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Vitamin D Modulates PAR-4 Expression in an in Vitro Model of Osteoarthritis

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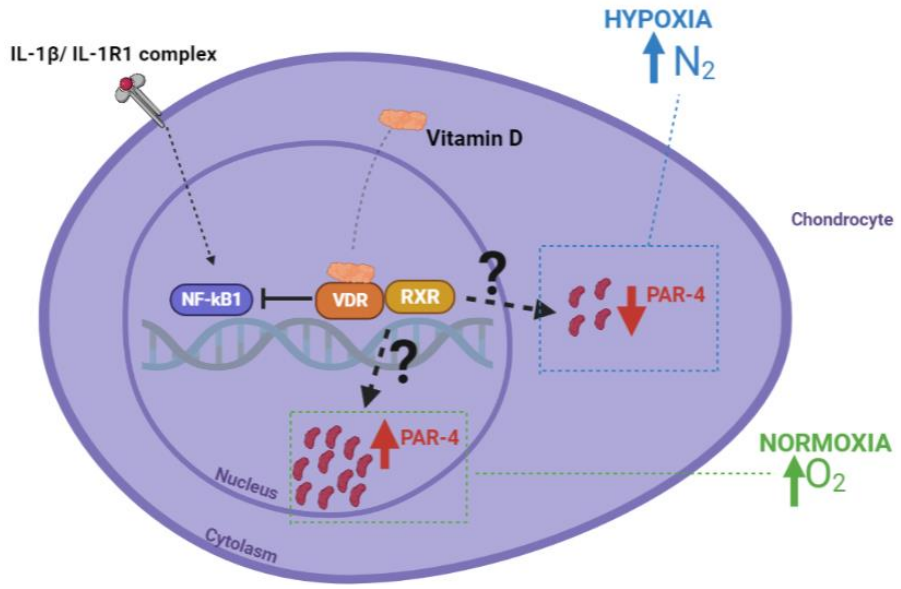
HIGHLIGHTS

- Chondrocytes express PAR-4 in the nucleus and cytoplasm;
- Vitamin D reduces the pro-apoptotic proteins PAR-4 and caspase-3;
- PAR-4 and caspase-3 may be potential therapeutic targets for osteoarthritis.

Abstract: Osteoarthritis (OA) encompasses degeneration of articular cartilage, subchondral bone erosions and sclerosis. Chondrocyte apoptosis and an oxygen-deprived microenvironment are essential factors in OA pathogenesis. PAR-4 (Prostate apoptosis response-4) is a pro-apoptotic protein implicated in many pathologies as well as in chondrocyte cell death mechanism. Vitamin D supplementation has been identified as a therapeutic tool for a variety of inflammatory pathologies. In the present manuscript, we investigated whether first, PAR-4 expression is influenced by chondrocytes in a model of OA, in vitro, and second, whether vitamin D modulates PAR-4 expression in the same model. To test our hypothesis, we used the primary culture of murine chondrocytes isolated from the femoral and tibial condyles of wistar rats. The expression of the pro-inflammatory effect interleukin IL-1β was evaluated in the presence and absence of vitamin D. Western blot and immunofluorescence analysis confirmed protein expression. In the normoxia condition, the chondrocytes expressed PAR-4 in the cell nucleus, and in the hypoxic condition, PAR-4 was expressed in the cell cytoplasm. We disclosed that the treatment with Vitamin D decreased PAR-4 ($p= 0.0137$) and caspase-3 ($p= 0.0007$) expression. Thus, the results suggested that PAR-4 and caspase-3 proteins could be potential targets for OA.However, we believe that research is needed to identify the mechanisms implicated in the regulation of PAR-4 in OA.

Keywords: Apoptosis; Hypoxia; Normoxia; Osteoarthritis; PAR-4; Vitamin D.

GRAPHICAL ABSTRACT



INTRODUCTION

Osteoarthritis (OA) is a chronic disease characterized by a rupture and loss of articular cartilage which lead to a reduction in the joint space and friction between the bones, causing chronic pain, functional impairment, deformity and desability [1]. In addition, inflammatory cytokines such as IL-1β and TNF stimulate catabolic pathways and contributes to disease progression [2].

