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Proteomic profiling of extracellular vesicles released from vascular smooth muscle cells during initiation of phosphate-induced mineralization.

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Abstract

Elevated serum phosphate is one of the major factors contributing to vascular calcification. Studies suggested that extracellular vesicles released from vascular smooth muscle cells significantly contribute to the initiation and progression of this pathology. Recently, we have demonstrated that elevated phosphate stimulates release of extracellular vesicles from osteogenic cells at the initiation of the mineralization process. Here, we used MOVAS cell line as an in vitro model of vascular calcification to examine whether vascular smooth muscle cells respond to high phosphate levels in a similar way and increase formation of extracellular vesicles. Vesicles residing in extracellular matrix as well as vesicles released to culture medium were evaluated by nanoparticle tracking analyses. In addition, using mass spectrometry and protein profiling, protein composition of extracellular vesicles released by MOVAS cells under standard growth conditions and upon exposure to high phosphate was compared. Significant increase of the number of extracellular vesicles was detected after 72 hours of exposure of cells to high phosphate. Elevated phosphate levels also affected protein composition of extracellular vesicles released from MOVAS cells. Finally, the comparative analyses of proteins in extracellular vesicles isolated from extracellular matrix and from conditioned medium identified significant differences in protein composition in these two groups of extracellular vesicles. In conclusion, results of this study demonstrate that exposure of MOVAS cells to high phosphate levels stimulates the release of extracellular vesicles and changes their protein composition.

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Keywords

extracellular vesicles; matrix vesicles; phosphate; mineralization; proteomics

Introduction

Vascular calcification, in particular mineralization of the elastic medial layer (known as medial vascular calcification, MVC), is a prevalent pathology commonly associated with chronic kidney disease, diabetes and aging (1–3). It has been shown that this pathological mineralization is accompanied by expression of osteogenic genes, elevated tissue-nonspecific alkaline phosphatase (TNAP/ALPL) and increased production of extracellular vesicles (EVs), which are positive for mineralization-related proteins (4–8). It has been suggested that EVs released from vascular smooth muscle of underlying mechanisms of vascular calcification. Alternatively, deposition of hydroxyapatite due to shift of the balance between mineralization promoters and inhibitors may precede osteogenic transdifferentiation of VSMC (9, 10). Although the exact mechanism of medial vascular calcification has not been fully elucidated yet, this pathological mineralization shows remarkable molecular similarities with physiological mineralization in skeletal and dental tissues.

A major contributing factor inducing vascular calcification is sustained elevation of serum phosphate levels (11–13). Phosphate plays a dual role in mineralization. First, inorganic phosphate ($\text{PO}_4^{3-}/\text{P}_i$) is a structural component of hydroxyapatite (HA) crystals, the primary mineral in hard tissues and in calcifying blood vessels. Second, P_i acts as a signaling molecule, which regulates expression of mineralization-related genes (14–16). Recently, we have demonstrated that elevated P_i levels stimulate release of mineralization-competent matrix vesicles (MVs) from osteogenic cells and change MVs protein composition (17). MVs have been first detected in the extracellular matrix (ECM) of the growth plate cartilage. It has been proposed that cartilage MVs are the initial sites of hydroxyapatite formation, hence facilitate the initiation of the mineralization process during endochondral bone formation (18–20). This specific function is determined by molecular composition of MVs. MVs are enriched in phospholipids and proteins that provide P_i and Ca^{2+} into MVs lumen, allowing for generating locally high concentration of these ions, which, in turn, facilitates nucleation of HA crystals. Consistently, mineral-containing MVs have been detected in hypertrophic cartilage, mantle dentin and woven bone (21–23), although the contribution of MVs to mineralization of these tissues in vivo remains to be determined.

The aim of this study was to gain better understanding of mechanisms of P_i -induced pathologic calcification driven by VSMC. We hypothesized that, similarly to osteogenic cells, elevated P_i levels stimulate release of EVs from VSMC and alter EVs protein composition.

Materials and Methods

Cell culture and EVs characterization

MOVAS cells were purchased from ATCC (Manassas, VA, USA) and grown in DMEM medium supplemented with 10% FBS and 50 µg/ml ascorbic acid and Na-Pi buffer (pH=7.4) at the final concentration as indicated. Mineralization experiments, alizarin red staining, EVs purification and characterization by nanoparticle tracking analyses (NTA) and cryo-electron microscopy (cryo-EM) were done as described previously (17) with a modification of the ECM digestion protocol by the addition of elastase (0.8U/ml). For the detection of cleaved caspase-3, confluent cells were treated with Na-Pi buffer for 24h.

