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Mechanical Loading Stimulates Ecto-ATPase Activity in Human Tendon Cells

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Abstract Response to external stimuli such as mechanical signals is critical for normal function of cells, especially when subjected to repetitive motion. Tenocytes receive mechanical stimuli from the load-bearing matrix as tension, compression, and shear stress during tendon gliding. Overloading a tendon by high strain, shear, or repetitive motion can cause matrix damage. Injury may induce cytokine expression, matrix metalloproteinase (MMP) expression and activation resulting in loss of biomechanical properties. These changes may result in tendinosis or tendinopathy. Alternatively, an immediate effector molecule may exist that acts in a signal-dampening pathway. Adenosine 5′-triphosphate (ATP) is a candidate signal blocker of mechanical stimuli. ATP suppresses load-inducible inflammatory genes in human tendon cells in vitro. ATP and other extracellular nucleotide signaling are regulated efficiently by two distinct mechanisms: purinoceptors via specific receptor–ligand binding and ecto-nucleotidases via the hydrolysis of specific nucleotide substrates. ATP is released from tendon cells by mechanical loading or by uridine 5′-triphosphate (UTP) stimulation. We hypothesized that mechanical loading might stimulate ecto-ATPase activity. Human tendon cells of surface epitenon (TSC) and internal compartment (TIF) were cyclically stretched (1 Hz, 0.035 strain, 2 h) with or without ATP. Aliquots of the supernatant fluids were collected at various time points, and ATP concentration (ATP) was determined by a luciferin-luciferase bioluminescence assay. Total RNA was isolated from TSC and TIF (three patients) and mRNA expression for ecto-nucleotidase was analyzed by RT-PCR. Human tendon cells secreted ATP in vitro (0.5–1 nM). Exogenous ATP was hydrolyzed within minutes. Mechanical load stimulated ATPase activity. ATP was hydrolyzed in mechanically loaded cultures at a significantly greater rate compared to no load controls. Tenocytes (TSC and TIF) expressed ecto-nucleotidase mRNA (ENTPD3 and ENPP1, ENPP2). These data suggest that motion may release ATP from tendon cells in vivo, where ecto-ATPase may also be activated to hydrolyze ATP quickly. Ecto-ATPase may act as a co-modulator in ATP load-signal modulation by regulating the half-life of extracellular purine nucleotides. The extracellular ATP/ATPase system may be important for tendon homeostasis by protecting tendon cells from responding to excessive load signals and activating injurious pathways. J. Cell. Biochem. 96: 117–125, 2005.

Key words: mechanical load; ecto-ATPase; tenocytes

Tendon cells receive mechanical signals from the load-bearing matrices as do cells in other musculoskeletal tissues, such as bone, cartilage, and ligament. Mechanical stimulation is crucial for normal tendon function. However, overloading tendon cells deteriorates matrix integrity by inducing intrinsic factors that are thought to be responsible for the matrix destruction commonly observed in tendinosis and tendinopathy (repetitive motion disorder) [Almekinders et al., 1995; Jozsa and Kannus, 1997; Archambault et al., 2002a,b; Ireland et al.,

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2001; Riley et al., 2002]. It has been reported that cyclic mechanical load induces inflammatory genes, interleukin 1-beta (IL-1β), cyclooxygenase-2 (COX2), and matrix metalloproteinase-3 (MMP-3), in human tendon cells in vitro, suggesting that tendon subjected to repetitive motion may be predisposed to resident fibroblast-mediated inflammatory cycles at the molecular level [Tsuzaki et al., 2003a,b]. Alternatively, however, there may exist an immediate effector that could act in a load signal-dampening pathway [Banes et al., 1995a, 2001; Tsuzaki et al., 2003b].

