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Calcification of vascular smooth muscle cells is induced by secondary calciprotein particles and enhanced by tumor necrosis factor-α

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ABSTRACT

Background and aims: Vascular calcification is prevalent in clinical states characterized by low-grade chronic inflammation, such as chronic kidney disease (CKD). Calciprotein particles (CPP) are calcium phosphate-containing nano-aggregates, which have been found in the blood of CKD patients and appear pro-inflammatory *in vitro*. The interplay of CPPs and inflammatory cytokines with regard to the calcification of vascular smooth muscle cells (VSMC), *in vitro*, has not been investigated yet.

Methods: Primary or secondary CPP were generated using phosphate-enriched culture medium (DMEM/10% FBS) incubated at 37 °C. Human VSMC were cultured with these media and mineralization was measured. Expression of TNF-α was detected by qPCR, ELISA and Western blot in calcified VSMC. To further characterize the significance of TNF-α and its receptors for the calcification of VSMC, RNA interference experiments using siTNF-α, siTNFR1 and siTNFR2 were performed.

Results: The addition of phosphate to cell culture medium containing DMEM/10% FBS led to the rapid formation of primary CPP, which underwent spontaneous transformation to secondary CPP. Exposure of VSMC towards secondary CPP led to pronounced and concentration-dependent calcification, whereas exposure towards primary CPP did not. Importantly, secondary CPP induced oxidative stress, and led to the up-regulation and release of TNF-α. Addition of TNF-α to the cell culture medium enhanced, whereas the suppression of endogenous TNF-α or TNF receptor type 1 (TNFR1) expression by siRNA, ameliorated calcification.

Conclusions: Secondary, but not primary CPP, induce VSMC calcification. Secondary CPP induce the expression and release of TNF-α, which enhances calcification via its receptor TNFR1.

1. Introduction

Vascular calcification is a common finding in chronic kidney disease (CKD) [13]. Medial calcification, the major form of vascular calcification in CKD, is associated with arteriosclerosis and vascular stiffening [22,30,43]. However, in patients with CKD, intimal and medial calcification may co-exist because of the presence of both uremia and traditional (Framingham) risk factors for atherosclerosis [10,27]. Vascular smooth muscle cells (VSMC) are the main cell type involved in medial calcification [11].

Inorganic phosphate induces calcification of VSMC *in vitro* [12,31]. When phosphate and calcium are added to artificial fluids containing the calcification inhibitor protein fetuin-A, calcium and phosphate do not sediment as crystalline hydroxyapatite, but instead form primary calciprotein particles (CPP), which contain amorphous calcium phosphate [15,26]. Over time, these primary CPP undergo spontaneous transformation to secondary CPP, which contain crystalline calcium phosphate. This transformation is associated with changes in CPP shape and an increase in particle diameter [26]. Interestingly, CPP have been found in the blood of patients with CKD and in the dialysate obtained from patients receiving peritoneal dialysis [6,16,36]. CPP have also been detected

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in the blood of patients with inflammatory rheumatoid diseases but normal renal function, a population at increased risk for the development of atherosclerosis [3,9].

When murine RAW 264.7 macrophages are exposed to secondary CPP, this induces the expression and secretion of inflammatory cytokines, like TNF-α [39]. Inflammatory cytokines have been associated with vascular calcification in isolated vascular cells *in vitro*, in experimental animals *in vivo* [2,42] and clinically in CKD patients [41]. Circulating CPP levels are associated with the concentration of inflammatory markers in patients with CKD [38] and CPP maturation time (T_{50}), a novel kinetic blood test that measures the overall calcification propensity of serum [26]. T_{50} is strongly associated with the survival of CKD and kidney transplanted patients [19,37].

Based on these observations, we hypothesized that both CPP and mediators of inflammation might directly be involved in the process of pathological biomineralization and therefore, aimed to investigate the effects of CPP and the inflammatory cytokine TNF-α on VSMC calcification *in vitro*.

2. Materials and methods

2.1. Generation and characterization of CPP

To generate CPP, calcification medium was prepared by adding 10% FBS, 3.5 mmol/L inorganic phosphate (2.14 mmol/L Na_2HPO_4, 1.36 mmol/L NaH_2PO_4, Sigma, Switzerland), 1 mmol/L calcium ($CaCl_2$, Sigma, Switzerland), 1% penicillin/streptomycin and 1% L-Glutamine to phenol-free DMEM. This calcification medium was stored at 37 °C for 1 and 7 days to generate primary and secondary CPP, respectively. The transformation from primary to secondary CPP was followed by monitoring the absorption at 570 nm and directly visualizing the CPP using transmission electron microscopy (TEM). The particle-containing calcification media were centrifuged at 25,000*g* for 120 min at 4 °C. The resulting pellet was resuspended by vigorous shaking, and calcium and phosphate contents were determined (QuantiChrom™ Calcium assay kit and QuantiChrom™ phosphate assay kit; Sococchim SA, Savigny, Switzerland). CPP concentrations were analyzed using Nanoparticle Tracking Analysis (NTA, Malvern, Switzerland).

2.2. Transmission electron microscopy (TEM) and electron energy loss spectroscopy (EELS)

Primary and secondary CPP were suspended in de-ionized water and applied to Formvar (polyvinyl formal)-coated nickel grids (Plano, Germany). The grids were dried at room temperature and primary and secondary CPP were visualized without staining using a Philips CM12 electron microscope, at 80 kV equipped with a Morada camera system, using iTEM software. Atomic analyses were performed by EELS for calcium, phosphate, carbon and oxygen.

2.3. Cell culture

Primary Human Aortic Smooth Muscle Cells (VSMC) were purchased from ATCC (PCS-100-012, USA); the cells were grown in vascular cell basal medium (PCS-100-030, ATCC, USA) supplemented with vascular smooth muscle growth kit components (PCS-100-042, ATCC, USA), according to the manufacturer's protocol. Cells were used from passages three to five.