Chondrocyte death can be of pathogenic relevance in the development of osteoarthritis. As the articular cartilage relies entirely on the chondrocytes for the maintenance of the extracellular matrix, the impairment of the function and survival of these cells will lead to a loss of the articular cartilage [3].

Caspases are a set of cysteines that after activation by the extrinsic or intrinsic pathways, enable a cascade of events that culminates in programmed cell death. Caspases and some proteins such as Fas, TRAIL and Beclin-1 have already been investigated in the OA cell death mechanism, as well as the Bcl-2 protein, that has anti-apoptotic action [4].

In contrast, PAR-4 protein (Prostate apoptosis response-4) exerts its pro-apoptotic functions according to the cellular context and post-translational modifications. Simultaneously, PAR-4 inhibits the transcriptional activity of the key survival factor NFκB, thus preventing the expression of pro-inflammatory and anti-apoptotic genes [5]. In addition, PAR-4 regulates apoptosis of neurodegenerative diseases [6-8]. However, there are few studies on the pro-apoptotic protein PAR-4 in the context of chondrocyte death. Futhermore, the PAR-4 has not yet been studied in the sense of rheumatic diseases, particularly OA, opening up new lines of study for the research of key proteins and the development of potential molecular drug targets.

Vitamin D is a steroid hormone that has several biological activities against various target tissues and is essential in the conduct of metabolic processes in bones, in which there are a variety of effects on the pathophysiology of the disease [9]. There are reports that deficiency the vitamin D was related to increased inflammation in chronic disease. It has been shown by Zanello and coauthors that vitamin D prevented osteoblast apoptosis. Understanding whether vitamin D acts on chondrocyte death mechanisms would be important [10]. In this context, we sought to investigate whether PAR-4 is expressed by chondrocytes in the OA in vitro model and whether is modulated by vitamin D.

MATERIAL AND METHODS

Ethics Statement

The study protocol was approved (No. 0007/2017) by the Animal Experimentation Ethics Committee, located at the Biosciences Center – Federal University of Pernambuco, Brazil. All Principles of Care for Laboratory Animals have been followed.

Animals

Six male, albino, Wistar rats were used, from the Animal Production Unit from the Physiology and Pharmacology Departament of the Federal University of Pernambuco. Animals were maintained under standard environmental conditions and fed with chow and water ad libitum. Experimental protocols to obtain murine chondrocytes were performed using to recommendations of the Brazilian College for Animal Experimentation guidelines.

Isolation and primary culture of murine chondrocytes

Initially, the rats were anesthetized with a solution of 2% Xylazine Hydrochloride (ANASEDAN) and 10% Ketamine (SYNTEC) intraperitoneally, in a total dosage of 0.2 mL of the solution for each 100g of animal weight [2:1]. Then, the animals were euthanized with 1 mL of Potassium Chloride (KCl) intracardiac. Cartilage samples were obtained from the femoral and tibial condyles of Wistar rats. After collecting the material, the cartilage samples were washed once with PBS and transferred to a 6-well plate containing 10 mL of DEMEN/F12 medium containing 3 mg / mL of type II collagenase and 5% SBF, where they were incubated overnight at 37 °C. The isolated chondrocytes were filtered through a 70µm nylon mesh to separate the extracellular matrix and cartilage residues. Subsequently, the cells were centrifuged at 2500 rpm for 3 minutes and resuspended in DEMEN / F12 medium, supplemented with 10% SBF. Chondrocytes were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. After a period of 10-15 days, they reached their maximum confluence (100%), to perform the proposed in vitro assays. Chondrocyte isolation validation was performed by immunofluorescence with primary antibodies COL2A1 (CusaBio) and CD44 (CusaBio).

In vitro inflammatory model of murine chondrocyte osteoarthritis

Murine chondrocytes were plated (1x105 cells / well) and stimulated in vitro, in 6-well plates with IL-1β (10 ng / mL eBioscience). Subsequently, they were treated with vitamin D at a dose of 100 nM and after 24 h of treatment the cells were collected for protein expression evaluation using Western Blot.