Adenosine 5′-triphosphate (ATP) is a candidate signal blocker. We have found that exogenous ATP suppresses load-inducible inflammatory gene expression in vitro [Tsuzaki et al., 2003b]. Intracellularly, ATP is an energy source for living cells. However, extracellularly ATP and its metabolites as well as uridine 5′-triphosphate (UTP) act as a signal transducer that plays critical roles via its cell surface receptors [Hansen et al., 1993; Harden et al., 1997; Homolya et al., 2000; Sauer et al., 2000; Housley et al., 2002], not only in the nervous system but in non-excitatory, connective tissues such as tendon. ATP serves not only in synaptic transmission and perception of senses in the nervous system, but also in vasomotor responses, platelet aggregation, immune defense, and other diverse mechanisms of cell functions [El-Moatassim et al., 1992; Ralevic and Burnstock, 1998]. ATP is released by a variety of cell types including those in non-excitatory tissues, such as chondrocytes, intervertebral disc annulus cells, and tendon cells, in response to mechanical or chemical stress [Lazarowski et al., 1997; Graff et al., 2000; Tsuzaki et al., 2003b; Yamazaki et al., 2003]. Human tendon cells in vitro naturally secrete ATP [Tsuzaki et al., 2003b]. ATP release is increased in response to cyclic stretching or addition of UTP [Tsuzaki et al., 2003b]. ATP and other extracellular nucleotide signaling are regulated by two distinct mechanisms: purinoceptors via specific receptor-ligand binding and ecto-nucleotidases via the hydrolysis of specific nucleotide substrates, which may be involved in ATP load-signal modulation in tendon.

Effects of extracellular ATP are mediated by two subfamilies of cell surface P2 type purinoceptors: the ligand-gated cation channels (P2X receptors) and the G-protein-coupled P2Y membrane receptors [Ralevic and Burnstock, 1998]. ATP metabolites, ADP, AMP, and adenosine, as well as pyrimidine nucleotides, UTP and UDP, also can bind to P2 receptors with different potency, to activate specific signal transducing pathways. Therefore, ATP and other extracellular nucleotides and nucleosides play key roles in a diversity of purinergic functions in different types of cells and tissues.

Extracellular ATP is hydrolyzed by ecto-nucleotidases and other extracellular enzymes, where the primary action of ATP terminates. There are three groups of enzymes with distinct characteristics that are involved in ATP hydrolysis: E-NTPDases (ecto-nucleoside 5′-triphosphate diphosphohydrolases, CD39 family) are classic, transmembrane apyrases that catalyze ATP as well as ADP at different substrate preferences. Another family is E-NPPs (ecto-nucleotide pyrophosphatases/phosphodiesterases) that are capable of cleaving ATP to AMP and PPi. E-NPP hydrolyzes both purine and pyrimidine nucleotides as substrates. The third consists of non-specific alkaline phosphatases [Zimmermann, 1996, 2000]. Many tissues express more than one type of ecto-nucleotidase family [Vollmayer et al., 2001; Vlajkovic et al., 2002]. Ecto-5′-nucleotidase catalyzes the final step of extracellular nucleotide degradation and the formation of adenosine from adenine nucleotides.

While the lifetime of ATP is short and its action is closely regulated by these enzymes, ATP metabolites, ADP, AMP, and adenosine, are pharmacologically active as ligands for their specific purinoceptors. Therefore, ATP release and load-signal blockade in tendon cells may be more complex. In concert with the selective binding of nucleotides to the purinoceptors, ecto-ATPase and ecto-nucleotidases may be involved in ATP modulation of load signal transduction by regulating extracellular nucleotide species. In the present study, we hypothesized that cyclic load might activate ecto-ATPase in human tendon cells and that the extracellular ATP/ATPase system might play a regulatory role in tendon.

MATERIALS AND METHODS

Tendon Primary Cell Culture

Human flexor digitorum profundus (FDP) tendons from three patients (3 male, 73 female, and 60 male) were obtained as discarded tissues after surgery at UNC Hospitals (IRB approved,

according to an established protocol). Tendon cells were isolated by sequential enzymatic (trypsin and collagenase), mechanical treatment, and selective plating: first releasing surface epitenon cells (TSC), and next more internal fibroblasts (TIF) [Banes et al., 1988a,b, 1995b]. Cells were grown in DMEM with 10% fetal calf serum, 20 mM HEPES, pH 7.2, 0.1 mM ascorbate-2-PO_4 and antibiotics, and used at passages 3–5.

