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The Therapeutic Potential of Exogenous Adenosine Triphosphate (ATP) for Cartilage Tissue Engineering

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Abstract

Objective: While mechanical stimuli can be used to enhance the properties of engineered cartilage, a promising alternative may be to directly harness the underlying mechanotransduction pathways responsible. Our initial studies on the adenosine triphosphate (ATP)–purinergic receptor pathway demonstrated that stimulation by exogenous ATP improved tissue growth and properties but elicited matrix turnover under high doses (250 μM) potentially due to the accumulation of extracellular inorganic pyrophosphate (ePPi). Therefore, the purpose of this study was to identify the mechanism of ATP-mediated catabolism and determine a therapeutic dose to maximize the anabolic effect. *Design:* Isolated bovine articular chondrocytes were seeded in high-density, 3-dimensional culture supplemented with varying doses of ATP for 4 weeks. The effects on biosynthesis, matrix metalloproteinase 13 (MMP-13) protein activity, and PPi accumulation were determined. Separate monolayer experiments were conducted to determine the effect of ePPi on MMP-13 activity. *Results:* High doses of ATP resulted in an increase in ePPi accumulation (by 54%) and MMP-13 activity (by 39%). Monolayer experiments confirmed a link between increased ePPi accumulation and MMP-13 activity, which appeared to require calcium and was inhibited by the MEK1/2 inhibitor U0126. Cultures supplemented with 62.5 to 125 μM ATP favored an anabolic response, which represented the therapeutic dose range. *Conclusions:* A therapeutic dose range of exogenous ATP to improve the properties of engineered cartilage has been identified, and a possible catabolic mechanism involving excess PPi was determined. Future research into PPi signal transduction and pathological crystal formation is necessary to maximize the beneficial effect of exogenous ATP on chondrocyte cultures.

Keywords

cartilage tissue engineering, adenosine triphosphate (ATP), calcium pyrophosphate dihydrate (CPPD), inorganic pyrophosphate (PPi), mechanotransduction

Introduction

The properties of articular cartilage allow for nearly frictionless motion in joints and the capacity to absorb large loads.[1] Unfortunately, when cartilage is damaged, it cannot adequately repair itself to recover its prior function.[1] Tissue engineering, a promising approach to repair damaged cartilage, currently falls short of creating functional tissue.[2] Typically, tissue-engineered constructs exhibit inferior mechanical properties in comparison to native articular cartilage primarily due to the insufficient accumulation of extracellular matrix (ECM) components.[3-6] Cartilage depends on mechanical forces for the development and maintenance of the ECM at a specific ratio of collagen to proteoglycans (typically between 2:1 and 3:1), which contributes to its unique mechanical properties.[1,5] However, it is neither simple nor efficient to mechanically stimulate tissues in culture due to the limitations on the size and shape of constructs that can be grown. Targeting the underlying mechanotransduction pathways responsible as a means to create functional tissue is an alternative strategy for tissue engineering that has been explored recently by our group.[7]

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One molecule that appears to be integral to one particular chondrocyte mechanotransduction pathway is adenosine triphosphate (ATP), termed the purinergic receptor pathway. ATP signaling has been implicated in mechanotransduction pathways in many different cell types including epithelial cells, astrocytes, osteoblasts, and chondrocytes.[8] In articular chondrocytes, ATP is the first known molecule to be released as a result of mechanical stimulation and acts as an autocrine/paracrine signaling molecule.[9] ATP acts on P2 receptors on the plasma membrane to promote ECM synthesis.[10] While other nucleotides also have similar affinity for P2 receptors, such as uridine triphosphate (UTP) and ATP analogs,[10] supplementation with exogenous ATP or UTP was shown to induce similar effects on ECM synthesis in bovine chondrocyte pellet cultures.[11] Previously, we also observed that the addition of exogenous ATP to engineered cartilage cultures *in vitro* stimulated both collagen and proteoglycan synthesis and significantly improved the mechanical properties of the developed tissue.[7] However, there appeared to be a dose effect of ATP on the cartilage cultures that warranted further investigation. High doses of ATP (250 μM), while eliciting increased matrix synthesis, did not result in increased ECM accumulation.[7] Additionally, gene expression of matrix metalloproteinase 13 (MMP-13 or collagenase 3) was 2 times greater under high doses of ATP. Similarly, in other studies, high doses of exogenous ATP are known to evoke the release of inflammatory mediators,[12,13] initiate matrix turnover,[14,15] and induce mineralization.[16] Undesirable effects, such as the mineralization of articular cartilage, have been associated with an accumulation of extracellular inorganic pyrophosphate (ePPi), which is a by-product of ATP degradation.[17] In physiological media, low concentrations of ePPi acts to inhibit mineralization, while excess ePPi has been implicated in the formation of calcium pyrophosphate dihydrate (CPPD) crystals.[17] Although the formation of CPPD crystals is poorly understood, these crystals can be a sign of pathological mineralization in the joint (chondrocalcinosis) and are associated with joint pain and subsequent cartilage degradation.[18,19]

There is evidence for 2 potential signaling pathways that connect the presence of CPPD crystals to increased matrix turnover. CPPD crystals either bind to toll-like receptors (TLRs) on the plasma membrane through a phosphatidylinositol 3kinase (PI3K)–dependent pathway[20,21] or can be potentially endocytosed and elicit changes through a mitogen-activated protein kinase (MAPK)–dependent pathway.[22] Therefore, the purpose of this study was to identify the mechanism of ATP-mediated catabolism and to determine a therapeutic ATP dose range for engineered cartilage to maximize the anabolic versus catabolic response.