One group was subjected to normoxia (oven at 5% CO_2 at 37 °C) and the other to hypoxia (chamber containing 94% N_2 and 5% CO_2 at 37 °C). After 48 hours of treatment, cells were collected for evaluation.

Western Blot Analysis

Total cellular proteins were extracted using nuclear and cytoplasmic lysis buffer and then centrifuged at 1500 rpm at 4 °C for 30 min. Protein dosage was performed using the Bradford method, according to the manufacturer's instructions (BioRad). After absorbance analysis, 40-60µg of proteins were electrophoresed on 12% polyacrylamide gel containing SDS (SDS-PAGE) and transferred to PVDF membrane (GE Healthcare Life Sciences). Blocking of nonspecific sites was performed by incubating the membrane with TBST-BSA 5%. Incubation with the primary antibodies PAR-4 (CusaBio), Bcl-2 (Santa Cruz), caspase-3 (Santa Cruz), GAPDH (Santa Cruz) and α-tubulin (Santa Cruz) diluted 1:1000 in TBST-BSA 5%, was performed at 4°C overnight. Membranes were incubated with respective HRP-conjugated secondary antibodies (1:10.000). HRP-conjugated immunolabelled proteins were detected by enhanced chemiluminescence method (ECL, GE Healthcare Life Sciences).

Immunofluorescence Analysis

To assess protein localization, chondrocytes were plated (1x105 cells / well) in a 24-well plate and after 24 hours, the IL-1β stimulus (10 ng/mL) and the combined form of the stimulus plus vitamin D [1.25 (OH) 2D3] (100 nM) were added.

Cells were seed on a glass slide and after 48 hours of treatment, were fixed in acetone and methanol 1: 1 for 10 minutes. After three washings in phosphate buffered saline (PBS), cells were blocked with 5% BSA for 1 h. Then, the coverslips were incubated with 25 µL of the primary antibodies COL2A1 (CusaBio), CD44 (CusaBio), PAR-4 (CusaBio) and HIF-1α (Imuny), all of them diluted 1: 200 at 4ºC overnight, under a humid chamber. After overnight incubation, slides were washed 3x with 100 µL of PBS / 0.5% BSA for 10 min and then incubated with DO5J (conjugate) and CSB-PA437981 (CusaBio). The slides were incubated with DAPI for 5 minutes in the dark and then added 10 µL of Glycerol + PBS (1:1). The coverslips were transferred with the cells facing downwards, where they were protected from light until the moment of the evaluation using the Eclipse Ts2® microscope (Nikkon).

Statistical Analysis

Statistical analysis were performed using Prism 6.0 (GraphPad Software Inc., San Diego, CA). D' Agostino test verified the normality of samples. Numerical data were expressed as mean ± SE if they were in normal distribution. Mann-Whitney U test was used to compare serum cytokine levels. A probability value of $p < 0.05$ was considered significant.

RESULTS

PAR-4 subcellular location in chondrocytes

To assess whether PAR-4 is expressed by chondrocytes in a context that mimics the OA microenvironment, chondrocytes were submitted to hypoxia condition. Figure 1 demonstrated that the chondrocytes expressed the pro-apoptotic protein PAR-4 (by green fluorescence) under normoxic and hypoxic conditions. In the first condition, PAR-4 is found majority in nuclear position while in the hypoxia condition is found majority in cytoplasmic position.

