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REGULAR ARTICLE

ATP promotes extracellular matrix biosynthesis of intervertebral disc cells

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Abstract We have recently found a high accumulation of extracellular adenosine triphosphate (ATP) in the center of healthy porcine intervertebral discs (IVD). Since ATP is a powerful extracellular signaling molecule, extracellular ATP accumulation might regulate biological activities in the IVD. The objective of this study was therefore to investigate the effects of extracellular ATP on the extracellular matrix (ECM) biosynthesis of porcine IVD cells isolated from two distinct anatomical regions: the annulus fibrosus (AF) and nucleus pulposus (NP). ATP treatment significantly promotes ECM deposition and corresponding gene expression (aggrecan and type II collagen) by both cell types in three-dimensional agarose culture. A significant increase in ECM accumulation has been found in AF cells at a lower ATP treatment level (20 μM) compared with NP cells (100 μM), indicating that AF cells are more sensitive to extracellular ATP than NP cells. NP cells also exhibit higher ECM accumulation and intracellular ATP than AF cells under control and treatment conditions, suggesting that NP cells are intrinsically more metabolically active. Moreover, ATP treatment also augments the intracellular ATP level in NP and AF cells. Our findings suggest that extracellular ATP not only promotes ECM biosynthesis via a molecular pathway, but also increases energy supply to fuel that process.

Keywords Intervertebral disc · Extracellular matrix · ATP · Pig

Introduction

Low back pain is a condition that causes distress and suffering to patients. The impact of low back pain also creates a major socio-economic burden in industrialized societies. As the leading cause of disability, low back pain affects more than 80 % of the US population at some point in life (How-Ran et al. 1999). Intervertebral disc (IVD) degeneration has been closely associated with low back pain, stimulating interest in finding the causes that lead to IVD degeneration. Therefore, an understanding of the mechanisms involved in the maintenance of IVD composition might help the development of novel therapies for IVD degeneration and low back pain.

The IVD provides the mechanical properties that allow flexion, bending, and torsion of the spine and transmission of loads through the spinal column. These biomechanical properties are maintained by the composition and organization of the extracellular matrix (ECM) of the disc. The interplay of the two main macromolecules of ECM, namely the highly hydrated proteoglycan (PG) gel and the fibrillar collagen network, determines the mechanical response of the IVD (Roughley 1976). The IVD cells, which populate the discs at low densities, are responsible for maintaining the proper homeostatic balance of biosynthesis, breakdown, and accumulation of ECM constituents (Ohshima et al. 1995). These cellular processes determine the quality and integrity of the ECM and thus, the mechanical response of the disc (Buschmann et al. 1995). In addition, a decreasing PG concentration has been found with increasing grade of IVD degeneration

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(Pearce et al. 1987). In an in vitro study, disc aggrecan (part of the PG family) has been shown to inhibit nerve growth; this has been linked with the development of low back pain (Johnson et al. 2002). Therefore, detrimental changes in the ECM have been suggested to be associated with IVD degeneration and low back pain.

Maintenance of the ECM is a high-energy-demanding process that requires glucose and oxygen consumption to produce energy in the form of adenosine triphosphate (ATP). Nutrients are supplied mainly by diffusion from blood vessels at the margins of the disc resulting from the avascular nature of the IVD and are transported through the dense ECM to IVD cells (Urban et al. 2004). This mechanism of transport might be restricted by factors such as the calcification of the endplate or changes in the composition of the ECM (Grunhagen et al. 2011), all of which result in detrimental effects on essential cellular activities (e.g., ATP production). A previous study has reported that intracellular ATP level declines during the development of spontaneous knee osteoarthritis in guinea pigs, indicating that depletion of ATP is associated with cartilage degeneration (Johnson et al. 2004). Hence, cellular energy production for the proper synthesis of ECM molecules might be crucial for sustaining the integrity and function of the IVD.

During daily activities, the spine is subjected to mechanical forces that influence cell metabolism, gene expression, and ECM synthesis in IVD cells (Kasra et al. 2006; Korecki et al. 2009; Maclean et al. 2004; Ohshima et al. 1995; Walsh and Lotz 2004). Our recent studies have demonstrated that compressive loading promotes ATP production and release in IVD cells in a three-dimensional agarose gel model (Czamanski et al. 2011; Fernando et al. 2011) and in situ energy metabolism in the IVD (Wang et al. 2013). Furthermore, high accumulation of extracellular ATP attributable to the avascular nature of the disc has been found in the center of young healthy porcine IVD (Wang et al. 2013). ATP is an extracellular signaling molecule that mediates a variety of cellular activities via purinergic pathways (Burnstock 1997), including ECM production (Croucher et al. 2000; Waldman et al. 2010). Hence, ATP metabolism mediated by compressive loading and extracellular ATP accumulation could be a potential pathway that regulates crucial biological activities in the IVD. Therefore, the objective of this study was to investigate the effects of extracellular ATP on the ECM synthesis of porcine IVD cells isolated from two distinct anatomical regions: the annulus fibrosus (AF) and nucleus pulposus (NP).

