

## Repetitively Stretched Tendon Fibroblasts Produce Inflammatory Mediators

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**We studied the expression of cytosolic phospholipase-A<sub>2</sub> and activity of secretory phospholipase-A<sub>2</sub> by human patellar tendon fibroblasts subjected to cyclic mechanical stretching. The effect of different stretching frequencies on the production of prostaglandin-E<sub>2</sub> and expression of cyclooxygenase enzyme were also examined. An in vitro system that can control alignment, shape, and mechanical loading conditions of tendon fibroblasts was used for this study. Cyclic stretching of fibroblasts increased the expression level of cytosolic phospholipase-A<sub>2</sub> by 88% and activity level of secretory phospholipase-A<sub>2</sub> by 190%, compared with those of non-stretched fibroblasts. Cyclic stretching of tendon fibroblasts at 0.1 Hz and 1.0 Hz also increased prostaglandin-E<sub>2</sub> production by 40% and 69%, respectively. Furthermore, cyclooxygenase-1 and cyclooxygenase-2 expression levels were increased in a stretching frequency-dependent manner, but cyclooxygenase-2 expression was increased more than that of cyclooxygenase-1. Because cytosolic phospholipase-A<sub>2</sub> and secretory phospholipase-A<sub>2</sub> are involved in the production of prostaglandin-E<sub>2</sub> and other inflammatory mediators, this study suggests that regulation of phospholipase-A<sub>2</sub> expression level may be an alternative approach to control in vivo tendon inflammation. The results of this study also may explain in part why activities that involve repetitive motion and high frequency loading of tendons are more likely to result in tendon inflammation.**

The term tendinopathy refers to a spectrum of tendon disorders characterized by pain, swelling, inflammation, and degeneration of a tendon.<sup>14</sup> Tendinopathy is commonly seen in athletic and occupational settings,<sup>10,13</sup> and the

symptoms of tendinopathy such as pain usually are exacerbated by an increase in the activity level.<sup>4,18</sup> Rest, therapeutic exercise, and antiinflammatory medications often are used as treatments, but they are largely empirical with little scientific data for justification.<sup>1,11,16</sup> Therefore, it is important to understand the cellular and molecular mechanisms involved in the development of tendinopathy so that better means of prevention and treatment can be devised.

Structurally, tendons are composed mostly of unidirectionally oriented collagen fibers interspersed with fibroblasts that are elongated. Because tendon fibroblasts are aligned along the collagen fibers, that is, the tendon's longitudinal direction, the cells are subjected to uniaxial stretching when the tendon is stretched. It has been shown that repetitive stretching of a tendon with exercise induces high levels of prostaglandin-E<sub>2</sub> (PGE<sub>2</sub>).<sup>12</sup> Phospholipase-A<sub>2</sub> (PLA<sub>2</sub>), a lipolytic enzyme, is involved in the production of PGE<sub>2</sub> and other inflammatory mediators by catalyzing the hydrolysis of membrane phospholipids and releasing arachidonic acid.<sup>9</sup> Arachidonic acid then is converted into prostaglandins by two isoforms of cyclooxygenase (COX), namely COX-1 and COX-2.<sup>20</sup> The PLA<sub>2</sub> family of enzymes consists of several isotypes, including cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) and secretory PLA<sub>2</sub> (sPLA<sub>2</sub>). The cPLA<sub>2</sub> is expressed ubiquitously and up-regulated by inflammatory stimuli.<sup>19,22</sup> The sPLA<sub>2</sub> is implicated in the regulation of regional blood flow to inflamed sites.<sup>23</sup> A previous study showed that cyclic stretching of human flexor tendon fibroblasts increases levels of PGE<sub>2</sub>.<sup>2</sup> However, there are important questions about the expression of PLA<sub>2</sub>, PGE<sub>2</sub>, and COX that remain to be answered. First, it is not known whether cyclic mechanical stretching affects expression levels of PLA<sub>2</sub> in human tendon fibroblasts. Second, it is yet to be determined whether the production of PGE<sub>2</sub> and the expression of COX depend on the frequency of cyclic stretching applied to the tendon fibroblasts.