Application of Mechanical Load to Cells

Cells were plated in collagen I-coated flexible-bottomed plates (BioFlex® culture plates, Flexcell International Co., Hillsborough, NC) at 25k cells/cm^2 in complete medium then growth-arrested by halving the serum content on days 3 and 5 [Tsuzaki et al., 2003a]. On day 6, medium was changed to fresh, serum-free, phenol red-free medium, and incubated for 3 h in the baseplate of the strain unit without stretch to allow extracellular ATP to return to basal levels [Tsuzaki et al., 2003b]. Cells were stretched equibiaxially with or without ATP (1, 10, or 100 μM) using a Flexercell® Strain unit with 31 mm diameter cylindrical loading posts (Flexcell International Co.) to apply a defined number of load cycles at 1 Hz and 0.035 (3.5%) strain to cells for 5, 10, 15, 30 min, 1 and 2 h. UTP (100 μM) was also used to stimulate ATP release from tendon cells [Ostrom et al., 2000]. Inhibitors including Suramin (100 μM, blocks P2 receptors), Gd^{3+} ($GdCl_3$, 20 μM, blocks stretch-activated (SA) channels [Yang and Sachs, 1989]), EDTA (10 mM, binds Ca^{2+} and Mg^{2+}) or Reactive Blue 2 (100 μM, blocks P2 receptors) were added 30 min before stretching. Aliquots of the supernatant fluid (100 μl) from each well were collected at each time point, placed on ice immediately then heated at 70°C for 5 min to inactivate ATPase and stored at −20°C until assay for [ATP].

ATPase Release Measurement

The supernatant fluid samples for ATPase release measurement were collected from tendon cell cultures with or without stretching at times 5, 10, 15, 30 min, 1 and 2 h. Forty microliters of each sample were added with 40 μl of 2 μM ATP in fresh, phenol red-free DMEM (final concentration of ATP was 1 μM), and incubated for 60, 90, or 120 min at 37°C. ATPase activity was terminated by heating at 70°C for 5 min, and [ATP] of the sample determined by luciferin-luciferase assay.

Luciferin-Luciferase Bioluminescence Assay

ATP concentrations in the samples (ATP) were determined by a fire-fly luciferin-luciferase bioluminescence assay in a 96-well, white microtiter plate using a series of ATP standards, plus medium and chemical reagent blanks including the inhibitors noted above. A luciferin-luciferase reagent was injected into each well and 562 nm emission was read using a luminometer (Labsystems, Helsinki, Finland) and spectrophotometry program (Fluoroskan Ascent, FL).

RT-PCR

Human tendon cell samples were collected in guanidinium denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethenol, 0.5% N-lauroylsarcosine) and total RNA was isolated. The cell lysate was passed sequentially through shredder and RNA separation columns (Qiagen, Valencia, CA), DNase digested, phenol-chloroform purified, and purity verified spectrophotometrically (A260 nm/A280 nm ~ 1.9). Oligonucleotide primers for ecto-nucleotidases were synthesized according to the published human sequences (Table I). Replicate total RNA samples (10 μg in 50 μl reaction) were reverse

TABLE I. Primer Sequences for PCR Analysis

ENTPD1	forward: GCCAGGATCATTACTGGCCAA reverse: GAAGGCACACTGGGAGTAAGG	(519 bp)
ENTPD2	forward: GGTGCCTCTACCCAGATCACT reverse: CTGACCCTGGCACTGCTGT	(271 bp)
ENTPD3	forward: GAAAGCCCTCTGATCCGTCTG reverse: TCAGCATGTGGGTAGTGAGGT	(373 bp)
ENPP1	forward: TCTTCAGTGGCTACAGCTTCC reverse: TCAGAGGGTCTCAATCGAGCT	(303 bp)
ENPP2	forward: AGAATCCTCGACATGGCAAGG reverse: GGTACACCGGCCTCATGTAGG	(610 bp)
ENPP3	forward: TGACACAGCCCAGCAGTCTCA reverse: ACTCTGGATACAAGCCCGTGA	(206 bp)
β-actin	forward: TCATCACCATTGGCAATGAG reverse: CACTGTGTTGGCGTACAGGTT	(125 bp)

transcribed, and the template cDNA (1 μl in 10 μl reaction) amplified by PCR with conditions as follows: hot start and denaturation at 94°C, annealing at 60°C, and extension at 72°C, for 40 cycles, were used (Perkin Elmer, Branchburg, NJ). The product cDNA bands were visualized after electrophoresis in a 1.8% agarose gel containing ethidium bromide.