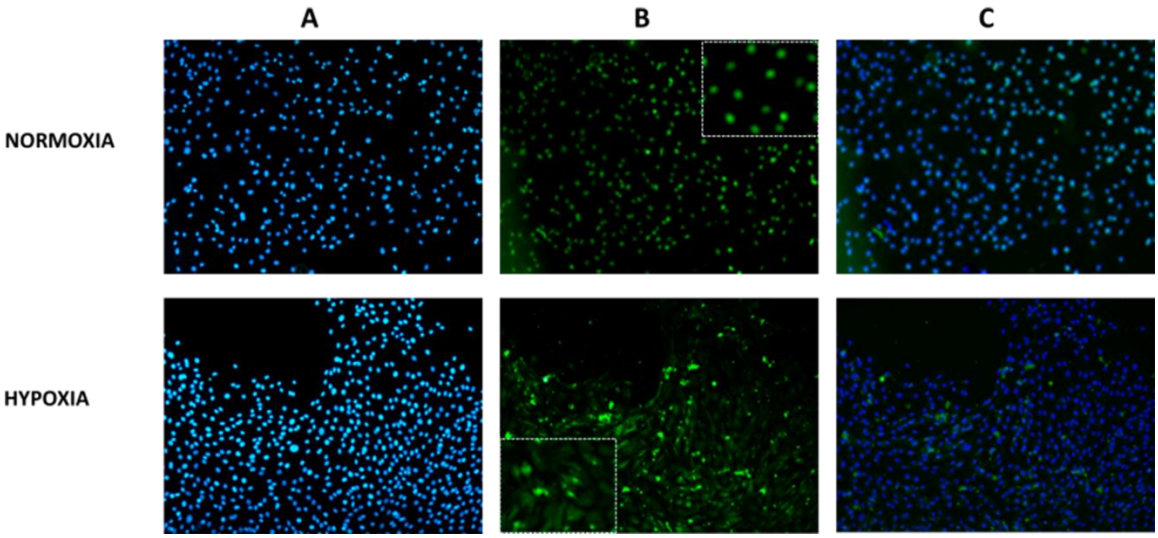


Figure 1. Subcellular location of the PAR-4 pro-apoptotic protein in chondrocytes under normoxic and hypoxic conditions. **A:** DAPI in normoxia and hypoxia conditions indicate the nuclear staining of chondrocytes. **B:** nuclear and cytoplasmic localization of PAR-4 under normoxia and hypoxia conditions, respectively. **C:** Overlay of the two images (A and B) to confirm that both belong to the same field and represent the same cell. Photos taken from the Eclipse Ts2® microscope (Nikkon) on the 10x objective.

Vitamin D reduces expression of PAR-4 and Caspase-3

It should be noticed that the 24-hour pro-inflammatory IL-1β stimulus was not sufficient to evoke the pro-apoptotic microenvironment. Treatment with 100 ng/mL of vitamin D resulted in reduction of pro-apoptotic proteins PAR-4 (Fig. 2 A; $p= 0.0137$) and caspase-3 (Fig. 2 B; $p= 0.0007$).

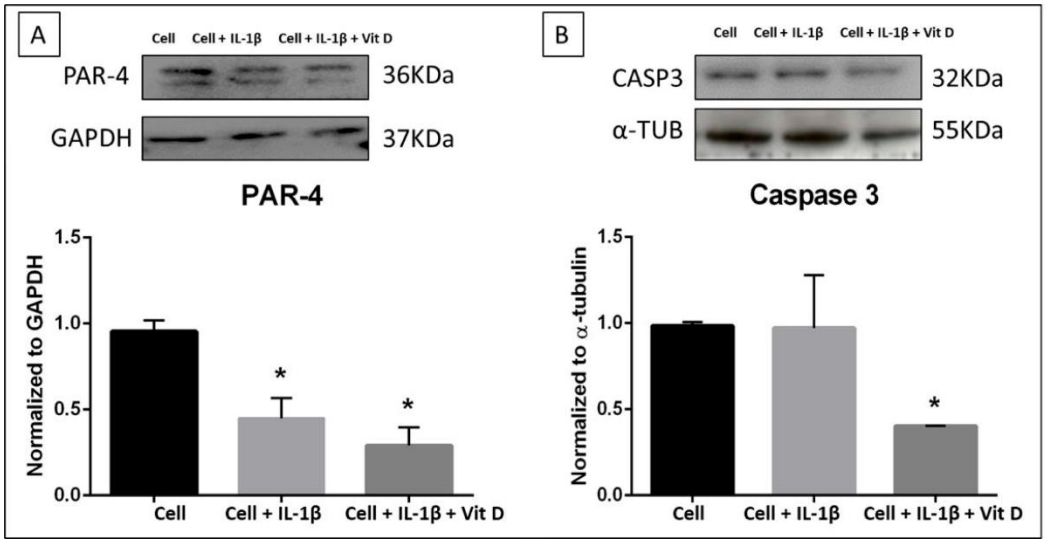


Figure 2. Protein expression of apoptotic proteins after 24 h of Vitamin D treatment. Expression and modulation by vitamin D of the pro-apoptotic protein PAR-4 **(A)** and caspase-3 **(B)** under different cell conditions. * p <0.05 vs control: Significant after analysis of variance (ANOVA).