Therefore, this study addressed the following two questions: (1) Does cyclic stretching of human tendon fibroblasts increase expression or activity level of cPLA<sub>2</sub> and sPLA<sub>2</sub>? and (2) Do the production of PGE<sub>2</sub> and the ex-

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pression of COX-1 and COX-2 depend on stretching frequency? To answer these questions, human patellar tendon fibroblasts were cyclically stretched at various frequencies. Then, the aforementioned inflammatory mediators were measured to determine their expression or activity levels. Herein we report the findings of these experiments.

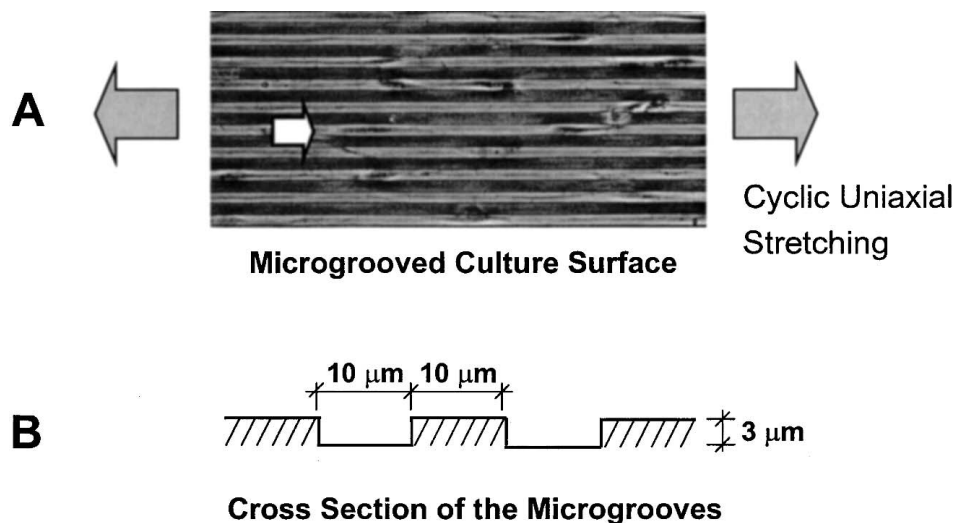
## MATERIALS AND METHODS

Custom-made silicone dishes were used in the current study for growing and stretching human patellar tendon fibroblasts. The silicone dishes were made by a molding process, as described previously.<sup>24</sup> A special feature of the dish is that it has a microgrooved surface (area  $3 \times 6$  cm), instead of a smooth surface. The microgrooves were rectangular, with a 10- $\mu$ m ridge and groove width and 3- $\mu$ m groove depth. For applying cyclic stretching to tendon fibroblasts, a custom-made stretching apparatus similar to one described previously was used.<sup>17</sup> This stretching apparatus can apply uniaxial stretching with various stretching magnitudes and frequencies to tendon fibroblasts in the microgrooved silicone dishes.

Human patellar tendon fibroblasts were derived from tendon samples obtained from two patients (a man, age 20 years and a woman, age 38 years), who had ACL reconstructions using patellar tendon autografts. Tendon fibroblasts from these two patients were chosen because they were healthy and because fibroblasts from this age group maintain their phenotype such as morphologic features and proliferation relatively unchanged compared with those from older patients ( $> 40$  years old). The protocol for collection of tendon samples was approved by the

institutional review board of our institution. The tendon samples were washed thoroughly with PBS (PBS; Life Technologies, Rockville, MD) and minced in Petri dishes. Then, 10 mL of DMEM, containing 10% heat inactivated FBS and 1% penicillin-streptomycin (Life Technologies), was added to each dish. These tendon explants were kept in an incubator with a humidified atmosphere of 5% CO<sub>2</sub> and a temperature of 37°C. After the tendon fibroblasts grew from the tendon explants and reached confluence, they were subcultured five to six times to obtain enough cells for stretching experiments. Because the tendon samples were from the midsubstance of the tendon grafts, the cells derived from the samples were tendon fibroblasts, which are the predominant cell type inside a tendon. In culture, these cells maintained their morphologic features during the experiments, and their doubling time did not change noticeably during the passages (as many as five or six times) used in this study.

