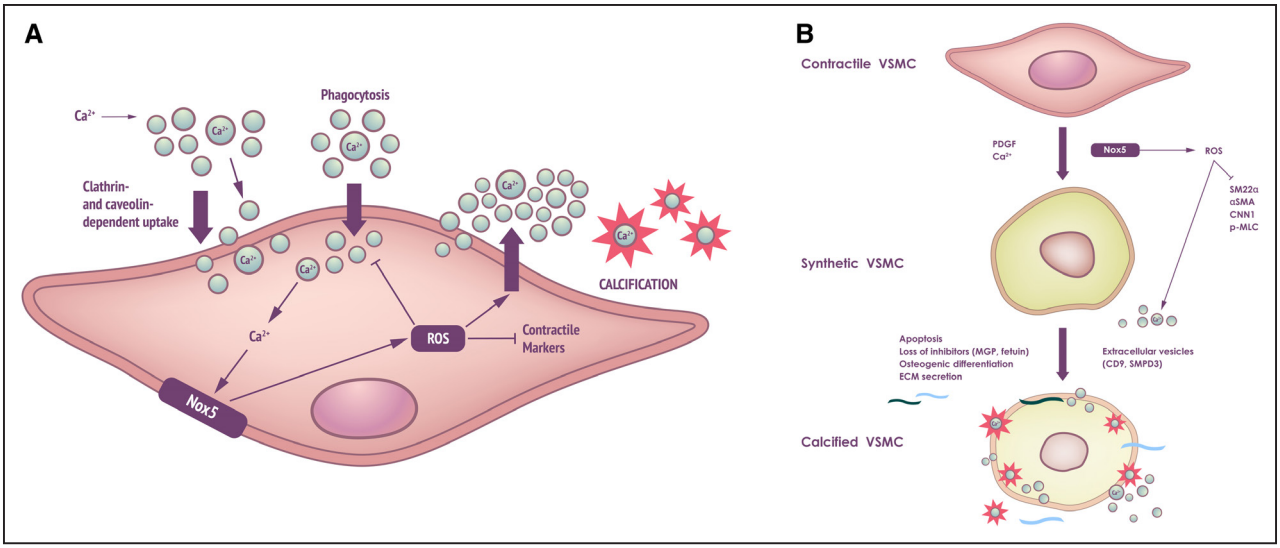

Figure 7. Regulation of vascular smooth muscle cell (VSMC) calcification by Nox5 (NADPH oxidase 5).
A, The synthetic phenotype is associated with increased Nox5 expression, which further exacerbates phenotypic switching to synthetic. Extracellular Ca^{2+} enters cells via clathrin- and caveolin-dependent uptake of extracellular vesicles (EVs), which results in a cytosolic Ca^{2+} rise. This activates Nox5, which is responsible for increased reactive oxygen species (ROS) production. Increased ROS leads to enhanced EV release and decreased phagocytosis. An increased amount of EVs in the extracellular matrix promotes calcification. **B**, Contractile VSMCs dedifferentiate to synthetic VSMCs. This process is induced by PDGF, high Ca^{2+} and mediated by Nox5. Synthetic VSMCs undergo further changes, including apoptosis and osteogenic differentiation, secrete increased amounts of extracellular matrix (ECM) and EVs. All these processes lead to increased calcification.

Contractile VSMCs Are Resistant to Calcification

In the present study, we demonstrate that contractile VSMCs are resistant to calcification in vitro suggesting that switching towards a synthetic phenotype is required for mineralization. We propose that this is, in part, due to differences in Nox5 expression. We show that Nox5 mRNA expression in advanced atherosclerotic plaques correlated with expression of some SMC markers and not of markers of other cell types. A negative correlation or no correlation was observed with CNN-1, SM22-α, MYOCD, and MYH11, suggesting that Nox5 is expressed in dedifferentiated VSMCs. In this data set, we also observed a negative correlation of Nox5 expression with S100A4 and KLF-4, previously associated with synthetic differentiation.[14,28] However, the atherosclerotic plaques in this study were of late stages (American Heart Association grade VI and VII). Therefore, it is possible that no active synthetic differentiation occurs at these stages, and the correlation is lost, although it may have been present during earlier stages of atherogenesis. Additionally, the negative correlation of S100A4 with Nox5 expression is in line with our results showing that overexpression of Nox5 or H_2O_2-induced oxidative stress downregulated expression of S100A4 protein in VSMCs. S100A4 is known to regulate cell proliferation and motility, but more recently, it has been shown to be released to the extracellular space where it has an important function in VSMC phenotype switching.[33] Neither of the methods which we used (mRNA analysis in plaque samples or Western blot of cell lysates) would have measured extracellular S100A4 protein accumulation, so its regulation by Nox5 and ROS cannot be fully excluded.