Materials and methods

IVD cell isolation and sample preparation

IVDs were obtained from mature pigs (~115 kg) within 2 h of sacrifice (Cabrera Farms, Hialeah, Fla., USA). NP and outer AF tissues were harvested and digested in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, Calif., USA) containing 1 mg/ml type II collagenase (Worthington Biochemical, Lakewood, N.J., USA) and 0.6 mg/ml protease (Sigma-Aldrich, St. Louis, Mo., USA) for 24 h at 37 °C, 5 % CO_2. The cell-enzyme solutions were filtered by using a 70-μm strainer (BD Biosciences, San Jose, Calif., USA), and cells were isolated by centrifugation. The IVD cells were then re-suspended in DMEM supplemented with 10 % fetal bovine serum (FBS; Invitrogen) and 1 % antibiotic-antimycotic (Invitrogen) and then mixed at a 1:1 ratio with 4 % agarose gel to obtain cell-agarose samples of 1×10^6 cells in 100 μl of 2 % agarose. Freshly isolated cells were used, since serial passaging was reported to cause phenotypic changes (Chou et al. 2006). Three-dimensional culture was chosen because of its minimal binding interaction with cells (Knight et al. 1998; Lee et al. 2000) and capability of maintaining cellular phenotype (Gruber et al. 1997). All the samples were cultured at 37 °C, 5 % CO_2 in DMEM supplemented with 10 % FBS and 1 % antibiotic-antimycotic for the duration of the experiments.

PG and collagen content measurements

Our previous study has found that NP cells reside in an environment that has an extracellular ATP level of ~165 μM (Wang et al. 2013). In the literature, the dose range of 62.5–125 μM ATP favors ECM production in articular chondrocytes in three-dimensional agarose culture (Usprech et al. 2012). Therefore, our experimental groups included the following: control (no ATP) and 20 μM and 100 μM ATP treatment groups (NP: $n=9$ for each group; AF: $n=9$ for each group). The samples were cultured for 21 days under ATP (Sigma-Aldrich). The cell culture medium was changed three times a week, and ATP was administered in each medium change. The duration of the experiment was chosen based on a previous study of chondrocytes (Croucher et al. 2000). After 21 days, each sample was lyophilized and then digested overnight in 1 ml papain at 60 °C. PG content was quantified by using the dimethylmethylene blue (DMMB) dye-binding assay as previously described (Farndale et al. 1986). Aliquots of 150 μl of the samples were further hydrolyzed overnight with 6 N hydrochloric acid at 105 °C and assayed for hydroxyproline (HYP) content, as previously described (Neuman and Logan 1950). As high HYP content is found in collagen, HYP levels were measured as an indicator of collagen content (Neuman and Logan 1950). DNA content was quantified in samples digested in papain by using a Quant-iT dsDNA HS Assay Kit (Invitrogen). The PG and collagen levels in each sample were normalized to its DNA content to account for variations in cell number. To evaluate the effects of long-term ATP treatment on PG and collagen contents, each ATP treatment group was normalized to its

respective control group. To evaluate the difference between NP and AF cells, values of the NP cells were normalized to the average of AF control groups. One-way analysis of variance followed by the post hoc Student Newman Keuls test (SPSS Statistics 20, Chicago, Ill., USA) was performed to compare PG and collagen contents between the different treatment groups of the same cell type. Student's *t*-tests were performed to compare PG and collagen levels between NP and AF cells with the same treatment. Significance was taken at $P<0.05$ in all statistical analyses. Additionally, cell viability was examined by using the LIVE/DEAD Cell Viability Assay (Invitrogen) as instructed by the manufacturer.