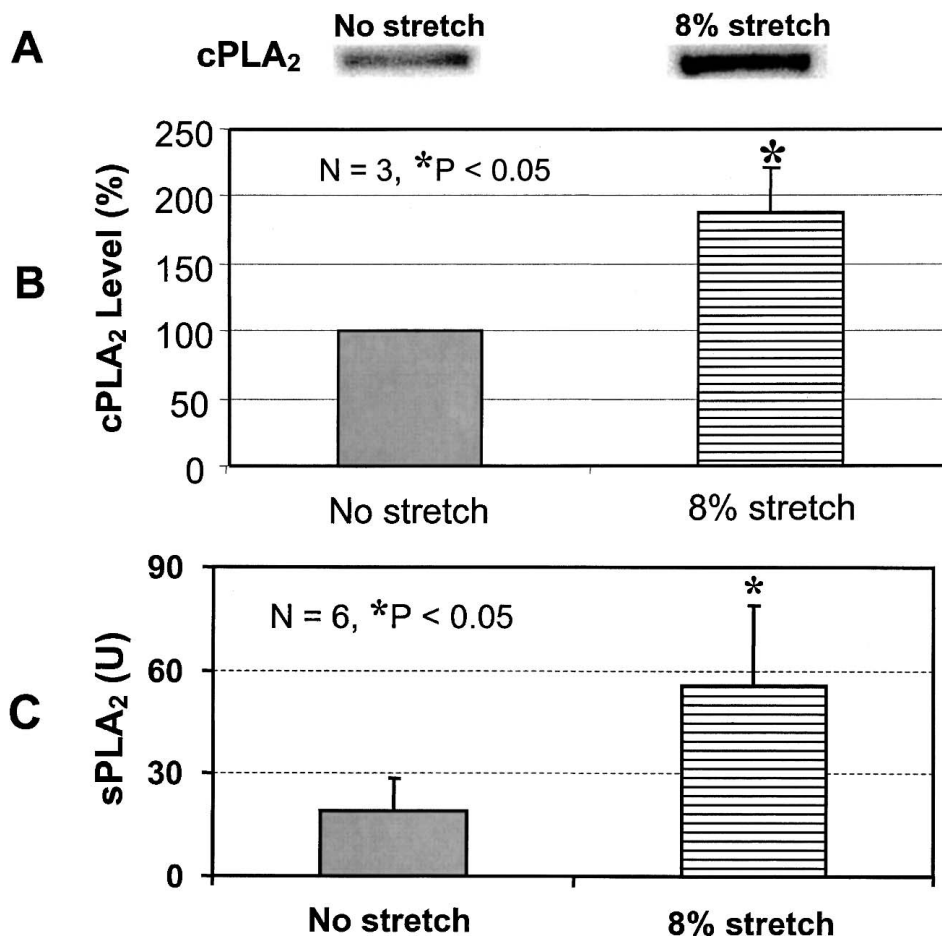
The human patellar tendon fibroblasts were plated onto the microgrooved silicone dishes, which had been coated with 10  $\mu$ g/mL ProNectin-F (BioSource International, Inc, Camarillo, CA) to promote cell attachment.<sup>24</sup> These fibroblasts aligned along the microgrooves of the silicone dishes with or without cyclic mechanical stretching. The plating density of the cells was  $2 \times 10^5$  cells per dish, and overall these cells were not visually in contact with each other to mimic the *in vivo* organization of these cells in the tendon (Fig 1). The cells were allowed to grow in DMEM with 10% FBS and 1% penicillin-streptomycin for 48 hours. During the time, the numbers of the tendon fibroblasts were only slightly increased ( $< 10\%$ ). The cells still resided individually in the microgrooved surfaces of the silicone dishes. One hour before application of cyclic stretching, the medium in each dish was replaced with fresh DMEM containing only 1% FBS to minimize the effect of serum on the production of in-



**Fig 1A–B.** An *in vitro* system used to study the effects of cyclic mechanical stretching on human patellar tendon fibroblasts is shown. The fibroblasts were grown in microgrooved silicone surfaces instead of commonly used smooth surfaces. (A) The cells were plated at a low density so that they did not visually contact each other to mimic *in vivo* conditions (white arrow points to a fibroblast residing in the microgrooves). (B) The width of the ridges and grooves is 10- $\mu$ m, and the depth is approximately 3- $\mu$ m.