Statistical Analysis

Levels of significance were tested by one way repeated measures analysis of variance (ANOVA) followed with Dunnett's *t*-test as well as appropriate post hoc procedures using SigmaStat software (SPSS Scientific, Chicago, IL). The $P < 0.05$ level was used to demonstrate statistical significance.

RESULTS

Normal quiescent human tendon cells secreted ATP in the culture medium (0.5–1.0 nM). ATP release was induced within 2 min upon changing media, [ATP] reaching above 150 nM at 5 min, even before stretching cells. Afterwards, [ATP] of the supernatant fluid declined to a basal level in 3–4 h. Cyclic mechanical load within physiological range (0.035 strain) stimulated tendon cells to release ATP (14–17 nM). [ATP] was increased then decreased to the basal level within 30 min of stretching [Tsuzaki et al., 2003b], suggesting that released ATP was degraded quickly. Exogenous UTP (100 μM) induced ATP release robustly (50–60 nM), and this case the [ATP] remained at a high level [Tsuzaki et al., 2003b], suggesting that ATP release or/and metabolism may be modified by the presence of UTP. UTP-stimulated ATP release was inhibited by Suramin, a P2 purinoceptor antagonist, whereas load-inducible ATP release was totally abrogated by gadolinium, a SA channel blocker [Tsuzaki et al., 2003b]. Since ATP released in response to load was hydrolyzed quickly, next we investigated ecto-ATPase expression and ATPase activity in tendon cell cultures.

Human tendon cells expressed mRNA for ecto-nucleotidase families (Fig. 1). Both cell types, surface epitenon (TSC) and internal fibroblasts (TIF), expressed mRNA for ATPases from different families as detected by RT-PCR at 40 cycles of amplification. In a preliminary experiment, we performed an optimization protocol for each gene by incrementing the cycle numbers up to 40 (data not shown). Since the purpose of this RT-PCR was to detect ecto-nucleotidase gene expression in tendon cells, we chose 40 cycles. Under the conditions used, ENTPD3 mRNA was expressed constitutively in TSC and TIF of all three patients' cell isolates (A, B, C), whereas expression of ENPP family members differed among patients as well as between the two cell types (Fig. 1).

ATPase activity was characterized by measuring degradation of ATP added to tendon cell culture. Exogenous ATP was hydrolyzed spontaneously in TSC or TIF culture (Fig. 2). Due to the sensitive bioluminescence assay, the

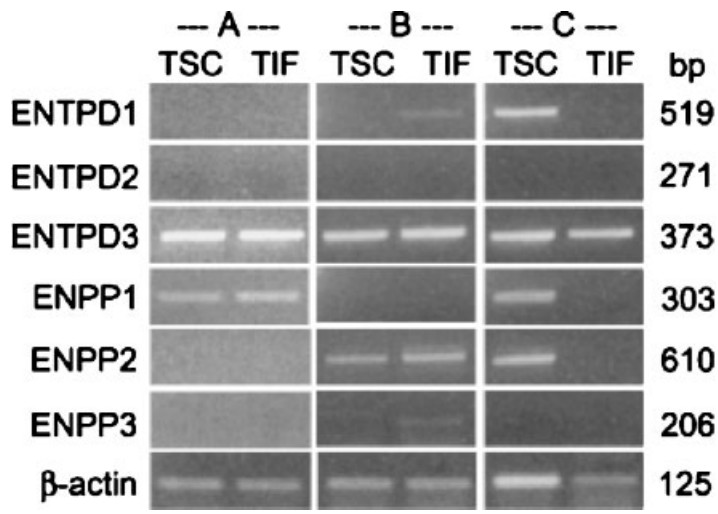

Fig. 1. Profile of ecto-nucleotidase mRNA expression in human tendon cells. Data shown are the representative PCR (40 cycles) amplimer cDNA bands from three patients (**A**–**C**) for ecto-nucleotidase families, ENTPD and ENPP. TSC and TIF expressed mRNA for ENTPD3 and ENPP1, ENPP2.