2.4. Induction of calcification and quantification of calcium deposition

VSMC were seeded in 24-well plates (3×10^4 per well) and grown to confluence. Calcification was induced by exposing cells to cell culture medium (normal growth medium: vascular cell basal medium supplemented with vascular smooth muscle growth kit components, 1 mL per well), supplemented with either primary or secondary CPP (final concentration equivalent to 100 μg/mL calcium) as previously established by Smith and colleagues [39]. Calcification was assessed after 24 h. VSMC were solubilized with 0.1 mol/L NaOH and 0.1% SDS. The protein content of the samples was measured with a BCA protein assay kit (Thermo Scientific, USA). The calcium content of the cell monolayer was normalized to the cell protein content and expressed as μg/mg protein. Mineralization was also assessed qualitatively by Alizarin red staining.

2.5. Cell viability

Cell viability and apoptosis were measured using Viability Dye eFluor® 450 and PE Annexin V and subsequently analyzed on a FACS (LSR II) flow cytometer. Furthermore, in some experiments, sulforhodamine B staining (Sigma, Switzerland) was used to assess cell viability [45]. VSMC cultured on 24-well plates were fixed with 4% formaldehyde for 60 min, Cells were then washed twice with phosphate buffered saline (PBS) and stained with 0.057% sulforhodamine in 1% acetic acid at 37 °C for 30 min. After staining, cells were washed four times with 1% acetic acid and air-dried. The stain was dissolved by washing with 10 mmol/L Tris base (400 μl) for 5 min. The absorbance of the samples was read at 570 nm (Tecan Microplate Reader, Switzerland). To assess cell viability, the percentage difference in absorbance with respect to control was calculated.

2.6. Determination of intracellular calcium by flow cytometry

Indo 1-AM (Sigma, Switzerland) was used as a fluorescent indicator for the determination of intracellular calcium. VSMC were loaded with 2 μg/ml Indo 1-AM and incubated for 1 h at 37 °C. Cells were then washed with DMEM, supplemented with secondary CPP for 30 and 60 min and subsequently analyzed on a FACS (LSR II) flow cytometer [4]. Data were analyzed using the FlowJo (Treestar) software.

2.7. Assessment of CPP localization

VSMC were fixed for TEM imaging using routine protocols. Briefly, adherent layers of VSMC grown to 80% confluence were fixed with 2.5% glutaraldehyde in 0.15 mol/L HEPES at 4 °C for 1 h, post-fixed with 1% osmium tetroxide, dehydrated and embedded in Epon812. Ultrathin sections were obtained at a thickness of 60 nm, stained with uranyl acetate and lead citrate and examined using a Philips CM12 electron microscope at 80 kV. Images were recorded with a Morada digital camera using iTEM software and contrast-adapted with Photoshop CS5.

2.8. Quantitative real time PCR

Total RNA was extracted from VSMC using the RNeasy Micro Kit (QIAGEN AG, Switzerland) according to the manufacturer's instructions. The extracted RNA was subjected to reverse transcription using a cDNA reverse transcription kit (Promega AG, Switzerland). PCR amplification was performed using the oligonucleotide primers (Suppl. Table S1 lists primer sequences) SYBR Green PCR Master Mix (Roche, Switzerland) [29]. Melt-curve analysis was performed to confirm the presence of a single specific product and non-template controls run to assess contamination. Target values were normalized to the house-keeping control gene (*GAPDH*). Real-time PCR reactions were performed in three

replicates. The fold difference for each samples were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) [21,28].

2.9. Protein extraction, detection and quantification

Proteins were extracted from VSMC by radioimmunoprecipitation assay (RIPA) buffer. The protein concentration was determined by Pierce BCA Protein Assay Kit from Thermo Scientific according to the manufacturer's instructions. Western Blotting was performed using primary antibodies against BMP2 (Rabbit polyclonal; 1: 500), TNF-α (Mouse monoclonal; 1: 500), TNFR1 (Rabbit polyclonal; 1/1000) and TNFR2 (Rabbit polyclonal; 1:200); all antibodies were purchased from Abcam, Switzerland (ab14933, ab83481, ab19139 and ab15563 respectively). GAPDH (Rabbit polyclonal: 1:1000; Cat No. 14C10; Cell Signalling, Switzerland) was used as a loading control. Primary antibody was detected using an appropriate horseradish peroxidase-conjugated secondary antibody (HRP conjugated anti-Rabbit IgG: 1: 10000; or anti-mouse IgG: 1:5000). Proteins were visualized by chemiluminescence agent using ECL Detection Reagents (GE healthcare, UK).

2.10. Enzyme-linked immunosorbent assay (ELISA)

Human TNF-α Ultrasensitive ELISA kit (Invitrogen, Switzerland) was used to quantify TNF-α in cell culture medium, according to the manufacturer's protocol. The assay was terminated with stop buffer and samples were read in triplicate at 450 nm using a microplate reader.

2.11. Determination of H_2O_2 in cell supernatants

VSMC were cultured in 24-well plates for 24 h followed by treatment with secondary CPP. Phenol red-free medium was used to avoid interference with the colorimetric hydrogen peroxide assay. Following the treatment period, the cell supernatant was centrifuged at 1,000 × *g* for 15 min within 30 min of collection. Samples were then filtered through a 10kD MWCO spin filter to remove the bulk of proteins. Thereafter, H_2O_2 was determined using a commercially available kit (Biovision, USA); In the presence of Horse Radish Peroxidase (HRP), the OxiRed probe reacts with H_2O_2 to produce red color, which was then detected at OD 570 nm H_2O_2 was normalized to the cell protein content and expressed as pmol/μg protein.

2.12. Gene silencing using RNA interference (siRNA)

Gene silencing was performed using the siRNA system (Santa Cruz Biotechnology, USA) with modifications of the manufacturer's instructions to increase silencing efficiency in VSMC. Cells were seeded at a density of 3×10^4 cells per well in 24-well plates in 1 mL antibiotic-free normal growth medium supplemented with FBS for 24 h before transfection. On the day of transfection 5 μl of 10 μmol/L siRNA (TNF-α siRNA, TNFR1 siRNA or ctrl siRNA) was mixed with 100 μl siRNA transfection medium and 5 μl lipofectamine LTX (Invitrogen, Switzerland). Reagents were then incubated at room temperature for 45 min. After washing, the transfection complexes were added to the cells in the presence of 800 μl antibiotic-free growth medium, supplemented with 5% FBS and incubated at 37 °C for 24 h. Thereafter, cells were exposed either to CPP-containing medium or to normal growth medium (control). The efficiency of the knockdown was confirmed by real-time PCR and Western blot. Human recombinant TNF-α was purchased from Sigma Chemical Co (Sigma, Switzerland).