flammatory mediators attributable to cyclic mechanical stretching. The fibroblasts were cyclically stretched at 0.5 Hz, with 8% stretching magnitude for 4 hours, followed by an additional 4 hours rest. This stretching protocol was chosen because a previous study showed that it can induce higher levels of PGE<sub>2</sub> production by human patellar tendon fibroblasts compared with those of nonstretched cells.<sup>25</sup> After the rest period ended, the medium was collected, and the cells in the silicone dish were washed quickly with ice-cold PBS. Next, lysis buffer (M-PER Mammalian Protein Extraction Reagent, Pierce, Rockford, IL) was added to each dish to collect cellular protein. Equal amounts of protein (20 μg) from each sample were loaded into a 10% acrylamide gel. After electrophoresis the separated proteins were transferred onto a nitrocellulose membrane using an Xcell Sure-Lock Cell and transfer module (Invitrogen, Carlsbad, CA). The membrane was blocked in 5% nonfat milk-PBS-Tween 20 solu-

tion in a cold room overnight, followed by application of cPLA<sub>2</sub> specific mouse monoclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) in 1% nonfat milk-PBS-Tween 20 (1:1000). After incubation at room temperature for 2 hours, the secondary antibody (goat antimouse IgG, Jackson Immunoresearch Laboratory, Inc, West Grove, PA) in 1% nonfat milk-PBS-Tween 20 (1:5000) was applied for 1 hour. The membrane was washed three times with 0.05% PBS-Tween for 15 minutes between application of the two antibodies. The cPLA<sub>2</sub> proteins on the nitrocellulose membrane were detected with the ECL Plus detection system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol. In addition, the collected medium was used to assay sPLA<sub>2</sub> activity levels using an ELISA kit (R&D Systems, Minneapolis, MN). Three sets of stretching experiments were done to assay cPLA<sub>2</sub>, therefore, the sample size was three. For assaying sPLA<sub>2</sub>, each of these three



**Fig 2A–C.** The effect of cyclic stretching on the cPLA<sub>2</sub> expression level and sPLA<sub>2</sub> activity level is shown. Human patellar tendon fibroblasts were stretched at 0.5 Hz with a stretching magnitude of 8% for 4 hours, followed by 4 hours rest. (A, B) Representative results of three experiments with Western blots showed that cyclic stretching of the tendon fibroblasts markedly increased the expression level of cPLA<sub>2</sub>, compared with that of nonstretched fibroblasts. (C) Cyclic stretching also markedly increased the activity level of sPLA<sub>2</sub> secreted into medium by tendon fibroblasts.

stretched experiments used two dishes for either the stretch group or the nonstretch group, and therefore the sample size was six.

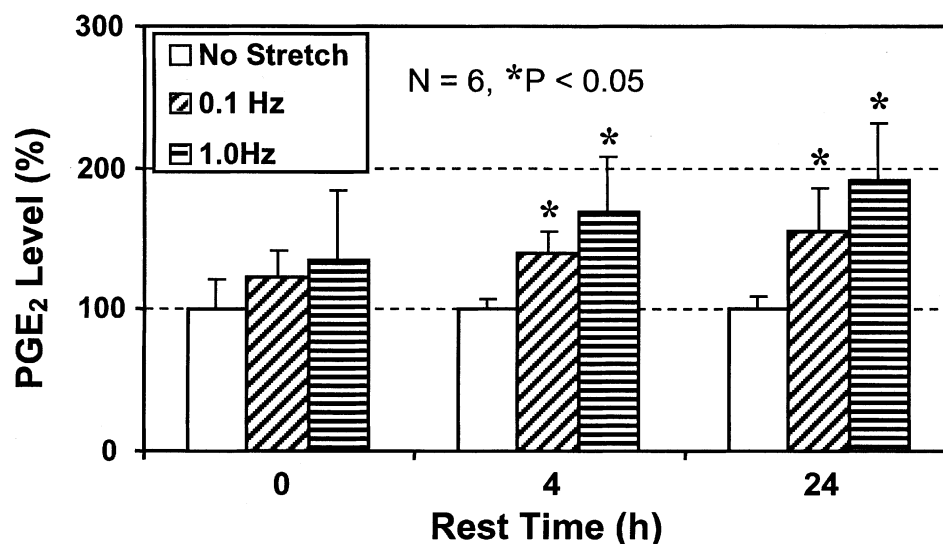
In separate experiments, fibroblasts were stretched cyclically at 8%, with one of three different stretching frequencies, 0 (no stretch), 0.1, or 1.0 Hz. The stretching duration for these cells was 4 hours, followed by varying rest times from 0 (no rest), 4, to 24 hours. Three such independent experiments were done, which involved two dishes of cells for each experimental condition above. Immediately after stretching, the media in the dishes were collected, and levels of PGE<sub>2</sub> were measured with ELISA kits (R&D Systems). In addition, after removal of the medium in the dish, total cellular protein was collected to assay for COX-1 and COX-2 expression levels with Western blot procedures similar to those used for assaying cPLA<sub>2</sub>. Cyclooxygenase-1 and COX-2 monoclonal mouse antibodies were used (1:1000 dilution in PBS, Cayman Chemical Co, Ann Arbor, MI). To semiquantify the levels of COX expression and cPLA<sub>2</sub>, the bands were measured on a densitometer (Model GS800, BioRad Laboratories, Hercules, CA). For statistical analysis, one-way ANOVA or a paired t test was used wherever appropriate, with  $p < 0.05$  considered statistically significant.