Materials and Methods

Cell Isolation, 3-Dimensional Culture, and Exogenous ATP Supplementation

Tissue-engineered cartilage constructs were generated from isolated chondrocytes harvested from calf (12-18 months old) metacarpal-phalangeal articular cartilage obtained from a local abattoir after slaughter (Brian Quinn's Meats Ltd., Yarker, ON, Canada) by sequential enzymatic digestion, as described previously.[5,23] To minimize interanimal variability, tissue was obtained from several joints (up to 4 per experiment) and pooled together. Isolated cells were seeded on the surface of type II collagen–coated Millicell filters (Millipore, Billerica, MA) in high-density, 3-dimensional (3-D) culture (2×10^6 cells/filter or 35,000 cells/mm^2)[23] and maintained in Ham's F12 media containing 10 mM glucose supplemented with 20% fetal bovine serum (FBS), 100 μg/mL ascorbate, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid) (Sigma Aldrich Ltd., St. Louis, MI). Two days after seeding, cultures were supplemented with varying doses of ATP (0, 62.5, or 250 μM) (Sigma-Aldrich Ltd.).[7] The culture medium was changed every 2 to 3 days, and fresh ascorbic acid and ATP were added at each change for a period of 4 weeks. For subsequent experiments involving the determination of MMP-13 activity, serum levels were reduced from 20% to 5% at the final media change to limit interference with the MMP-13 activity assay. Cultures were grown in an incubator maintained at 37 °C and 95% relative humidity supplemented with 5% CO_2:95% atmospheric air. Experiments were repeated twice using separate cell isolations ($n = 8$).

MMP-13 Protein Activity

Intracellular protein was extracted from the 3-D cultured constructs according to a protocol adapted from Nielsen *et al*.[24] After harvest, samples were snap frozen in liquid nitrogen and pulverized. Samples were then homogenized (twice for 30 seconds each) (Power Gen 125, Fisher Scientific, Hampton, NH) in the presence of extraction buffer (50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, and 0.1% Triton X-100 (v/v)) supplemented with one Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics Canada, Laval, QC, Canada) per 10 mL of buffer. The samples were centrifuged at 1700*g* for 10 minutes at 4 °C. Afterwards, the supernatants were harvested and further centrifuged at 10,000*g* for 10 minutes at 4 °C and then stored at –20 °C until analysis.

To assess the amount of active MMP-13 in the tissue, a FRET-based assay outlined by Knauper *et al.* was used.[25] The Mca MMP-13 FRET fluorogenic substrate (MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2) (AnaSpec Inc., Fremont, CA) consists of a fluorophore coupled to a quencher, which

fluoresces when cleaved by MMP-13. Equal volumes of the protein extracts and Mca MMP-13 FRET substrate in 20% DMSO (v/v) were mixed with assay buffer (1:4, 200 mM NaCl, 50 mM Tris base $CaCl_2$, 20 μM $ZnSO_4$, and 0.05% BRIJ 35 (w/v), pH 7.5) and incubated at 37 °C with 95% humidity for 18 hours in the dark. After incubation, fluorescence was measured at 325-nm excitation and 393-nm emission with Mca MMP FRET fluorescence peptides (MCA-Pro-Leu-OH) reconstituted in 20% DMSO (v/v) used as assay standards.

Accumulation of ePPi

The amount of ePPi was measured from tissue digests using a protocol modified from Justesen *et al.*, which makes use of a coupled enzymatic reaction resulting in the formation of NADPH from PPi.[26] Briefly, aliquots of papain-digested tissues were incubated with triethanolamine acetate buffer (70 mM triethanolamine, 20 mM magnesium acetate, 20 mM potassium acetate, 2 mM EDTA, 2 mM DTT, pH 7.6) and an enzyme mixture (30% triethanolamine acetate buffer (v/v), 3 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 2 mM uridine diphosphoglucose (UDPG), 30 μM glucose 1,6-diphosphate, 7.5 U UDPG pyrophosphorylase from beef liver, 5 U phosphoglucomutase from rabbit muscle, and 4 U glucose-6-phosphate dehydrogenase from yeast) for 5 minutes at 37 °C. Fluorescence was measured at 340-nm excitation and 460-nm emission, with NaPPi in papain digestion buffer used as assay standards. ePPi was also measured from the conditioned culture media using the same methods outlined above, with NaPPi in culture media as standards.

