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Original

Proteomics applied to the study of vascular calcification in chronic kidney disease

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Abstract

Introduction: vascular calcification (VC) is associated with increased mortality in the general population, as well as in patients with chronic kidney disease, in whom prevalence is much higher. The need for effective and early diagnosis of VC to improve preventive and prognostic strategies has driven research on biomarkers. The aim of this study is to examine the differential expression of proteins associated with the VC process using proteomics techniques.

Materials and methods: vascular smooth muscle cells were cultured under non-calcifying and calcifying conditions. Differential protein expression was performed using 2D-DIGE and LC-ESI-MS/MS, and identification was conducted using with the MASCOT search engine.

Results: after 6 days of culture, a total of 121 protein spots with differential expression were detected. A total 21 out of all these proteins were identified in 24 spots. In the cells cultured in the calcifying medium, a total of 4 showed a significantly increased expression; collagen type I exhibited the greatest change (3.49 times) vs those cultured in a non-calcifying medium. Other muscle and structural proteins showed a decrease in their expression. Furthermore, changes were reported in the expression of nucleobindin-1 and endoplasmin, which had not previously been associated with VC.

Conclusion: the results confirmed the reduction in the expression of typical muscle and cytoskeletal proteins during VC. Additionally, changes in the expression of proteins that had not previously been associated with VC were identified, and these may be involved in the process.

Keywords:
Vascular calcification.
Chronic kidney disease.
Proteomics.
2D-DIGE.

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INTRODUCTION

In patients with chronic kidney disease (CKD), the mechanisms regulating Ca and phosphorus homeostasis are compromised, leading to what is known as "Chronic Kidney Disease-Mineral and Bone Disorders" (CKD-MBD), which includes vascular calcification (VC) (1).

VC is associated with an increased risk of mortality in the general population (2) and especially in patients with CKD (3,4), in whom it is more prevalent than in individuals of the same age with preserved renal function (5,6).

The high morbidity and mortality of CKD have been attributed to both traditional and non-traditional risk factors. As in the general population, traditional risk factors (smoking, hypertension, diabetes, and male sex) are largely responsible for the progression of VC. However, these factors cannot explain the high prevalence of cardiovascular complications in CKD patients (7). Among non-traditional factors, hyperphosphatemia is one of the most studied, being related to the increased VC and mortality in CKD patients (8,9).

VC occurs through a complex, active, and regulated process involving different mechanisms (10-12). Several studies have been published to date that have advanced knowledge on the underlying mechanisms of the VC process (7,10). However, the inability to detect VC early has driven research into potential new biomarkers that could be used for early diagnosis of VC and improve preventive and prognostic strategies. Mass spectrometry applied to proteomics studies allows for the characterization of the proteomic profile of a biological sample rapidly at a specific point in time. Therefore, the main objective of this work was to study the differential expression of proteins associated with the VC process using proteomics techniques, employing an in vitro VC model with vascular smooth muscle cells (VSMCs).

MATERIALS AND METHODS

CELL CULTURE AND CALCIFICATION CONDITIONS

The A7r5 cell line from rat aorta (ATCC CRL-1444™) was used as VSMCs. The cells were cultured in DMEM (1.8 mM Ca [Ca] and 1 mM phosphate [P]; Lonza) supplemented with fetal bovine serum (FBS) (10 %), glutamine (1 %) (Biochrom), non-essential amino acids (NEA) (1 %), penicillin and streptomycin (1 %) (Biochrom).

The A7r5 cells were seeded into 20 plates of 15 cm in diameter (152 cm^2 surface area). For Ca content analysis, 6-well plates (9.6 cm^2 surface area) were used. When cells reached 60 %-70 % confluence, the experimental conditions were added. For calcification experiments, DMEM-F12 culture medium was used to maximize the differences between non-calcifying and calcifying conditions. Non-calcifying medium (control): DMEM-F12 (1 mM Ca and P) supplemented with BSA (0.1 %), penicillin, and streptomycin (1 %). Calcifying medium: a calcifying stimulus was added to the non-calcifying medium to change the A7r5 cells phenotype to osteoblast-like; in this case, it was supplemented with Ca and P to final concentrations of 2 mM and 3 mM, respectively. In our experimental conditions, concentrations > 2 mM Ca and 3 mM phosphate induce spontaneous precipitation of $Ca_3(PO_4)$.