## RESULTS

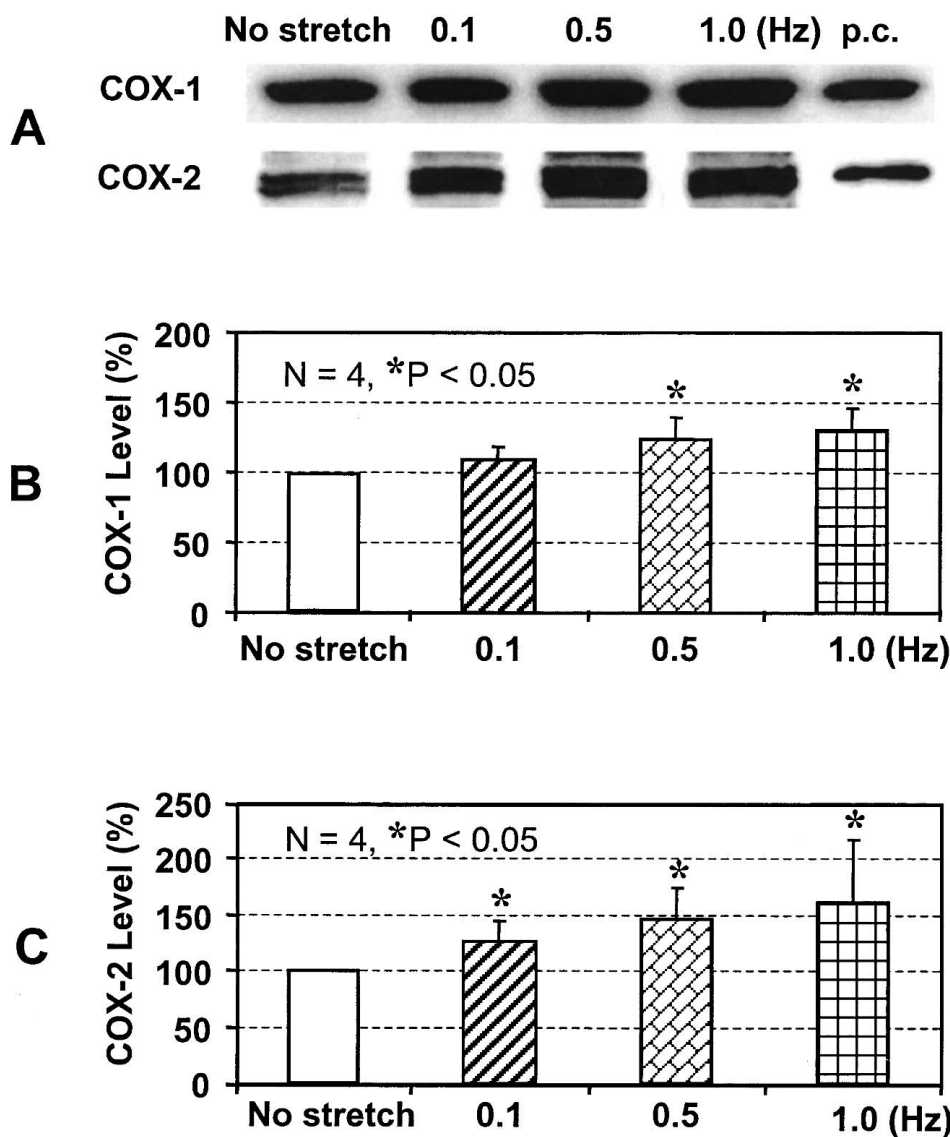
Compared with the nonstretched control group, the tendon fibroblasts subjected to 4 hours of stretching followed by 4 hours rest increased cPLA<sub>2</sub> expression level by 88% ( $p = 0.009$ ) and sPLA<sub>2</sub> activity level by 190% ( $p = 0.011$ ) (Fig 2).