By immunohistochemistry, we have shown that Nox5 expression in synthetic VSMCs in the vessel wall, consistent with its role as a regulator of phenotypic switching. In line with this, Nox5 expression was previously shown to be highly upregulated in VSMCs in advanced human atherosclerotic plaques,[34] which are characterized by VSMC phenotypic switching and calcification. Additionally, polymorphisms in the Nox5 gene are linked to changes in blood pressure,[35] which are associated with VSMC-mediated vascular remodeling. It remains to be established what causes the increase in Nox5 expression in synthetic VSMCs in the first place. Inflammation-related stimuli have been shown to regulate Nox5 expression,[36] however, this has not been investigated in the context of phenotypic switching.

We also found that contractile VSMCs had significantly higher levels of MGP, an inhibitor of vascular calcification. Carboxylated MGP is known to block both crystal formation and BMP signaling.[37] An increased level of this inhibitor might offer another explanation to the observed resistance of contractile VSMCs to calcification.

Regulation of VSMC Phenotype by Nox5

We show here that Nox5 is a marker and (via increased ROS production) an inducer of the synthetic phenotype. Consistent with our findings, Nox5-derived ROS was previously identified as a driving force for coronary

SMC migration, consistent with the synthetic phenotype, via upregulation of intermediate-conductance Ca^{2+} activated K^+ channels.[38] Additionally, Nox5-produced ROS have been shown to mediate PDGF-induced proliferation of VSMCs.[39] However, in other contexts ROS were shown to be required for VSMC contractile differentiation[40] and maintenance of the contractile phenotype via Nox4-derived ROS.[41,42] ROS are also required for VSMC contraction.[43] It is possible that the discrepancy between our results and previous studies is due to different Nox enzymes mediating divergent effects. The overall outcome for the cells would then depend on the balance between the activity of these enzymes. Another possibility is that ROS form a negative feedback loop for phenotype control, with synthetic cells producing more ROS, which among other effects, help the cells regain a contractile phenotype. This is in line with research showing that Nox5 is inactivated by oxidation of its Ca^{2+}-binding domain.[44] These control mechanisms possibly become dysregulated in vascular pathologies when many adverse factors are at play, as oxidative stress is a causative factor in vascular disease.[45]

Our results with regards to Nox5-produced ROS promoting a synthetic phenotype in VSMCs are contrary to recent findings by Montezano et al.[46] In this study, human Nox5 was constitutively expressed under the SM22-α promoter in mice, as Nox5 is absent in the murine genome. The authors observed systemically increased oxidative stress in the transgenic mice, however, this was accompanied by increased mesenteric artery contractility and increased expression of contractile markers p-MLC and p-MYPT1. One possible explanation for this contradiction with our results is that in this model Nox5 expression occurred also during development. The continued presence of Nox5 since early development might render different effects than increased expression occurring as a result of vascular disease and remodeling in the adult. Nevertheless, this study confirms the detrimental effects of increased ROS in the vasculature,[47] since the authors postulate that the uncovered mechanisms are likely relevant to the pathology of hypertension. Additionally, these results are in line with our conclusion that Nox5 is a regulator of VSMC phenotype, but the exact effects of increased Nox5 are possibly dependent on the physiological context.

Nox5 Is the Connection Between Ca^{2+} and Oxidative Stress

It has been shown before that treating cells with high Ca^{2+} leads to changes in Ca^{2+} that result in increased calcification.[21] In this study, we propose uptake via EVs as a new mechanism of extracellular Ca^{2+} influencing cytosolic Ca^{2+} levels. It has been suggested before that high Ca^{2+} can cause increased loading of Ca^{2+} into EVs.[21] As this effect was blocked by the cytosolic Ca^{2+} chelator BAPTA, Ca^{2+} uptake into cells was suggested to happen before loading into EVs. However, we demonstrate that Ca^{2+} uptake into EVs can happen in the absence of cells. We also provide evidence that Ca^{2+}-loaded EVs increase cytosolic Ca^{2+} and that Ca^{2+} uptake is mediated by clathrin- and caveolin-mediated EV uptake. Taken together, this suggests that 2 mechanisms of Ca^{2+} uptake exist—EV-dependent and EV-independent, via Ca^{2+} channels.[6] Interestingly, a recent study, which focused on EVs from high-phosphate treated VSMCs demonstrated that these EVs induced an increase in cytosolic Ca^{2+} partially via release from cytosolic stores.[48] Furthermore, it was reported that exosomes bound to autotaxin induced the release of Ca^{2+} from cytosolic stores.[49] We did not investigate the mechanism of Ca^{2+} elevations in our model. However, it is tempting to speculate that these rises in cytosolic Ca^{2+} lead to increased Nox5 activity.