Gene expression of aggrecan and type II collagen

Samples were cultured for 16 h with 100 μM ATP (NP: $n=12$ for control and ATP treatment group; AF: $n=9$ for control and ATP treatment group). According to our pilot study, the highest increase in gene expression induced by ATP was found at 16 h post-treatment. Additionally, samples from three independent experiments were cultured for 21 days with and without ATP (100 μM) to examine whether agarose culture influenced gene expression and maintained cell phenotype. Total RNA from each sample was obtained by using a modified version of the Trizol (Tri-Reagent, Molecular Research Center, Cincinnati, Ohio, USA) protocol. To improve the yield of RNA, 2 ml Trizol was added to the samples to facilitate agarose homogenization. After homogenization, vortexing, and incubation for 5 min at room temperature, the samples were centrifuged for 10 min at 5,000 rpm. The supernatants were collected, and the Trizol protocol was followed starting from the phase separation step. At the end of the procedure, the RNA pellets were left to dry for 5 min at room temperature, and 20 μl DNase/RNase-free water were added. The RNA pellets were left to swell for 5 min at room temperature and then stored at −80 °C overnight. The following day, the pellets were homogenized and centrifuged at 12,000 rpm for 20 min at 4 °C to collect the supernatant containing the RNA. RNA was quantified by using the Qubit RNA BR assay kit (Life Technologies, Carlsbad, Calif., USA) and reverse-transcribed to cDNA by using the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster, Calif., USA) according to the manufacturers' specifications. The levels of mRNA of the anabolic genes aggrecan and type II collagen were measured by using real-time polymerase chain reaction (PCR; One step Plus, Applied Biosystems) and normalized to that of the endogenous control (18 s) and the average of the internal controls. The $2^{-\Delta\Delta C_T}$ method was applied assuming that the amplification efficiencies of the target and the reference genes were approximately equal (Livak and Schmittgen 2001). Student's *t*-tests were performed to compare relative changes in gene expression between the control and the treatment group of the same cell type and between the various time points. The primer sequences were as follows: aggrecan forward primer, AGAC AGTGACCTGGCCTGAC; aggrecan reverse primer, CCAG GGGCAAATGTAAAGG; type II collagen forward primer, TGAGAGGTCTTCCTGGCAAA; type II collagen reverse primer, ATCACCTGGTTTCCCACCTT; 18S forward primer, CGGCTACCACATCCAAGGA; 18S reverse primer, AGCTGGAATTACCGCGGCT. The sizes of PCR products for aggrecan, type II collagen, and 18S were 151, 161, and 188 bp, respectively.

Intracellular ATP measurements

Samples were cultured for 2 h with 100 μM ATP (NP: $n=9$ for control and ATP treatment group; AF: $n=9$ for control and ATP treatment group). The time point was selected based on a previous study of endothelial cells, which indicated a maximal

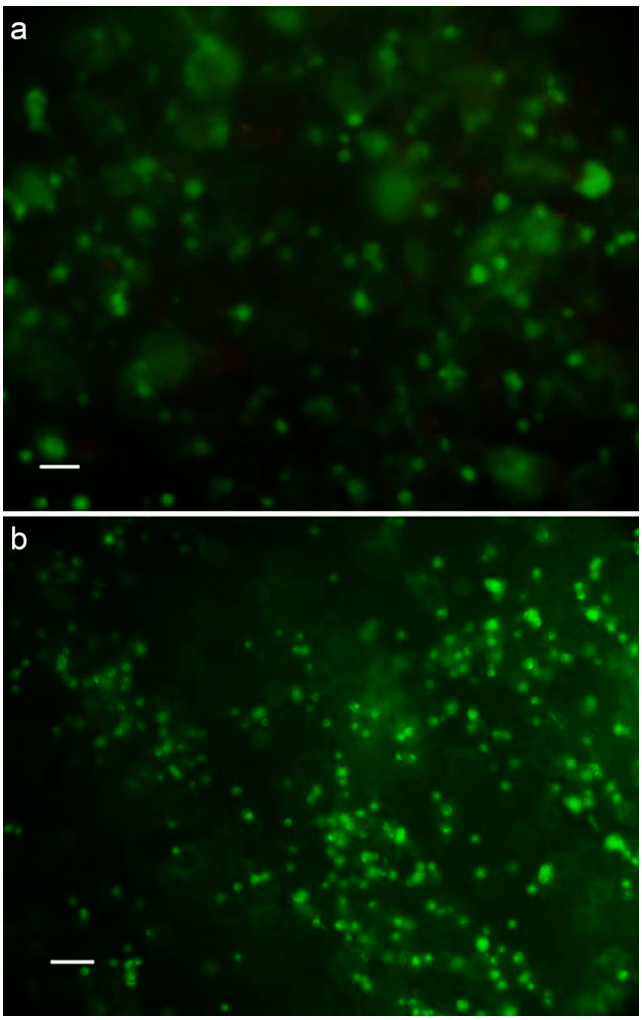

Fig. 1 Viability of intervertebral discs (IVD) cells treated with 100 μM ATP for 21 days (*green*/*red*: alive/dead cells). **a** Nucleus pulposus (NP) cells. **b** Annulus fibrosus (AF) cells. *Bar* 100 μm