The 2 studies were conducted in parallel, and the culture medium was replaced every 2 days.

CALCIUM CONTENT

The degree of mineralization was assessed by Alizarin Red staining at day 6 of culture under calcifying and non-calcifying conditions. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed with formaldehyde (10 % in PBS) at 4 °C for 45 minutes, and after a wash with deionized water, they were stained with 2 % Alizarin Red (pH 4.2) for 5 minutes.

PROTEOMICS STUDY

After 6 days of culture and confirming the presence of calcification, the cells were collected in 20 mL of PBS and pooled to obtain 4 samples cultured in non-calcifying medium (C1, C2, C3, and C4) and 4 samples from the culture in calcifying medium (P1, P2, P3, and P4). In both groups, the samples were solubilized by sonication in lysis buffer (7M urea, 2M thiourea, 4 % CHAPS, and 30 mM Tris). The total protein concentration was measured using the Bradford method (13) (Bio-Rad, Hercules, CA, USA).

Following the manufacturer's recommendations, 400 pmol of CyDye™ DIGE FluorDyes (GE Healthcare, Uppsala, Sweden) were added per 50 µg of protein (fluorochromes Cy3 and Cy5 were used to stain the samples and Cy2 for the internal standard, resulting from the mix of equal amounts of protein from the samples cultured under non-calcifying and calcifying conditions). The samples were stained in pairs (non-calcifying and calcifying conditions) to avoid labeling differences.

The separation of the labeled protein extracts was performed in 2D-DIGE gels (2D Fluorescence Difference Gel Electrophoresis), following the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). First dimension: the samples were loaded on IPG strips (24 cm; pH 3-10 NL) (GE Healthcare). After the first dimension, the IPG strips were incubated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30 % glycerol,

2 % SDS, and traces of bromophenol blue) containing 0.5 % dithiothreitol (DTT) for 15 minutes and then, in the same buffer, with 4.5 % iodoacetamide for another 15 minutes. For the second dimension, the strips were loaded onto polyacrylamide gels (12.5 %) (Ettan DALT six system, GE Healthcare, Uppsala, Sweden) and run (2W/gel) for 4 hours and 30 minutes until the bromophenol blue reached the gel's bottom. Then, the 2D gels were scanned (EttanDigerImager, GE Healthcare, Uppsala, Sweden) at 100 µm resolution with $\lambda_{ex}/\lambda_{em}$ of 488/520, 532/580, and 633/670 nm for Cy2, Cy3, and Cy5, respectively.

Three independent experiments were conducted for each condition. The image analysis was conducted with the DeCyder2D v7.0 software (GE Healthcare, Uppsala, Sweden). For spot selection, volume quantification, and normalization of the samples in the gel, the differential in-gel analysis (DIA) module was used. The biological variation analysis (BVA) module was used to compare protein spots across different gels and identify those showing significant differences.

A preparative gel in polyacrylamide (12.5 %) with 500 µg of protein (the same amount from each sample) was performed following the same procedure described above. The proteins were visualized by staining with Oriole™ Fluorescent Gel Stain (Bio-Rad, Hercules, USA); the images were acquired using a gel documentation system, ChemiDoc™ XRS+ (Bio-Rad, Hercules, USA).

Differentially expressed spots were manually excised. The digestion of the spots was performed with 12.5 ng/µL trypsin in 50 mM ammonium bicarbonate for 12 hours at 37 °C. For peptide extraction, a solution of formic acid (1 %) and acetonitrile (50 %) (Sigma Aldrich, St Louis, USA) was used.

PROTEIN IDENTIFICATION

The protein extracts were analyzed using a nanoHPLC system (Ultimate 3000, Dionex/LC Packings) with an autosampler connected to a Q-trap ion mass spectrometer (Applied Biosystems) with a nanoelectrospray ionization (ESI) source. The chromatographic column was C-18 with 75 µm diameter (Dionex/LC Packings) connected to a silica capillary to generate the electrospray. The sample injection volume used was 1 µL, and the mobile phase flow rate was 0.125 mL/min (split mode).

The mass spectrometry (MS/MS) spectra obtained were analyzed using the Analyst software and matched to the SwissProt database for protein identification using the MASCOT search engine (MatrixScience). Search parameters included a tolerance of ± 1.2 Da and possible protein changes, such as deamidation, carbamylation, and oxidation of methionine. A protein was considered identified when, at least, 2 different peptides were detected. The function and location of the identified proteins were assigned based on information from the PubMed and ExPasy databases.

