

Vitamin C is Required To Allow Sustained IL-1 β -Induced Suppression of The Pyrophosphate Regulators ANKH and ENPP1 in Bovine Nucleus Pulposus Cells

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Abstract

The extracellular concentration of pyrophosphate (PPi) is increased by the activity of proteins like ectonucleotidepyrophosphatase/phosphodiesterase (ENPP1) and progressive alkalosis protein homolog (ANKH), whereas the concentration of PPi is decreased by tissue non-specific alkaline phosphatase (TNAP) through hydrolysis. Since PPi prevents calcium-phosphate deposition, ANKH and ENPP1 inhibit tissue mineralization and TNAP enhances mineralization. The presence of the inflammatory cytokine Interleukin-1 (IL-1) β alters expression of important regulatory proteins in many cells. The objective was to determine the effect of IL-1 β on the gene expression of ANKH and ENPP1 in bovine Nucleus pulposus (bNP) cells, and the importance of vitamin C. A dose-dependent increase in calcium staining of TNAP-transduced bNP cell cultures was observed with alizarin red, indicating increased calcium deposition in the matrix at 3 and 7 days when treated with IL-1 β .

A dose-dependent decrease in the transcripts for the pyrophosphate regulating proteins ANKH and ENPP1 was also observed with IL-1 β treatment in cultured naïve cells. A possible cause of the enhancement in matrix mineralization seen with IL-1 β treatment is this down-regulation of ANKH and ENPP1 transcript in bovine NP cells treated with IL-1 β . The expression of both in cells treated with 2ng/mL IL-1 β is statistically lower than the untreated cells at 1 day. However, only in the presence of vitamin C do we see a sustained decrease in gene expression. ANKH has been implicated in several studies as an important protein for homeostasis and maintenance of the cell phenotype. ENPP1 expression could also be important in the NP niche. We show IL-1 β down-regulates expression of ANKH and ENPP1 which could play a significant role in pathology of DDD, and vitamin C prolongs this response.

Keywords: IL-1 β ; ANKH; ENPP; Vitamin C; Calcification; Nucleus Pulposus

Abbreviation: Inorganic Pyrophosphate (PPi); Inorganic Phosphate (Pi); Ectonucleotidepyrophosphatase/Phosphodiesterase (ENPP1); Progressive Ankylosis Protein Homolog (ANKH); Tissue Non-specific Alkaline Phosphatase (TNAP); Nucleus Pulposus (NP); Interleukin-1 (IL-1) β ; Tumor Necrosis Factor (TNF).

Introduction

Mineralization in soft tissue is a complex process determined primarily by the extracellular concentrations of a chemical inhibitor, inorganic pyrophosphate (PPi), and a chemical promoter, inorganic phosphate (Pi) [1]. The extracellular concentration of PPi is increased by the activity of proteins like ectonucleotidepyrophosphatase/phosphodiesterase (ENPP1) and progressive alkalosis protein homolog (ANKH) [2], whereas the concentration of PPi is decreased by tissue non-specific alkaline phosphatase (TNAP) by hydrolysis. Therefore ANKH and ENPP1 actively inhibit tissue mineralization, whereas TNAP enhances and is essential for mineralization.

Previous studies have shown that the presence of the inflammatory cytokines Interleukin-1 (IL-1) β and tumor necrosis factor (TNF) alter expression of important mineralization-regulatory proteins in nucleus pulposus (NP) cells [3], vascular smooth muscle cells [4], primary chondrocytes [5], mesenchymal stem cells and osteoblasts [6]. ANKH in particular has been identified as an important gene in the maintenance of a soft tissue phenotypes in articular cartilage [7] and the NP [8]. A reduction in ANKH therefore could disrupt homeostatic maintenance of the soft tissue phenotype.