Immunostaining

MOVAS cells were plated at 1×10^5 cells/ chamber in 2-chamber slides (Lab Tek II) and grown to the confluency for 3 days. Fibrillin was detected using anti-fibrillin I antibody (1:50 dilution, Abcam) with anti-rabbit IgG HRP secondary antibody (1:2000 dilution, Abcam) and DAB Peroxidase Substrate detection kit (Vector Labs) according to the manufacturer instructions. No counterstaining was used. Cells were imaged under the Nikon TE2000 microscope.

Mass spectrometry and bioinformatics analyses

EVs were homogenized in NuPAGE LDS buffer, sonicated for 20min, followed by centrifugation at $12,000 \times g$, 4°C for 20min. 5µg of protein per sample were denatured and separated (half-way) on a 10% SDS Bis-Tris gel. The gels were stained, and four molecular weight fractions were cut out, digested with Trypsin Gold (Promega), and peptide extracts were reconstituted in 0.1% formic acid/ ddH₂O at ~0.1µg/µL. Mass spectrometry was carried out, and the data were processed, searched, filtered, grouped, and quantified, as previously reported in detail (under section 2.5 nLC-ESI-MS2 and Protein IDs for GeLC) (24). Following protein identification, and relative quantification by normalized spectral counting, a three-fold cut off was applied to each pairwise comparison to be further analyzed using gene ontology assignments. PANTHER 11.0 software and tools were used to generate gene ontology (GO) assignments for identified proteins. To identify overrepresented and underrepresented terms in each EVs group, Panther statistical overrepresentation test was employed (25, 26).

Results

Phosphate stimulates release of extracellular vesicles from MOVAS cells

To understand the mechanisms of P_i-induced vascular calcification, the MOVAS cell line (mouse aortic smooth muscle cells) was used as an in vitro model of MVC (27). First, the effect of elevated P_i levels on mineralization of MOVAS cells was evaluated. Alizarin red staining for calcium deposits demonstrated that in all 3 tested concentrations of P_i, mineralization of MOVAS cells can be detected as early as on day 7 of growth in the osteogenic medium (**Fig. 1A**). On day 14, there was an apparent correlation between the extent of mineralization and the P_i concentration. Importantly, exposure of MOVAS cells to elevated P_i levels did not result in increased cell apoptosis as demonstrated by cleaved

caspase-3 (**Fig. 1B**). There was no difference between the levels of cleaved caspase-3 in cells cultured under standard growth conditions and cells treated with high P_i for 24h.

Next, the effect of elevated P_i levels (10mM) on the release of EVs was analyzed. Since in our previous studies on P_i -induced EV release from osteogenic cells we determined that the majority of EVs reside in ECM (17), EVs were isolated from both ECM and from conditioned media. Of note, the ECM produced by MOVAS cells has not been characterized yet. Using immunostaining, we detected fibrillin (**Fig. 1C**), a characteristic protein of elastic tissues ECM. We did not detect elastin nor collagen type I in MOVAS cells (data not shown). Quantification of EVs by NTA revealed that the significant increase of the number of EVs in both ECM and conditioned medium fractions occurred at 72h of exposure to elevated P_i (**Fig. 1D**). In addition, NTA analysis showed that the size of EVs does not change in response to 10mM P_i (**Fig. 1E**). Cryo-EM imaging showed that purified nano-particles used for subsequent molecular analyses were indeed membrane vesicles (**Fig. 1F**).

Elevated phosphate changes protein composition of MOVAS extracellular vesicles in conditioned medium and in extracellular matrix

To characterize protein profiles of EVs released by VSMC and to determine the effect elevated extracellular P_i on EVs molecular composition, four experimental groups of EVs released from MOVAS cells were analyzed by tandem mass spectrometry: 1) EVs released by cells to medium within 24h of exposure to 10mM P_i (P_i -med group); 2) EVs released to medium by cells grown without additional P_i (Ctrl-med group); 3) EVs released to ECM by cells within 24h of exposure to 10mM P_i (P_i -ECM group); 4) EVs released to ECM by cells grown without additional P_i (Ctrl-ECM group). A total of 1222 proteins were detected in MOVAS EVs, 27% of which were detected in all 4 EVs groups (**Fig. 2A and Suppl. Table 1**). 37% of all identified proteins were detected only medium EVs and 11% only in ECM EVs (**Figure 2A**). Each group had a set of unique proteins; the Ctrl-med group had the highest percentage of unique proteins (10% of all Ctrl-med proteins), while the P_i -med group had the fewest unique proteins (4% of all P_i -med proteins).

