Nano-Hydroxyapatite Accelerates Vascular Calcification by Inhibiting Lysosomal Acidification in Smooth Muscle Cells.

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Research

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Abstract

Background: Vascular calcification (VC) is a common characteristic of aging, diabetes, chronic renal failure, and atherosclerosis. The basic component of VC is hydroxyapatite (HAp). Nano-sized HAp (nHAp) has been identified as the initiator of pathological calcification of vasculature. However, whether nHAp can induce calcification *in vivo* and the mechanism of nHAp in the progression of VC remains unclear.

Results: We discovered that nHAp existed both in vascular smooth muscle cells (VSMCs) and their extracellular matrix (ECM) in the calcified arteries from patients. Synthetic nHAp had similar morphological and chemical properties as natural nHAp recovered from calcified artery. nHAp induced rapid progression of VC by stimulating osteogenic differentiation and accelerating mineralization of VSMCs *in vitro*. Synthetic nHAp could also directly induce VC *in vivo*. Mechanistically, nHAp was internalized into lysosome, which impaired lysosome vacuolar H⁺-ATPase for its acidification, therefore blocked autophagic flux in VSMCs. The accumulated autophagosomes and autolysosomes were converted into calcium-containing exosomes which were secreted into ECM and accelerated vascular calcium deposit. Inhibition of exosome release in VSMCs decreased calcium deposition.

Conclusions: Our results illustrated a novel mechanism of nHAp-induced vascular calcification. Understanding the role of nHAp in autophagy-lysosome-exosome pathway in SMCs could have great clinical significance in preventing the progression of VC.

Introduction

Vascular calcification (VC) is a detrimental pathology highly prevalent in aging, diabetes mellitus, atherosclerosis, and chronic renal failure. Development of VC involves aberrant calcium deposition in the vessel wall, apoptosis of vascular smooth muscle cells (VSMCs) and the phenotypic transformation of VSMCs into osteoblast-like cells.[1, 2]. During calcification, cytoplasmic Ca²⁺ and phosphate (Pi) incorporate with alkaline phosphatase (ALP) into exosomes that bud off the plasma membrane and associate with extracellular proteins, such as collagen. Crystals in the exosomes finally grow into nano-sized hydroxyapatite (nano-HAp or nHAp) [Ca₁₀(OH)₂(PO₄)₆][3]. Studies have shown that nHAp stimulated osteogenic transdifferentiation of SMCs *in vitro* and induced calcification on extracellular matrix (ECM) [4-6], but there was no experimental result *in vivo*. The above studies suggested that nHAp may lead to a pathological feedback loop causing more inflammation, cell death, phenotypic change, matrix degradation, and calcification. This may explain why calcification progresses rapidly once microcalcification exists. Whether nHAp can induce calcification *in vivo* and the underlying mechanism by which nHAp affects the VSMCs remain unclear.

Autophagy is a “housekeeping” subcellular process for lysosome-mediated turnover of damaged cytosolic materials that are engulfed by a double membraned phagophore to form an autophagosome. The autophagosomes fuse with single membraned acidic lysosomes to form autolysosomes, where the engulfed content is degraded and recycled [7]. Autophagy has been shown to regulate Ca²⁺ homeostasis

in VSMCs. Studies proved that autophagy dysfunction contributes to VC[8, 9]. These studies demonstrated a protective role of autophagy in vascular disorders. Mice with VC had increased expression of the autophagy markers microtubule-associated light chain 3 II (LC3-II) and sequestosome 1 (SQSTM1/ p62), etc. in aortic VSMCs as compared to that in normal mice[10]. Evidence also demonstrated that increased cytosolic concentrations of Ca^{2+} induced autophagosome accumulation, which was caused by a decline in autophagic clearance via lysosomes[11, 12]. These studies indicated that impaired autophagy may contribute to VC, and lysosomal function may involve in VC. These data suggest that autophagy-lysosomal function plays an important regulatory role in vascular calcification.

Lysosomes are single membrane-enclosed organelles present in all eukaryotic cells that contain large numbers of hydrolytic enzymes. Lysosomes exhibit their maximal enzymatic activity at a low pH ($\text{pH} \leq 5$) and digest extracellular material from the endocytic pathway and intracellular material from the autophagic pathway. Disorders of lysosome acidification disrupt the clearance of autophagy[13]. Nanoparticles could lead to impairment of both autophagy and lysosome[14]. However, whether and how nHAp affects autophagy and lysosome in VSMCs remain to be elucidated.

Recently, secretory autophagy as a new concept was presented in addition to the traditional degradative autophagy [15]. Autophagic proteins were found on matrix vesicles (MVs) that were secreted out of cells[15]. MV membranes are enriched in phosphatidylethanolamine[16, 17] which is also a main constituent of the mature autophagosome membrane[18]. These data suggest that autophagic vesicles can form MVs to be released into matrix outside of cells. In addition, one study proved that the calcium-containing MVs in osteoblasts were transported via lysosome and secreted by exocytosis, indicating that lysosome plays a central role in mineralization[19]. Studies have shown that MVs are enriched with a concoction of calcifying enzymes and crystals of calcium phosphate HAp mineral[16, 20]. Such MVs are released from osteogenic cells and facilitate HAp formation in ECM[21]. In context of atherosclerosis, VSMCs and macrophages are the primary source of these calcified MVs which are released into the collagen rich matrix in the intima to promote atherosclerotic calcification, directly leading to the formation of calcified plaques[22]. It is reasonable to speculate that MVs may be entwined with the network of autophagic vesicles either at the stage of their formation or release during the process of vascular calcification. Accumulating evidence indicates that matrix vesicles are secreted from multivesicular bodies and are enriched with the exosome markers CD63, CD9, CD81, and MHC I. Secretion of these exosome-like structures is regulated by sphingomyelin phosphodiesterase 3, and inhibition of this enzyme prevents vascular calcification[23]. Comparative proteomics showed that VSMC derived exosomes were compositionally similar to exosomes from other cell sources but also shared components with osteoblast-derived MVs including calcium-binding and extracellular matrix proteins[24]. Despite the clinical importance of exosomes in triggering vascular calcification, little is known of the link between exosomes biogenesis and VC induced by nHAp.

In this study, we demonstrated that nHAp induced VC through lysosomal impairment and blockage of autophagy flux in VSMCs and enhanced the release of calcifying-exosomes. We for the first time

illustrated the effect of autophagy-lysosome impairment on VC, and highlighted the link between nHAp-induced deficiencies of lysosome activity and calcium deposition of VSMCs.