There are many molecules that play a role in the inhibition and induction of mineralization. Bone gamma-carboxyglutamic acid protein (BGP), or Osteocalcin (OCN), which is under RUNX2 regulation works by binding calcium at mineral nucleation sites in collagen. OCN is a uniquely osteoblast-specific gene, under control of osteoblast specific factors, and it is essential for mineral formation [9]. Meanwhile matrix gla protein (MGP) is a potent inhibitor of matrix mineralization – the absence of this protein leads to Keutel Syndrome, marked by abnormal cartilage calcification [10]. Osteopontin (OPN), or Secreted Phosphoprotein (SPP)1, is a regulator of tissue mineralization found abundantly in atherosclerotic lesions but absent in soft vascular beds [11]. PHEX is a protein involved in osteoblast-regulated bone formation which enzymatically activates OPN [12]; mutation of PHEX has been linked with hypophosphatemic Rickets and full length OPN accumulation. Circulating phosphate is also essential for regulating hypertrophic chondrocyte and endothelial cell apoptosis [13-15].

Mineralization is a process commonly studied *in vitro*. To achieve calcium-phosphate deposition *in vitro* most formulations of mineralizing media include a phosphate donor, such as β -glycerophosphate, and vitamin C (ascorbic acid) to allow collagen synthesis. β -glycerophosphate is a substrate for TNAP [16], and in the absence of another phosphate source mineralization cannot occur without it. Vitamin C is a common media component for differentiation and mineralization media. The presence of vitamin C is essential for collagen synthesis and TNAP activity [17]; it is unclear if it is required for cytokine stimulation. Vitamin C is a free radical scavenger which relieves the inflammatory effects of induced nitrous-oxide synthase (iNOS), NF- κ B activation and cytokines like TNF, acting through p-38 MAPK [18,19].

The NP is a unique connective soft tissue found in the center of the intervertebral disc. In a healthy state the NP does not mineralize. TNF and IL-1 β are both implicated in the progression of Degenerative Disc Disease (DDD) [20]. Tissue mineralization and a catabolic gene expression profile are hallmarks of DDD. Calcification of the nucleus-adjacent endplate tissue in the intervertebral disc is associated with decreased ANKH and ENPP1 expression [21].

Transducing cells from NP tissue with the TNAP gene in a viral expression vector leads to a mineralizing phenotype (manuscript in preparation) [22]. These TNAP-modified bovine NP cells (bNP-TNAP) provide a useful model for analyzing modulation in phosphate regulation of the NP cells with short time-scale mineralization results. This model can be used to assess the role of IL-1 β and vitamin C on the expression of regulatory genes and the accumulation of mineral in NP cultures.

The objective of this study was to determine the effect of IL-1 β on the gene expression of ANKH and ENPP1 in bNP cells *in vitro*, and to assess the effect cytokine exposure has on

mineralization. Further, we hoped to establish the importance of vitamin C with regard to duration of gene expression response to cytokine *in vitro* in bNP cells.

Methods

Cell Culture

Bovine nucleus pulposus (bNP) cells were obtained from three bovine tails (Max Insel Cohen, Livingston, NJ) by overnight digestion in Collagens Type II (500U/mL, Worthington, Lakenwood NJ) at 37°C. After digestion, cells were plated in monolayer in Dulbecco's Modified Eagle Media (DMEM), 10% Fetal Bovine Serum (FBS), 1% antibiotic-antimycotic and 10uM HEPES (Gibco, Grand Island, NY).

Generating Transduced cells

bNP cells were transduced with retroviral constructs as described by Krzyzanowska et al. Briefly, passage 2 bNP cells were transduced with in-house generated pMXs-IRES-Bsd-TNAP constructs to overexpress human TNAP (1649bp), generating bNP-TNAP cells. A pMXs-IRES-Bsd-LacZ construct was used to assess transduction efficacy. Transduced cells were selected using 4ug/mL Blasticidin (*In vivo* Gene, San Diego, CA).

Culture Conditions

Naive and transduced bNP cells were seeded at 200,000 cells/cm² in Falcon 12-well plates (BD) in complete media (10% FBS, 1% antibiotic) or medium supplemented with: 50mM β -glycerophosphate and 50 μ g/mL of L-Ascorbic acid (Sigma-Aldrich, St Louis, MO), and treatment groups with .01 (low dose) or 2 (high dose) ng/mL IL-1 β (R&D Systems), changing media every 3 days. Cells were cultured 1, 3, or 7 days in 5% CO₂ in a Cryostar (Westbury, NY) incubator. Media was also made with complete media + 50 μ g/mL L-ascorbic Acid (vitamin C) alone, 2ng/mL IL-1 β alone, or both together.