Optimization of ATP Dose and Assessment of ECM Synthesis

ECM synthesis was initially assessed in the 3-D cultured constructs as a result of 0, 62.5, and 250 μM ATP stimulation ($n = 8$). In 2 additional experiments investigating ECM synthesis (n = 7-8 samples per group), engineered cartilage constructs were cultured in a range of exogenous ATP doses (0, 31.25, 62.5, 125, or 250 μM) to determine the optimal therapeutic dose based on maximizing the anabolic response and minimizing the catabolic response. After 4 weeks of culture, half of the cultures were assessed for MMP-13 activity (using the protocol described previously), and the remaining half of the cultures were assessed for ECM synthesis, as follows. During the final 24 hours of the 4-week culture period, tissue constructs were incubated in the presence of both [^{35}S] SO_4 (5 μCi/culture) to label proteoglycans[27] and [^{3}H] proline (5 μCi/culture) to label collagen.[28] Unincorporated isotopes were removed by gently washing the samples 3 times in phosphate-buffered saline. Constructs were then digested by papain (40 μg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM DTT) for 48 hours at 65 °C. The accumulation of newly synthesized proteoglycans and collagen was then estimated by quantifying radioisotope incorporation from aliquots of the papain digest using a β liquid scintillation counter (Beckman Coulter LS6500, Mississauga, ON). The amount of synthesized molecules was calculated relative to the DNA content of the tissue, determined from aliquots of the papain digest using the Hoechst dye 33258 assay[29] and expressed as a percentage of the unstimulated controls.

The Effect of PPi on MMP-13 Activity

Chondrocyte monolayer cultures were established using primary isolated cells from calf (12-18 months old) metacarpal-phalangeal articular cartilage (as described previously). Cells were seeded in 6-well plates (100,000 cells/well) with Ham's F12 media containing 5% FBS. Culture media were changed every 2 to 3 days until a final cell density of approximately 300,000 cells/well was obtained (typically 1 week). Due to interference of serum with the MMP-13 activity assay, serum concentrations were gradually reduced prior to addition of PPi and determination of MMP-13 activity. Cultures were then washed and incubated in Ham's F12 media containing 1% FBS 18 hours before the addition of PPi. One hour before the addition of PPi, cultures were washed and incubated with serum-free Ham's F12 media containing 1X ITS. After 1 hour of incubation, NaPPi (0, 1, or 10 μM) was added to the cell cultures and incubated for a period of 24 hours. Conditioned media from the cell cultures were then harvested and stored at –80 °C prior to analysis of MMP-13 activity. Individual cell cultures were subsequently incubated with trypsin and scraped, and cell counts were obtained using a hemocytometer (Hausser Scientific Bright-Line, Horsham, PA). Additional monolayer experiments were conducted to investigate potential underlying mechanisms responsible for increased MMP-13 activity in response to ePPi. One hour prior to the addition of NaPPi, cultures were incubated in the presence of either 50 μM LY294002 (PI3K pathway inhibitor) or 20 μM U0126 (MEK1/2 pathway inhibitor) with the equivalent volume of carrier DMSO as a control. To assess the contribution of calcium, separate monolayer experiments were also carried out in the presence of calcium-free Ham's F12 media ($N = 3$; $n = 10$ samples per group)

Transmission Electron Microscopy (TEM)

To determine the presence of CPPD crystal accumulation in the engineered tissue constructs as a result of exogenous ATP, separate engineered cartilage constructs from each exogenous ATP condition were fixed in 2% glutaraldehyde (v/v) in 0.1 M sodium cacodylate buffer, pH 7.3, after

harvest. After 1 hour at room temperature, samples were transferred to 4 °C overnight. Samples were then processed for embedding, sectioning, and TEM. After fixation, the samples were rinsed with 0.1 M sodium cacodylate buffer with 0.2 M sucrose, pH 7.3, and then postfixed with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium cacodylate buffer, pH 7.3, for 1.5 hours. Samples were rinsed again in 0.1 M sodium cacodylate buffer with 0.2 M sucrose, pH 7.3, dehydrated in a series of graded ethanol solutions, and infiltrated with a 1:1 propylene oxide:Quetol-Spurr resin mixture. After infiltration, samples were placed in embedding molds and polymerized in an oven at 65 °C overnight. Thin sections (90 nm) were cut using an ultramicrotome (RMC model MT600, Boeckeler Instruments Inc., Tucson, AZ) and stained with uranyl acetate and lead citrate. TEM imaging was performed using a FEI Tecnai 20 transmission electron microscope (FEI, Hillsboro, OR).

Statistical Analyses

All results were expressed as the mean ± standard error of the mean (SEM). Collected data were analyzed statistically using a 1-way ANOVA (independent samples) and the Fisher least significant difference *post hoc* test (SPSS version 16, SPSS Inc., Chicago, IL). The data were checked prior to performing statistical tests for both normality and equal variance. Significance was associated with P values less than 0.05. Trends were noted with P values between 0.1 and 0.05.

Results

Effect of Exogenous ATP on MMP-13 Protein Activity and Accumulation of PPi

MMP-13 protein activity was assessed from the 4-week-old cartilage cultures using a FRET-based assay. There was a dose-dependent effect of ATP supplementation on MMP-13 activity in the engineered cartilaginous tissues (**Fig. 1**). MMP-13 activity was increased by 19% ± 3% and 39% ± 7% with 62.5 and 250 μM of ATP, respectively ($P < 0.05$) (**Fig. 1**).

