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# Inflammatory Response of Human Tendon Fibroblasts to Cyclic Mechanical Stretching

Zhaozhu Li, MD, Guoguang Yang, MS, Mustafa Khan, MD, David Stone, MD, Savio L-Y. Woo, PhD, and James H-C. Wang,\* PhD  
*From the Musculoskeletal Research Center, Department of Orthopaedic Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania*

**Background:** The cellular and molecular mechanisms for the development of tendinopathy are not clear, but inflammatory mediators produced by tendon fibroblasts in response to repetitive mechanical loading may be an important factor.

**Hypotheses:** (1) Cyclic stretching of tendon fibroblasts affects the production of leukotriene B<sub>4</sub> and the expression of 5-lipoxygenase; and (2) the production level of leukotriene B<sub>4</sub> is inversely related to that of prostaglandin E<sub>2</sub>.

**Study Design:** Controlled laboratory study.

**Methods:** Human patellar tendon fibroblasts were uniaxially stretched in the presence of indomethacin (25 μM) or MK-886 (10 μM). After stretching for 4 hours, followed by 4 hours rest, levels of prostaglandin E<sub>2</sub>, leukotriene B<sub>4</sub>, and expression of 5-lipoxygenase were measured.

**Results:** Stretched tendon fibroblasts increased the levels of leukotriene B<sub>4</sub> but did not appreciably change the expression of 5-lipoxygenase. Indomethacin decreased the cellular production of prostaglandin E<sub>2</sub> but caused increased leukotriene B<sub>4</sub> levels. MK-886 caused decreased production of leukotriene B<sub>4</sub> but increased production of prostaglandin E<sub>2</sub>.

**Conclusions:** Cyclic stretching of human tendon fibroblasts increases the production of prostaglandin E<sub>2</sub> and leukotriene B<sub>4</sub>. Blocking prostaglandin E<sub>2</sub> production leads to increased leukotriene B<sub>4</sub> levels and vice versa.

**Clinical Relevance:** The use of nonsteroidal anti-inflammatory drugs for the treatment of tendon inflammation might increase the levels of leukotriene B<sub>4</sub> within the tendon, potentially contributing to the development of tendinopathy.

**Keywords:** stretching; microgrooves; tendon fibroblasts; leukotriene B<sub>4</sub>; 5-lipoxygenase

Tendinopathy is a common medical problem that accounts for approximately 48% of occupational illnesses in the United States.<sup>16</sup> It is generally believed that excessive, repetitive mechanical loading on the tendon is the initial event in the development of tendinopathy, leading to tendon inflammation, degeneration, and even rupture.<sup>4</sup> To understand the pathophysiology of tendinopathy, *in vivo* studies have been conducted to examine the role of mechanical loading in the development of tendinopathy. For example, after rabbit tendons were passively flexed and extended for 2 hours per day, 3 times a week, over a total period of 6 weeks, the tendons were found to be gross-

ly inflamed and fibrillated.<sup>5</sup> Histological examination revealed the presence of inflammatory cell infiltrates, fibroblast proliferation, and increased vascularity. However, the study did not attempt to examine what inflammatory mediators were responsible for these findings. To address this question, animal models injected with various inflammatory agents have been used. For example, rabbit patellar tendons injected with a cytokine mixture were shown to produce fibrosis, hypercellularity, and increased angiogenesis.<sup>21</sup> Also, peritendinous injection of prostaglandin E<sub>1</sub> into rabbit Achilles tendons has been shown to cause peri- and intratendinous degeneration and fibrosis.<sup>22</sup> Although injection of inflammatory agents is useful for demonstrating that they can induce tendon pathology, it is unclear whether they are actually involved in the pathophysiological processes of the tendinopathy development.

To elucidate the cellular and molecular mechanisms for the development of tendinopathy, we have developed an *in vitro* model system.<sup>25</sup> Using this model system, we have shown that cyclic stretching of human patellar tendon

\* Address correspondence to James H-C. Wang, Musculoskeletal Research Center, Department of Orthopaedic Surgery, University of Pittsburgh Medical Center, E1641 Biomedical Science Tower, 210 Lothrop Street, P.O. Box 71199, Pittsburgh, PA 15213 (e-mail: wanghc@pitt.edu).