Expression of HIF-1α and PAR-4 by chondrocytes

Figure 3 demonstrates qualitatively the expression of the hypoxia-inducible factor 1α (HIF-1α) and the pro-apoptotic PAR-4 protein in chondrocytes when subjected to two types of cellular microenvironment.

In the hypoxic microenvironment (Fig. 3 A) that mimics OA, the reduction of PAR-4 by vitamin D seems to be more evident when compared to the normoxic condition (Fig. 3 B). In addition, chondrocytes stimulated with IL-1β, seem to positively modulate the expression of HIF-1α in both normoxic and hypoxic conditions.

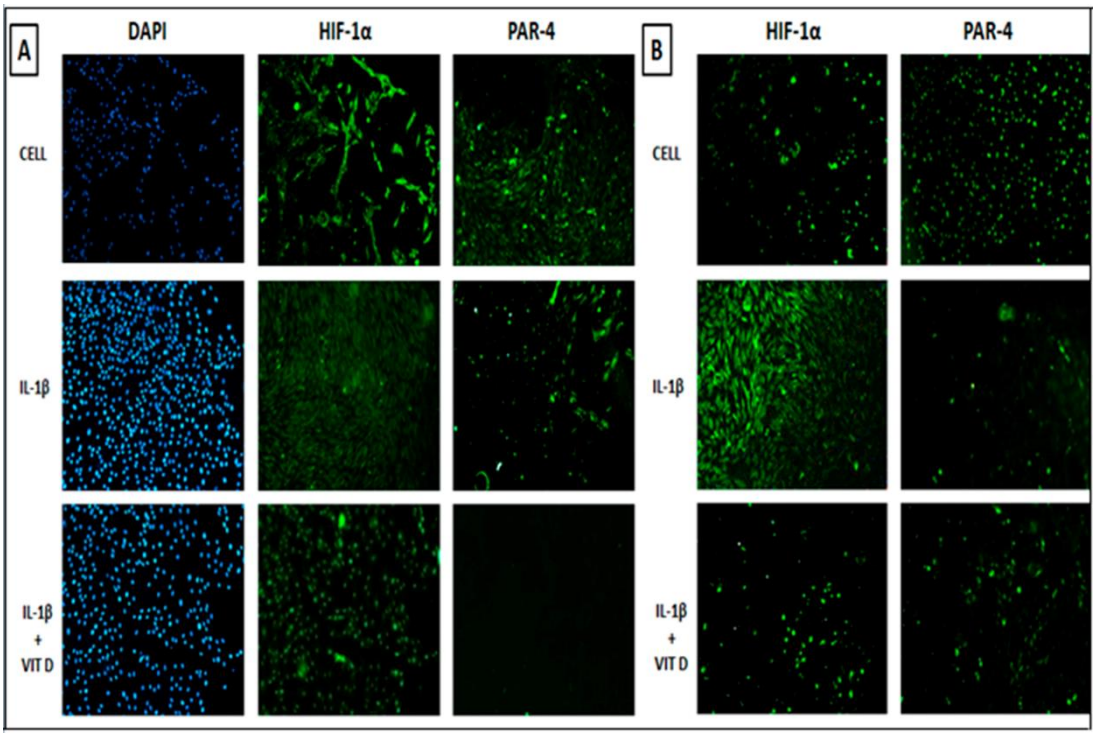


Figure 3. Qualitative analysis of the expression of HIF-1α and PAR-4 by chondrocytes in two types of cellular microenvironment. **A:** Expression of HIF-1α and PAR-4 by chondrocytes in the hypoxic microenvironment and under different cell conditions. **B:** Expression of HIF-1α and PAR-4 by chondrocytes in the normoxic microenvironment and under different cell conditions. Photos taken from the Eclipse Ts2® microscope (Nikkon) on the 10x objective.