Next, we focused the analyses on the influence of P_i on the molecular composition of EVs. In this set of analyses, the effect of P_i was evaluated separately for ECM EVs proteins and medium EVs proteins. These comparative analyses revealed that a majority of proteins were present in both control and treatment groups (**Fig. 2B**). Of these shared sets of proteins, 14% were significantly (3 fold difference) changed by P_i treatment in both ECM EVs and medium EV groups (**Fig. 2B**). To gain insight into the biological and functional significance of P_i -induced changes, the Panther statistical overrepresentation test with all 1222 identified proteins as a reference list was employed (25, 26). For this test, proteins significantly upregulated by P_i were combined with proteins detected only in P_i -treated group, and proteins significantly downregulated by P_i were combined with proteins detected only in control group. This generated 4 sets of P_i -regulated proteins: i) ECM EVs ↓ by P_i (201 unique proteins + 39 significantly downregulated); ii) ECM EVs ↑ by P_i (88 unique proteins + 29 significantly upregulated); iii) medium EVs ↓ by P_i (174 unique proteins + 59 significantly downregulated); iv) medium EVs ↑ by P_i (102 unique proteins + 55 significantly upregulated). Gene ontology terms significantly overrepresented in each group

in comparison with all identified proteins were delineated (**Fig. 2C and 2D**). Due to space constraints, 18 of the least changed and redundant GO Biological Process terms were excluded from the **Figure 2C**. In summary, mass spectrometry in combination with gene ontology analyses and Panther overrepresentation test revealed differences in protein composition of ECM EVs and medium EVs and demonstrated that P_i treatment significantly alters the protein composition of both types of EVs produced by MOVAS cells. Furthermore, the regulation pattern of specific functional groups of proteins is different in ECM and medium EVs.

Discussion

In this study, we took a comprehensive proteomic approach to understand the consequences of the elevated extracellular P_i on EVs released by vascular smooth muscle cells. Results of this study demonstrate that high P_i levels have a similar effect on MOVAS cells as previously reported for osteogenic cells (17). First, P_i stimulates the release of EVs from MOVAS cells. Second, P_i influences the molecular composition of MOVAS EVs.

The increase of the EVs release was detected 72h after exposure to high P_i , which is similar to osteoblast precursor cells MC3T3-E1 and ST2 and over 48h later than observed in already committed 17IIA11 osteogenic cells (17). This suggests that in MOVAS cells the increase of the EVs release requires extended exposure to high P_i levels. This mimics the chronic hyperphosphatemic state in vivo. Of note, unlike osteogenic cells, where the vast majority of EVs reside in ECM, the EVs released by MOVAS cells were equally distributed between ECM and medium. This may reflect the differences in the abundance and composition of ECM produced by MOVAS cells in comparison with osteogenic cells. ECM produced by osteogenic cells is rich in collagen type I, while ECM of MOVAS has characteristics of elastic tissues (**Fig. 1C**). Alternatively, the function of P_i -induced EVs from MOVAS cells may be different than the function of osteogenic EVs, which support the initiation of mineralization. For example, very low levels of tissue-nonspecific alkaline phosphatase were detected in MOVAS ECM EVs and none in medium EVs, while this mineralization-supporting enzyme is highly abundant in MVs released by chondrocytes and osteogenic cells. However, it is important to note that in this study the protein composition of EVs released from vascular smooth muscle cells was analyzed after only a short exposure to high P_i . To address the role of MOVAS EVs in the mineralization process, EVs released after an extended period of the exposure to high P_i should also be analyzed to allow MOVAS cells to activate the osteogenic program.

All four groups of MOVAS EVs were highly positive for annexins, which can function as calcium channels. Although this function implicates annexins in the mineralization process, these proteins are commonly detected in a variety of EVs released by many different cell types, thus are not considered specific for mineralization-supporting EV. Histones and cytoskeleton proteins, such as actins and tubulins, are also commonly detected in EVs of different origin. These proteins were also highly abundant in MOVAS EVs, demonstrating that MOVAS EVs share common molecular features with EVs released from other cell types.