Initially, there were no significant differences in PGE<sub>2</sub> production seen between the high frequency stretching group and the low frequency stretching group. However, the levels of PGE<sub>2</sub> increased more in the high frequency group compared with the low frequency group as the rest time increased. Specifically, immediately after 4 hours of stretching, the stretched fibroblasts produced consistently higher levels of PGE<sub>2</sub> compared with nonstretched fibroblasts, but there were no statistical differences between the stretched and nonstretched fibroblasts. However, after 4 hours rest, PGE<sub>2</sub> levels were increased on average by 40% (0.1 Hz group;  $p = 0.014$ ) and 69% (1.0 Hz group;  $p = 0.002$ ), compared with the nonstretched control group. After 24 hours rest, PGE<sub>2</sub> levels were increased by 55% (0.1 Hz group;  $p = 0.006$ ) and by 90% (1.0 Hz group;  $p = 0.0001$ ) compared with the nonstretched control group (Fig 3).

After 8% stretching at 0.1 Hz, the COX-1 level was not significantly increased ( $p = 0.082$ ), whereas the COX-2 level did increase significantly ( $p = 0.030$ ), compared with the nonstretched control group. However, after 8% stretching at 0.5 Hz, both COX-1 and COX-2 levels were significantly increased ( $p = 0.029$ , and  $p = 0.025$ , respectively), compared with the nonstretched control group. Similarly, 8% stretching at 1.0 Hz significantly increased COX-1 ( $p = 0.021$ ) and COX-2 levels ( $p = 0.032$ ). However, the increase in COX-2 level was more pronounced than that of COX-1. For example, with cyclic stretching at



**Fig 3.** The effect of stretching frequency on PGE<sub>2</sub> production by human patellar tendon fibroblasts is shown. The cells were stretched at 8% at either 0.1 Hz or 1.0 Hz for 4 hours, followed by rest for 0, 4, and 24 hours. Prostaglandin-E<sub>2</sub> production by the fibroblasts increased with increased stretching frequency. With increased rest time, the stretching frequency effect became more pronounced.



**Fig 4A–C.** (A) A representative Western blot shows the effect of stretching frequency on COX expression levels by human patellar tendon fibroblasts (p.c.—positive control for COX). (B, C) With increased stretching frequency from 0.1, 0.5, to 1.0 Hz, COX-1 and COX-2 expression levels also increased. The level of the increase in COX-2 expression was markedly higher than that of COX-1. Four separate experiments were done, and consistent results were obtained. Cyclooxygenase-1 and COX-2 levels were normalized with respect to their own controls without stretching for each of the four experiments.