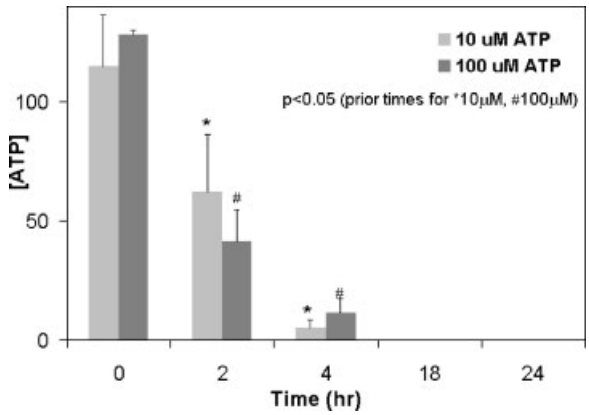

Fig. 2. ATP hydrolysis by human tendon cells. Exogenous ATP is hydrolyzed by half within 2 h by human tendon cells. ATP was added (10 μl/2 ml well, final concentration 10 or 100 μM), and [ATP] in the culture medium assayed at times 0, 2, 4, 18, and 24 h ($n = 6$, $P < 0.05$). The representative data depict [ATP] of the samples diluted to 1/100 (10 μM ATP) or 1/1,000 (100 μM ATP).

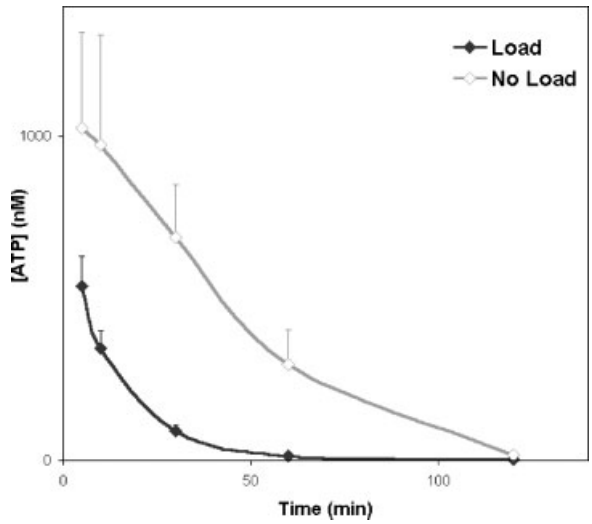

Fig. 3. Stimulation of ATPase activity by cyclic mechanical loading. Load upregulates ATP hydrolysis by human tendon cells. ATP was added (1 μM), then cells were stretched (Load) or non-stretched (No Load), and [ATP] was assayed at times 5, 10, 30, 60, and 120 min in the culture medium ($n = 6$, $P < 0.05$).

samples were diluted so that [ATP] determined would fall within a liner range. Regardless of the initial ATP concentration at time 0, ATP was hydrolyzed, but not to zero, to maintain [ATP] at the basal level (0.5–1.0 nM). On the other hand, ATP added to cell-free medium was quite stable and remained undegraded for up to 24 h at 37°C. Therefore, ATP stimulated tendon cell ATPase activity.

Upon cyclic stretching of tendon cells, [ATP] in the supernatant fluid samples from stretched cells declined at a significantly greater rate compared to that for no load controls (Fig. 3). ATP was hydrolyzed by 50% within the first 5 min. Afterwards [ATP] continued to decline until it reached at the base level. Since some ecto-ATPase types are released by proteolytic cleavage from the plasma membrane and act as free ATPase, mechanical loading may induce ATPase release (shedding). In order to detect ATPase release or the presence of free ATPase in the culture medium, we measured ATP hydrolyzing activity of the supernatant fluids collected from stretched and non-stretched tendon cells at various time points (Fig. 4). Exogenous ATP (1 μM) was not hydrolyzed for up to 2 h at 37°C in any of the samples tested, suggesting that tendon cells do not secrete free ATPase from the plasma membranes naturally, furthermore, that mechanical load does not induce ATPase release (Fig. 4).