fibroblasts (HPTFs) increases cyclooxygenase (COX) expression levels and production of prostaglandin  $E_2$  ( $PGE_2$ ). This suggests that the production of inflammatory mediators by fibroblasts due to repetitive mechanical loading might be an important step in subsequent tendon inflammation and injury. However,  $PGE_2$  is not the only known inflammatory mediator. Leukotrienes, such as leukotriene  $B_4$  ( $LTB_4$ ), are also important in the inflammatory response.<sup>12,13</sup> Interestingly, both prostaglandins and leukotrienes are synthesized from the same precursor, arachidonic acid (AA), via a complex series of steps and intermediates.<sup>7</sup> AA is converted by the COX enzyme into prostaglandins, whereas the enzyme 5-lipoxygenase (5-LO) converts AA into leukotrienes.<sup>7,17,18,20</sup> A cofactor protein known as 5-lipoxygenase activating protein (FLAP) is required for the activity of 5-LO.<sup>18</sup> Besides being a major product of the leukotriene pathway,  $LTB_4$  is a potent inflammatory mediator, and reliable ELISA assays for its measurement are available; yet whether cyclic stretching of human tendon fibroblasts affects  $LTB_4$  production and 5-LO expression is not known. Furthermore, because both COX and 5-LO pathways use AA to synthesize  $PGE_2$  and  $LTB_4$ , respectively, how alterations in the activity of 1 pathway under cyclic mechanical stretching will affect the other pathway is also unknown. Therefore, the aforementioned studies provide us a rationale to investigate whether the leukotriene pathway is affected by in vitro cyclic stretching of HPTFs.

Therefore, the objective of this study was to test the following 3 hypotheses: (1) Cyclic stretching of HPTFs affects the production of  $LTB_4$  and the expression of 5-LO; (2) blocking the COX pathway will lead to increased end products of the 5-LO pathway, such as  $LTB_4$ ; and (3) blocking the 5-LO pathway will lead to increased end products of the COX pathway, such as  $PGE_2$ . To test these hypotheses, we used an in vitro model system, which can control the alignment, shape, and mechanical loading conditions of the tendon fibroblasts.

## MATERIALS AND METHODS

### Cell Culture

HPTFs from young, healthy donors (20-40 years old) were isolated using explant tissue culture techniques. The tendon samples were obtained from fresh surgical wastes of normal tendon grafts for reconstruction of the anterior cruciate ligament. The protocol for obtaining the tendon samples was approved by the Institutional Review Board of the University of Pittsburgh Medical Center (IRB # 0108109). The tendon samples were minced aseptically, transferred to 100 mm polystyrene Petri dishes, and cultured with Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), penicillin (50 U/mL), and streptomycin (50 U/mL). The culture was maintained in a humidified atmosphere of 5%  $CO_2$  at 37 C. To obtain sufficient numbers of fibroblasts for stretching experiments, the cells were subcultured up to 4 times. For stretching

experiments, fibroblasts were plated on culture surfaces in custom-made silicone dishes (culture area 3 cm  $\times$  6 cm), which were transparent, elastic, and nontoxic to cultured cells.<sup>24</sup> A unique feature of the dish was that the culture surface was fabricated with microgrooves, with 10  $\mu$ m groove width, 3  $\mu$ m groove depth, and 10  $\mu$ m wide ridges between the grooves (Figure 1). To promote cell attachment, the microgrooved surfaces of the dishes were coated with 10  $\mu$ g/ml ProNectin-F (Sigma, St Louis, Missouri) before they were plated with tendon fibroblasts.

### Stretching Experiments

A total of  $4 \times 10^5$  human tendon fibroblasts were plated on each dish. The cells were grown with 3 mL of regular growth medium (ie, DMEM + 10% FBS and antibiotics) per dish for 2 days. The growth medium was replaced with a low serum (1% FBS) DMEM prior to mechanical stretching. The cells were cyclically stretched at 1 of 3 predetermined magnitudes (4%, 8%, 12%) at 0.5 Hz, for 4 hours, using a custom-made stretching apparatus as described previously.<sup>25</sup> Six sets of experiments were performed to determine the relationship between stretching magnitude and  $LTB_4$  production. Unstretched cells were used as controls. In other stretching experiments, 25  $\mu$ M indomethacin (Sigma), a specific nonsteroidal anti-inflammatory drug (NSAID) inhibitor for COX, was added to the medium ( $n = 6$ ). This concentration was chosen because it was the lowest dose that inhibited  $PGE_2$  production in our experiments. Similarly, 10  $\mu$ M MK-886 (Sigma), which is a specific inhibitor of FLAP,<sup>19</sup> was added in other stretching experiments ( $n = 3$ ). After stretching, the cells were rested for an additional 4 hours. The medium was then collected to assay for  $LTB_4$  ( $n = 6$ ) and  $PGE_2$  ( $n = 3$ ). The cell lysates were also harvested to assay for 5-LO ( $n = 4$ ). For the set of experiments performed to study the effects of indomethacin on  $LTB_4$  production, the media obtained at the end of each experiment were frozen in a freezer ( $-80^\circ\text{C}$ ) for all 3 groups (no stretch, 8%, 8% + indomethacin;  $n = 6$  each).