2.13. Statistical analysis

All statistical analyses were calculated with GraphPad Prism software (GraphPad software, USA). Significance of differences was analyzed using Student's *t*-test. A difference between experimental groups was considered significant when the *p* value was <0.05.

3. Results

3.1. Supplementation of cell culture medium with calcium and phosphate leads to the spontaneous formation of calciprotein particles (CPP)

To establish our *in vitro* calcification system, VSMC were exposed towards DMEM supplemented with 10% FBS, 3.5 mmol/L inorganic phosphate and 1.0 mmol/L calcium. Using this approach however, the occurrence of calcifications was variable (data not shown).

We, therefore, monitored the calcification medium for changes during the incubation for seven days at 37 °C (Suppl. Fig. 1). When calcium and phosphate were added to DMEM/10%FBS, the initially clear medium turned slightly turbid in the first hour (Fig. 1A). This faint turbidity was maintained for 3 days when the medium started to become distinctly more turbid. These changes were visible with the naked eye (Fig. 1A) and also detectable using absorptiometry at 570 nm (Fig. 1B).

TEM imaging revealed the presence of spherical primary CPP in the first and spindle-shaped secondary CPP in the second phase (Fig. 1C). Qualitative EELS confirmed the presence of calcium, phosphorus, carbon and oxygen in the particles (Fig. 1D). The calcium and phosphate contents increased upon transformation, whereas the particle count remained stable (Fig. 1E).

These results indicate that (i) primary CPP are formed in the calcification medium and undergo transformation to secondary CPP, and that (ii) the presence of primary or secondary CPP in the cell culture medium at a given time point might provide an explanation for the variability of our calcification results.

3.2. Secondary CPP induce calcification in vitro, whereas primary CPP do not

Exposure of VSMC towards cell culture medium supplemented with secondary CPP (100 μg calcium per mL) led to a pronounced and consistent accumulation of cell-bound calcium within one day, whereas supplementation with primary CPP (100 μg calcium per mL) did not. Calcium accumulation was confirmed by Alizarin red stain (Fig. 2A) and quantitatively by direct calcium determination (Fig. 2B). Cell viability was not affected by the amount of CPP used in our experiments however secondary CPP lead to early apoptosis in VSMC (Fig. 2C). The extent of CPP-induced VSMC calcification was concentration-dependent (Suppl. Fig. 2).

3.3. Calcification induced by secondary CPP is an active cell-mediated process

We investigated whether *in vitro* calcification depends on the functional integrity of cellular processes. Calcification of VSMC was only observed in living cells but not after fixation of cells with 4% paraformaldehyde (Fig. 3A). Increased intracellular calcium concentration occurred as early as 30 min following the exposure towards secondary CPP (Fig. 3B). Consistent with these results, CPP-bound calcium decreased in the supernatant and cell-bound calcium increased (Fig. 3C). Secondary CPP were detected intracellularly using TEM (Fig. 3D). These results indicate that calcification induced by secondary CPP is an active cell-mediated process, which