Alizarin Red Staining

Alizarin red staining was performed and quantified as described [23]; briefly, plates were fixed in 4% paraformaldehyde (Sigma-Aldrich), rinsed for 5 minutes in d DI water, and stained with 2% alizarin solution (Sigma-Aldrich) at pH 4.1-4.3. The plates were then rinsed once for 20 minutes in d DI water, and a 10x image of each well was taken on a Nikon (Tokyo, Japan) Eclipse T100 microscope as well as a full plate scan at 300dpi with an Epson color scanner. The staining was then quantified after 10% acetic acid dissolution and ammonium hydroxide quenching on a TECAN (Mannedorf, Switzerland) Spectra Fluor Plus photo spectrometer at 405nm.

RT-qPCR Analysis

Total RNA was extracted from bNP or bNP-TNAP monolayer cultures in various culture conditions at 24 or 72 hours. Amplifications were carried out with Bio-Rad SYBR Green mix on a CFX96 RT System and C1000 Thermo cycler using qPCR

primers specific for bovine ANKH and ENPP1 (Table 1), for 40 cycles annealing at 60 C. Data were calculated as the ratio of each gene to RPL13a (housekeeping gene control) compared to a reference sample in complete media via $2(-\Delta\Delta Ct)$ qPCR

normalization. Replicates were different stocks of pass aged primary cells, plated in separate experiments and processed separately.

Table 1: Primers used for qPCR analysis (FWD/REV).

Gene	Sequence FWD/REV
Bovine ANKH	CCATGTGGATGAGTCAGTGG / GCACATCCAACCAGGAAACT
Bovine ENPP1	AATTGAGCGCTTGACGTTCT / TCAGTGCTGTGCTTGAATCC

Statistics

For alizarin testing, absorbance values were compared

within experiments through pair-wise t-t-test analysis. RT-PCR $\Delta\Delta Ct$ values were compared by student’s t-test.