Supplementary data shows a diagram of the processes performed in the proteomics study (Fig. 1) and representative images of the gels labeled with the fluorochromes Cy2, Cy3, and Cy5, and the resulting image from the combination of the three fluorochromes (Fig. 2).

STATISTICAL ANALYSIS

The analysis of the DIGE results was performed with the DeCyder2D v7.0 software (GE Healthcare, Uppsala, Sweden) using Student's t-test ($p < 0.05$). Protein spots showing expression changes above a threshold of 1.5 were selected.

RESULTS

Experimental conditions for VSMC cultivation were maintained until the day 6 of treatment, at which point an increase in Ca deposition in the cultures treated with calcifying medium was observed, as determined by Alizarin Red staining (Fig. 3).

To assess the differential protein expression in VSMCs treated with calcifying medium vs cells cultured under non-calcifying conditions, a 2D-DIGE comparative analysis was performed. Approximately a total of 1860 protein spots were detected, 121 of which were differentially expressed ($p < 0.05$), and 101 of these spots were analyzed by LC-ESI-MS/MS (Fig. 4). The obtained mass spectra were matched vs databases, and an individual score > 32 in Mascot indicated identity or homology ($p < 0.05$). A total of 20 different proteins were identified in 24 spots.

Four of all identified proteins showed increased expression in the cells cultured in calcifying medium vs those cultured in non-calcifying medium (Table I). Type I collagen and annexin A2 exhibited the largest differences between non-calcifying and calcifying conditions (ratios of 3.49 and 2.17, respectively) after 6 days.

Table II lists 16 proteins whose expression was inhibited in cells cultured in calcifying medium vs those cultured in non-calcifying medium. Tropomyosins (beta chain, alpha-1, and alpha-3) were the proteins that showed the most significant decrease when VSMCs were cultured in a calcifying medium. Additionally, some of them were identified in multiple spots, suggesting possible changes to their structure or post-translational changes. Other muscle-specific proteins that