Methods And Materials

Characterization of Nanoparticles

Synthetic nHAp (purity 97%; particle size less than 100 nm) was obtained from Aladdin Biochemical Technology (Shanghai, China). Human-derived HAp crystals were prepared from samples of human carotid arteries removed by endarterectomy. The HAp crystals were dissected as described previously[27]. The hardened, obviously calcified regions were dissected from the atherosclerotic vessels and digested with collagenase (3 mg/mL) and elastase (1 mg/mL) overnight at 37°C. After digestion, the crystals were washed 3 times in Hanks' Balanced Salt Solution. Samples were then placed on a 70- μ m sieve (Gibco) and washed further. The remaining crystals ($\approx$ 70 μ m) were crushed using a pestle and mortar into Hanks' Balanced Salt Solution. Washing of the crystals at this stage was achieved using ultracentrifugation at 100000 g to capture very small particles. The morphology and size of the crystals were characterized by scanning electron microscope (SU8010, HITACHI, Japan). The phase composition of the prepared nano-sized crystals was confirmed by X-ray diffraction (XRD, PANALYTICAL, The Netherlands) with Cu-K α radiation and Fourier transform infrared (FT-IR) spectroscopy (FTR-8400S, SHIMADZU, Japan).

Cell Culture

Vascular SMCs were isolated from aortas of 8-week-old wild-type C57BL/6 mice (Slac Laboratory Animal). Briefly, the adventitia and endothelium were removed and the median layers were digested with 1 mg/ml collagenase type I (Worthington) and 0.714 U/ml elastase (Worthington) for about 1.5 h at 37°C. Cells were maintained in Dulbecco's modified Eagle's medium (Hyclone) containing 20% fetal bovine serum (Hyclone) and 100 U/mL penicillin, 100mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Cells were used between passage 3-5 in the experiments.

To determine the calcium deposition in vitro, VSMCs were treated with osteogenic medium (OM) containing 10 nmol/L dexamethasone (Sigma-Aldrich), 0.25 mmol/L L-ascobic acid (Sigma-Aldrich), 10 mmol/L β -glycerophosphate (Sigma-Aldrich), or/and 100 mg/ml HAp for 24 h, then washed with phosphate-buffered saline (PBS) to remove HAp, then continue to be cultured in OM or DMEM for 2 weeks.

Measurements of activity of Alkaline phosphatase (ALP).

The ATP activity of VSMCs was determined by using a luminescence ATP detection kit in accordance with the manufacturer's instructions (Beyotime Biotechnology,

Shanghai, China). After SMCs were cultured with HAp for 7 days, SMCs were washed with cold PBS, scraped into 100 μ L of lysis buffer (0.2% NP-40 with 1 mM MgCl₂) and incubated for 30 min on ice. Then

the lysis was centrifugated at 5000rpm for 5 min and the supernatant was collected. All samples were stored under -80°C until use for ALP assay which was performed following the manufacturer's instructions (Beyotime Biotechnology, Shanghai, China). By catalyzing p-nitrophenyl phosphate (pNPP) to pNP, ALP activity is referred as the change of absorbance at 405nm, and then normalized to the protein level. Unit Definition: One ALP DEA unit equals hydrolyzation of 1 mole of p-nitrophenyl phosphate per minute at pH 9.8 at 37°C.

Alizarin Red Staining

After cultured at specified conditions for 14 days, SMCs on dishes were fixed with 10% formalin for 30 min, then washed with double distilled water (ddH₂O) twice and incubated with Alizarin Red (Solarbio Life Sciences, China) for five minutes and washed with ddH₂O twice to remove the excessive dye. After examination and photography under a microscopy, the dye on the cells was extracted with 100ul Hexadecyl Pyridinium chloride (Sigma-Aldrich) and the optical density (OD) at 560 nm was measured using a microplate reader (Spectra MAX 190, Molecular Devices, USA).

von Kossa staining

A segment of the abdominal aorta was excised and fixed in 10% formalin for 24 h, then dehydrated, embedded in paraffin, and cut into 5-μm -thick sections. The slides were deparaffinized, dehydrated and washed in distilled water (ddH₂O), the samples were incubated with reagent A of von Kossa staining Kit (Solarbio Life Sciences, China) and placed under ultraviolet light for 10 min ,and then placed into reagent B for 2 min followed by staining with hematoxylin for the demonstration of cell nucleus.

Acridine orange (AO) staining

VSMCs were exposed to 100 μg/mL sHAP for 24 h. The cells were then stained with 5μg/mL acridine orange (Aladdin, China) for 20 min at 37°C and 5% CO₂. Then the cells were washed with PBS, followed by the addition of DMEM without serum to the cells. The fluorescence images of the cells were immediately acquired on a fluorescence microscope (Leica).

Analysis of EGFR degradation

After treated with nHAp for 24 h, VSMCs were incubated with serum-free DMEM containing 50 ng/ml of EGF for 0, 1, 2 and 3 h at 37 °C, and then the cells were either lysed and analyzed by western blot, or were fixed in 4% formaldehyde, stained with anti-EGFR antibody and imaged by fluorescence microscopy (Leica).

Calcium Quantification

To determine the calcium deposition in cells or exosomes, PBS-washed VSMCs on a dish or purified exosomes were dissolved in 100 ul 0.6 N HCl for 48 h. Calcium content was measured by Calcium Assay

Kit (Cayman Chemical, USA). The amount of calcium was normalized to the total amount of protein or the number of cells.

Exosome isolation

Exosomes were harvested by following a modified Exosome isolation protocol[52]. Confluent VSMCs were washed twice with PBS and cultured with DMEM medium with sHAp (100 µg/ml) for 3 days, and then the medium was collected and centrifuged at 10,000x g for 1 h to remove cells and apoptotic bodies. Exosomes were then harvested from the supernatant by centrifugation at 110,000x g for 70 min twice at 4°C in an Ultracentrifuge (Optima L-90K; Beckman Coulter, USA). Exosomes were resuspended with PBS or 0.6N HCl for protein or calcium content measurement, respectively. Exos were observed by TEM (Tecnai G2 spirit, Thermo FEI, USA) which demonstrated typical cup shapes with diameters around 100 nm (Fig S11A). Dynamic light scattering analysis further confirmed that the average diameter of Exos was approximately 133nm (Fig S11B). Western blot confirmed the expression of the Exos' marker proteins Alix, TSG101 and CD9 in Exos, but no GAPDH expression. While the VSMCs have no expression of CD9. And the above markers all don't exist in the supernatants after ultracentrifugation (Fig S11C).

mRFP-GFP-LC3 Adenovirus Puncta Assay

For the analysis of autophagic flux, VSMCs were transfected with stubRFP-sensGFP-LC3 Adenovirus (JIKAI, China) following the manufacturer's instructions. After 36 h the transfected cells were starved or treated with 100ug/ml sHAp for 24 h. Then the cells were fixed with 4% paraformaldehyde in PBS and then stained with DAPI, and imaged using a fluorescence microscope (Leica, Germany).