### Assay for $LTB_4$ and $PGE_2$ Levels

A commercially available ELISA kit (R&D Systems, Minneapolis, Minnesota) was used to assay for  $LTB_4$  levels. The assay was based on the competitive binding technique, in which  $LTB_4$  present in a sample competes with a fixed amount of alkaline phosphatase-labeled  $LTB_4$  for sites on a rabbit polyclonal antibody. During incubation, the antibody becomes bound to the goat antirabbit antibody coated on the microplate. After incubation, the plate was washed to remove excess conjugates and unbound sample. Next, a substrate solution was added to the wells to determine the activity of the alkaline phosphatase. The resulting optical density was measured at 405 nm with a spectrophotometer (model GS800, Bio-Rad Laboratories, Hercules, California). This absorbance was inversely proportional to the concentration of  $LTB_4$  in the sample. Using the standard curve generated by testing of known

concentrations of  $\text{LTB}_4$  supplied by the manufacturer, the amount of  $\text{LTB}_4$  in each sample was determined using a standard curve generated for  $\text{LTB}_4$  at the same time. The sensitivity of this  $\text{LTB}_4$  kit is about 10 pg/ml.

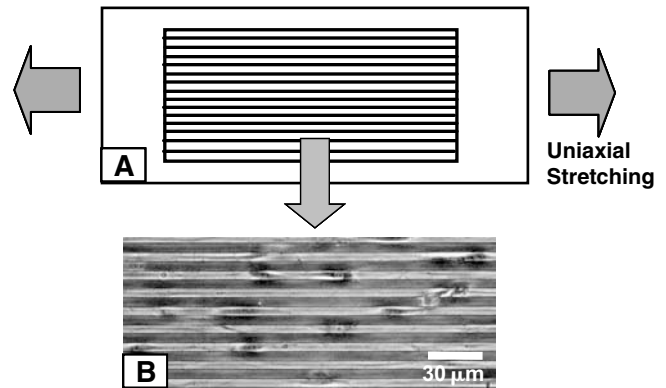
Similarly, the levels of  $\text{PGE}_2$  were measured using a commercially available kit (R&D Systems, Minneapolis, Minnesota). The protocol was similar to the one described above for measurement of  $\text{LTB}_4$ . The only exception was that a mouse monoclonal antibody specific for  $\text{PGE}_2$  and a goat antimouse antibody coated on the microplate were instead used. The sensitivity of this  $\text{PGE}_2$  kit is about 36 pg/ml.

### Western Blot for Measuring 5-LO Expression

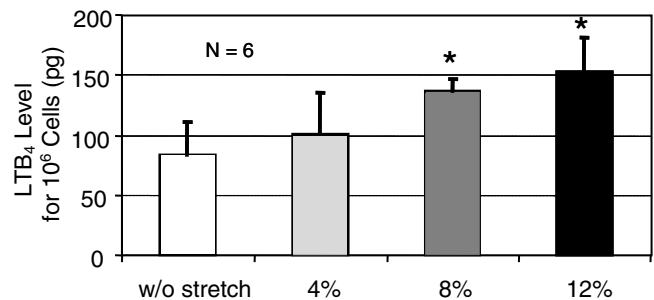
To determine the expression level of 5-LO, a standard Western blotting technique was used. First, 75  $\mu\text{L}$  of CLAP (chymostatin, leupeptin, antipain, and pepstatin A) solution containing protease inhibitors was added to 5 mL of lysis buffer (15 mM HEPES, 145 mM NaCl, 0.1 mM  $\text{MgCl}_2$ , and 1 mM EGTA) to make a lysis solution. Then, the media were removed from the silicone dishes after the cells had incubated for 4 hours after stretching. The cells were then washed twice with ice-cold PBS. Then, 300  $\mu\text{L}$  of the lysis solution was added to the dish, and the cells were scraped from the dish. The resulting protein sample from total cell lysate was pipetted into a 1.5-mL Eppendorf tube. The protein concentration of the sample was measured using the BCA Protein Assay kit (Pierce, Rockford, Illinois). Then, 20  $\mu\text{g}$  of protein from each sample with loading buffer was loaded into a 7% SDS-PAGE gel for electrophoresis. The separated proteins were then transferred to a nitrocellulose membrane, using a Mini-Protean 3 cell and transfer module (Bio-Rad Laboratories). The membrane was incubated in 5% nonfat milk/PBS-Tween 20 solution in a cold room overnight, followed by application of the primary monoclonal antibody (concentration 1:2000) to 5-LO (Research Diagnostics Inc., Flanders, New Jersey) in 1% nonfat milk/PBS-Tween 20. After 2 hours of incubation at room temperature, the secondary antibody, goat antimouse IgG (Research Diagnostics Inc.) in 1% nonfat milk/PBS-Tween 20 (concentration 1:5000), was applied with 1 hour of incubation. The membrane was washed 3 times with 0.05% PBS/Tween for 15 minutes after application of each antibody. The 5-LO protein bands on the nitrocellulose membrane were detected with the ECL Plus Western blot detection system (Amersham Pharmacia Biotech, Piscataway, New Jersey) according to the manufacturer's protocol. The resulting protein bands were quantified on an imaging densitometer (Model GS800, BioRad Laboratories).