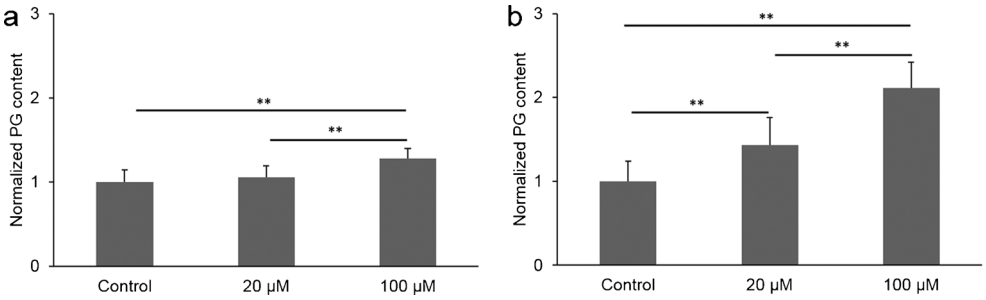

Fig. 2 Proteoglycan (*PG*) content of IVD cells treated with ATP at various concentrations for 21 days. **a** NP cells. **b** AF cells (n=9; **P<0.01 indicates statistically significant differences between groups)

increase of intracellular ATP generation after 2 h of ATP treatment (Andreoli et al. 1990). After incubation with ATP, the samples were dissolved in lysis buffer, consisting of 15 % 1.5 M NaCl, 15 % 50 mM EDTA, 1 % Triton-X 100, and 10 % 100 mM TRIS–Cl at pH 7.4, by heating at 65 °C. The lysates were centrifuged for 10 min at 9,000 rpm, and the supernatants were collected for intracellular ATP and DNA content measurements. Intracellular ATP was measured by using the luciferin-luciferase method (Sigma-Aldrich) and a plate reader (DTX880, Beckman Coulter, Brea, Calif., USA). Values of intracellular ATP were quantified and normalized to DNA content. To compare variations of intracellular ATP in each cell type, Student's *t*-test was performed between the control and the treatment group. To evaluate differences between NP and AF cells, values of NP cells were normalized to the average of AF control groups, and Student's *t*-tests were performed.

Results

Effects of ATP treatment on accumulation of PG and collagen

Cell viability staining confirmed no detrimental effects on cells after 21 days of 100 μM ATP treatment (Fig. 1). In NP cells, 100 μM ATP treatment significantly increased PG and collagen as compared with both the 20 μM ATP and control groups after 21 days of treatment (Figs. 2a, 3a). No significant difference was found in ECM deposition between the control and the 20 μM ATP groups. In AF cells, both ATP treatment groups exhibited significantly higher PG and collagen levels than the control group, whereas the 100 μM ATP group showed a significantly higher increase in the contents of PG and collagen than the 20 μM ATP group after 21 days of treatment (Figs. 2b, 3b). The PG content deposited by NP cells was significantly higher than that of AF cells under all treatment conditions (Fig. 4a). The collagen content accumulated by NP cells was significantly higher in the control and the 100 μM ATP groups compared with their respective AF groups. No significant difference in collagen content was found between NP and AF cells treated with 20 μM ATP (Fig. 4b).

Effects of ATP treatment on gene expression of aggrecan and type II collagen

The gene expressions of aggrecan and type II collagen in NP and AF cells were significantly higher after 16 h of 100 μM ATP treatment compared with control conditions (Fig. 5). In addition, long-term culture in agarose upregulated the gene expression of aggrecan and type II collagen in both cell types. However, no significant differences were found in gene expression between the 100 μM ATP and control groups at 21 days of culture (Fig. 6).

Effects of ATP treatment on intracellular ATP content

Intracellular ATP content significantly increased in NP and AF cells after 2 h of 100 μM ATP treatment compared with their respective control groups (Fig. 7a). In addition, a comparison between NP and AF cells showed that NP cells had a significantly higher intracellular ATP content than AF cells under all conditions (Fig. 7b).