Murine models of calcification

Two different murine models of calcification were created for this study. One is ectopic calcification on soft tissue inducing via injection of Matrigel mixed with GFP-labeled VSMCs and sHAp. The other is by smearing HAp on the abdominal aorta. Mice (C57BL/6, 3-month old) were anesthetized with isoflurane. The mice were shaved and the skin was disinfected with 75% ethanol prior to operation. For the first one, three longitudinal skin incisions about 1cm in length on the dorsal surface of each mouse were made, and subcutaneous pouches were created by blunt dissection. The implants were 400 µl in volume containing 5mg sHAp or 6×10^6 SMCs or both, which was thoroughly mixed in advance, and were placed into pouch as following: left pouch, 200 µl sHAp + 200 µl Matrigel; right pouch, 100 µl sHAp + 100 µl GFP-labeled SMCs+200 µl Matrigel; lower pouch, 200 µl GFP-labeled SMCs+200 µl Matrigel. The incisions were closed with surgical sewing. For the second model, a longitudinal skin incision about 2cm in length on the abdominal surface of a mouse was made, and the abdominal aorta was isolated (between renal artery and the bifurcation of common iliac artery), the vascular adventitia was torn off. The artery was incubated with 0.9% NaCl, 0.5 M/l CaCl_2 , or sHAp (25 mg/ml 0.9% NaCl) for 15min with the aid of Stereo Microscope (SZ61, Olympus, Japan). The incisions were closed with surgical sewing. Calcification in vivo was evaluated using micro-CT (U-CT-OI, Milab, USA).

Statistical analysis.

Data were expressed as means $\pm$ standard deviation (SD) and analyzed by Program Graph-Pad Prism version 6.0. Multiple comparisons were performed by one-way ANOVA followed by Bonferroni correction. A value of $p < 0.05$ was considered as statistically significant.

Results

Nano-sized hydroxyapatite exists in human calcified aorta

To study the relationship between nHAp and vascular calcification, human calcified aorta specimen was collected. Calcium deposition in both media and adventitia of the artery was detected (Fig 1A). Nano-sized HAp was observed on the surface of the vascular cells by scanning electron microscope (SEM) (Fig. 1B). Interestingly, we found that nHAp was adhered to the VSMC surface and there was nHAp in the lysosome of the cell (Fig 1C). In addition, abundant α -SMA positive cells expressing osteogenic marker Runx2 were observed on the calcified artery (Fig 1D). These findings indicate that nHAp internalization potentially contribute to the rapid progression of VC.

Characterization and cellular internalization of HAp crystals

To analyze the effect of HAp on calcification in SMCs, we used synthetic nHAp (sHAp) to mimic human naturally crystallized HAp (hHAp) that was derived from atherosclerotic plaques of human aorta. The crystalline structures of synthetic and hHAp crystals were similar in morphology under SEM (Fig 2A), both were homogeneous and rod-like crystals. The size is about 100 nm for sHAp and ~ 80 nm for hHAp crystals. The X-ray diffraction (XRD) spectrum of synthetic and hHAp crystals showed both had eight diffraction peaks of crystals (Fig 2B), consistent with the standard HAp (JCPDS09-0432)[25]. The samples were further investigated by Fourier transform infrared spectrum (FT-IR) (Fig 2C). Both crystals had the vibration peaks at 563, 603 and 1029 cm^{-1} which belonged to the asymmetric stretching vibration peaks of P-O in the PO_4^{3-} groups. Vibration peak at 3421 cm^{-1} were attributed to the O-H stretching vibration in HAp. Both XRD and FT-IR spectra data confirmed that synthetic and hHAp are similar and well crystallized in pure-phase.

To study engraftment of the HAp crystals by VSMCs, HAp was dyed bright green by the fluorexon (Fig S1). As shown in Fig 1D, both hHAp and sHAp were internalized into VSMCs after the HAp crystals were mixed with VSMCs for 24 h. Under EM, irregularly aggregated HAp crystals in different size were visible inside cytoplasm of VSMCs after mixing cells with HAp for 24 h (Fig 2E). The images show that a number of HAp crystals were gathered in the lysosomes, autophagosomes and autolysosomes.

Nano-HAp induced osteogenic differentiation of VSMCs and calcification deposition

To evaluate the effect of HAp on osteogenic differentiation of VSMCs, osteogenic marker proteins were examined. Expression of ALP, Runx2 and OPN was all increased significantly after VSMCs were treated

with sHAp (Fig 3A) or hHAp (Fig S2A) for 5 days as compared with those of the controls. The activity of ALP in VMSCs was also increased significantly after treated with HAp (Fig S2B).

After VSMCs were treated with osteogenic medium (OM) or/and sHAp (VSMC^{sHAp}) for 14 days, significantly more calcium deposition in VSMC^{sHAp} was observed (Fig 3B-D) as compared with VSMCs cultured in OM, whereas VSMCs under normal medium showed no calcium deposition. VSMCs treated with both OM and sHAp showed the maximal calcium deposition. Similar phenomena were also observed for VSMCs cultured with hHAp (Fig S2C-E). These data indicated that both sHAp and hHAp crystals could induce calcification of VSMCs, and HAp crystals accelerated the development of calcification compared with the normal OM. Such capacity was notably enhanced with OM. In view of the fact that human samples are hard to obtain, we may use sHAp instead of hHAp to conduct the following experiments on the basis of the above experiments.

Nano-HAp induced ectopic tissue calcification in vivo

To investigate whether nHAp can directly contribute to mineralization of ECM in vivo, Matrigel mixed with VSMCs or/and sHAp were subcutaneously implanted into the dorsum of C57BL/6 mice (Fig 4A). Greater calcification was detected using micro-CT at the region injected with both VSMCs and sHAp as compared to that with sHAp only (Fig 4B-C) after implantation for 1 month. No calcification was observed at the site injected with VMSCs only. Likewise, the calcium content in the recovered subcutaneous tissues with both VSMCs and sHAp was the highest among 3 groups (Fig 4B-C). Calcific deposits and cells were identified in the recovered subcutaneous tissues (Fig S3). Most of the cells in the recovered tissues with VSMCs and sHAp were GFP positive, indicating that they were the originally implanted VSMCs, and they were strong positive for osteogenic marker Runx2; whereas in the tissue with VSMCs only, significantly less Runx2 was detected in the GFP⁺ VSMCs (Fig 4D). As expected, neither GFP⁺ cells nor Runx2 were detected in the recovered tissues with sHAp only. These results confirmed that nHAp possessed the ability to induce osteogenic differentiation of VSMCs and ectopic matrix mineralization in vivo.