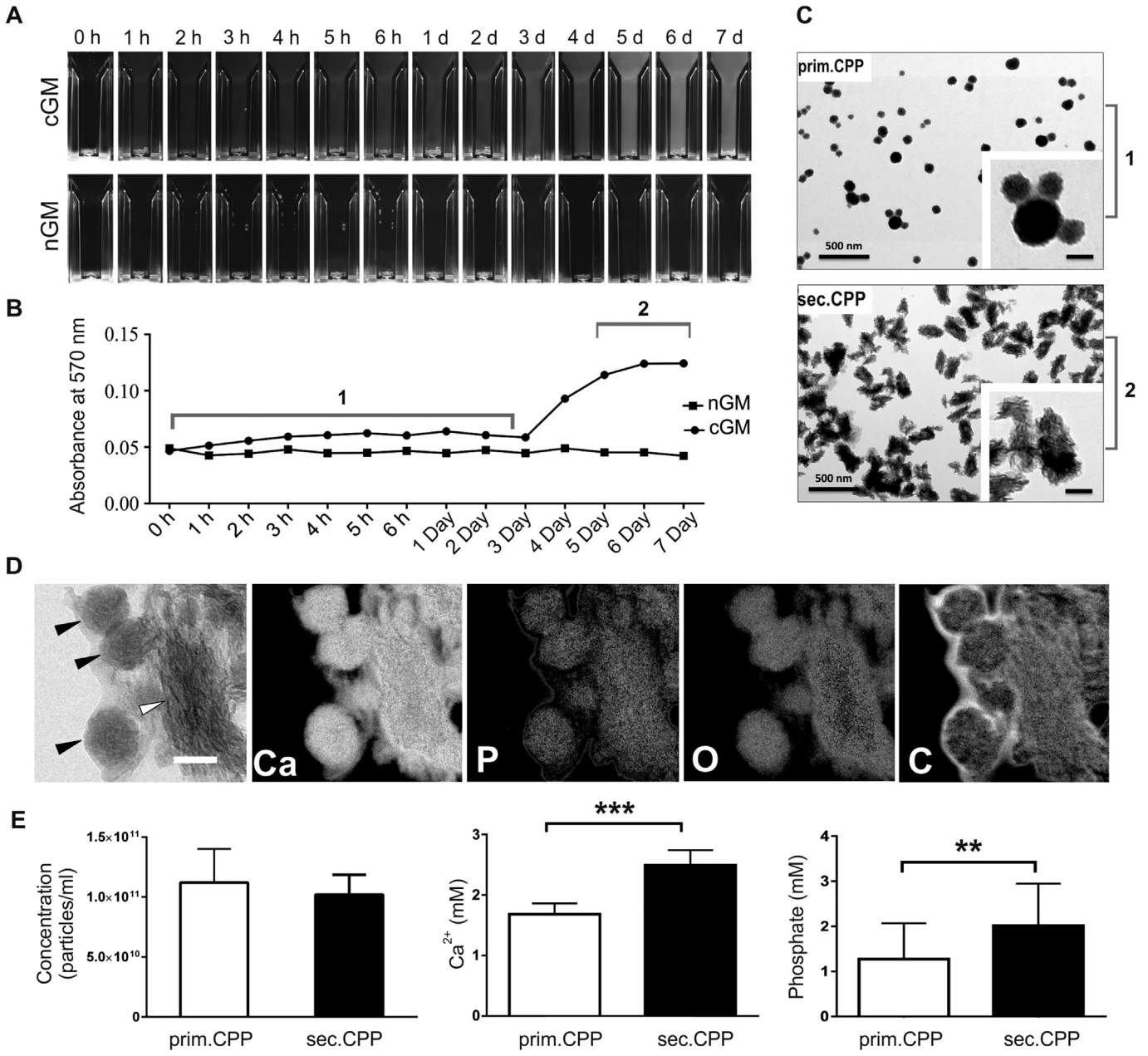

Fig. 1. CPP form spontaneously in cell culture medium containing high phosphate. (A) Change of turbidity of medium spiked with phosphate (3.5 mM) and calcium (1.0 mM) over time. (B) Detection of turbidity by monitoring the light absorbance at 570 nm. (C) TEM imaging of the pellets after sharp centrifugation (25,000 × *g* for 120 min at 4 °C). Data are representative of three independent experiments. (D) Characterization of CPP, first left, TEM imaging of pellets of primary CPP (black arrowheads) and secondary CPP (white arrowhead), EELS imaging showing calcium (Ca), phosphorus (P), oxygen (O) and carbon (C) signals in primary and secondary CPP. (E) CPP concentration in the standard calcification medium containing DMEM/10% FBS and 3.5 mM added phosphate and 1.0 mM added calcium was analyzed using Nanoparticle Tracking Analysis; the calcium and phosphate contents of the CPP-containing pellets (derived from 1 mL growth medium centrifuged at 25,000 × *g* for 120 min at 4 °C) were measured after dissolution of CPP in HCl (0.6 mM) (mean ± SD; N = 3; $^{**}p < 0.01$, $^{***}p < 0.001$). Scale bar = 500 nm for TEM. nGM: Normal Growth Medium; cGM: Calcification Growth Medium.

involves the uptake of CPP by VSMC.

3.4. Secondary CPP induce oxidative stress and an inflammatory response of VSMC

Soluble TNF-α was released into the cell supernatant upon treatment with secondary CPP, as demonstrated by ELISA (Fig. 4A). RT-PCR and Western-blotting demonstrated the upregulation of TNF-α expression (Fig. 4A). Treatment with secondary CPP led to an increase in extracellular H_2O_2 and to the up-regulation of bone morphogenetic protein-2 (BMP-2) and Nuclear Factor Kappa-B (NF-κB) (Fig. 4B). These results demonstrate that secondary CPP induce oxidative stress, i.e. the production of reactive oxygen species, and an inflammatory response in VSMC.

3.5. TNF-α and its receptor type 1 enhance the calcification of VSMC

We investigated whether the addition of exogenous TNF-α to the cell culture medium aggravated the pro-calcifying effect of secondary CPP towards VSMC. To this end, VSMCs were exposed to TNF-α (10 ng/ml) for 24 h in the presence or absence of primary or secondary CPP. Calcification was augmented when TNF-α was