Interestingly, ROS have been shown to increase intracellular Ca^{2+} by stimulating IP3-mediated Ca^{2+} mobilization, SERCA (ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting) inhibition, and by stimulating Ca^{2+} channels.[43] This has interesting implications for Nox5 activation and suggests the presence of a positive feedback loop between Ca^{2+}, Nox5, and ROS.

Ca^{2+} uptake into the arterial vessel wall has been shown to be involved in the pathogenesis of arteriosclerotic lesions in vivo[50] and imbalanced Ca^{2+} metabolism is known to contribute to medial calcification.[6] Here, we provide a new mechanism by which Ca^{2+} induces VSMC calcification, showing that it plays a role in Nox5-dependent ROS generation. Thus, we demonstrate that increased Ca^{2+} can induce oxidative stress. These findings have implications for vascular disease, as a mineral imbalance in circulation is known to be a manifestation of chronic kidney disease, which is accompanied by vascular calcification.[11] Additionally, hitherto advanced glycation end products and oxidized low-density lipoproteins were shown to be the main inducers of oxidative stress in the vasculature.[29] Although mineral imbalance in chronic kidney disease is known to be associated with ROS accumulation,[51] we demonstrate here that in synthetic VSMCs it is Ca^{2+} itself that contributes to this.

Nox5-Mediated Oxidative Stress Induces EV Release and Inhibits Their Re-Uptake

EVs are a well-established factor contributing to vascular calcification.[17,52,53] Our data indicate that EVs alone, without cells, are sufficient to induce extracellular matrix calcification, demonstrating how potent calcification inducers they are.

We show here that phenotypic switching is associated with changes in EV release. This is consistent with previous studies[25] where contractile VSMCs showed reduced and synthetic VSMCs increased EV release. However, our study demonstrates for the first time that

Nox5 is the molecular link between Ca^{2+}, oxidative stress and EV release. ROS have been shown to promote EV release from other cell types,[54,55] however, this has not been reported in VSMCs before. Interestingly, Ca^{2+} can directly stimulate EV release from cancer cells via Ca^{2+}-dependent Munc13-4.[56] Whether this mechanism is relevant for VSMCs remains to be tested.

It has been shown previously that VSMCs exhibit phagocytic capacity[24] towards phosphatidylserine expressing apoptotic cells and bodies[19] and that EVs present phosphatidylserine.[57] Additionally, it has been demonstrated that increased oxidative stress inhibits phagocytosis of apoptotic bodies.[58] Our results extend this effect also to secreted EVs and suggest that the increased expression of Nox5 in concert with increased Ca^{2+} levels fuels the production of H_2O_2 and subsequently limits the phagocytic capacity of VSMCs which together with increased EV release may culminate in increased numbers of EVs in the extracellular matrix.

Limitations and Conclusions

In this study, we used both human and porcine VSMCs. Although we established that cells of both species react similarly in the experimental conditions in terms of phenotypic switching and calcification, we cannot exclude the possibility of differences. Most importantly, there are species differences in the Nox5 gene with regards to isoforms.[39,59] It remains to be established which human isoforms resemble closest the porcine orthologues.

Another limitation of this study is the use of heparin, which is known to inhibit EV uptake,[60] to induce the contractile phenotype in VSMCs. However, in our experiments, cells treated with heparin secreted fewer EVs than untreated or synthetic cells. If heparin inhibited EV uptake we would expect to observe an accumulation of EVs and, therefore, possibly a net increase. That suggests that in our model heparin's EV-blocking activity is weaker than contractile phenotype-promoting activity. However, it cannot be excluded that decreased EV uptake is connected to maintaining contractile differentiation, as these pathways are poorly described in VSMCs.

In conclusion, we showed that switching phenotypes to synthetic is required for VSMC calcification, and that in synthetic cells increased levels of Ca^{2+}-regulated Nox5 play a key role in the generation of oxidative stress and EV release. Inhibition of VSMC phenotypic switching in pathological contexts may be of therapeutic benefit for vascular calcification in vivo and this research identifies Nox5 as a potential therapeutic target to achieve that.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Materials

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