To further confirm the effect of nHAp on vessel calcification, we applied sHAp to the out surface of mouse abdominal aorta whose adventitia was torn off. Calcification dots on the aorta being applied with either sHAp or CaCl₂ were detected by enhanced micro-CT (Fig 4E) or directly viewed under stereomicroscope (Fig 4F) 14 days after the surgery, while application of control NaCl to aorta resulted in no calcium deposits, which was further confirmed by the von Kossa staining of the vessels (Fig 4G). Significantly more Runx2 was co-localized with SMCs marker α -SMA on the aorta treated with sHAp or CaCl₂ as compared to that treated with NaCl (Fig 4H). These results indicate that nHAp could induce osteogenic differentiation of SMCs and vascular calcification in vivo.

Nano-HAp induced accumulation of autophagosomes in VSMCs

Under TEM, we observed increased number of double-membrane autophagosomes in VSMC^{HAp} (Fig 5A and Fig S4A) as compared to the control cells, indicating that internalization of HAp could cause the

accumulation of autophagic organelles. Indeed, almost 4-fold more LC3, a classical marker for autophagy, was detected on the calcified artery than that on the control (Fig 5B). More puncta formation of endogenous LC3 was observed in VSMC^{sHAp}, while less and evenly dispersed LC3 throughout the cytoplasm was seen in the control cells (Fig 5C). Furthermore, significantly higher LC3-II was detected in VSMC^{HAp} than the untreated controls (Fig 5D, and Fig S4B), indicating that HAp stimulated the conversion of cytoplasmic LC3-I to membrane-conjugated LC3-II. Taken together, these data demonstrate that both types of HAp crystals induced accumulation of autophagosomes in VSMCs.

The classical pathway of autophagy induction involves inhibition of mTOR, usually induced by energy reduction. We found that sHAp did not alter the phosphorylation of either mTOR or AMPK (Fig S5A), suggesting that sHAp stimulates formation of autophagosomes through a pathway different from starvation-induced mTOR inhibition. Rapamycin, an inhibitor of mTOR, which induces autophagy, additively increased LC3-II level in VSMC^{sHAp} (Fig S5B), further confirming that sHAp-stimulated production of autophagosomes involves a pathway independent of mTOR inhibition.

Nano-HAp inhibited autophagic degradation without effecting fusion of autophagosome with lysosome.

Accumulation of autophagosomes could be a result of autophagy induction or blockage of autophagosome clearance which involves fusion of autophagosomes with lysosomes and thereafter degradation of the resulted autolysosomes[26]. S-HAp did not increase the mRNA of LC3 (Fig S6A), indicating that HAp-induced increase in LC3 was not due to enhanced LC3 expression for more autophagosome formation.

Protein sequestosome 1 (p62) is a selective autophagic receptor that is incorporated into autophagosomes and preferentially degraded along with other substrates by lysosomal hydrolysis. Level of p62 protein was increased after VSMCs were treated with sHAp or hHAp (Fig 6A, and Fig S4B). Immunostaining showed more cytoplasmic p62 proteins were assembled into aggregates in VSMC^{sHAp} (Fig S7). Such effect of HAp on accumulation of p62 was not due to more p62 expression as its mRNA level was not different from that in the untreated cells (Fig S6B). When chloroquine (CQ), an inhibitor of autophagosome-lysosome fusion, was added into the culture, both LC3II and p62 were additively elevated (Fig 6B). These data indicate that nHAp could block the autophagy flux.

When 3-methyladenine (3-MA), an inhibitor of autophagosome synthesis, was added to the starved cells, LC3-II was significantly reduced; however, such 3-MA mediated reduction of LC3-II did not occur in VSMC^{sHAp} (Fig 6C), also suggesting that nHAp blocks autophagosome degradation.

Autophagic flux was further examined by transferring mRFP-GFP-LC3 fused genes using adenoviral vector. Under starvation, fusion of autophagosomes with lysosome lowered the pH that quenches the GFP signal (Fig 6D). When the cells were cultured with sHAp, starvation-induced quenching of GFP fluorescence was significantly reduced, confirming that sHAp blocks autophagic flux and autophagic degradation.

To investigate the formation of autolysosomes, co-localization of autophagosomal LC3 with lysosome-anchored Ras-related protein 7 (Rab7), a GTPase, was examined to implicate the fusion of autophagosome with lysosome. LC3 was poorly colocalized with Rab7 in control VSMCs, while starvation enhanced the colocalization, demonstrating the formation of autolysosome (Fig 6E). Nano-HAp treatment of VSMCs resulted in a similar colocalization of LC3 and Rab7 as that of starved cells, indicating that the formation of autolysosomes was not affected by nHAp. This was further confirmed by colocalization of LC3 with the lysosomal associated membrane protein 1 (LAMP1) (Fig S8).

Nano-HAp impaired lysosomal degradation through inhibiting lysosomal acidification.

Autophagy is a degradation process in cooperation with lysosomes. Since nHAp does not affect the fusion of autophagosome with lysosome, we then examined the function of lysosomes. The expression of early endosomal antigen 1 (EEA1), and LAMP1 both increased in VSMCs after sHAp treatment (Fig S10), indicating that sHAp may enter VSMCs through endosome/lysosome pathway. Entry of sHAp into lysosome was observed after mixing sHAp with VSMCs for 24 h (Fig 7A), which could impair the function of lysosomes. We then evaluated the ability of lysosomal degradation using a degradation assay of epidermal growth factor receptor (EGFR). EGFR was localized primarily on cell surface and was internalized and clustered into puncta after binding with its ligand EGF. Clustered EGFR was observed at 1 h post EGF treatment, and then was degraded later in the control VSMCs (Fig 7A). However, such EGFR puncta were retained in VSMC^{sHAp}, indicating weakened lysosomal proteolysis. This was further confirmed by western blot analysis showing that total cellular EGFR was retained in VSMC^{sHAp} while a timely reduction of EGFR was observed in the control cells (Fig 7B). These data indicate that nHAp impairs lysosomal degradation capacity.