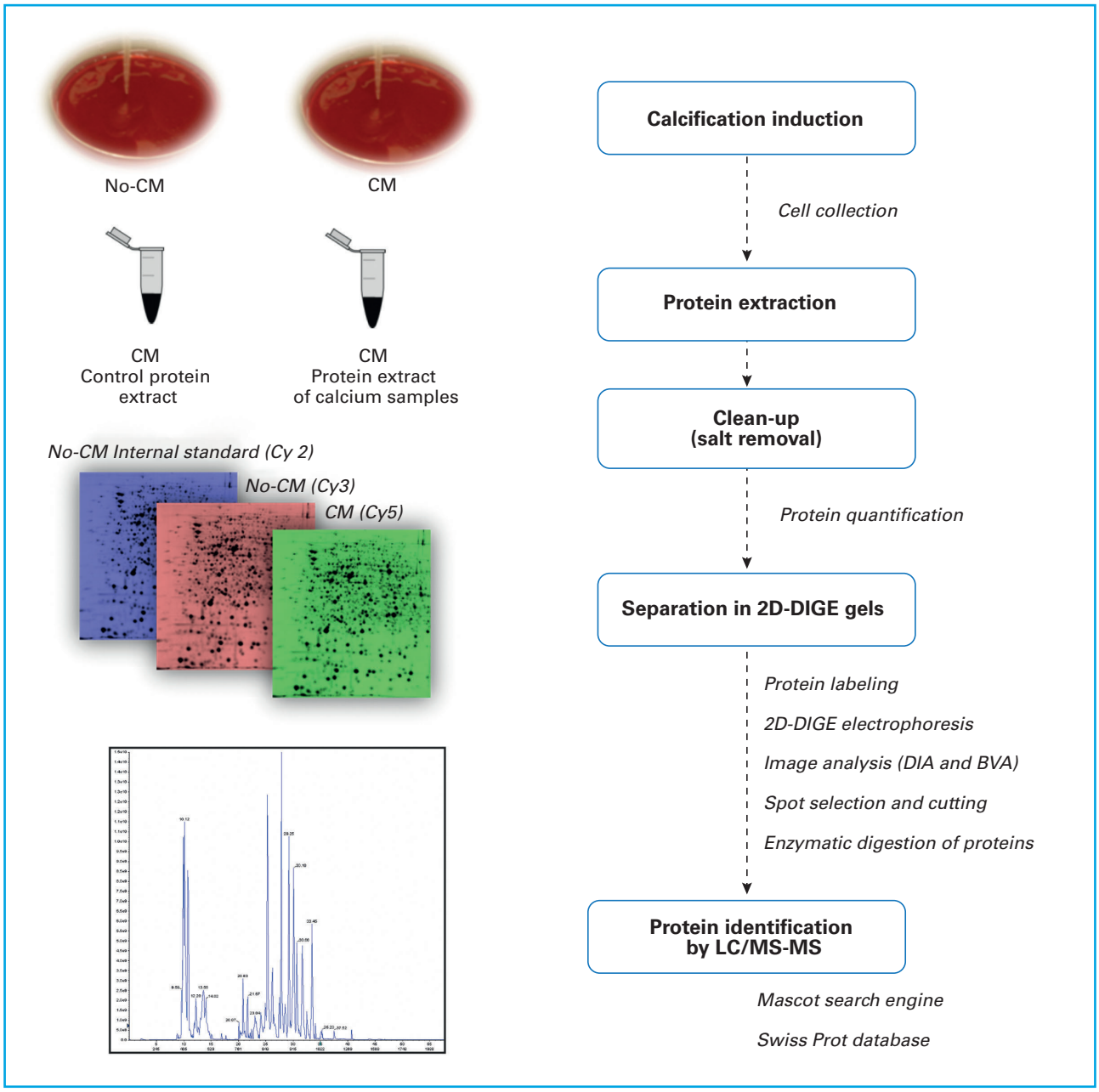

Figure 1. Sample treatment for proteomic analysis by LC-MS/MS.

reduced their expression included actin, myosin-9, desmin, alpha-actin, and other cytoskeletal proteins such as vimentin and alpha-1-A chain of tubulin.

Disulfide isomerase proteins A6 and A1 and endoplasmin were 3 chaperones localized in the endoplasmic reticulum that reduced their expression more than 6, 3, and 2 times, respectively, under calcifying vs non-calcifying conditions. A decrease in the expression of nucleobindin-1, a Ca-binding protein localized in the Golgi apparatus, and P3H1, which has a structural function and is a component of the extracellular matrix, was also observed.

DISCUSSION

The publication of numerous studies on VC and CKD has partially contributed to understanding the process of CKD-related VC. In this regard, omics techniques (genomics, transcriptomics, and proteomics, along with other analysis techniques) provide a powerful tool to obtain and integrate biological information about the VC process (14). Proteins—the main effectors of most biological processes—are the most suitable molecules to be used as biomarkers or targets for the treatment of diseases due to the close relationship between proteins and phenotypes. For this reason, results obtained

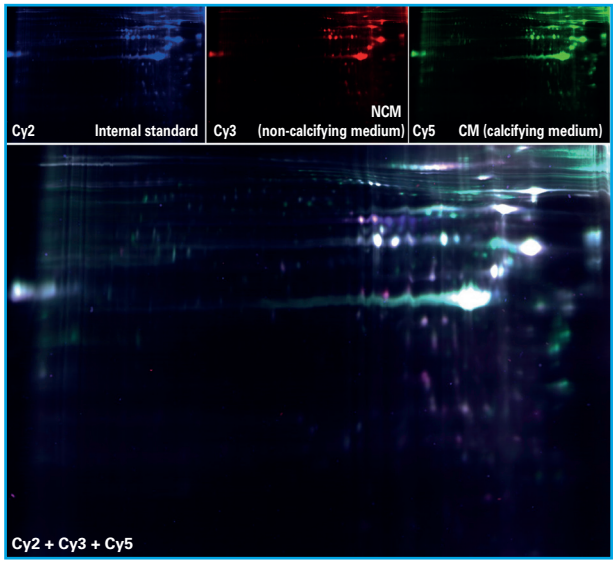

Figure 2. Representative images of the gels resulting from the electrophoresis of proteins from cells cultured in non-calcifying (Cy3) and calcifying medium (Cy5), the internal standard (Cy2), and the resulting combination of the 3 (Cy2 + Cy3 + Cy5).

in proteomic studies could be more reproducible than those in genomics and transcriptomics (14).