Levels of PPi were measured in the conditioned media and tissue constructs after long-term culture (4 weeks) to elucidate the effect of ATP stimulation on PPi homeostasis. Only under the high dose of ATP (250 μM) was there a significant increase in media PPi (increase of 54% ± 11%, $P < 0.01$) compared to the low dose (62.5 μM) and controls (**Fig. 2A**). Alternatively, tissue PPi did not appear to be affected by ATP stimulation and exhibited no discernable effect among the exogenous ATP conditions (**Fig. 2B**). While the baseline (control) level of PPi in the media and in the tissue varied with each experiment, media PPi was typically higher on average compared to tissue PPi (media PPi: ~0.5 μg PPi/μg DNA; tissue PPi: ~0.2 μg PPi/μg DNA).

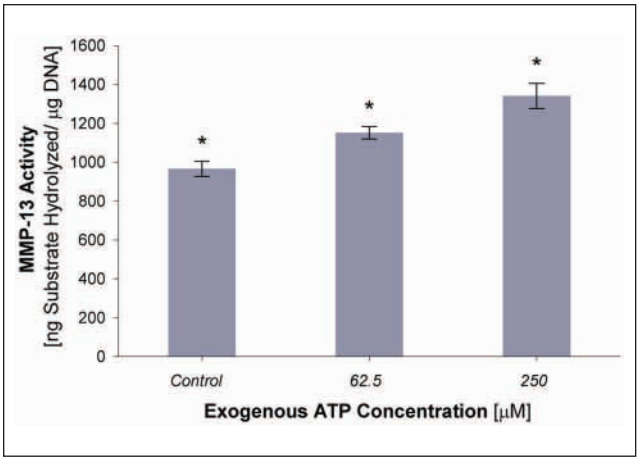

Figure 1. Effect of long-term (4 weeks) ATP supplementation on MMP-13 activity. Data are presented as the mean ± SEM ($N = 2$, $n = 8$ samples per group). *Denotes a significant difference between all other groups ($P < 0.05$).

Determination of Optimization of ATP Dose

To determine a therapeutic dose of ATP, a range of doses (0, 31.25, 62.5, 125, and 250 μM ATP) were supplemented to the engineered cartilage constructs over a 4-week period. Harvested constructs were either assessed in terms of ECM synthesis (collagen and proteoglycans) or MMP-13 activity.

Both collagen and proteoglycan synthesis (determined from [^{3}H] proline and [^{35}S] sulfate incorporation, respectively) appeared to follow the same general trends in response to exogenous ATP. ECM synthesis was slightly inhibited under low doses of ATP (31.25 μM) and was increased in response to intermediate doses of ATP (62.5 and 125 μM). Upon higher doses of exogenous ATP (250 μM), ECM synthesis was further increased compared to lower doses and the unstimulated controls ($P < 0.05$) (**Fig. 3**).

MMP-13 activity was also affected by exogenous ATP and appeared to follow the same general trends of ECM synthesis. MMP-13 activity increased (between 20% ± 4% and 13% ± 3%) under intermediate doses of ATP (62.5 and 125 μM), which was further increased (41% ± 6% increase) in response to higher doses of ATP (250 μM) compared to the lower doses and the unstimulated controls ($P < 0.01$) (**Fig. 4**).

Potential Mechanism of ATP-Mediated Catabolism

Chondrocyte monolayer cultures were established to determine the effect of PPi on MMP-13 activity. In response to