Next, we examined the acidification ability of lysosomes since acidification is essential for the maturation and activation of most lysosomal enzymes. Acridine orange (AO) staining was used to assess the acidification of lysosomes. In cytosol, AO exists in nonprotonated monomeric form and emits green fluorescence. When it enters acidic lysosomes, the protonated form of AO aggregates and fluoresces bright red. As shown in Fig 7C, red fluorescence of AO was dramatically reduced in VSMC^{sHAp} as compared with that in control cells, indicating a reduced acidified- compartments in VSMC^{sHAp}.

In addition, more pre-matured cathepsin D (CTSD) at 43-kDa, a lysosomal protease, and less matured CTSD were detected in VSMC^{sHAp} in comparison with that in the control cells (Fig 7D), indicating a suppression of the conversion from pre-matured to matured form.

To understand the alteration of acidification and subsequent lysosome immaturation in VSMC^{sHAp}, the subcellular localization of V-ATPase, an ATP-driven proton pump that imports protons into lysosomal lumen for the acidification of the compartment was examined. V1D subunit of V-ATPase was colocalized strongly with lysosome membrane protein LAMP1 in the control cells, while this colocalization was disrupted in VSMC^{sHAp} (Fig 7E), suggesting a failure in the targeting of V-ATPase V1D subunit to lysosomes in VSMC^{sHAp}. These results demonstrated that nHAp impairs lysosomal acidification.

To investigate the relationship between autophagy and calcification, we utilized autophagy stimulator rapamycin (RAPA) and autophagy inhibitor chloroquine (CQ) [Fig S9]. RAPA and CQ themselves did not affect calcification in VSMCs (Fig 7G). When sHAp was added along with either RAPA or CQ, stronger Alizarin red staining with more calcium deposition in VSMCs was detected (Fig 7G), suggesting that both RAPA and CQ enhanced the calcification induced by sHAp.

Nano-HAp promoted release of exosomes containing LC3 and LAMP1.

Since exosome budding off plasma membrane has been proposed as a mechanism of calcification, and the interplay of autophagy and exosomal secretion was reported recently, we postulated that the accumulated autophagosomes and autolysosomes can be converted into exosomes and then secreted out of cells to lead calcification. To confirm that, exosomes (Exos) were purified from the conditioned medium of VSMCs and characterized (Fig S11). More Exos ($18.8 \pm 0.6 \text{ mg}/10^7 \text{ cells}$) were harvested from VSMC^{sHAp} than that from VSMCs ($7.5 \pm 0.5 \text{ mg}/10^7 \text{ cells}$) (Fig 8A). There were more exosome specific marker CD9 as well as autolysosome-associated LC3 and LAMP1 proteins in Exos from VSMC^{sHAp} than VSMCs (Fig 8B). Calcium content in Exos from VSMC^{sHAp} was significantly higher ($139.7 \pm 1.6 \text{ ng}/10^7 \text{ cells}$) than that from VSMCs ($64.4 \pm 1.6 \text{ ng}/10^7 \text{ cells}$) (Fig 8C). When GW4869, an inhibitor of neutral sphingomyelinase (nSMase), was added to the culture to block Exos release, significantly less Exos were harvested from VSMC^{HAp}. Exosome specific protein CD9, and autolysosome-associated LC3 and LAMP1 were correlatively reduced in Exos from VSMC^{sHAp} treated with GW4869 (Fig 8D and E), as well as the calcium content (Fig 8F). Accordingly, when VSMC were treated with sHAp in the presence of GW4869, calcium deposits were significantly reduced as compared with VSMC^{sHAp}.

To confirm that sHAp could be carried out of the cells via Exos after they were internalized into VSMCs, fluorexon-labeled sHAp were cultured with VSMCs. Fluorescence signal was detected in the Exos purified from the conditional medium of VSMC^{sHAp}. These data suggest that nHAp promoted release of Exos from VSMCs, and these Exos were at least partly originated from the accumulated autophagic vesicles which were resulted from the blockage of autophagy flux by nHAp (Fig 8H).

Discussion

In this study, we demonstrated nHAp existed in calcified artery, and first observed internalization of nHAp into VSMC. Nano-HAp stimulated osteogenic differentiation and accelerated mineralization of VSMCs *in vitro*, and enhanced calcium deposits in artery *in vivo*. Such effect of nHAp on promoting VC was through altering lysosomal acidification and impairing the degradation function of thereafter formed autolysosomes. We first discovered that the blockage of autophagy flux by nHAp resulted in accumulation of autophagosomes and autolysosomes in VSMCs, which were turned into more calcium-containing exosomes released into ECM.

Nano-HAp could be both a result and cause of vascular calcification processes. HAp crystals had been isolated from human carotid atherosclerotic plaques[27] and suggested to contribute to early osteoblastic

transdifferentiation of cardiovascular cells[28]. Here we detected nano-crystalline deposits in human calcified aorta, onset of Runx2 expression, and nHAp existence in the VSMC (Fig 1). And for the first time demonstrated that synthetic and human-derived HAp crystals were similar in morphology and characteristics. Both can accelerate calcium deposition of VSMCs via blockage of autophagy flux and accumulation of autophagic vacuoles. Studies have shown that nHAp promoted osteogenic differentiation of mesenchymal stem cells (MSCs) and osteoblasts with the increased expression of Runx2, OCN and ALP[29-33]. Such effect of nHAp on mineral deposition has been translated into clinical use of nHAp for bone repair[34]. The effect of HAp on VSMCs had also been studied, and upregulation of BMP-2 and OPN gene expression was found in VSMCs after treated with Pi-induced nanocrystals[35]. Calcifying nanoparticles (CNPs) extracted from human serum was found to induce accumulation of apoptotic bodies and accelerate vascular calcification[5]. CNPs were detectable in calcific but not healthy human arteries. They could propagate and induce mineral deposition in vitro with sufficient Pi[36] [4]. However, the above studies are lack in clinical evidence, thorough mechanism study and in vivo experiments. Our study confirmed that nano-sized HAp rested in calcified aorta, attached to the surrounding cells and internalized to the VSMC. Either sHAp or hHAp alone elevated the expression of Runx2 and OPN and the activity of ALP (Fig 2A and FigS3A-B), and expedited calcium deposition in VSMCs (Fig 2B). With additional Pi supplied in the osteogenic medium, HAp-induced calcification was significantly enhanced (Fig 2B), which is supported by the recently published report[6].