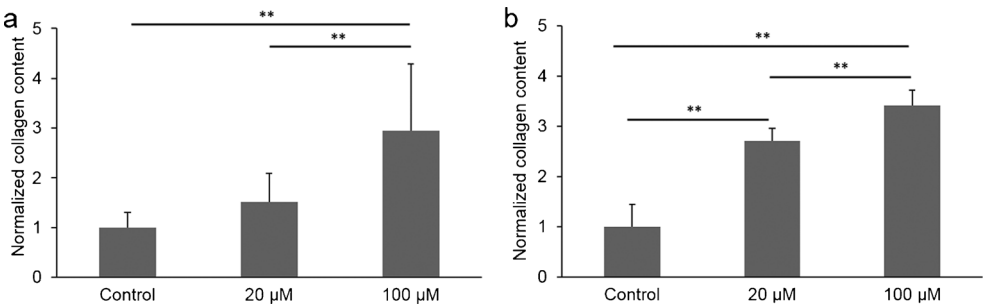

Fig. 3 Collagen content of IVD cells treated with ATP at various concentrations for 21 days. **a** NP cells. **b** AF cells (n=9; **P<0.01 indicates statistically significant differences between groups)

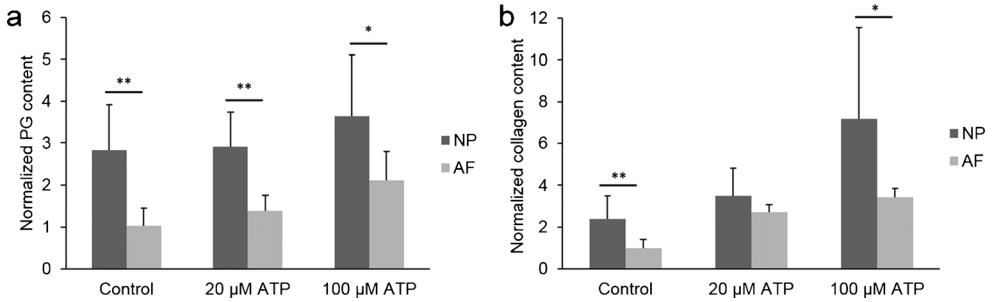

Fig. 4 Comparative extracellular matrix (ECM) macromolecule content of NP and AF cells treated with ATP at various concentrations for 21 days. NP cell values were normalized to the average of the control groups of AF cells. **a** PG content. **b** Collagen content (n=9; *P<0.05 and **P<0.01 indicate statistically significant differences between groups)

Discussion

Increased matrix breakdown, altered matrix synthesis (reduced synthesis of aggrecan and synthesis of type I collagen instead of type II collagen), and apoptosis are among the metabolic changes that contribute to IVD degeneration (Adams and Roughley 1976; Freemont 2009). Moreover, aggrecan has been shown to inhibit nerve growth in vitro, suggesting that loss of aggrecan is associated with the ingrowth of nerves that might cause low back pain in degenerated IVDs (Johnson et al. 2002). The proper biosynthesis of ECM in the IVD is a complex process that requires an extensive amount of ATP, especially for PG biosynthesis, which uses ATP as an energy source and building block (Hirschberg et al. 1998). The findings that high levels of extracellular ATP promote ECM biosynthesis and intracellular ATP production in IVD cells suggest that the high accumulation of extracellular ATP found in the NP (Wang et al. 2013) plays an important role in maintaining the healthy ECM structure of the IVD. Furthermore, to our knowledge, this is the first study to demonstrate that extracellular ATP influences ECM biosynthesis and intracellular ATP content in IVD cells.

In skin cells, galactosyltransferase-I, an enzyme that synthesizes the linkage region between the core protein and the glycosaminoglycan chains of PGs, enhances its activity after incubation with ATP (Higuchi et al. 2001). In addition, chondrocytes cultured with ATP demonstrate increased PG and collagen deposition (Croucher et al. 2000; Waldman et al. 2010). These previous studies support our findings suggesting that extracellular ATP can mediate cellular ECM biosynthesis. In addition, the upregulation of ECM synthesis by exogenous ATP is diminished by an antagonist of P2 receptors suggesting an involvement of a purinergic signaling pathway (Waldman et al. 2010).