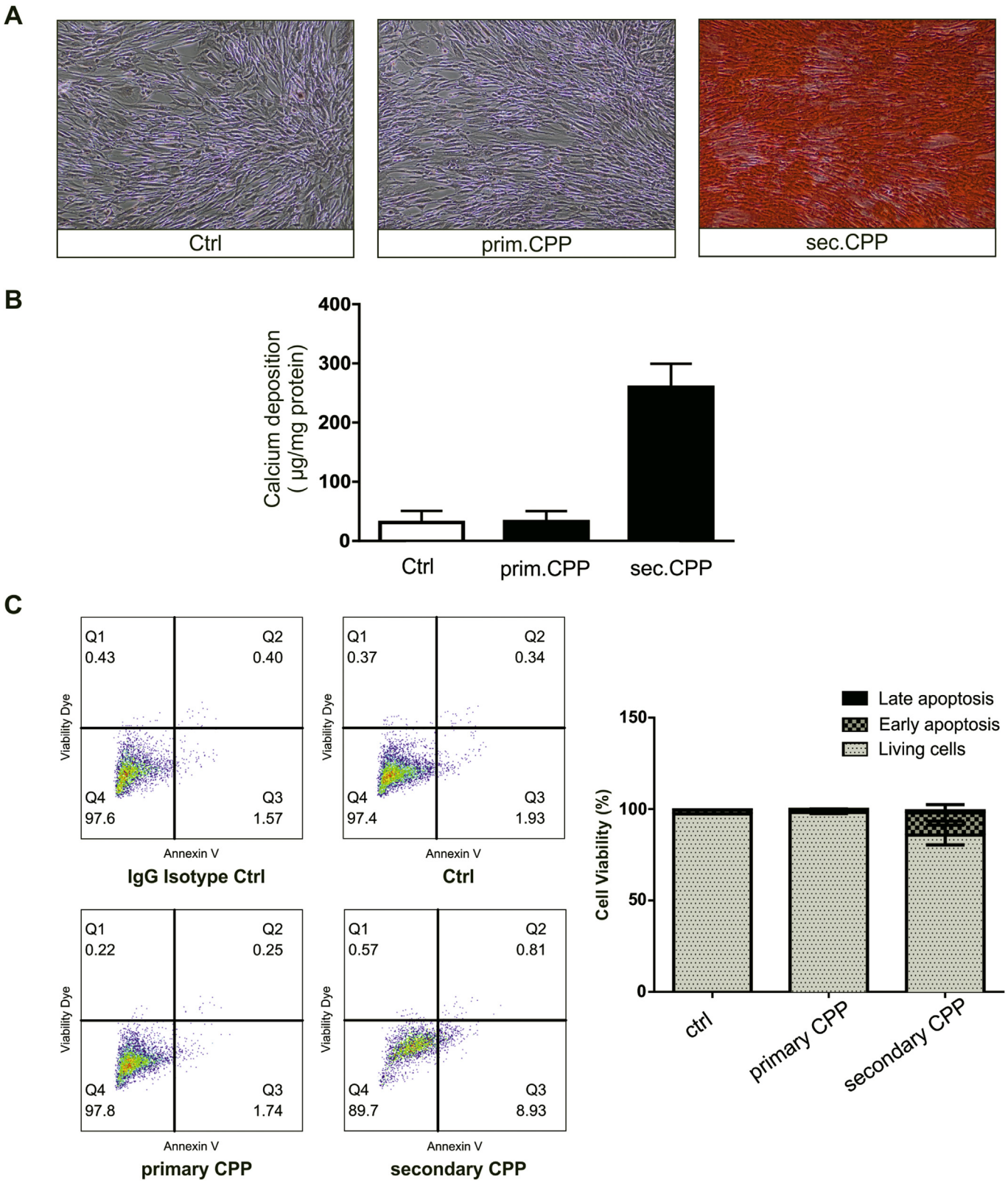

Fig. 2. Exposure of VSMC towards primary or secondary CPP. (A) VSMC were cultured in growth medium supplemented with primary or secondary CPP (final concentration equivalent to 100 µg/mL calcium) and Alizarin red staining was performed after 24 h. (B) Quantification of calcium deposition (mean ± SD; N = 3; ****$p < 0.0001$). (C) Cell viability and apoptosis were measured using Viability Dye eFluor® 450 and PE Annexin V and subsequently analyzed on a FACS (LSR II) flow cytometer.

added to VSMC treated with secondary, but not with primary CPP (Fig. 4C and D). Again, cell viability was not affected by the amount of CPP and TNF-α used in our experiments (Fig. 4E). Concentration-response experiments using different amounts of secondary CPP or TNF-α confirmed these results (Suppl. Fig. 3).

To further characterize the significance of TNF-α and its

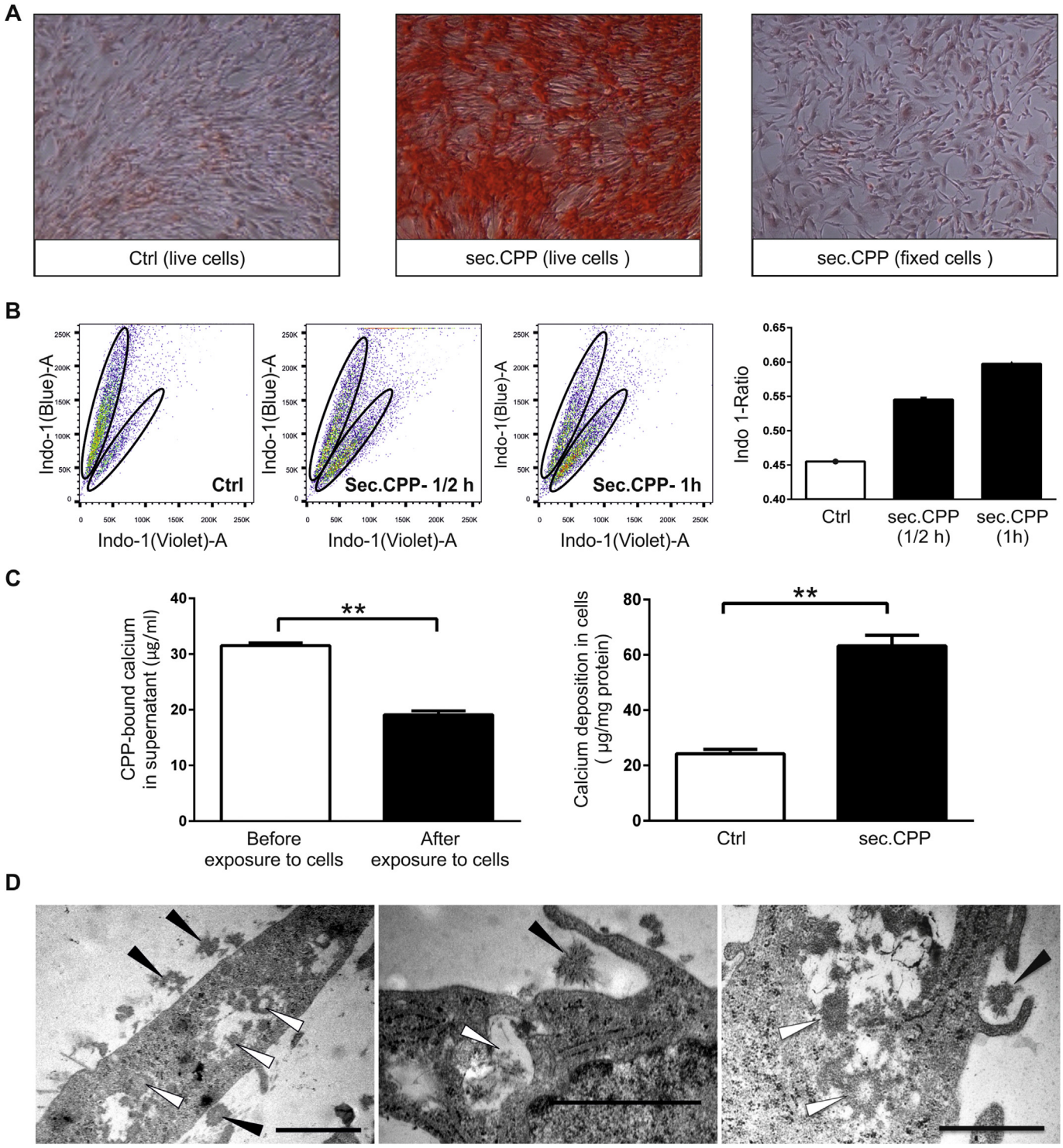

Fig. 3. Vascular calcification is an active cell-mediated process. (A) Effect of secondary CPP on living or paraformaldehyde-fixed VSMC after 24 h. (B) Increase of intracellular calcium following secondary CPP-exposure of VSMC. Cells were loaded with 2 µM Indo-1. Unbound Indo-1 has a peak emission at 485 nm, which shifts to 410 nm upon Ca^{2+} binding. This shift was measured over time by flow cytometry and represented as a ratio of the two emission wavelengths. (C) Decrease of CPP-bound calcium concentrations in the supernatant (starting concentration: 30 µg/ml of CPP-bound calcium) and increase of cell-bound calcium upon secondary CPP treatment. (D) TEM imaging of secondary CPP-exposed VSMC showing crystalline particles intra- (white arrowheads) and extracellularly (black arrowheads). Scale bars = 500 nm (graphs represent mean ± SD, N = 3, $^{**}p < 0.01$).