To confirm the effect of nHAp on calcification in vivo, sHAp was implanted underneath the skin or outside of aorta. Implantation of sHAp directly induced calcium deposition (Fig 3A-C). When nHAp was implanted along with VSMCs, more calcium deposition was observed (Fig 3C) and the implanted VSMCs were differentiated in osteogenic phenotypes with more Runx2 expression (Fig 3D), demonstrating that nHAp can induce VSMCs osteogenic differentiation in soft tissue. When nHAp was applied to the outside of abdominal aorta, significant calcification was observed in the vascular wall after 2 weeks (Fig 3E-G). According to previous studies, both CaCl_2 and CaPO_4 could induce calcification in the arterial segments[37]. Here, we employed CaCl_2 as a positive control which indeed showed substantial calcification in artery (Fig 3A). This is the first time to demonstrate that nano-sized HAp induced direct vascular calcification in vivo.

Autophagy is a key regulator of cellular metabolism and homeostasis. Emerging evidence has demonstrated that autophagy plays an important role in vascular calcification. It has been documented that atorvastatin protected VSMCs from TGF- β 1-stimulated calcification by inducing autophagy through suppression of the β -catenin pathway[9]. Another study demonstrated that estrogen inhibited osteoblastic differentiation of VSMCs in vitro and prevented arterial calcification in vivo by promoting autophagy through the ER α signaling pathway[38]. These studies demonstrated a protective role of autophagy in vascular calcification [10].Recent investigations point to the critical and defensive role of autophagy in the behavioral function of vascular cells against different insults[39], which was mainly focused on the death and phenotypic change of VSMCs without precise mechanism. Here, we found that nHAp was internalized by VSMCs (Fig 2B) and caused accumulation of autophagosomes (Fig 5A & S4A). It has

great clinical significance to figure out whether blockage of autophagy flux contributes to nHAp-induced calcification.

In our study, we found that both sHAp and hHAp crystals increased intracellular autophagosomes (Fig 5A, and Fig S4A) and elevated autophagic cargo proteins, including LC3-II and p62 in the VSMCs (Fig 5C,6A and Fig S4B), and the process is mTOR-independent. This was due to blockage of autophagic flux by nHAp-induced dysfunction of autolysosome degradation. The underlying mechanism was further elucidated that acidification of lysosome was inhibited by sHAp (Fig 7D). However, Wang, et al proved that nHAp modulated osteoblast differentiation by mediating

Autophagy via mTOR signaling pathway[40], which is inconsistent with our results. Our data suggest an involvement of an inhibitory effect of nHAp on the late stage of autophagy. This conclusion was supported from 3 lines of evidence. First, nHAp had an additive effect on rapamycin-induced production of LC3-II (Fig S5B); second, nHAp arrested the degradation of LC3 and p62, the selective autophagy substrates (Fig 6); third, nHAp prevented the maturation of lysosomes, leading to lysosomal dysfunction (Fig 7). Our results first demonstrate that nHAp-induced autophagy dysfunction by lysosomal impairment resulted in vascular calcification. Lysosomal impairment could be a general toxic mechanism of nanoparticles to cells [41, 42]. Previous studies testified lysosomal dysfunction exacerbates vascular calcification[43, 44]. This provides evidence to focus on lysosomal function in our study. Mechanism underlining nHAp-induced dysfunction of autolysosome degradation was further studied by analyzing lysosomal acidification. We found that nHAp impaired lysosome maturation by inhibiting lysosomal acidification without disturbing fusion between autophagosome and lysosome (Fig 6E). Degradation of internalized EGFR was impaired (Fig 7A & B). Maturation of lysosomal protease CTSD was inhibited (Fig 8C). Based on the dampened acidity of lysosome with a deficiency in CTSD maturation, we considered V-ATPase be a potential target of damage by nHAp. V-ATPases are membrane-embedded protein complexes that function as ATP hydrolysis-driven proton pumps. V-ATPases are the primary source of organellar acidification in all eukaryotes, making them essential for many fundamental cellular processes[45]. In our study, we detected a disruption in lysosomal anchoring of V-ATPase (Fig 7E). Although further studies are required, our data first suggest that nHAp-caused defect of lysosomal acidification may involve impairment in V-ATPase transport to lysosomes. This may provide us new idea that cyclic-3',5'-adenosine monophosphate (cAMP) which restores lysosomal pH may control the progression of vascular calcification. As nHAp blocks autophagy flux by impairing autolysosome degradation (Fig 6), the accumulated autophagic vacuoles promoted calcium deposits. This was supported by our observation that either autophagy stimulator rapamycin or inhibitor CQ aggravated nHAp-induced calcification (Fig 7G), and both had an additive effect on the accumulation of LC3 when they were used with nHAp (Fig 6B and Fig S5B).

Previous study proved that blocking autophagy promotes more rampant calcification by promoting release of MVs[46], and VSMC vesicle release contributed to calcification[47]. It has been shown that MVs played a central role in skeletal mineralization as they served as a nidus for initiation of hydroxyapatite crystal formation[48]. MVs were also identified in human aortic calcification, demonstrating that vascular

calcification and bone mineralization occur by similar mechanisms[49]. Proteomic studies showed similarities between exosomes from VSMCs and skeletal osteoblasts, supporting that vascular calcification recapitulates skeletal mineralization[50]. We found that the exosomes released from VSMCs^{nHAp} contained more LC3 and LAMP1 than that from control VSMCs (Fig 8B). It has been reported that more “calcifying” exosomes were secreted from VSMCs after pro-calcifying stimulation, which induced VC procession[46]. Calcium binds with Pi to form hydroxyapatite nodes on the inner and outside of “calcifying” exosomes membranes, which further initializes mineral deposition[46]. Nollet et al. found that autophagosomes from calcified mouse osteoblasts are packed with calcified hydroxyapatite[51]. Our study also showed there were HAp crystals in autophagosomes in VSMCs^{nHAp} (Fig 1E). Autophagic proteins were detected in MVs released from valve interstitial cells [20]. These results suggest that exosomes may have a crosstalk with the network of autophagic vesicles either at the stage of their formation or release during the process of VC[7]. Our study showed the accumulation of autophagosomes and autolysosomes, the enhancement of ALP activity and the increased release of exosomes from VSMCs induced by HAp (Fig 8A). Based on these previous studies, we assume that the autophagic vesicles in VSMCs may be secreted out of the cells in the form of exosomes. We found that there were more exosomes released from VSMC^{HAp} as compared with the control (Fig8A, Fig S11A&B), which resulted in more calcium contents released into ECM (Fig 3B, &8C). GW4869 reduced not only the number of exosomes but also the protein content of LC3 and LAMP1, and calcium deposition as well (Fig 8D-G). All these confirmed our postulation that accumulated autophagosomes and autolysosomes could be converted into calcium-containing exosomes that were released into ECM (Fig 9). This agrees with the report showing that VSMC calcification is mediated by regulated exosome secretion[24]. However, more studies are needed to further explore the relationship between autophagosomes and exosomes in regards the VC process. Our study unveiled that once nano-crystals were formed, they would facilitate the more rapid progression of calcification, showing the positive feedback of nHAp in the development of VC. Therapies aim to either prevent initial nHAp formation or nHAp-induced autophagy blockage or exosome releasing may be useful to control VC.