Results

Cytokine IL-1 β Increases Calcification *In Vitro*

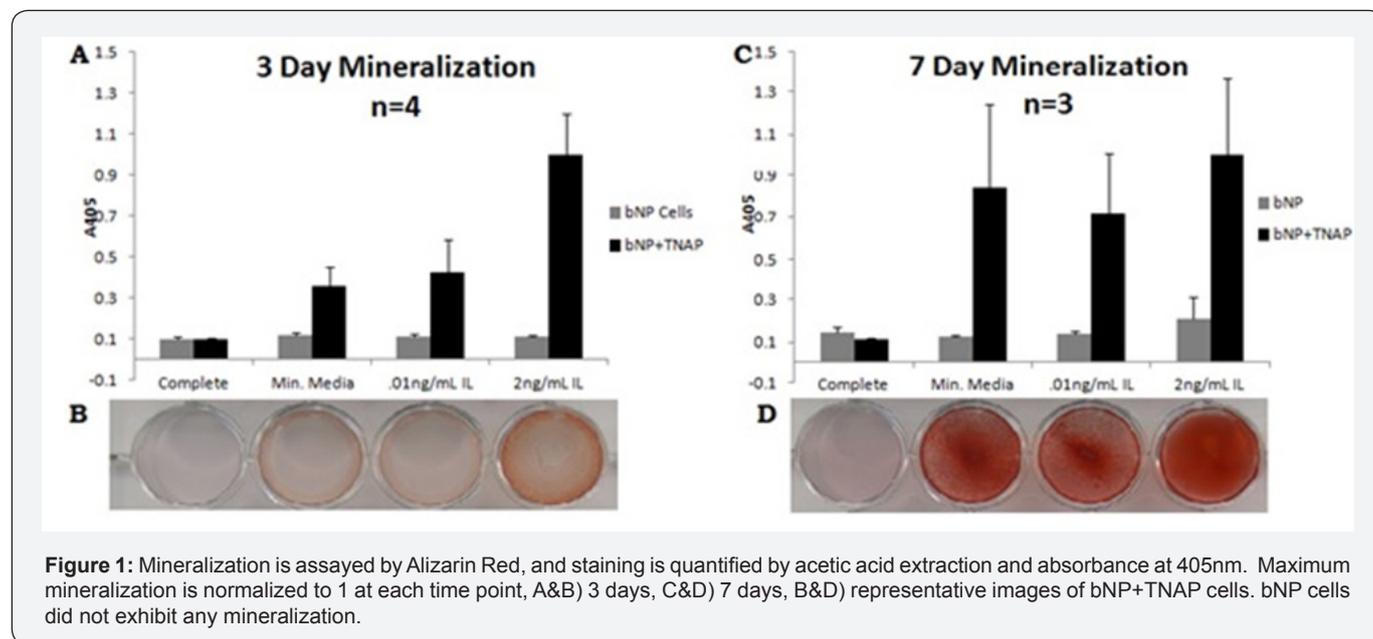


Figure 1: Mineralization is assayed by Alizarin Red, and staining is quantified by acetic acid extraction and absorbance at 405nm. Maximum mineralization is normalized to 1 at each time point, A&B) 3 days, C&D) 7 days, B&D) representative images of bNP+TNAP cells. bNP cells did not exhibit any mineralization.

To assess the mineralizing potential of TNAP-transduced cells we cultured bNP-TNAP cells in media enriched with β -glycerophosphate and vitamin C (Min Media) and doses of .01 or 2 ng/mL of IL-1 β . Calcification was observed in all wells containing mineralizing media whereas there was no calcification in wells with complete un-enriched (Comp) media alone; these wells stained positive for calcium deposition with Alizarin Red. The quantification (Figure 1A) of the plate stains (Figure 1B) showed a trend of increased mineralization at 3 days with cytokine vs without cytokine. Pair wise testing was performed on these samples as the variability between cell platings creates differences in the absolute magnitude of the staining. Students T tests yielded a p value of .06 describing the difference between cells in mineralizing media and mineralizing media with 2ng/mL IL-1 β . This effect did not persist at 7 days *in vitro* under the same conditions (Figure 1C & 1D) at which point all mineralizing treatment groups had more comparable calcium

deposition. The significance between the mineralizing media and 2ng/ml IL group yielded a p value of only .12 at 7 days.

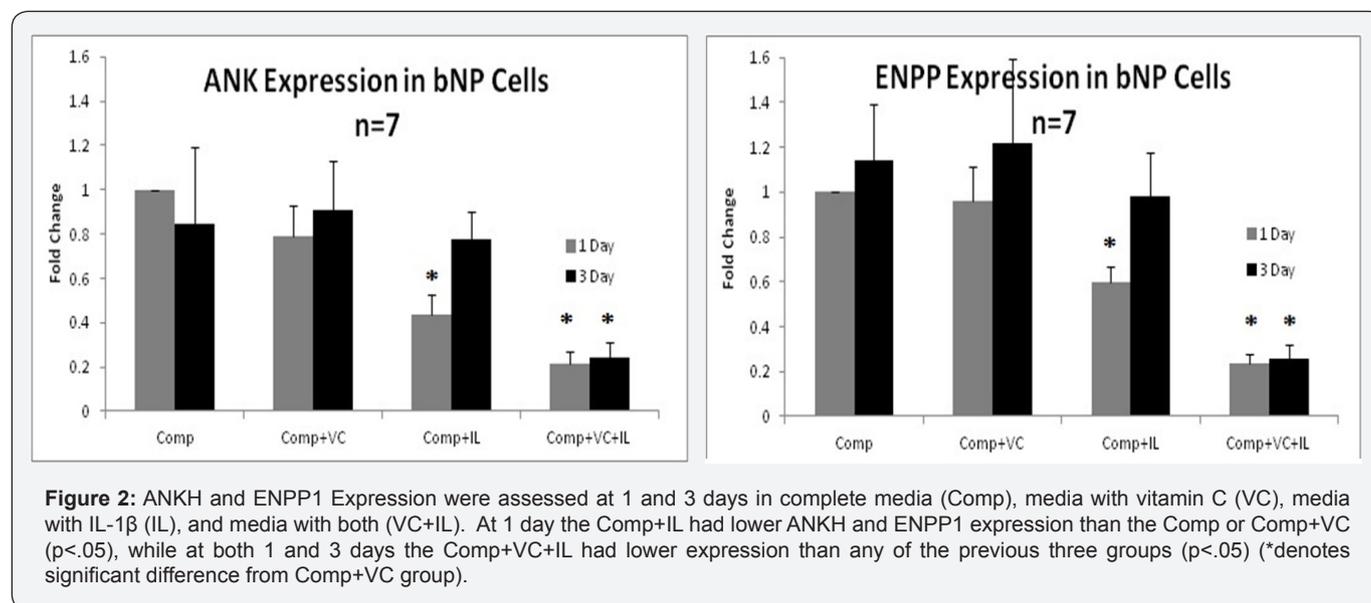
Cytokine IL-1 β Decreases Expression of Ankh And Enpp1, and Vitamin C Prolongs The Effect