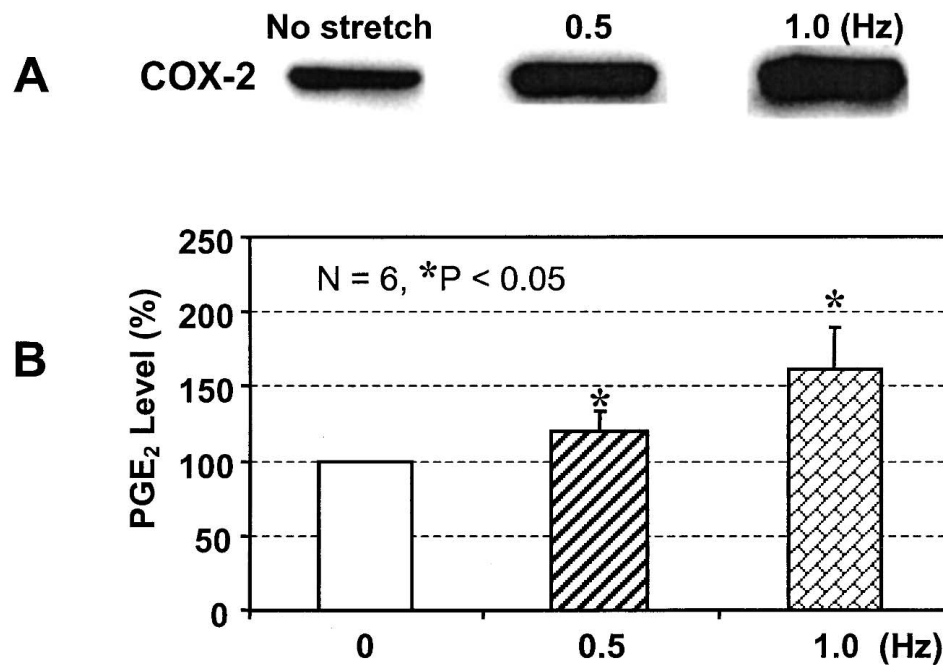
1.0 Hz, the COX-2 level was increased on average by 62%, whereas the COX-1 level increased by 30% (Fig 4).

We also found that stretching frequency, instead of the total number of stretching cycles, was the main determinant of COX-2 expression and PGE<sub>2</sub> production (Fig 5). This was determined by using two groups of fibroblasts that were stretched at two frequencies (0.5 Hz and 1.0 Hz) for two lengths of time such that the total number of cycles was the same (0.5 Hz for 2 hours versus 1.0 Hz for 1 hour). The results showed that the group of fibroblasts stretched

at 1.0 Hz still produced higher levels of COX-2 (Fig 5A) and PGE<sub>2</sub> compared with that at 0.5 Hz (Fig 5B, *p* = 0.014).

## DISCUSSION

Previous work showed that the *in vitro* system used in the current study aligns tendon fibroblasts along the stretching direction and subjects the cells to cyclic uniaxial stretching similar to *in vivo* conditions.<sup>25</sup> Using this system, the cur-



**Fig 5A–B.** The effect of stretching-frequency on PGE<sub>2</sub> production and COX expression is shown. Two stretching frequencies (0.5 Hz and 1.0 Hz) with the same number of total cycles (2 hours stretching for 0.5 Hz and 1 hour for 1.0 Hz) were applied to the human tendon fibroblasts. (A) Stretching at 1.0 Hz induced a higher level of COX-2 expression and (B) PGE<sub>2</sub> production than at 0.5 Hz. Prostaglandin-E<sub>2</sub> levels at both frequencies were normalized with respect to controls without stretching for each experiment.

rent study showed that cyclic stretching of human patellar tendon fibroblasts increased levels of cPLA<sub>2</sub> expression and sPLA<sub>2</sub> activity. Furthermore, cyclic stretching increased COX (COX-1 and COX-2) expression and PGE<sub>2</sub> production. This increase in COX and PGE<sub>2</sub> levels was likely stretching-frequency dependent, because stretching at 1.0 Hz still induced higher levels of COX and PGE<sub>2</sub> than at 0.5 Hz when the number of cycles was the same.

This study was designed to study the mechanobiology of tendon fibroblasts *in vivo* using an *in vitro* system. Although this system controls many variables, such as alignment of fibroblasts and the mechanical loading conditions, other factors, such as extracellular matrix composition and cytokines that can influence the *in vivo* behavior of tendon fibroblasts, were not considered in this study. It is possible that these factors exert additional effects on the response of fibroblasts to cyclic mechanical stretching, and future studies are needed to determine their effects.

Previous studies have shown that mechanical stretching of fibroblasts increases PGE<sub>2</sub> levels.<sup>2,3</sup> The PGE<sub>2</sub> production by cyclically stretched human flexor tendon fibroblasts also was shown to depend on stretching frequency.<sup>2</sup> Therefore, our finding of stretching frequency effect on PGE<sub>2</sub> production by human patellar tendon fibroblasts