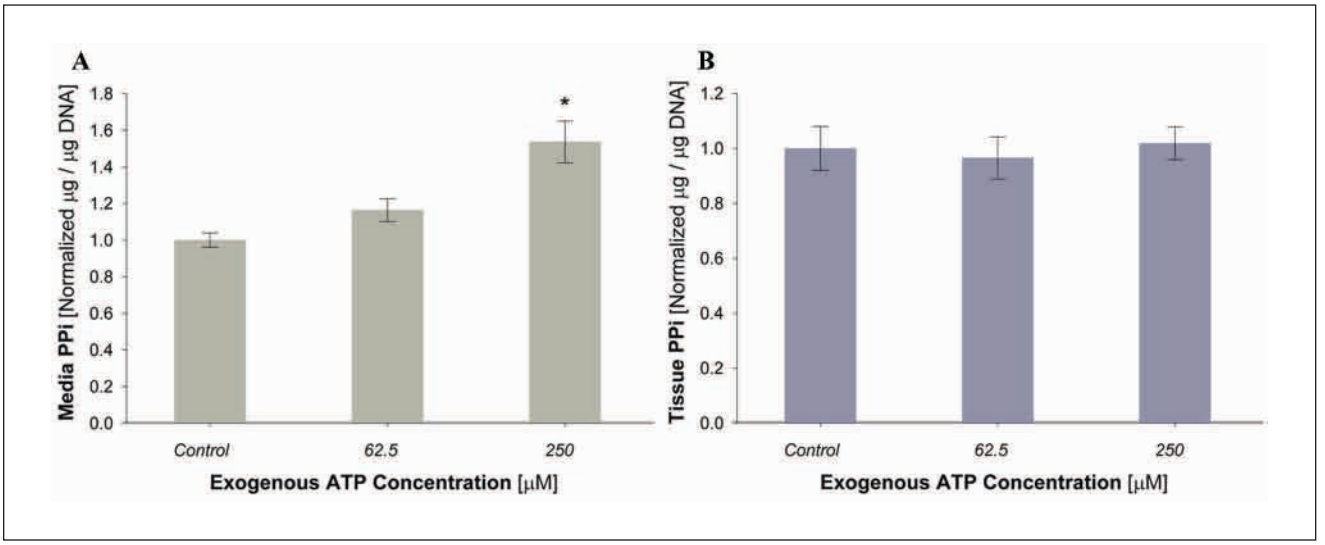

Figure 2. Effect of long-term ATP supplementation on PPi levels in the (**A**) conditioned culture media and (**B**) within the developed tissues. Data (normalized to unstimulated controls) are presented as the mean ± SEM ($N = 4$, $n = 14$-16 samples per group). *Denotes a significant difference between all other groups ($P < 0.05$).

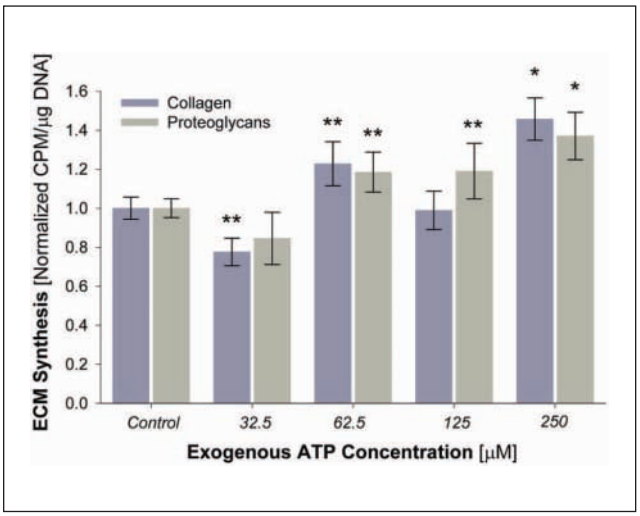

Figure 3. Combined ECM synthesis results for the optimization of ATP dose. Data (normalized to unstimulated controls) are presented as the mean ± SEM ($N = 2$, $n = 7$-8 for 31.25- and 125-µM groups; $N = 4$, $n = 15$ for 0-, 62.5-, and 250-µM groups). *Denotes a significant difference from control ($P < 0.05$). **Denotes a trend from control ($P < 0.09$).

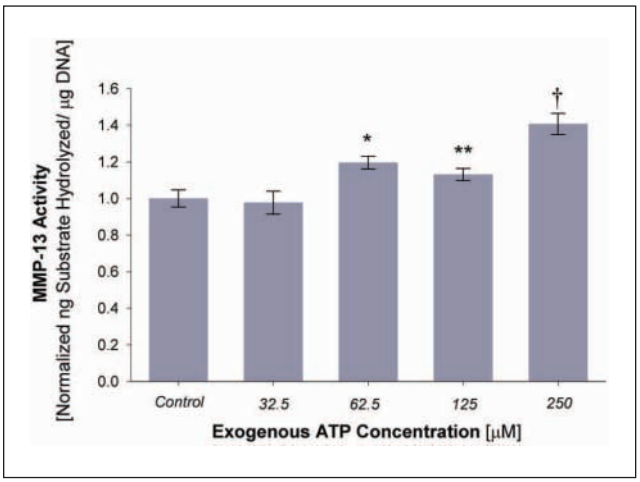

Figure 4. MMP-13 activity optimization. Cultures were grown from the same batches of cells as for the ECM synthesis optimization data. Data (normalized to unstimulated controls) are presented as the mean ± SEM ($N = 2$, $n = 8$ samples per group). †Denotes a significant difference from all other doses ($P < 0.01$). *Denotes a significant difference from control ($P < 0.05$). **Denotes a trend from control ($P < 0.08$).

10 µM PPi, chondrocyte cultures expressed a 32% ± 10% increase in MMP-13 activity compared to the unstimulated controls ($P < 0.05$). In contrast, lower doses of PPi (1 µM) did not appear to have any differential effect on MMP-13 activity (**Fig. 5**).