Previous work has indicated that reduction in certain phosphate regulating genes may be observed with cytokine treatment³. Such proteins, like ANKH and ENPP1, increase extracellular pyrophosphate; a reduction in these proteins would lead to decreased pyrophosphate which would create an environment more amenable to mineralization. To determine whether this was the case in our bNP cells treated with IL-1 β , RNA was isolated at 1 and 3 days, and qPCR analysis was performed for ANKH and ENPP1.

There was no change in the expression of ANKH or ENPP1 between the complete media cell group and the Vitamin C treated group. The expression of both ANKH and ENPP1 was lower in the

IL-1 β and IL-1 β +Vitamin C group at 1 day (Students T test, $p < .05$). At 3 days only the treatment with IL-1 β +vitamin C maintained the low expression ($p < .05$), and none of the complete, vitamin C alone, nor IL-1 β alone had this reduction (Figure 2). Vitamin C alone did not significantly change the expression of either gene at either time. By 3 days, the IL-1 β treated group had recovered

its ANKH and ENPP1 expression (Figure 2), Comp+ IL, grey vs black bar. Both genes responded to the treatments in the same way suggesting a common regulation; the gene expression with IL-1 β +vitamin C was only 20% of what is in complete media, a drastic reduction in message production.



Discussion

IL-1 β enhances the calcium deposition of cells over-expressing TNAP

IL-1 β is a well-characterized inflammatory cytokine believed to be a master regulator of catabolic processes in the intervertebral disc [24,25]. Naive bNP cells in monolayer culture will not mineralize at any time due to a phenotypic gene expression maintaining soft tissue. The bNP-TNAP cell cultures show detectable calcium uptake *in vitro* beginning as early as 3 days with mineralizing culture media [22], quicker than most mineralizing cell lines [26]. The induction of mineralization in these cells is enhanced in the presence of 2ng/ml IL-1 β compared to a lower dose (.01ng/ml) or no cytokine at all. This makes these cells a compelling model for studying soft tissue mineralization *in vitro*.

Deposition at 7 days was comparable between doses of IL-1 β (0, .01, & 2ng/mL) suggesting that the role cytokines play in altering mineralization is acute. There are several suggested mechanisms for how this alteration works. Lencel suggests that cytokines such as TNF and IL-1 β directly alter alkaline phosphatase activity making TNAP more enzymatic ally active in vascular smooth muscle cells, but less active in in situ ethereal chondrocytes and *in vitro* mouse and human chondrocytes. These findings make it difficult to explain changes in mineralization by TNAP effects alone.

The same group published that cytokines in mesenchymal stem cells increase alkaline phosphatase activity but

block expression of RUNX2, a transcriptional regulator of mineralization, and OCN, the calcium-regulating modulator of mineral formation [16]. Our work and others' show that cytokines can alter expression of mineralization-regulating genes like ANKH and ENPP1, which could change the extracellular environment sufficiently to affect mineralization [3]. Reduction in ANKH protein levels could explain a loss of the characteristic soft-tissue phenotype in NP cells. The decrease in ANKH expression and activity is not sufficient to induce mineralization but it may counteract the negative regulation on mineralization in the NP milieu.