receptors for the calcification of VSMC, RNA interference experiments using siTNF-α, siTNFR1 and siTNFR2 were performed. VSMC were transfected with these specific or scrambled (control) siRNAs, leading to a reduction of the mRNA levels of TNF-α, TNFR1, and TNFR2 by 79%, 73% and 74%, respectively and protein levels (Fig. 5A). Concomitant with the down-regulation of TNF-α or TNFR1, calcification of VSMC exposed to secondary CPP was reduced, whereas the down-regulation of TNFR2 or the treatment with scrambled siRNA did not reduce calcification (Fig. 5B). Combining *TNF-α* and *TNFR1* siRNA, enhanced this anti-calcifying

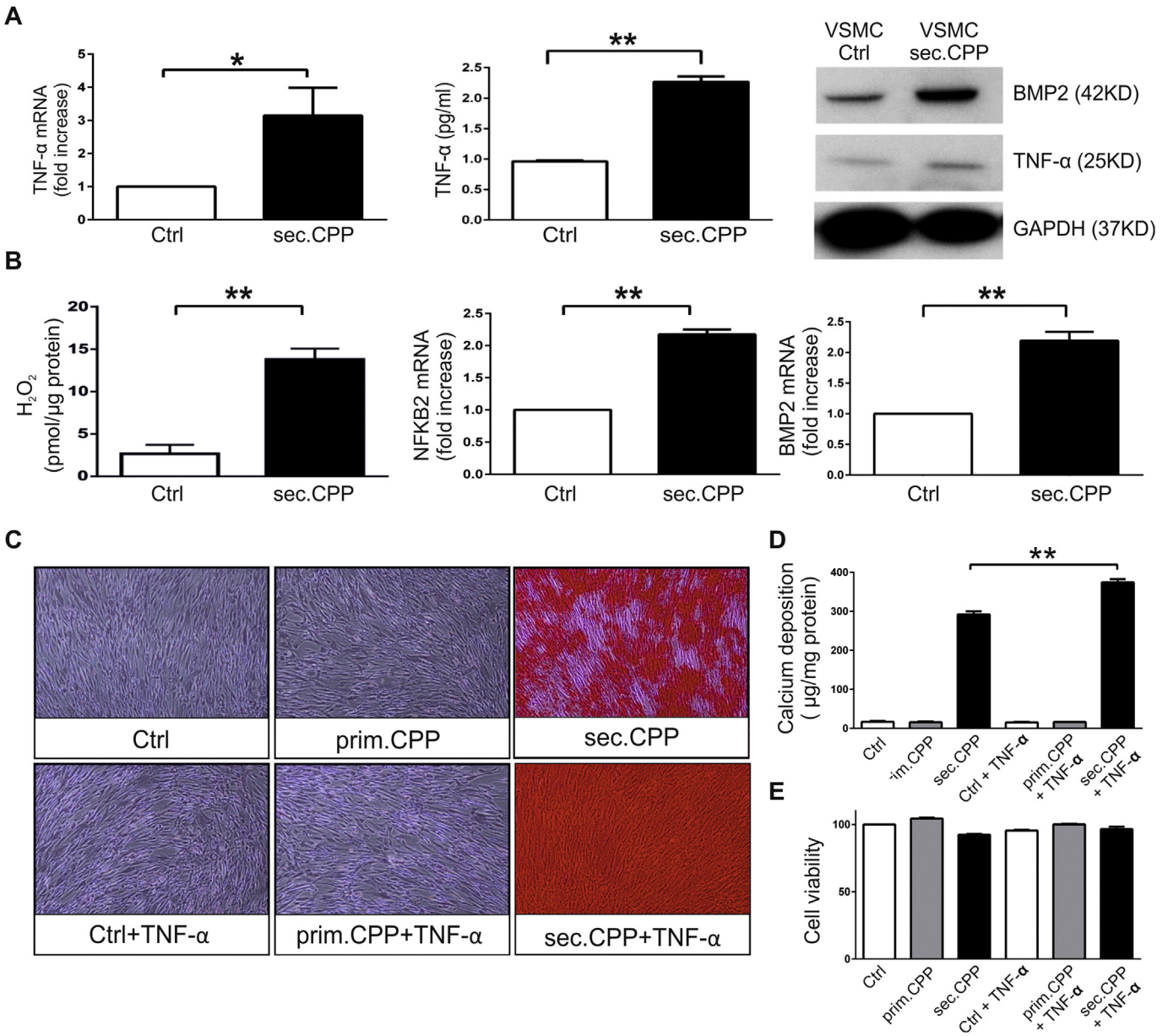

Fig. 4. Tumor necrosis factor-α and reactive oxygen species are expressed upon treatment with secondary CPP. (A) Up-regulation of TNF-α detected by qPCR, ELISA and Western blot, expression of BMP2 by Western blot. (B) Exposure of VSMC to secondary CPP *in vitro* significantly increased H_2O_2 generation after 24 h and subsequent expression of BMP-2 and NF-κB. (C) VSMC treated with secondary CPP for 24 h, in the presence of 10 ng/ml TNF-α, showed increased calcification. However, no calcification was observed in cells that were treated with control medium, primary CPP, TNF-α alone or TNF-α and primary CPP together, as determined by Alizarin red staining. (D) Quantification of calcium deposition. (E) No decline in viability was observed in VSMC challenged with CPP and TNF-α (mean ± SD; N = 3; $^{*}p < 0.05$, $^{**}p < 0.01$).

effect (Suppl. Fig. 4). The transfection of VSMC with siRNA against *TNF-α*, *TNFR1*, or *TNFR2* or scrambled siRNA followed by incubation without secondary CPP had no effect on cellular calcium content and the resultant calcification.