After mass spectrometry analysis of the protein extracts obtained from VSMCs cultured for 6 days in calcifying and non-calcifying media, 20 proteins were identified with high reliability.

Of the 4 proteins identified that showed increased expression in calcifying medium, the one with the greatest change was the alpha chain of type I collagen. Several studies relate the increase in collagen I

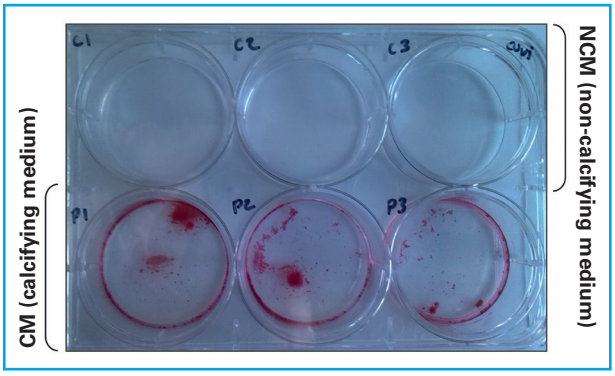

Figure 3. Alizarin red staining of CMLV after 6 days of culture in non-calcifying and calcifying conditions.

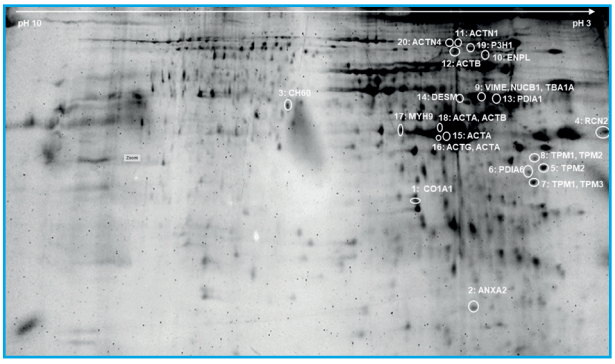

Figure 4. Map of the differential protein expression profile in VSMC cultures under calcifying vs non-calcifying conditions. Preparative gel. The spot number and the MASCOT symbol of the identified proteins are indicated.

levels to VC. Collagen I and III are the main components of the extracellular matrix whose levels remain relatively stable under physiological conditions.

Table I. Overexpressed proteins in CMVL A7r5 cells cultured under calcifying conditions vs those cultured under non-calcifying conditions, identified by LC-MS/MS

Spot No.	Mascot symbol	Protein name	Function	Localization	*p-value*	Fold change ratio	Mascot score	Mass (Da)	IP	Peptides
1	CO1A1_RAT	Alpha-1 collagen chain (I)	Structural	Extracellular matrix	0.0023	3.49	80	137869	9.28	2 (1)
2	ANXA2_RAT	Annexin A2	Ca binding and interaction with cytoskeleton	Extracellular matrix	0.018	2.17	111	38654	7.53	3 (3)
3	CH60_RAT	Mitochondrial 60 kDa heat shock protein	Cellular stress (chaperone)	Mitochondrial matrix	0.0072	1.57	136	60917	5.91	2 (2)
4	RCN2_RAT	Reticulocalbin-2	Ca binding	RER	0.017	1.54	131	37410	4.23	3 (2)

RER: rough endoplasmic reticulum. The spot number, Mascot symbol, protein name, function, localization, theoretical mass (Da), isoelectric point (IP) obtained from the databases (ie, without considering possible post-translational alterations or changes), as well as other mass spectrometry analysis data, such as the number of peptides identified (with the number of unique peptides indicated in parentheses), are shown.

Table II. Proteins inhibited in CMVL A7r5 cell cultures subjected to calcification treatment vs control