It has been demonstrated that EVs carry a molecular footprint of the type of cells they originate from. Consistently with this observation, vascular smooth muscle cells ECM proteins were among the most represented peptides detected in MOVAS EVs. Interestingly, they were differentially distributed between ECM and medium EVs. For example, perlecan (basement membrane-specific heparan sulfate proteoglycan core protein) and fibronectin were highly enriched in medium EVs in comparison with ECM EVs, while fibrillin, the essential protein for formation of elastic fibers, was specifically detected in ECM EVs. This highlights the molecular differences between EVs retained in ECM and those, which escaped ECM. Further studies are required to establish, if these proteins have an active role in determining the target location of EVs released by MOVAS cells or they are specifically distributed to EVs that are already destined for different extracellular locations.

Before the increase of the number of released EVs in response to elevated P_i occurs, changes in the EVs protein composition could be detected. For example, clathrin heavy chain 1 protein, a protein involved in endocytosis and membrane traffic (28), is significantly downregulated by P_i specifically in ECM EVs, while it remains at high levels in medium EVs. This suggests that P_i -induced ECM EVs may be formed by clathrin-negative membrane domains. An interesting example of a protein upregulated by P_i is Golgi apparatus protein 1 (also known as E-selectin ligand 1). This ECM protein, which regulates TGF- β signaling and bone mineralization (29) is not detected in Ctrl medium EVs but is present in medium EVs released upon P_i treatment, and is one of the highly abundant proteins in P_i -ECM EVs. This suggest that P_i -induced EVs may regulate mineralization indirectly by affecting signaling molecules residing in the ECM.

Overall, elevated P_i had a significant influence on protein composition of both ECM and medium EVs. However, Gene Ontology analyses revealed that different functional groups of proteins were overrepresented in P_i -treated ECM EVs and P_i -treated medium EVs (**Fig. 2C and 2D**). Similarly, nontreated ECM EVs and non-treated medium EVs shared very few overrepresented protein groups. Taken together, this suggests that ECM EVs are different from medium EVs on both molecular and functional level. This finding has a particular importance for designing studies searching for biomarkers of cellular metabolic changes as well as for studies addressing biological functions of EVs, as the results of such studies may vary depending on the extracellular compartment from which EVs are obtained.

In conclusion, these studies demonstrate that MOVAS cells respond to elevated P_i by increasing the release of EVs and changing their molecular composition. Further studies are required to determine whether these changes are driven by a gene expression program or P_i directly effects signaling pathways involved in release of EVs and/or protein trafficking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

P_i -induces mineralization of MOVAS cells and stimulates release of EVs. **A.** Alizarin red staining of cells cultured in the osteogenic medium containing 5, 7 or 10 mM P_i , and cells cultured in standard growth medium (Ctrl). **B.** Western blot analyses of cleaved caspase-3 (casp-3) in cells treated with 5, 7, and 10 mM P_i for 24h, and non-treated cells (Ctrl). Alpha-tubulin (α -tub) was used as the protein loading control. **C.** Representative microscopic images of MOVAS immunostaining with antifibrillin antibodies (Fbn1), and control staining without the primary antibody (IgG (-)ctrl). No counterstain was used. **D.** Results of nanoparticle tracking analyses showing the number of EVs isolated from conditioned medium (medium) or extracellular matrix (ECM) of MOVAS cells grown in the presence of 10 mM P_i for 24, 48 and 72h. Data are represented as the mean values of three independent experiments $\pm$ SD, * p <0.01. **E.** Results of nanoparticle tracking analyses showing the size distribution of EVs. **F.** Representative cryo-electron microscopy images of four experimental groups of MOVAS EVs (arrows). Scale bar = 100nm.

Figure 2.

Summary of proteomic analyses of EVs released from MOVAS cells under standard growth conditions (Ctrl) and upon 24h treatment with 10 mM P_i (P_i). EVs were isolated either from conditioned medium (medium) or from the extracellular matrix (ECM). **A.** Venn diagram showing distribution of identified proteins in specific EVs groups. **B.** Venn diagrams summarizing the effect of high P_i on EVs protein composition. **C.** Protein profiling: GO annotations on molecular function and biological process of proteins significantly changed by P_i (≥ 3 fold difference) and proteins present only in either Ctrl or in P_i group. All 1222 proteins identified by mass spectrometry were used as a reference to determine the overrepresented GO terms. Significantly overrepresented GO terms are listed (p<0.05).