Conclusion

In summary, we report nHAp could directly induce VC in vivo and put new light on the mechanism by which nHAp promotes VC. Nano-HAp internalizes into VSMCs, damages lysosomal V-ATPase and then impairs lysosomal acidification, thus causing blockage of autophagy flux and release of Ca²⁺-containing exosomes, eventually resulted in calcium deposition on ECM. Our results suggest that targeting this novel autophagy-lysosome-exosome pathway may help to control vascular calcification development.

Abbreviations

Nano-HAp, nHAp: Nano-sized hydroxyapatite; VC: Vascular calcification. VSMCs: vascular smooth muscle cells. ALP: alkaline phosphatase; Runx2: Runt-related transcription factor 2; OPN: Osteopontin; MVs: Matrix vesicles; SEM: Scanning electron microscope; TEM: Transmission electron microscopy; XRD: X-ray

diffraction; FTIR: Fourier transform infrared; SQSTM1, p62: sequestosome 1; EEA1: Early endosomal antigen 1; RAB7: Ras-related protein 7; LAMP1: Lysosomal-associated membrane protein 1; EGFR: Epidermal growth factor receptor; CQ: Chloroquine; RAPA: Rapamycin; 3-MA: 3-methyladenine; CTSD: Cathepsin-D; AO: Acridine orange; Exos: Exosomes.

Declarations

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Authors' contributions

QL and YL performed experiments, analyzed data, and prepared the manuscript; QL, YL, YZ and PPX analyzed data and prepared the figures; YZ, JYZ, PPX, WWJ, WJJ and MYC performed statistical analysis; RKT designed the experimental study, and revised the manuscript; HY designed the experimental study, prepared and revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate

Animals were treated humanely and all experimental protocols were approved by the Committee on Animal Use and Care of Zhejiang University. All the methods in the present study were performed according to the approved guidelines.

Consent for publication

Both authors of this paper have read and agreed to its content and are accountable for its accuracy and integrity. This research is original and is not currently under consideration by another journal.

Declaration of competing interest

The authors declare no competing financial interest.

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Figures

Figure 1

Characterization of human calcified aorta. A) Representative images of the human aorta sections stained with von Kossa to detect calcium deposit (black arrows). Scale bar, 100 μm . B) Representative image of SEM showing HAp (Blue) on the surface of vascular cells (brown) in the calcified aorta. Scale bar, 1 μm . C) Representative images of TEM showing nano-sized HAp (black arrows) located intracellularly (middle) and extracellularly (right). Scale bar, 2 μm and 500 nm. D) Immunofluorescent staining of aorta sections shows expression of α -SMA and Runx2. Scale bar, 250 μm .

Figure 2

Characterization, cellular uptake and cytotoxicity of HAp crystals. A) SEM of synthetic nHAp and human plaque derived HAp. Scal bar, 100 nm. B) XRD pattern of sHAp and hHAp. C) FT-TR of sHAp and hHAp. D). Confocal microscopic images of VSMCs after mixed with 100 µg/ml fluorexon-labeled HAp for 24 h. Scale bar: 50 µm. E) TEM images of VSMCs after cultured with 100 µg/ml HAp for 24 h. Scale bar: 1 µm. Red arrow points lysosome, green arrows point autophagosomes, blue arrow points autolysosome and yellow arrows point aggregated HAp.

Figure 3

nHAp induced osteogenic transdifferentiation of VSMCs and calcification deposition. VSMCs were mixed with sHAp for 1 day and then washed with PBS to remove HAp and further cultured for specified days. A) Protein expression of ALP, RUNX2 and OPN in VSMCs 5 days post sHAp treatment was analyzed by western blot. The bar graphs show quantification of ALP, Runx2 and OPN expression. B) VSMCs were stained for mineralization by Alizarin red to show calcium deposition after 14 days' treatment. C) Quantification of Alizarin Red extracted from the cultures in B to represent the amount of calcium. D) Total calcium in the dishes was quantified. Data are expressed as the mean $\pm$ SD (* p < 0.05, ** p < 0.01, *** p < 0.001).

Figure 4

Nano-HAP induced ectopic tissue calcification in vivo. A) Ectopic tissue calcification experimental strategy showing that Matrigels mixed with 1) GFP transduced VSMCs, 2) sHAp, 3) GFP transduced VSMC plus sHAp, were separately injected into 3 subcutaneous pockets fashioned on the dorsum of mice. B) CT scan to detect calcification in the Matrigels after implantation for 1 month. Robust calcification was detected in the areas injected with Matrigels containing sHAp, while the calcium content

was higher in the region with VSMCs (blue box) than that without cells (green box). No calcification was observed in the region of matrigel with VSMCs only (red box). C) Calcification was quantified as a fold change in comparison with the region with VSMCs only. Data are expressed as mean $\pm$ SD, * $p < 0.05$. D) Immunofluorescent staining of dissected subcutaneous tissue detected the implanted GFP+ cells and expression of Runx2. Scale bar: 50 μ m. The bar graph shows Runx2 expression. N=6. Data are expressed as mean $\pm$ SD, * $p < 0.05$. E) Enhanced CT scan detects calcification on abdominal aorta in mice 14-day after adventitia was torn off, and sHAp, or CaCl₂ (a positive control), or NaCl (a negative control) were applied to the out surface of the mouse abdominal aorta. F) Representative images of CT scan showing calcium deposition on the aorta. Red boxes are the position of aorta with calcification. The right panel shows quantification of calcification on the aorta from the CT scan. G) Representative images of the aorta under stereomicroscope to show the calcium deposits (white). H) Representative images of the aorta sections stained with von Kossa to detect calcium deposit (brown). I) Representative images of the aorta sections after immunofluorescent staining for Runx2 and α -SMA. Scale bar: 25 μ m. The right panel shows quantification of Runx2 expression on the vessel. N=6. Data are expressed as mean $\pm$ SD, * $p < 0.05$.