The potential mechanism behind the PPi-mediated increases in MMP-13 activity was investigated through the use of the MEK1/2 and PI3K pathway inhibitors U0126 and LY294002, respectively. Increased MMP-13 activity in response to exogenous PPi was not abolished in the presence of the PI3K pathway inhibitor (LY294002) ($P < 0.05$) but was effectively abolished in the presence of the MEK1/2 pathway inhibitor (U0126) (**Fig. 6**). To determine whether the presence of calcium influenced PPi-mediated MMP-13 activity, additional experiments were performed in the presence of calcium-free media. In the absence of calcium, there was no significant effect of PPi on MMP-13 activity (10 µM PPi: 55 ± 3 ng substrate hydrolyzed per 10,000 cells; 0 µM

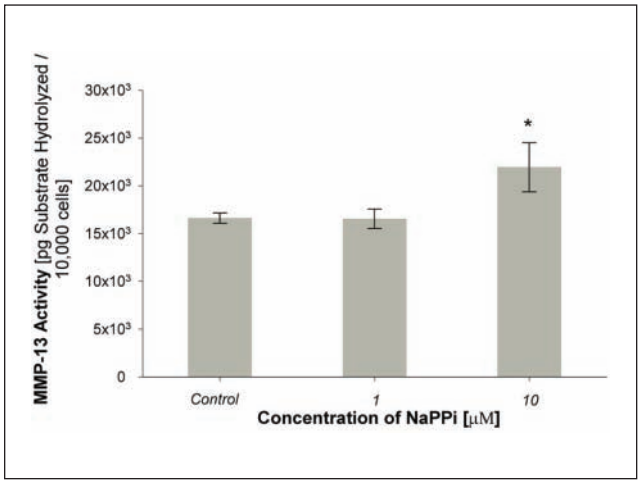

Figure 5. Effect of NaPPi supplementation on chondrocyte monolayer cultures. Data are presented as the mean ± SEM ($N = 2, n = 5$-6 samples per group). *Denotes a significant difference from control ($P < 0.05$).

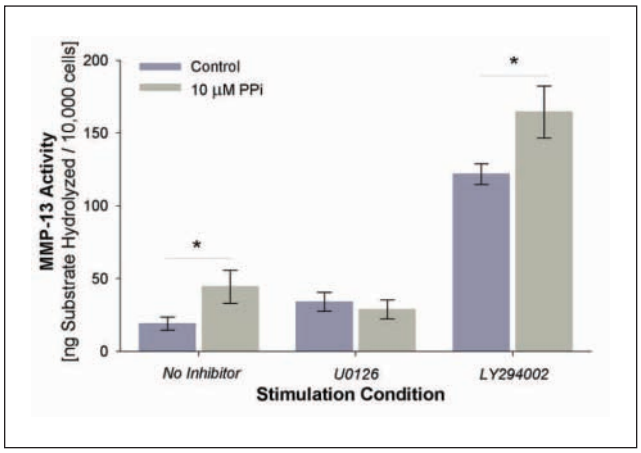

Figure 6. Effect of U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor) on PPi-induced MMP-13 activity. Data are presented as the mean ± SEM ($N = 2$, $n = 6$ samples per group). *Denotes a significant difference between control and PPi conditions ($P < 0.05$). **Denotes a significant difference between no inhibitor and inhibitor conditions ($P < 0.05$).

PPi (control): 57 ± 8 ng substrate hydrolyzed per 10,000 cells) ($P > 0.4$).

TEM imaging was conducted to detect the presence of CPPD crystals in engineered cartilaginous tissues supplemented with 0, 62.5, and 250 μM ATP; however, no mineralization was observed (**Fig. 7**). The appearance of cells within the constructs was also similar between the different ATP conditions.

Discussion

The application of mechanical loading to engineered cartilage constructs is a widely used method to enhance tissue growth and mechanical properties.[3-6] Although this approach has been highly successful, there are limitations in applying mechanical stimuli to anatomically shaped constructs with irregular geometry and/or high radii of curvature. Alternatively, by harnessing the known mechanotransduction pathways responsible, it may be possible to achieve the same effect in the absence of externally applied forces. In a recent study, we demonstrated that direct stimulation of the ATP-purinergic receptor pathway through exogenous supplementation of ATP can elicit a comparable anabolic response and be used to improve both tissue growth and mechanical properties of the developed tissue.[7] However, high doses of ATP (250 μM) resulted in a simultaneous catabolic response characterized by an increase in MMP-13 expression, potentially due to the accumulation of ePPi.[7] In the present study, we have determined a therapeutic dose range of exogenous ATP to maximize the anabolic response and ascertained that ATP-mediated matrix turnover was most likely a result of the formation and endocytosis of calcium-containing crystals from accumulated ePPi in the culture media.

ATP can be catabolized by soluble (e.g., tissue nonspecific alkaline phosphatase, tissue transglutaminase) and/or membrane-bound (e.g., nucleotide pyrophosphatase/phosphodiesterases, ecto-5′-nucleotidase) nucleotide-degrading enzymes after P2 (purinergic) receptor binding, both leading to the formation of ePPi. PPi, measured from the conditioned culture media after 4 weeks of nucleotide exposure, was significantly increased in response to ATP stimulation. This is consistent with previous studies that have also detected an increase in ePPi from conditioned culture media of both porcine explants and chondrocytes in monolayer culture as a result of P2 receptor signaling.[30] There is a very narrow range for the physiological ePPi concentration in normal human articular cartilage, with synovial fluid in the knee containing 10 ± 0.5 μM PPi (determined over 50 individuals).[31] The average basal level of PPi detected in the conditioned media surrounding 3-D cultures in the present investigation was similar to these values, at around 10 μM. Because 250 μM ATP supplementation increased MMP-13 activity along with the increased presence of ePPi, accumulated ePPi could have contributed to the catabolic effects observed under high doses of exogenous ATP. PPi levels, determined from the digests of the 3-D cultured constructs, were also investigated to gain a better understanding of PPi homeostasis in the vicinity of cells. No significant differences were observed in the amount of PPi present in the tissue digests, suggesting that excess ePPi was most likely being utilized and prompted our mechanistic investigation into how PPi was contributing to ATP-mediated catabolism.