We have found that a lower ATP concentration (i.e., 20 μM) induces a significant increase in the accumulation of both studied ECM molecules by AF cells compared with NP cells. This finding suggests that AF cells are more sensitive to low concentrations of extracellular ATP than NP cells. This difference in cellular responses to ATP between NP and AF cells can be explained by our previous study, which has found that NP cells reside in an environment with a higher level of extracellular ATP (~165 μM) than that of AF cells (<10 μM; Wang et al. 2013). Furthermore, our findings of higher PG and collagen accumulations and intracellular ATP content by the NP groups compared with the AF counterpart groups are consistent with those of our previous studies, which have suggested that NP cells are more metabolically active than AF cells (Czamanski et al. 2011; Fernando et al. 2011). The differences in the metabolic activities between AF and NP cells can be explained by differences in cell phenotypes, as AF

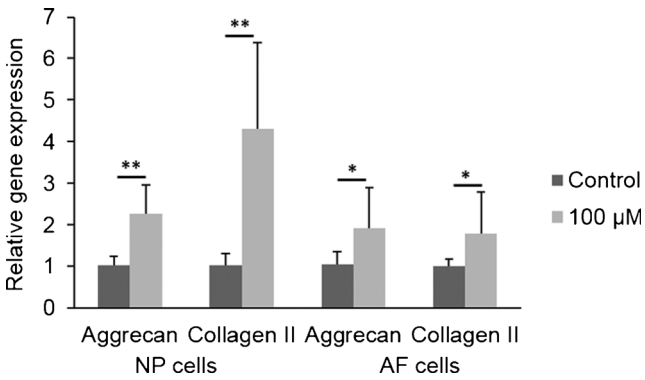

Fig. 5 Aggrecan and type II collagen gene expression of NP and AF cells treated with 100 μM ATP for 16 h (n=12 for NP, n=9 for AF; *P<0.05 and **P<0.01 indicate statistically significant differences between groups)

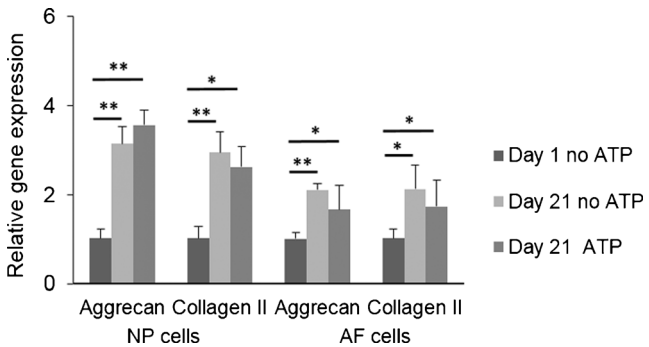

Fig. 6 Effect of long-term agarose culture on gene expression in IVD cells with ATP treatment (100 μM) and without ATP (n=3; *P<0.05 and **P<0.01 indicate statistically significant differences between groups)

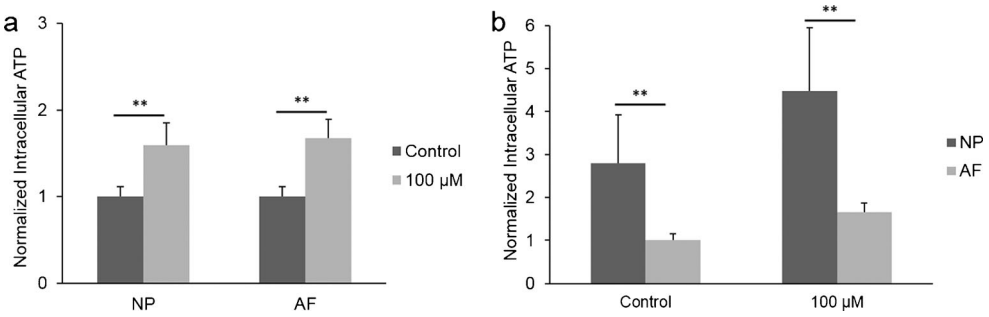

Fig. 7 Intracellular ATP content of IVD cells treated with 100 μM ATP for 2 h. **a** Comparison between experimental groups of the same cell type. **b** Comparison between NP and AF cells (n=9; **p<0.01 indicates statistically significant differences between groups)

cells are elongated and resemble fibroblasts, whereas NP cells are spheroidal and chondrocyte-like (Buckwalter 1995). Moreover, both cell types have distinct embryonic origins; NP cells are derived from the notochord, and AF cells are derived from the mesenchyme (Roughley 1976).