EDTA inhibited ATP hydrolysis by binding Mg^{2+} and Ca^{2+} that are required for ATPase action (Fig. 5). EDTA did not reverse the initial reduction of [ATP] when the loading regimen began. However, [ATP] was maintained at half the original level while [ATP] in the load control quickly declined to a basal level (Fig. 5). Purinoceptor antagonists such as Suramin or Reactive Blue 2 (RB2) also inhibited ATPase activity (Fig. 6). Suramin treatment partially inhibited ATP hydrolysis but not significantly so. On the other hand, Reactive Blue 2 completely blocked ATP hydrolysis by tendon cells

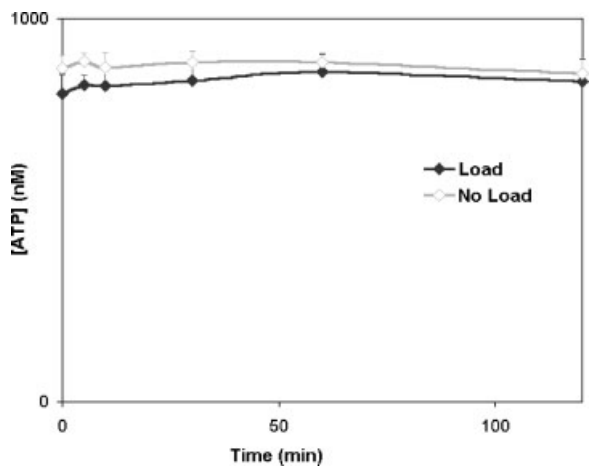

Fig. 4. ATPase release in the supernatant fluids from tendon cells. Tendon cells did not release ATPase by stretching. The supernatant fluids from tendon cell culture with or without stretching were collected at times 0, 5, 10, 30, 60, and 120 min, incubated with exogenous ATP (1 μM) for 2 h, and [ATP] was determined. The representative data are shown ($n = 6$, $P < 0.05$).

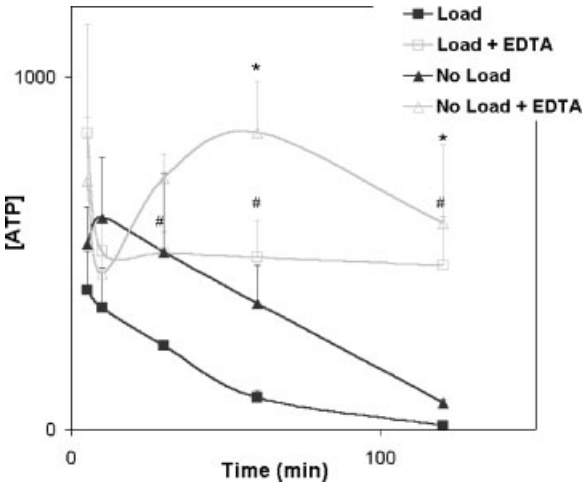

Fig. 5. Inhibition of ATPase activity by EDTA. EDTA (10 mM) inhibited ATP hydrolysis by tendon cells. EDTA bound divalent cations (Ca^{2+}, Mg^{2+}) that are required for ecto-ATPase action (n = 6; Significantly different from No Load (*) or Load (#) control at each time point, $P < 0.05$).

(Fig. 6). We tested Gd^{3+}, which completely blocked load-inducible ATP release [Tsuzaki et al., 2003b], to see if load stimulation of ATPase activity was via ATP release induced by mechanical loading of tendon cells. Gadolinium had no effect on load-stimulated ATP hydrolysis (data not shown), suggesting that ATPase was activated directly by mechanical load independent of ATP release. These data suggest that cyclic mechanical loading upregulated ATP hydrolysis by stimulating the membrane-bound, ecto-ATPase.