Spot No.	Mascot symbol	Protein name	Function	Localization	*p-value*	Fold change ratio	Mascot score	Mass (Da)	IP	Peptides
5	TPM2_RAT	Beta tropomyosin	Cytoskeleton (structural)	Cytoplasm	0,0075	-10,4	170	32817	4,66	6 (5)
6	PDIA6_RAT	Disulfide isomerase A6 protein	Chaperone	RER	0,016	-6,45	83	48100	4,95	2 (1)
7	TPM1_RAT	Alpha-1 tropomyosin chain	Cytoskeleton (structural) and cell adhesion	Cytoplasm	$6\ e^{-006}$	-6,39	299	32661	4,69	11 (9)
	TPM3_RAT	Alpha-3 tropomyosin chain	Cytoskeleton (structural)	Cytoplasm			100	28989	4,75	5 (3)
8	TPM1_RAT	Alpha-1 tropomyosin chain	Cytoskeleton (structural) and cell adhesion	Cytoplasm	0,0055	-6,21	89	32661	4,69	2 (2)
	TPM2_RAT	Beta tropomyosin	Cytoskeleton (structural)				89	32817	4,66	2 (2)
9	VIME_RAT	Vimentin	Cytoskeleton	Cytoplasm	0,018	-5,17	248	53700	5,05	7 (7)
	NUCB1_RAT	Nucleobindin-1	Ca binding	Golgi apparatus			138	53474	5,01	4 (3)
	TBA1A_RAT	Alpha-1 tubulin	Cytoskeleton	Cytoplasm			132	50104	4,94	2 (2)
10	ENPL_RAT	Endoplasmin	Chaperone (oxidative stress), Ca binding	RER	0,02	-3,49	235	92713	4,69	4 (4)
11	ACTN1_RAT	Alpha-actinin-1	Cytoskeleton and internal cell motility	Cytoplasm	0,0098	-2,63	523	102896	5,23	16 (13)
12	ACTB_RAT	Actin (cytoplasmic) 1	Cytoskeleton (structural)	Cytoplasm	0,0098	-2,63	53	41710	5,21	2 (1)
13	PDIA1_RAT	Disulfide isomerase A1 protein	Chaperone (stress)	RER	0,016	-2,37	278	56916	4,77	5 (5)
14	DESM_RAT	Desmin	Structural	Cytoplasm	0,0043	-2,32	47	53424	5,21	2 (1)
15	ACTA_RAT	Smooth muscle actin (aorta)	Cytoskeleton and cell motility	Cytoplasm	0.032	-2,3	159	41982	5,24	7 (5)
16	ACTG_RAT	Actin (cytoplasmic) 2	Cytoskeleton and cell motility	Cytoplasm	0,032	-2,3	317	41766	5,31	7 (5)
	ACTA_RAT	Smooth muscle actin (aorta)					145	41982	5,24	7 (3)
17	MYH9_RAT	Myosin-9	Cytoskeleton	Cytoplasm	0,017	-2,15	168	226197	5,49	5 (3)
18	ACTA_RAT	Smooth muscle actin (aorta)	Cytoskeleton and cell motility	Cytoplasm	0,015	-1,93	127	41982	5,24	4 (3)
	ACTB_RAT	Beta-actin (cytoplasmic) 1					354	47710	5,24	9 (8)
19	P3H1_RAT	Prolyl 3-hydroxylase 1	Structural, Extracellular matrix component	RER secreted to the extracellular matrix	0,05	-1,88	224	82338	5	6 (6)
20	ACTN4_RAT	Alpha-actinin-4	Cytoskeleton and internal cell motility	Cytoplasm	0,049	-1,5	326	104849	5,27	6 (6)

RER: rough endoplasmic reticulum.

However, elevated phosphate and Ca levels increase collagen I expression, favoring the differentiation of VSMCs into osteoblast-like cells (15). Another protein involved in collagen biosynthesis and maturation is prolyl-3-hydroxylase (P3H1), a proteoglycan with enzymatic activity necessary for the proper formation of collagen helices, whose expression was reduced under calcifying conditions, consistent with other studies analyzing protein expression profiles in VSMCs under calcifying conditions (16). Furthermore, mice deficient in this protein showed hypermineralization of the bone matrix (17). Changes in the characteristics of the extracellular matrix and behavior among its constituents influence not only the mechanical properties of connective tissues but also contribute to modulating the cellular phenotype, altering protein expression, cytoskeleton organization, and, consequently, intracellular signaling pathways.

Other proteins from the rough endoplasmic reticulum (RER) whose expression was altered include disulfide isomerase proteins A1 and A6 (PDIA1, PDIA6), involved in disulfide bond formation, isomerization, and reduction, acting alongside chaperones in the regulation of misfolded proteins. The expression of these proteins was found to be inhibited under calcifying conditions, and although there is no evidence of PDIA6 role in VC, changes in PDIA1 expression have been associated with increased VC and aortic calcification (18,19).