The IVD is subjected to static and dynamic loading at various magnitudes and frequencies during daily activities. Mechanical loading activates diverse mechanotransduction pathways, which can lead to the modification of cell function, metabolism, and gene expression (Chowdhury and Knight 2006; Maclean et al. 2004). Previous studies have shown that mechanical loading mediates the ECM biosynthesis of IVD cells (Kasra et al. 2006; Korecki et al. 2009; Maclean et al. 2004; Ohshima et al. 1995; Walsh and Lotz 2004). The mRNA expression of aggrecan and collagens in NP and AF regions is altered by specific mechanical loading regimens (Hutton et al. 1999; Maclean et al. 2004; Neidlinger-Wilke et al. 2006), whereas similar effects of mechanical loading have been observed on the incorporation of [^{35}S]-sulfate and [^{3}H]-proline (measures of protein

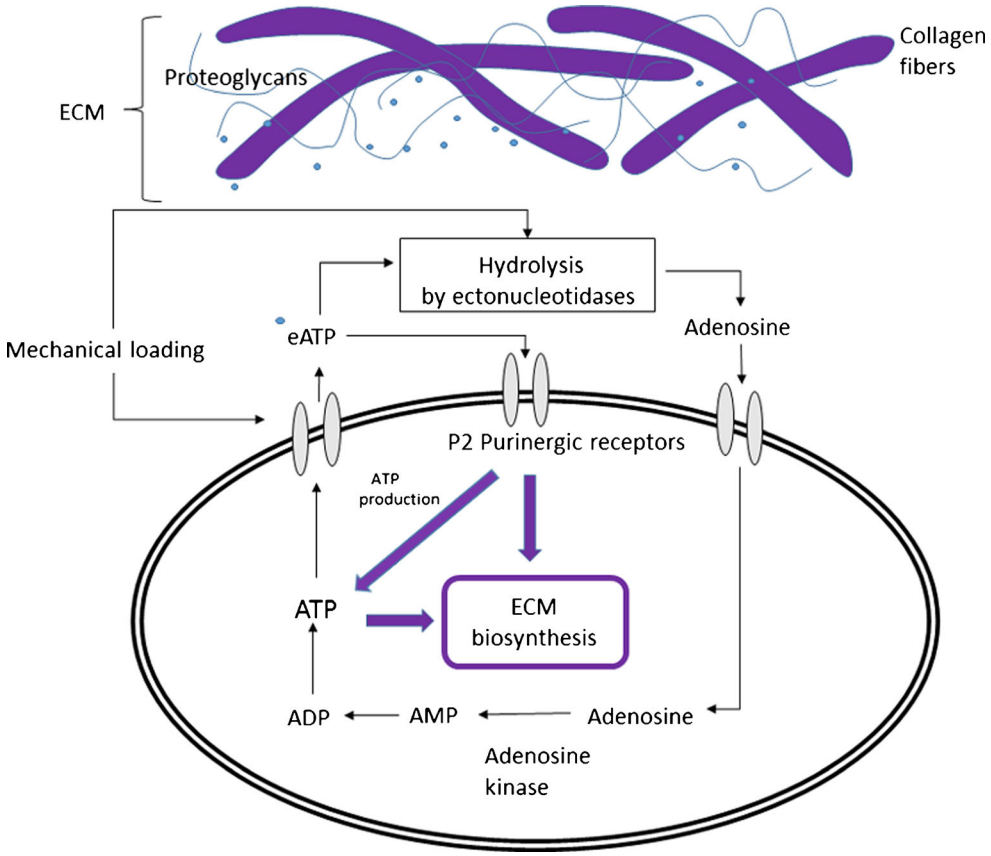

Fig. 8 Postulated mechanobiological pathway regulating ECM biosynthesis in IVD cells via ATP metabolism. Mechanical loading stimulates ATP release (Czamanski et al. 2011; Fernando et al. 2011) via a transport mechanism through a membrane channel or by leakage through a damaged cellular membrane (Graff et al. 2000). Extracellular ATP (*eATP*) activates P2 purinergic receptors that lie on the cell membrane and that are involved in the ECM biosynthesis process (Chowdhury and Knight 2006) and in the production of ATP (Kwon 2012; Kwon et al. 2012). Mechanical loading might promote eATP hydrolysis (Wang et al. 2013). Adenosine, which results from the hydrolysis of eATP, is taken up into the cell, and adenosine kinase rephosphorylates adenosine to AMP, which is subsequently rephosphorylated into ATP (Andreoli et al. 1990; Lasso de la Vega et al. 1994), which serves as an energy source and building block for ECM biosynthesis

synthesis) into collagens and PGs, respectively (Hutton et al. 1999). In our study, the mRNA levels of aggrecan and type II collagen were upregulated by extracellular ATP; this also correlated with their corresponding protein synthesis. Since our previous studies have shown that static and dynamic loading alter ATP production and release in IVD cells (Czamanski et al. 2011; Fernando et al. 2011) and in situ energy metabolism in the IVD (Wang et al. 2013), the finding of this study suggests that mechanical loading affects the ECM production of IVD cells via an extracellular ATP pathway.