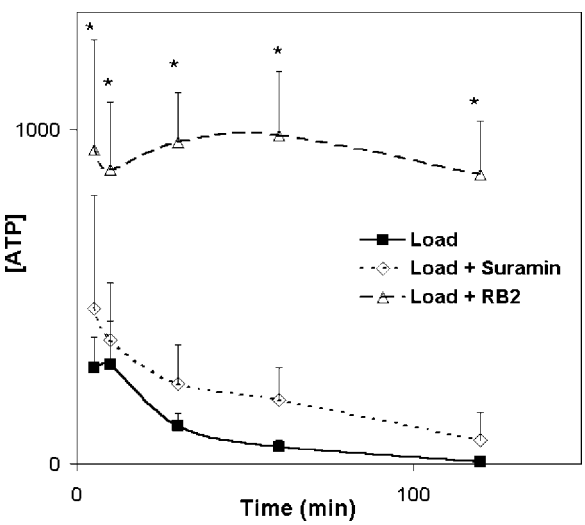

Fig. 6. Inhibition of ATPase activity by purinoceptor antagonists. Purinoceptor antagonists that bind to ecto-ATPase, such as Suramin (100 μM) or Reactive Blue 2 (RB2, 100 μM), also inhibited ATP hydrolyzing activity by tendon cells (n = 6; Inhibition of RB2 was significant at all times, whereas that of Suramin was not, $P < 0.05$).

DISCUSSION

Response to mechanical stimuli is crucial for tendon cell survival, growth, and the tissue-specific functions [Banes et al., 1995a, 2001]. Tendon healing is significantly promoted by motion therapy [Koob et al., 1992; Banes et al., 1995b], and loss of mechanical stimulation results in matrix degeneration and atrophy [Hannafin et al., 1995]. However, overloading could cause repetitive motion disorder including tendinosis and tendinopathy [Almekinders et al., 1995; Carpenter et al., 1998]. A mechanical signal could activate either anabolic or catabolic pathways or both at the same time [Banes et al., 1995a, 2001; Hannafin et al., 1995]. In our tendon overuse and tendinopathy model, it was hypothesized that overuse injury and excessive mechanical loading may induce cytokine IL-1β, as has been suggested for cartilage and periodontal ligament [Shimizu et al., 1994; Fujisawa et al., 1999; Moos et al., 1999]. Endogenous IL-1β may trigger tissue destructive pathways by inducing MMPs in a positive feedback loop, in a resident, fibroblast-derived cytokine production [Tsuzaki et al., 2003a]. It was also hypothesized that concomitant autocrine/paracrine release of ATP, a load-signal blocker, may serve in a negative feedback loop to protect tendon [Tsuzaki et al., 2003b]. Data from the present study suggest that ecto-ATPase and ecto-nucleotidases may be involved closely in ATP load-signal blockade as a regulatory mechanism by limiting the availability of extracellular ATP as well as producing other nucleotide species. Repetitive motion stimulation in tendon, the extracellular ATP/ATPase system, and coordinated activation/inactivation of specific purinergic receptors may together modulate the responsiveness of tendon cells to load/cytokine stimuli. Attenuation or failure of this blocking mechanism, where load signals are transduced out of regulation, resulting in activation of matrix destructive pathways, may drive the progression to tendinosis and tendinopathy.

Human tendon cells secrete ATP in vitro, maintaining an extracellular [ATP] of 0.5–1.0 nM. Mechanical stimulation causes ATP release, upon which membrane-bound ecto-ATPase is also activated to hydrolyze ATP

quickly. Furthermore, UTP increased ATP release robustly, sustaining [ATP] relatively high. ATP induces ATP release [Bodin and Burnstock, 1996], therefore, UTP-induced ATP release may result in more ATP release. UTP may be a second effector molecule by occupying certain P2 receptors (P2Y2 and P2Y4) as well as modifying ATP metabolism [Harden et al., 1997; Lazarowski et al., 1997]. UTP-inducible ATP release was inhibited by Suramin, suggesting that P2 purinoceptors may be involved in the UTP/ATP positive feedback mechanism [Harden et al., 1997]. On the other hand, load-inducible ATP release was abrogated by Gd^{3+}, suggesting the involvement of cation channels such as SA channels [Guharay and Sachs, 1984; Lansman et al., 1987].