Figure 5

Nano-HAp induced accumulation of autophagosomes in VSMCs. A) Autophagic vacuoles in cells with or without 100 μ g/ml sHAp treatment were observed by TEM. The arrow indicates autophagic vacuoles. Scale bar: 1 μ m. Data are presented as mean $\pm$ SD. B) Representative images of immunofluorescent staining for LC3 on the recovered aorta whose outside has been applied with NaCl or sHAp. Expression of LC3 was quantified by measuring the relative fluorescence intensity from 10 sections. Scale bar: 25 μ m. C) VSMCs were treated with different concentrations of sHAp for 24 h, and the cellular LC3 expression levels were detected by western blot. The relative LC3-II protein level was quantified in the bar graph.

D) Images of immunofluorescence analysis show the LC3 distribution patterns in the control and crystals-treated cells. Scale bar: 50 μ m. The number of punctate LC3 per cell were quantified. Data are expressed as mean $\pm$ SD. (* $p < 0.05$).

Figure 6

Nano-HAp inhibited autophagic degradation. A) Western blot analysis of p62 in VSMCsHAp at the specified concentration of sHAp for 24 h. The relative p62 protein level was quantified in the bar graph. B) Western blot analysis of p62 and LC3-II in VSMCs that were treated with sHAp for 24 h, with or without CQ (10 μ mol/L) cotreatment for the last 3 h. The relative protein levels were quantified in the bar graph. C) Western blot analysis of LC3-II in VSMCs that were treated with sHAp at 100 μ g/mL for 24 h or starvation medium, and then treated with 1mM of 3-MA. The relative LC3B-II protein level was quantified in the bar graph. D) Representative immunofluorescence images show LC3 in VSMCs that were transduced with mRFP-GFP-LC3 adenoviral vector and cultured under starvation condition for 24 h or sHAp (100 μ g/ml) treatment. GFP fluorescence is pH sensitive while RFP is not. Starvation-induced autophagy flux caused the fusion of autophagosome with lysosome, which resulted in quenching GFP fluorescence signal; when autophagy flux was blocked by sHAp, much less GFP fluorescence signal was quenched. Relative GFP fluorescence intensity over RFP was quantified in the bar graph. Scale bars: 50 μ m. E) Immunostaining

with antibodies against Rab7 and LC3. Cells were starved, or treated with or without HAp for 24 h. Scale bar: 50 μ m. The colocalization coefficient of RAB7 and LC3 was represented as percentage of punctate signals of LC3 that were positive for RAB7.

Figure 7

Nano-HAp impaired lysosomal degradative capacity and inhibited lysosomal acidification. A) Representative confocal immunofluorescence images show colocalization of sHAp and LAMP1 in

VSMCs. The cells were treated with 100 µg/ml fluorexon labeled sHAp for 24 h, and then stained with LAMP1 antibody. The yellow dots represent the colocalization of sHAp and the lysosome. B) VSMCs were treated with 100 µg/mL of sHAp for 24 h, and then treated with 50 ng/ml EGF for 0, 1, 2 and 3 h which was removed after the specified time. The cells were fixed and immunostained with anti-EGFR antibody. Scale bars: 25µm. Quantitative analysis of EGFR was performed by normalization to the green fluorescence intensity of cells with EGF treatment for 1 h. C) Western blot analysis of EGFR protein in the cells treated as in (A) and their quantification. D) VSMCs treated with or without sHAp were labeled with acridine orange (AO). Red fluorescence of AO staining per cell was quantified. Scale bar: 100 µm. E) Western blot analysis of CTSD protein in VSMCs which had been treated with 100 µg/mL nHAp for 24 h. The ratio of mature-CTSD to premature-CTSD was quantified in the bar graph. F) Representative immunofluorescence images show V-ATPase V1D and LAMP1 in VSMCs which were treated with or without 100 µg/ml sHAp for 24 h, and then stained with antibodies against V-ATPase V1D and LAMP1. The colocalization coefficients of V-ATPase V1D and LAMP1 were quantified as percentage of punctate signals of V-ATPase V1D that were positive for LAMP1. Data are presented as mean ± SD (*p<0.05). G) Alizarin red staining of VSMCs that were pretreated for 3 days with RAPA or CQ followed by addition of sHAp for 14 days. H) Alizarin red and calcium content on the cells were quantified. Data are expressed as the mean ± SD. (*vs control, #vs sHAp. *p < 0.05, ***p<0.001, #p<0.05, ###p<0.001).

Figure 8

Nano-HAp promoted release of exosomes which contained LC3 and LAMP1. A) Total protein was assessed in the Exos that were purified from the conditioned medium of VSMCs treated with or without sHAp. B) Western Blot analysis for detection of CD9, LC3 and LAMP1 expression in the purified exosomes. Relative protein levels were quantified in comparison with the control. C) Calcium content in Exos was measured. D) Total protein was assessed in the Exos that were purified from the conditioned medium of VSMCs that were treated with 100 µg/ml sHAp or/and 10 µm/l GW4869 for 3 days. E) Western blot analysis of CD9, LC3B and LAMP1. F) Analysis of calcium content of Exos that were treated with sHAp or/and GW4869 for 5 days. Data are expressed as the mean ± SD. (*vs control, #vs HAp, *p < 0.05, ** p < 0.01, ***p<0.001, #p<0.05). G) Alizarin red staining for calcification detection in VSMCs that were with sHAp or/and GW4869 for 14 days. The bar graphs show the quantification of Alizarin red and the calcium content in the cells. Data are expressed as the mean ± SD. (***p<0.001). H) FACS analysis of Exos from the conditioned medium of VSMCs treated with or without 100 µg/ml fluorexon-labeled sHAp. The fluorexon labeled sHAp was appointed as a positive control.

Figure 9

Schematic illustration of the autophagy-lysosome-exosome pathway that mediates nano-HAp-induced calcification of vascular smooth muscle cell. Nano-HAp is endocytosed and located in early endosomes. Nano-HAp enters lysosomes through fusion of endosome with lysosome, impairs the lysosome vacuolar H⁺-ATPase and therefore inhibits lysosomal acidification. Nano-HAp does not affect autophagosome-lysosome fusion, but blocks autophagic degradation. The autophagic flux blockage promotes release of exosomes which are loaded with undissolved nHAp, Ca²⁺, Pi and ALP. The exosomes bud off the plasma membrane, deposit within ECM, and form calcium nodules.

Supplementary Files

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