Among this group of proteins involved in folding, which showed an impaired expression in this study, is the 60 kDa mitochondrial heat shock protein (HSP60) and endoplasmin (ENPL). The expression of HSP60 in vascular disease has been correlated with the severity of atherosclerosis and the anti-inflammatory response (20). Several studies have demonstrated that HSP expression is closely related to the VC process (21-23). On the other hand, although no association between ENPL and VC has been described, this protein participates in the normal differentiation of cardiac, smooth, and skeletal muscle cells (24), so changes in its expression could alter their normal differentiation.

In the present study, an increase in Ca^{2+}-binding proteins, such as reticulocalbin-2 (Rcn-2) and annexin A2, was also observed. Ca-binding proteins (CaBP) participate actively in many cellular processes through specific domains, such as Ca^{2+} homeostasis or signaling pathways (25). Mineralization initiation requires the entry of Ca^{2+} into matrix vesicles. Specifically, Rcn-2 has been described as a mediator of VC, increasing the expression of osteogenic markers and reducing the expression of contractile phenotype markers in VSMCs (26). On the other hand, annexins are a family of Ca-dependent membrane-binding proteins. Different studies have shown the importance of annexins in matrix vesicle formation through Ca entry and the initiation of mineralization (27) and the similarities between the process of matrix vesicle formation from VSMC membranes and chondrocytes, including the enrichment of annexins A2, A5, and A6 as triggers for the mineralization process (28). Another Ca-binding protein, but one that was inhibited in this study, is nucleobindin 1 (NUCB1), localized in the Golgi apparatus, where it plays an important role in Ca homeostasis (29), although no studies have yet associated it with VC.

Additionally, in line with previous work conducted in in vivo and in vitro VC models and proteomic studies, the results of this work showed a decrease in the expression of structural proteins and the deregulation of cytoskeletal proteins in VSMCs (16,30,31). Among this group of proteins, those that experienced the greatest decrease were typical muscle proteins (tropomyosin, actin, desmin, myosin, etc.). This may be interpreted as a loss of the muscle phenotype of VSMCs as a result of exposure to high concentrations of phosphate and Ca, as previously described in other studies (32).

One of the issues with VC is that it is diagnosed at late stages, once it is already established, and no therapies specifically targeting VC have been described to date. Furthermore, the diagnostic techniques used, mainly based on imaging techniques, may be limited due to factors such as cost, availability, and radiation exposure (33). Therefore, the identification of minimally invasive, sensitive, and specific circulating biomarkers that could identify the presence of VC in early stages would present a highly interesting alternative. The proteins identified in this study could help in the search for biomarkers to identify patients at higher risk of developing VC or could even be used as therapeutic targets once validated in other studies.

This type of study presents several limitations, as protein identification is limited by the quantity and quality (low concentration of salts, nucleic acids, lipids, etc.) of the obtained protein extract, which directly depends on the effectiveness of the extraction method, and the sample preparation, which is crucial for the quality of the results (34). In this study, in addition to the proteins identified with high reliability, others with low scores were also detected, which could be due to various reasons (35): in the cut spots, the concentration of some proteins may be much lower than that of others, and post-transcriptional changes or proteolysis of some proteins could alter the availability of peptides for identification. Of note, being an in vitro calcification model, it does not allow for a direct comparison between the CKD state and a healthy control, as CKD involves systemic factors and physiological conditions that are not fully captured in this model. Another limitation to consider is that the cell line used was from rats, so the translation of the results to humans is limited. On the other hand, the characterization of the protein profile of the cells was carried out in a calcification model that was maintained for 6 days. Analyses at other points in the process would contribute to a deeper understanding of vascular calcification. It is possible that in models where calcification could be

maintained for longer periods, the levels of some proteins could have been higher.

CONCLUSIONS

The results obtained confirmed a change in the phenotypic expression of muscle cells, with a decrease in typical muscle and cytoskeletal proteins. Moreover, it was possible to identify changes in the expression of proteins previously not linked to VC, which could participate in this process. Considering the results obtained in this study, it would be very useful to conduct complementary experiments to clarify the role of these and other differentially expressed proteins in the VC process. These proteins could be used as markers of the early stages of the disease or as target molecules for the development of new therapeutic strategies that could reduce the morbidity and mortality of patients with CKD.

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