These results demonstrate that the activation of the TNF-α/TNFR1-system enhances the calcification of VSMC exposed towards secondary CPP.

4. Discussion

Here we demonstrate for the first time the formation and calcification-inducing properties of secondary CPP on VSMC *in vitro*, as well as the resulting expression of TNF-alpha by VSMC and its calcification-enhancing effects. Our data provide a conceptual framework on the interplay of spontaneously formed calcified matter with live cells and the resulting cellular responses.

Based on the present work, the process of calcification of VSMCs *in vitro* is characterized by the characteristics summarized in Fig. 5C. As a first and inevitable step in this VSMC *in vitro* calcification model, secondary CPP have to be formed. This is a cell-independent physico-chemical process. The second step is the uptake of the CPP by the VSMCs, which causes a detectable rise in intracellular calcium and induces the calcification process. The third step is the cellular response, which is characterized by the expression or upregulation of markers of oxidative stress and inflammation. The final step is the activation of the TNF-α/TNFR1 system, which enhances the calcification process and influences and determines the extent of calcification.

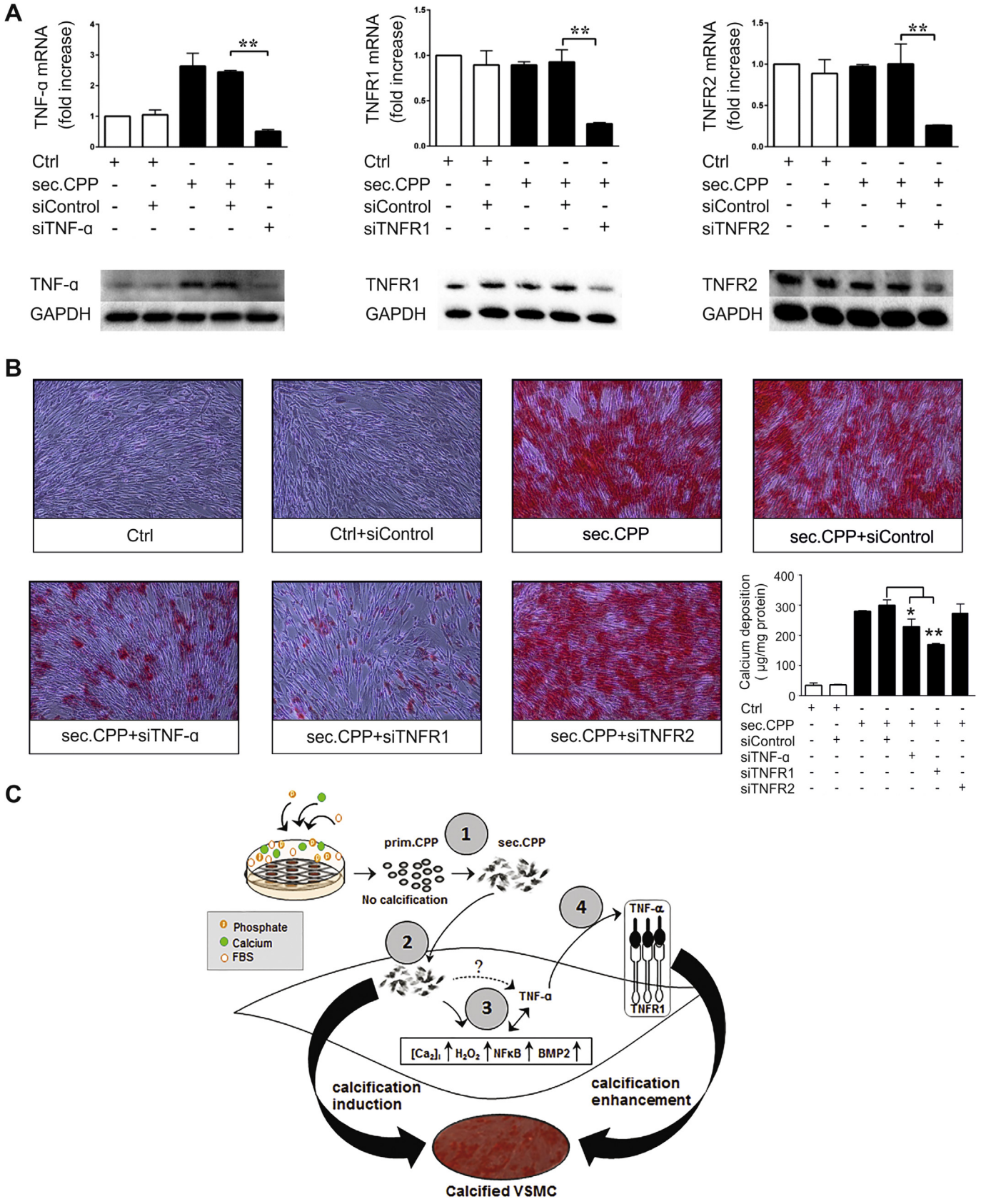

Fig. 5. The effect of TNF-α on calcification is mediated via TNFR1. (A) VSMC were transfected with small interference RNA (siRNA) specific for *TNF-α*, *TNFR1*, *TNFR2* or with scrambled siRNA (siControl) for 24 h. The mRNA levels are presented as fold-increase relative to cells incubated in the absence of secondary CPP. Data are expressed as mean ± SD of 3 independent experiments ($p < 0.05$ compared with siControl). Reduced levels of the target proteins were confirmed by Western blot. (B) Alizarin Red staining and direct measurement of cell-associated calcium showed reduced calcification of secondary CPP-treated VSMC upon down-regulation of TNF-α or TNFR1 (mean ± SD; N = 3; $^{*}p < 0.05$, $^{**}p < 0.01$). (C) Schematic illustration of the calcification process in VSMCs. The first step is a cell-independent physicochemical process (formation of secondary CPP). The second step is the interaction and uptake of secondary CPP by the VSMC. Step three is the cellular response, which is characterized by the expression or upregulation of oxidative stress as well as inflammation markers. Step four is the activation and auto-/paracrine self-stimulation of the TNF-α/TNFR1 system.

