

# P2 Receptors Mediate Nucleotide-Induced Calcium Signalling and Gene Expression Changes in Tenocytes – Implications for Tendon Mechanotransduction

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**INTRODUCTION:** To adapt to the repeated mechanical load produced by movement, tenocytes (the resident cell-type in tendons) translate mechanical signals into biological responses. This process is known as mechanotransduction [1]. Previous literature indicates that mechanical loading of tendons alters intracellular signalling and extracellular matrix synthesis; however, the mechanisms mediating this process remain to be fully elucidated [2]. Mechanical stimuli induce the release of nucleotides into the extracellular space [3]. These extracellular nucleotides then signal in an autocrine and paracrine manner through ionotropic P2X and metabotropic P2Y receptors expressed by target cells. P2 receptors are essential for mechanotransduction in musculoskeletal tissues, such as bone; however, the expression and role of P2 receptors in tendon was unknown before this study. As such, we *hypothesized* that murine tenocytes express P2 receptors that signal through intracellular calcium to modulate gene expression in response to exogenous nucleotides (**Figure 1**). We conducted this study to characterize, for the first time, the P2 receptors expressed by tenocytes and their role in tendon mechanobiology.

**METHODS:** This study was conducted per the policies and guidelines of the Canadian Council on Animal Care and was approved by the Animal Use Subcommittee of the University of Western Ontario. We first isolated primary tenocytes from the Achilles, patellar, and tail tendons of 1-to-2-month-old wildtype C57BL/6N mice and then grew these cells in 2-D culture. To investigate P2 receptor expression, we extracted RNA from Achilles, patellar, and tail tenocyte cultures and performed qPCR using gene-specific primers. To test for the activation and function of specific P2 receptors, we loaded Achilles, patellar, and tail tenocyte cultures with the calcium-sensitive ratiometric indicator Fura-2-AM and evaluated changes in calcium signalling following treatment with Vehicle (PBS), ATP (100  $\mu$ M), or UTP (100  $\mu$ M) by live-cell fluorescence microscopy. In addition, we treated Achilles and tail tenocytes with Vehicle, ATP (10  $\mu$ M), or UTP (10  $\mu$ M) in the presence or absence of the P2Y<sub>2</sub>-specific antagonist AR-C 118925XX (1  $\mu$ M) to assess whether P2Y<sub>2</sub> is the principal P2 receptor functioning in tenocytes. To examine the effect of nucleotide-induced calcium signalling on tenocyte gene expression, we treated tail tenocyte cultures with Veh, ATP (100  $\mu$ M), or UTP (100  $\mu$ M) and performed qPCR to analyze gene expression of tenocyte-specific genes at various time points. Differences among two groups were assessed using Student's t-tests, and differences among three or more groups were assessed using one-way ANOVA followed by Tukey's multiple comparisons test.

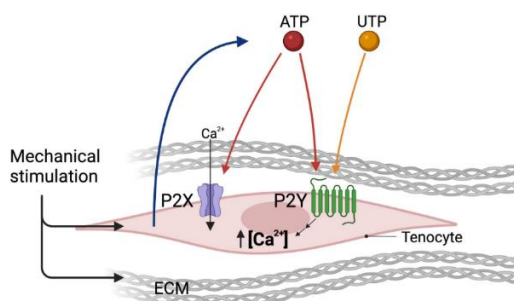
**RESULTS:** Our gene expression analysis of 2-D primary Achilles, patellar, and tail tenocyte cultures showed similar receptor profiles, with P2X<sub>4</sub>, P2Y<sub>2</sub>, P2Y<sub>6</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub> being most abundantly expressed in all three tissues (n=3). These results are consistent with bulk RNA-seq data obtained previously from 1-month wildtype murine Achilles and patellar tendons [4]. In this regard, 100  $\mu$ M ATP or 100  $\mu$ M UTP treatments elicited transient intracellular calcium signalling compared to Veh in Fura-2-loaded Achilles, patellar, and tail tenocyte cultures (n=3-5). As P2Y<sub>2</sub> is the only ATP- and UTP-sensitive receptor expressed by these cells, we further hypothesized that P2Y<sub>2</sub> mediates nucleotide-induced calcium signalling in Achilles, patellar and tail tenocytes. We first performed a cross-desensitization experiment to assess whether ATP could blunt a UTP-induced calcium response and vice versa – a strategy that takes advantage of the fact that metabotropic P2Y<sub>2</sub> receptors experience desensitization. Indeed, we observed marked cross-desensitization of secondary calcium signalling when 100  $\mu$ M ATP was added before 100  $\mu$ M UTP and vice versa (n=3-5). Additionally, AR-C 118925XX, a P2Y<sub>2</sub>-specific antagonist, reduced the intracellular calcium response to ATP and UTP treatment, further supporting that P2Y<sub>2</sub> mediates the calcium response (n=2-3). Finally, treatment of tail tenocyte cultures with 100  $\mu$ M ATP or 100  $\mu$ M UTP acutely increased gene expression of interleukin-6 and prostaglandin E2 at 1 h post-treatment and increased expression of Scleraxis at later time points (n=2-3), suggesting that nucleotide treatment alone is sufficient in driving expression of markers of matrix turnover and anabolism.

**DISCUSSION:** Taken together, we show, for the first time, that tenocytes express an ensemble of P2X and P2Y receptors. Moreover, intracellular calcium signalling elicited by treatment with exogenous ATP or UTP is likely mediated through P2Y<sub>2</sub>. Finally, the gene expression changes elicited by ATP or UTP treatment suggest that P2 receptor signalling may play a functional role in tendon mechanotransduction. Future studies underway are investigating tenocyte gene expression changes in response to mechanical loading *ex vivo* and *in vivo* to further characterize the role of mechanical stimulation in a physiological context.

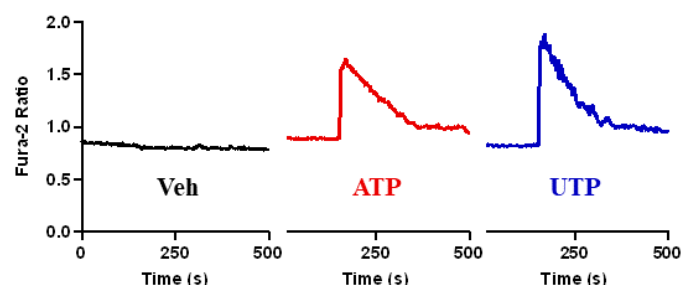
**SIGNIFICANCE/CLINICAL RELEVANCE:** Tendons and ligaments play a critical role in the function of the skeleton, with dysfunction of these tissues underlying joint dysfunction and disability. Understanding how tendons and ligaments respond to mechanical loading may improve our ability to engineer tendon or ligament repair constructs and how these tissues contribute to joint dysfunction in diseases such as osteoarthritis.

**REFERENCES:** [1] Grol et al. 2009. [2] Felsenthal and Zelzer. 2017. [3] Suzuki et al. 2003. [4] Grol et al. 2021

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**Figure 1.** Simplified schematic of the P2 receptor hypothesis for tendon mechanotransduction. Image created with BioRender.com.



**Figure 2.** Treatment with ATP or UTP elicits a greater calcium response in tenocytes compared to Veh (PBS). Representative single-cell calcium trace (from tail tenocytes) showing change in Fura-2 Ratio (F340/F380) (n=3-5).