DISCUSSION

There are no studies in the literature that correlate the involvement of pro-apoptotic PAR-4 protein with OA. According to Burikhanov and coauthors, PAR-4 is expressed in a diversity of tissues and is located both in the nucleus and in the cytoplasm of cells [11]. However, the studies do not correlate the expression of PAR-4 and apoptosis.

There is controversy if PAR-4 subcellular location induces apoptosis. In cells derived from the embryonic body, it was observed that it was located in the nucleus and in the cytosol, but none of them were correlated with apoptosis due to the absence of condensed nucleus and cell morphology corresponding to non-apoptotic cells. Notably, in most types of cancers, nuclear entry of PAR-4 is essential for direct apoptosis but not for normal or immortalized cells [12].

In contrast, stress-inducing agents in the Endoplasmic Reticulum induce the secretion of PAR-4. Further, TRAIL-induced apoptosis depends on the extracellular signaling of PAR-4 through the GRP78 receptor [13]. Our results suggest that in chondrocytes under hypoxia, PAR-4 is located in cytoplasm and suggesting that PAR-4 induces apoptosis by GRP78 pathway. Further assays should be carried out to better investigate the role of PAR-4 in chondrocytes submitted to hypoxia, since this is the first study described.

In vitro studies have shown that TNF and IL-1β potentiate the impact of cartilage [14,15]. However, in our study, the pro-inflammatory cytokine IL-1β, for 24 h, was not sufficient to mimic a pro-apoptotic microenvironment. Armada-Lópes and caouthors pointed that although IL-1β and TNF are important cytokines in the pathophysiology of OA, they act indirectly in the degradation of cartilage and in apoptosis of chondrocytes [16].

However, the IL-1β seemed to positively modulate the expression of HIF-1α in both normoxic and hypoxic conditions. Studies have already shown that vitamin D modulates HIF-1α expression in several types of tumor [17,18]. In our research, vitamin D also demonstrated an important modulatory role for HIF-1α expression in OA.

In contrast, Murata and coauthors [19] reported high levels of HIF-1α in human OA chondrocytes cultured under hypoxia and normoxia conditions after treatment with IL-1β for a period of 24 h. Consequently, they suggested that in chondrocytes, as in other cell lines, IL-1β act as a positive modulator of HIF-1α at the post-transcriptional level, corroborating our results [19].

In a cross-sectional clinical study, it was found that vitamin D deficiency was associated with OA development [20,21]. Our study demonstrated that vitamin D act as a good modulator involved in apoptosis, significantly modulating PAR-4 expression and caspase 3 proteins. Interestingly, vitamin D reduced PAR-4 expression in a hipoxic microenvironment associated with the presence of IL-1β, compared to normoxic conditions.

Vitamin D may have an anti-inflammatory effect by modulating the function of human monocytes or by the VDR (Vitamin D Receptor) signaling pathway. Furthermore, in the inflammatory process, VDR signaling intrinsically suppresses NF-κB activation, and this inhibitory activity is likely to be elevated in the presence of vitamin D ligands [22].

In parallel, studies have shown that PAR-4 acts as an NF-κB modulator. PAR-4 inhibits the transcriptional activity of NF-κB promoting cell apoptosis [23]. At the same time, there are few studies on the role of vitamin D in the regulation of genes involved in the apoptotic pathway. Somayeh et al. have shown that vitamin D in association with exercise negatively modulated gene expression of Bax and caspase 3 proteins in lung tissue submitted to an in vivo of pulmonary stress model [24]. However, this is the first study that demonstrated that vitamin D could modulate PAR-4 expression in OA. As PAR-4 negatively modulates NF-κB, further studies would be important to assess whether vitamin D via VDR may directly modulate PAR-4 expression or whether this modulation is via the NF-κB pathway.

In summary, in the OA in vitro model, chondrocytes express PAR-4 at the cytoplasmic level; postulating that it is involved in the chondrocyte cell death process, primordial for the pathogenesis of OA. In addition, vitamin D negatively modulates PAR-4 and caspase 3 expression. Finally, further assays must be performed to define the pathways involved in the modulation of PAR-4 in OA progression.

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