The upregulation of ECM gene expression observed in both cell types without ATP treatment after 21 days of culture indicates that the agarose culture is capable of maintaining cellular phenotypes; this is consistent with the findings in a previous study of three-dimensional alginate culture (Baer et al. 2001). Increased gene expression in agarose culture over time might be attributable to changes in nutrimental conditions (i.e., higher level of nutrients in the culture media compared with the avascular in vivo condition of the IVD) and in the ECM environment in agarose culture (i.e., more ECM deposition around cells). Moreover, a previous study has shown that a single dose of ATP on the first day of culture promotes ECM biosynthesis in bovine chondrocyte pellets cultured over 7 and 21 days (Croucher et al. 2000). Hence, in our study, ATP does not upregulate gene expression after 21 days of culture, suggesting that short-term ATP treatment might be adequate to elicit significant effects on IVD cells. In addition, a high content of PGs has been reported to have a potential role as an inhibitor of ATP hydrolysis (Vieira et al. 2001). Therefore, the lack of effects of ATP seen on gene expression after 21 days of culture might be attributable to the accumulation of ATP resulting from an overall increase in ECM deposition over time.

In this study, we have also found that extracellular ATP treatment promotes intracellular ATP production in IVD cells. This finding is consistent with previous studies showing that treatment with extracellular nucleotides or adenosine increases the concentration of intracellular ATP (Andreoli et al. 1990; Lasso de la Vega et al. 1994). In cancer cells, the action of exogenous ATP appears to be mediated by the hydrolysis of extracellular ATP and subsequently the uptake of adenosine into cells, which increases the intracellular ATP content (Lasso de la Vega et al. 1994). In human umbilical vein endothelial cells, treatment with 25 μM of ATP, ADP, AMP, or adenosine significantly raises intracellular ATP levels through the same mechanism (i.e., adenosine uptake; Andreoli et al. 1990). In addition, previous studies have also reported that extracellular ATP signaling via $P2X_4$ receptor mediates intracellular ATP oscillations, which are involved in prechondrogenic condensation in chondrogenesis (Kwon 2012; Kwon et al. 2012). Hence,this evidence suggests that intracellular ATP production is mediated by the hydrolysis of extracellular ATP, the subsequent uptake of adenosine into cells, and/or the activation of purinergic receptors on the cell membrane.

Because of the avascular nature of the IVD, the delivery of nutrients to IVD cells relies on diffusion. In humans, about 25 % of water is extruded from the disc because of high loads during daily activities (Paesold et al. 2007). A decrease in disc hydration reduces the supply (diffusion) of oxygen and glucose for cellular ATP production, which is essential for maintaining cell viability and normal ECM production, especially in the center of the disc (i.e., NP region). Since mechanical loading might promote the hydrolysis of extracellular ATP, which is greatly accumulated in the NP (Wang et al. 2013), intracellular ATP levels in IVD cells might be increased via the adenosine-uptake mechanism described in the previous section, compensating for the effects of mechanical loading on nutrient supply. When disc hydration recovers during rest at night (Boos et al. 1993), cells can produce more ATP, which might be released and accumulated in the ECM. Therefore, the large accumulation of extracellular ATP in the NP region (Wang et al. 2013) might play an important role in maintaining the normal activities of IVD cells. This also suggests a mechanobiological pathway for the regulation of ECM biosynthesis via ATP metabolism (Fig. 8). Since the porcine model might not exactly simulate the conditions in human discs, future studies are required to confirm our findings in human IVD cells.

In summary, this study demonstrates that extracellular ATP promotes the biosynthesis of ECM and intracellular ATP production in IVD cells. The gene expression of aggrecan and type II collagen in NP and AF cells is also upregulated by extracellular ATP. In addition, NP cells appear to be less sensitive to low concentrations of extracellular ATP than AF cells, whereas NP cells exhibit a greater accumulation of PG, collagen, and intracellular ATP compared with AF cells.

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