IL-1 β Suppression of Ankh And Enpp1 creates A Calcifying Environment By Altering Ppi Levels

Calcium phosphate is generated from inorganic phosphates and calcium in a hydrolytic reaction. Inorganic pyrophosphate itself blocks the formation of calcium phosphate crystals [1]. At the gene expression level we observed that treatment of bNP cells in mineralizing media with IL-1 β reduces the mRNA levels of ANKH and ENPP1 at 1 and 3 days. This supports the hypothesis that cytokines alter the extracellular environment of mineralizing cells by suppressing the proteins responsible for maintaining high extracellular PPI, preserving soft tissue characteristic. Lowering PPI outside the cell relieves the inhibition of mineralization normally present in soft tissue.

Further experimentation is required to determine the actual change in pyrophosphate concentration and whether that alone accounts for the changes in calcium uptake. Both Pi and PPI can

become toxic at high doses; phosphate-induced apoptosis has been described related to nitric oxide generation [1,27]. Thus there is strict physiological regulation of both Pi and PPI. This process is also regulated by several other factors, such as TGF- β in tandem with calcium²⁷ therefore it will be informative to study the interplay of cytokines and growth factors on the overall effects on mineralization, especially when trying to describe *in vivo* behavior.

This study did not address the roles of PHEX, OPN, BGP, or MGP although literature suggests cytokines would play a role in regulating them either directly or through modulation of transcription factors such as RUNX2 or DLX. The regulations described here directly affect pyrophosphate levels, though obviously mineralization may be regulated through phosphate or calcium modulation.

Vitamin C is required in the media for a prolonged suppression of ANKH and ENPP1

The data show that IL-1 β alone does reduce the expression of both ANKH and ENPP1 mRNA at 1 day; however that suppression is stronger in the presence of vitamin C. By three days the reduction in ANKH and ENPP1 mRNA is reversed with IL-1 β treatment alone, however the suppression is maintained at close to 20% of baseline expression with vitamin C and IL-1 β . Vitamin C alone does not have a notable effect altering the gene expression or the detectable calcium deposition; however vitamin C has been noted for its anti-inflammatory effects. Bowie et al. showed that vitamin C acts through p38 to inhibit NF- κ B [18]. This suggests that the cytokines role in reducing ANKH and ENPP1 may not be linked to the inflammatory response; this phenomenon could rather be from a stabilization of an alternative cytokine effect.

Vitamin C is also essential to chondrocytes undergoing hypertrophy as it regulates Collagen II and X expression and alkaline phosphatase activity [17]. While bNP-TNAP cells have high constitutive AP activity, vitamin C may well still act as a stimulator of further AP activity. Unlike humans, most animals including cows synthesize their own vitamin C through the enzymatic activity of L-gulonolactone oxidase (GULO), however the primary vitamin C-producing cells are hepatocytes in the liver [28]; it is not surprising that there is no detectable vitamin C synthesis in the bNP cells under any conditions. Therefore the high vitamin C supplemented in the media is required for the effect noted and is unrelated to endogenous vitamin C.

Conclusion

The bNP-TNAP cells are a model of rapid soft tissue calcification. Cytokines can increase the strength of the phenotypic shift towards calcified tissue through reduction of soft-tissue regulators ANKH and ENPP. The presence of vitamin C may relieve the inflammatory component of cytokine signaling and stabilize the change in mRNA expression, as well as stimulate alkaline phosphatase activity and enhance

necessary collagen synthesis. This work should be of interest to basic scientists and clinician-scientists for of understanding the mechanisms underlying the mineralization of soft tissue, namely the NP in the intervertebral disk. Further description of how vitamin C contributes to the regulation of these important proteins could inform treatment of fractures, fusion surgery, and DDD in general.

Availability of Data and Material

Data will not be made available yet, pending publication of the remaining work in the lab, reference #22.

Competing Interests

We have no competing interests, financial or otherwise, to declare.

Authors Contribution

R. Frawley performed the majority of these experiments and analyses. A. Krzyzanowska prepared the cell lines and guided experimental design. M. Cunningham oversaw and funded all experiments.

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