ATP hydrolysis was stimulated by cyclic mechanical loading as well as ATP itself. ATPase is a Ca^{2+}/Mg^{2+}-dependent enzyme. Since extracellular Ca^{2+}/Mg^{2+} are abundant in the culture medium and our data showed that load-stimulated ATP hydrolysis was independent of load-induced ATP release, mechanical load may activate ecto-ATPase directly. Among ecto-nucleotidase families, ENPP have a common structure with a short, single cytosolic extention so that extracellular domain including enzyme activity could be cleaved into a soluble form [Zimmermann, 2000]. We expected ATPase release from tendon cell plasma membranes by stretching as it has been reported by fluid sheer in vascular cells [Yegutkin et al., 2000; Bobalova and Mutafova-Yambolieva, 2003]. However, no free, soluble ATPase was detectable in the stretched tendon cell culture medium. Therefore, cyclic loading may stimulate membrane-bound ecto-ATPase. Released ATP in response to mechanical stimuli must be ended a short life, being caught either by P2 purinoceptors or ecto-ATPases at the membrane site. ATPase activity in stretched tendon cells was not completely inhibited by EDTA, suggesting a possibility that Ca^{2+}/Mg^{2+}-independent, unknown ecto-ATPase may be present and activated by mechanical loading. Purinoceptor antagonists have been shown to inhibit ATP hydrolysis by binding to ecto-ATPase at different binding sites and with different kinetics, while competing with ATP for P2 purinoceptors on the membrane surface [Yegutkin and Burnstock, 2000; Yegutkin et al., 2000]. Lack as well as excess of ATPase may also result in an imbalance in nucleotide and nucleoside species in extracellular spaces and discordance in purinergic signaling. Therefore, load-activation of ATPase may be involved in a regulatory mechanism for load-inducible ATP and P2 receptor signaling in an extracellular ATP/ATPase system where ecto-ATPase is a co-effector.

Human tendon cells express P2Y class purinoceptors and respond to extracellular ATP as well as UTP by signaling with a rise in intracellular calcium ($[Ca^{2+}]_{ic}$) [Francke et al., 1998]. However, prolonged exposure to ATP causes a transient desensitization [Francke et al., 1998; Minchew et al., 1999]. ATP suppressed mechanical load-inducible inflammatory genes, IL-1β, COX2, and MMP-3, suggesting that mechanotransduction may intersect with purinergic- as well as cytokine pathway(s) [Hamada et al., 1998; Ostrom et al., 2000]. In the present in vitro study, $[ATP]_{ec}$ resulting from ATP ($\sim$15 nM) release from tendon cells was relatively low compared to the levels requiring to block load signals ($10 \sim 100\ \mu M$). This disparity in effective concentrations is likely due to dilution of ATP in supernatant fluids as well as capture at the membrane surface by ATPase and P2 purinoceptors and hydrolysis. Therefore, an instantaneous rise of real-time [ATP] in the pericellular microenvironment must be greater than our measurements indicate. Indeed, recent studies have indicated that ATP is released by mechanical stimulation at high concentration in the extracellular space, activating purinoceptors robustly [Lazarowski et al., 1997; Hazama et al., 1998; Arcuino et al., 2002]. Moreover, UTP and its metabolites may be involved in the ATP modulation of load signals, although little has been known about release, concentration and metabolism of extracellular UTP.

Our data suggested that human tendon cells express mRNA for ecto-nucleotidases from the ENTPD family as well as the ENPP family (Fig. 1). Released ATP may be immediately cleaved at the membrane surface by these ecto-ATPases, then by ecto-5′-nucleotidase responsible for the formation of adenosine. Within minutes after ATP release, therefore, the extracellular space may be populated by adenine nucleotide derivatives varying in their concentration and proportion. While activated ecto-ATPase terminates primary ATP signaling, these products, ADP, AMP, and adenosine, activate different classes of purinoceptors that transduce different pathways [Ralevic and

Burnstock, 1998]. Therefore, mechanical load-activation of ATP/ATPase is complex. For normal cell function, however, elimination of ATP and the subsequent production of purine nucleotides may be as important as the initial ATP release and action. Ecto-ATPases may be a regulatory mechanism of purinergic signaling by controling extracellular purine nucleotides.

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