is in agreement with the previous study. Because levels of PGE<sub>2</sub> after cyclic mechanical stretching of tendon fibroblasts are increased *in vitro*, there is a possibility that such an increase of PGE<sub>2</sub> also might occur *in vivo*. Prostaglandin-E<sub>2</sub> levels in the Achilles tendons of human subjects during exercise were found to be markedly increased.<sup>12</sup> The stretching-induced PGE<sub>2</sub> production may cause tendon degeneration *in vivo* in addition to known tendon inflammation by PGE<sub>2</sub>. Evidence supporting this possibility is that injection of PGE<sub>1</sub> around the Achilles tendons of rats results in degenerative changes in the tendons.<sup>21</sup> The results of the current study suggest that tendons that undergo high rates of stretching might be more susceptible to inflammation. Therefore, activity modification, such as avoidance from activities with high rates of loading on tendons, may be a beneficial preventive strategy.

The current study differs in experimental conditions from previous studies in several ways.<sup>2,3</sup> First, our study applied uniaxial stretching, as opposed to biaxial stretching in the previous studies. We also applied a much smaller strain (8%) compared with that used in the previous studies (as much as 25%). Finally, our study allows the shape and alignment of tendon fibroblasts to be controlled, both of which are important in influencing cell metabolism

and responsiveness to mechanical loading.<sup>5</sup> It is likely that overall biologic responses of tendon fibroblasts to uniaxial stretching as used in the current study would be expected to differ from those of the previous studies.

We also found that COX-1 and COX-2 expression levels of tendon fibroblasts are stretching-frequency dependent, although the increase in COX-2 level is larger than that of COX-1 (Fig 4). It generally is thought that in response to inflammatory stimuli, such as cytokines and bacterial products, COX-2, but not COX-1, is responsive by upregulation.<sup>6</sup> Nevertheless, it has been reported that like COX-2, COX-1 can be upregulated, contributing to high levels of production of prostaglandins in humans when stimulated with lipopolysaccharide.<sup>15</sup> Our finding that the levels of both COX isoenzymes increase in response to cyclic mechanical stretching indicates that they both are responsive to mechanical stimuli. It also is consistent with the concept that COX-2 is more of an inducible inflammatory enzyme compared with COX-1, because COX-2 levels were higher than those of COX-1 in response to the same cyclic mechanical stretching.

Cyclooxygenase-1 and COX-2 play important roles in regulating cellular inflammatory responses, because they are responsible for the conversion of arachidonic acid into prostaglandins.<sup>8,20,26</sup> Inhibiting COX by indomethacin, a nonsteroidal antiinflammatory drug, decreased PGE<sub>2</sub> production by tendon fibroblasts that were subjected to cyclic stretching.<sup>3</sup> Therefore, stretching-induced increased COX expression may be responsible in part for increased PGE<sub>2</sub> production by tendon fibroblasts subjected to stretching. This is because some of the increased PGE<sub>2</sub> production could be attributable to the increased production of arachidonic acid through the action of PLA<sub>2</sub>, in addition to the increased level or activity (or both) of COX.

It is known that PLA<sub>2</sub> catalyzes the release of arachidonic acid from the cell membrane, which is a precursor for many inflammatory mediators such as PGE<sub>2</sub>, and therefore it mediates cellular inflammatory responses to exogenous stimuli. Phospholipase-A<sub>2</sub> plays an important role in initiating tissue inflammation. For example, intratracheal, intradermal, and intraarticular injection of PLA<sub>2</sub> in rabbits induces profound inflammatory lesions.<sup>7</sup> Blocking the action of PLA<sub>2</sub> may stop the production of prostaglandins, thereby potentially slowing or halting the progression of tendinopathy. However, given the multiple physiologic roles of PLA<sub>2</sub> in vivo,<sup>22</sup> complete blockage of its expression or activity might have unintended consequences. Similarly, prostanoids mediate a wide variety of functions in vivo, and therefore these functions also might be disrupted if PLA<sub>2</sub> function is inhibited.

The current study showed that cyclic mechanical stretching of human tendon fibroblasts produces high levels of cPLA<sub>2</sub> expression and increases sPLA<sub>2</sub> activity. The

cyclically stretched fibroblasts also produce high levels of PGE<sub>2</sub> and COX in a stretching-frequency dependent manner. These findings may be useful in devising new strategies for prevention and treatment of tendinopathy. Additional studies are needed to investigate the role of these inflammatory mediators (PLA<sub>2</sub>, COX, and PGE<sub>2</sub>) in animal models.

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