In order to determine an optimal dose of exogenous ATP for the 3-D cultured constructs, additional doses flanking 62.5 μM were investigated (31.25 and 125 μM). MMP-13 activity was chosen as the measure of catabolism because it

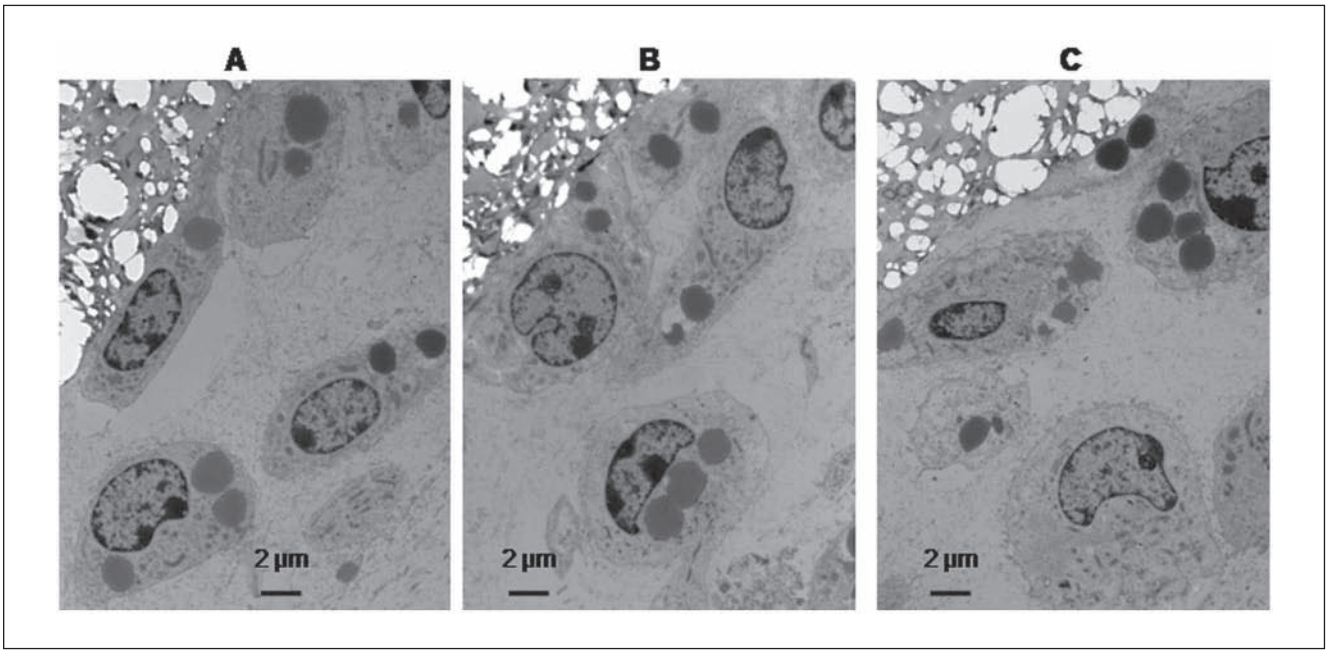

Figure 7. Representative TEM images of cartilaginous tissues grown for 4 weeks in (**A**) the absence of ATP, (**B**) the presence of 62.5 μM ATP, and (**C**) the presence of 250 μM ATP ($N = 2$, $n = 3$ samples per group). Magnification: 5,000x. The top left corner of each image was the porous Millipore filter that the cartilaginous tissues were grown on.

was the only matrix-degrading enzyme that was observed to be stimulated as a result of ATP stimulation.[7] However, the limitation of this approach is that MMP-13 activity may not necessarily reflect all of the catabolic processes at work. Similarly, ECM synthesis determined at the end of the culture period (4 weeks) only reflects the anabolic response over the final 24-hour period after long-term ATP exposure. For this reason, conclusions were derived by comparing patterns of MMP-13 activity and ECM synthesis with the long-term tissue formation and properties observed previously.[7] The patterns for both ECM synthesis and MMP-13 activity as a result of ATP stimulation were strikingly similar. ECM synthesis and MMP-13 activity were observed to increase with increasing ATP dose and stayed relatively stationary between 62.5 and 125 μM. Previously, it was observed that ATP-stimulated cultures (62.5 and 250 μM) yielded tissue with properties that were more indicative of native cartilage, with indentation moduli 5 to 6.5 times larger than the unstimulated controls.[7] Although the long-term accumulation of collagen and proteoglycans was not investigated at 125 μM, the mechanical properties as a result of 62.5 and 250 μM did not significantly differ from each other, leading to the assumption that 125 μM ATP would elicit a similar effect on mechanical properties. Therefore, the dose range of ATP between 62.5 and 125 μM was determined to be optimal for maximizing the anabolic effect and minimizing the catabolic effect of exogenous ATP for engineered cartilaginous constructs in our particular experiments. It should be mentioned that due to interference with the MMP-13 activity assay, serum concentrations needed to be reduced in these studies. While this may affect comparison of the results, serum concentrations were only reduced during the final media change (48 hours) of the 4-week culture period, suggesting that any potential effects would be minimal.