In vitro calcification of VSMC has been widely studied using calcification medium, i.e. cell culture medium supplemented with phosphate, calcium and FBS [25,40,47,48]. Using this approach, however, calcification induction was initially variable in our hands. Here, we identified the primary-to-secondary CPP transformation as a major source of this variability. When using secondary CPP, we were able to substantially stabilize our *in vitro* calcification model.

CPP form spontaneously when calcium and phosphate are added to serum or artificial solutions containing fetuin-A and albumin [15,26], but also in cell culture calcification medium as demonstrated here. The formation of primary CPP was a rapid and robust process which occurred within the first hour at 37 °C upon addition of calcium and phosphate to the FBS-containing medium. In contrast, their transformation into *secondary* secondary CPP is variable and sensitive to small inconsistencies of the incubation conditions. Adding preformed secondary CPP to media instead of waiting for their more or less variable formation under the routine cell culture conditions eliminated the inconsistencies of mineralization induction in our VSMC calcification system. This indicates that the presence of secondary CPP was a necessary prerequisite for the induction of calcification in our system. It may therefore be reasoned that the timing of the cell-independent process of primary-to-secondary CPP transformation reflects the global likelihood of the calcification process to start. Of note, the transformation time point is also determined by a novel blood test, which measures the calcification propensity in human blood [26]. A high calcification propensity in this test is strongly related with relevant clinical endpoints (survival, cardiovascular survival and graft loss) in CKD patients and kidney-transplanted patients [19,37].

Calcification started when secondary CPP were present. Our finding is in line with previously published data showing that artificial calcium phosphate crystals drive calcification *in vitro* [32,44]. Furthermore, this result is consistent with *in vitro* data showing increased exosome secretion and enhanced calcification upon exposure of VSMC towards CaP crystals and artificial CPPs generated using pure fetuin-A [18].

Our refined analysis of the spontaneous formation of nanoparticles in calcification medium, the type of particle required for calcification induction, and, the controllability for particle type is of relevance for cell culture-based research investigating mechanisms of biomineralization.

The specific reason(s) for the absence of an effect of primary CPP on calcification is not known yet. It may however be due to its low/absent crystallinity, its low calcium- and phosphate contents, its protein contents and/or specific protein composition, its mineral-to-protein ratio and/or the size/diameter of the particles, or specific combinations of these properties. Future studies should further elucidate the specific causes why prim. CPP do not induce calcification.

Our data indicate that calcification was an active cell-mediated process in our system and related to the processing of secondary CPP by VSMC. The cell-dependent nature of CPP-induced VSMC mineralization is consistent with previously published work [33,35] and is indicated by three main findings: (i) mineralization was only observed in the presence of living but not formalin-fixed cells, (ii) intracellular calcium increased upon exposure towards secondary CPP, and (iii) calcified crystalline structures were found intracellularly using TEM imaging.

CPP have been found circulating in the blood of renal patients [39] and in the dialysate of patients receiving peritoneal dialysis [16]. In both these studies, secondary CPP could be directly visualized using TEM. Indicating a relevant clinical role, circulating CPP were furthermore found to be associated with vascular calcification and aortic stiffness [14,38]. Here, the interaction of VSMC with secondary CPP *in vitro* triggered the expression and release of the inflammatory cytokine TNF-α. Secondary CPP are known to possess pro-inflammatory properties and to induce the expression and secretion of TNF-α when exposed to macrophages *in vitro* [39] Furthermore, previous studies demonstrated that the inflammatory cytokine TNF-α promotes VSMC calcification *in vitro* [2,34,42]. While in these studies TNF-α had been added to the growth medium [42], we also investigated the endogenous expression and functional relevance of VSMC-derived TNF-α in our system. We found, that TNF-α mRNA and protein were increased when VSMC were exposed towards secondary but not primary CPP. Demonstrating a causal role for TNF-α, calcification was decreased when the expression of TNF-α or its receptor type 1 were suppressed by siRNA. Combining these interventions augmented the inhibitory effect on calcification, which reached approximately 50%, whereas the down-regulation of the TNF-α receptor 2 or the use of scrambled siRNA had no effect. This indicates an important calcification-enhancing role of CPP-induced upregulation of TNF-α and the stimulation of its receptor type 1 *in vitro* in primary human VSMC.

Interestingly, both inflammation and vascular calcification commonly co-exist in patients with CKD [41], and several mediators of inflammation such as oxidation, C-reactive protein, and cytokines have been suspected to directly stimulate vascular calcification and coronary events [20,46]. Moreover, inflammation is associated with lower circulating fetuin-A levels [24], Since fetuin-A retards the transformation of primary to secondary CPP, inflammation may well promote the maturation of CPP [17].

A further important effect of secondary CPP in our system was the induction of oxidative stress (H_2O_2), a condition also commonly found in patients with CKD [1,23]. In addition, exposure of VSMC towards calcification medium supplemented with H_2O_2 leads to enhanced calcification [5]. Furthermore, in line with recent studies which reported that BMP-2 expression is governed by H_2O_2 and NF-κB-dependent pathways [7,8], we observed that the expression of BMP2 and NF-κB was increased in response to secondary CPP. The induction of the TNF-α system may have also contributed to the NF-κB activation [7,8]. A detailed temporal analysis of these activations and of the respective cause-and-consequence relations was, however, beyond the scope of our current work and awaits clarification in future studies.

The same applies to further questions, which can be addressed using this stable calcification system. Such research may e.g. in more detail address the mechanism of particle uptake, the mechanism of calcification including the potential involvement of matrix vesicle formation, the question why primary CPP do not induce calcification, and the mechanism by which TNF-alpha enhance calcification.

In conclusions, we demonstrate for the first time that calcification of VSMC is induced by the presence of secondary CPP. The extent of calcium deposition is then determined by the amount of these CPP and furthermore by the activation of the TNF-α/TNFR1 system. The latter is stimulated by secondary CPP and markedly enhances the calcification process. These findings may provide a conceptual framework for the development of future therapeutic interventions aimed at the prevention of the calcification process in the vasculature.

Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.05.044.

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