Excess ePPi has been shown to induce an increase in MMP-13 gene expression in monolayer culture of bovine articular chondrocytes.[20] In the present study, MMP-13 activity was increased in response to 10 μM PPi, but not 1 μM PPi, through a MAPK-dependent pathway. This provides evidence that relatively small changes in ePPi concentration can lead to significant increases in MMP-13 activity of chondrocytes. Although PPi may appear to be a potential candidate for eliciting catabolism, its method of action is unclear. There is no known direct route of entry for ePPi into the cell,[17] and the accumulation of intracellular PPi as a result of multiple intracellular reactions[32] is only cleared from the cytosol into the extracellular space by the 1-way transmembrane channel, progressive ankylosis protein (ANK).[17,33] In addition, there is currently no known channel or receptor that actively transports or binds ePPi to induce intracellular signaling pathways.

When monolayer cultures were exposed to ePPi in calcium-free media, the previously observed PPi-mediated increases in MMP-13 activity did not manifest. This result implicates calcium and pyrophosphate as key factors involved in the PPi-mediated catabolic response. PPi mineralization could potentially explain this phenomenon. CPPD crystals have been shown to spontaneously form at relatively low concentrations of PPi (>10 μM) in the

presence of calcium (as well as being influenced by pH and the concentrations of sodium and magnesium).[34] CPPD crystals can bind to TLRs, specifically TLR2, on the plasma membrane and elicit MMP-13 gene expression through a PI3K-dependent pathway[20,21] or, alternatively, potentially be endocytosed and elicit changes through a MAPK-dependent pathway.[22] Activation of the TLR pathway has been associated with inflammation, characterized by increased nitric oxide (NO) and prostaglandin E_2 (PGE_2) release.[21] In our previous study, PGE_2 and NO release was unaffected by ATP stimulation under all doses investigated (up to 250 μM).[7] Similarly, inhibition of the PI3K-dependent pathway by LY294002 did not abolish the relative induction of MMP-13 activity from exogenous PPi. Alternatively, inhibition of the MAPK-dependent pathway (specifically MEK1/2) by U0126 abrogated PPi-induced MMP-13 activity, suggesting that crystal endocytosis and activation of MEK1/2 (upstream of MAPK) could be the predominant mechanism of action of catabolism as a result of ATP stimulation. A potentially confounding result was the lack of direct identification of CPPD crystals in the 3-D cultured constructs by TEM. However, it may be possible that CPPD crystals were first endocytosed and subsequently degraded, which has been demonstrated for hydroxyapatite crystal–induced metalloproteinase activity.[35] The detection of CPPD crystals is also often fraught with difficulty[18,36] because the crystals can be small and sparse, requiring more sophisticated methods of analysis.[36] Amorphous forms of calcium pyrophosphate (more than 30 types have been identified) induced by excess PPi could also potentially elicit catabolic responses from chondrocytes through a similar mechanism.[37] Studies that have positively identified CPPD crystals in articular cartilage as a result of exogenous ATP utilized significantly greater concentrations than what was used in the present study (>1 mM ATP)[16,38,39] and specifically set out to induce a state of pathological mineralization or chondrocalcinosis (pathological presence of CPPD crystals in the joint space).[19] Future work is required to confirm the presence of CPPD crystals in the 3-D cultured constructs. In addition, it should be noted that LY294002, in the absence of exogenous PPi, appeared to upregulate MMP-13 activity. While the exact reason for this is currently unknown, PI3K signaling pathway is a major regulator of cell function and remodeling of the ECM and can influence other downstream signaling pathways involved in inflammation and matrix turnover.[40] While future work is required to gain a better understanding of the effects of LY294002, inhibition of the PI3K signaling pathway had no apparent effect on the induction of MMP-13 activity in response to exogenous PPi.

These findings suggest that calcium-containing crystals, potentially formed from excess ePPi in the presence of Ca^{2+}, may upregulate MMP-13 through a mechanism involving crystal endocytosis, and possibly subsequent dissolution, and the MAPK pathway. If indeed CPPD crystals are causing increased MMP-13 activity as a result of ATP stimulation, mineralization inhibitors, for example, phosphocitrate,[41] could potentially be utilized in conjunction with ATP to further extend the therapeutic range. Phosphocitrate is a naturally occurring inhibitor of mineralization that has been used in several *in vitro* studies to inhibit both CPPD and hydroxyapatite crystal formation.[41] Phosphocitrate acts by restricting the nucleation, growth, and aggregation of calcium salts[41] and has also been shown to block crystal-induced MMP synthesis.[42]

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