### Data Analysis

To determine whether the amount of  $\text{LTB}_4$  production by the stretched tendon fibroblasts was dependent on the stretching magnitude, 1-way analysis of variance (ANOVA) was used, followed by Fisher's paired least significant difference test for multiple comparisons to deter-



**Figure 1.** Silicone dishes were used for growing and stretching tendon fibroblasts in this study (A). A special feature of the dish is that it contains a microgrooved culture surface of 3 cm  $\times$  6 cm. The tendon fibroblasts on the microgrooved surface were elongated in shape, aligned in the direction of the microgrooves, and subjected to cyclic uniaxial stretching (horizontal arrows) (B).

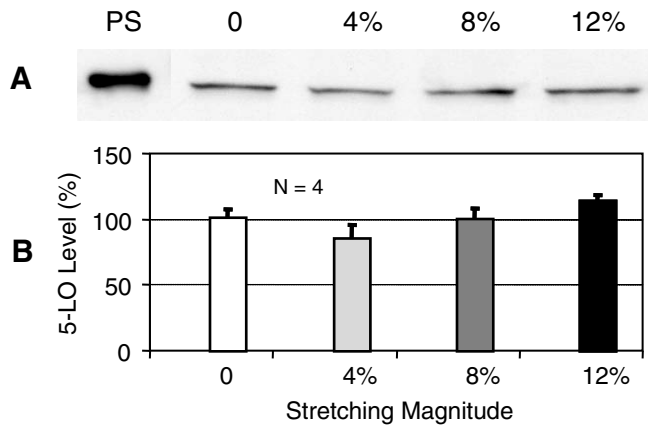


**Figure 2.** The effect of cyclic mechanical stretching on  $\text{LTB}_4$  production by human tendon fibroblasts. Cyclic stretching at 8% and 12%, but not 4%, significantly increased  $\text{LTB}_4$  production compared to the cells without stretching. In addition,  $\text{LTB}_4$  levels at 8% were significantly increased compared with 4% but not so compared with 12% stretching.

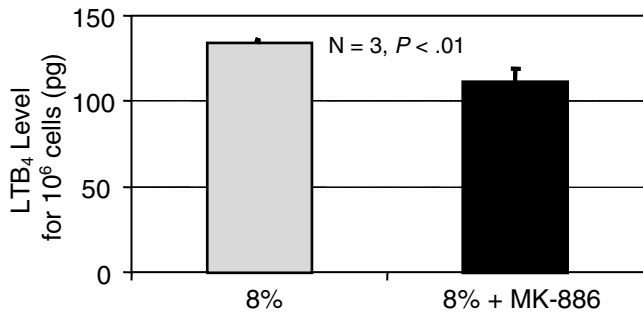
mine the effect of a specific stretching magnitude (eg, 8%) on  $\text{LTB}_4$  production by the cells. A difference between 2 groups was considered to be statistically significant if the  $P$  value was less than .05.

### RESULTS

In the *in vitro* model system, the HPTFs grew on the microgrooves, mimicking the *in vivo* alignment, shape, and mechanical loading conditions of the tendon fibroblasts (Figure 1B). It was found that the  $\text{LTB}_4$  levels increased significantly ( $P < .05$ ) with stretching compared to the unstretched control (Figure 2). For both 8% and 12% stretching, but not 4%,  $\text{LTB}_4$  production increased significantly compared to the control without stretching ( $P < .05$ ). Furthermore,  $\text{LTB}_4$  levels at 8% or 12% stretching were significantly increased compared to 4% ( $P < .05$ ), but the  $\text{LTB}_4$  level at 8% was not significantly different from 12%



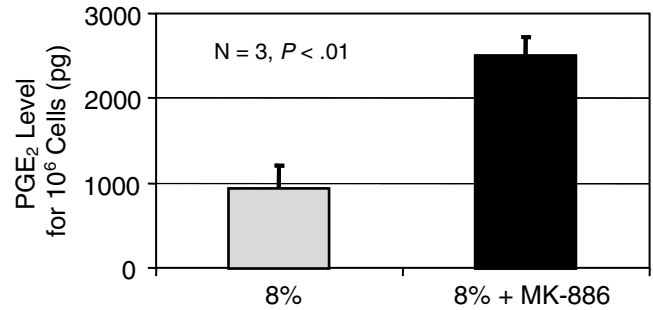
**Figure 3.** A representative result of stretched-induced 5-LO expression of the human tendon fibroblasts by Western blot is shown. The cells were stretched in the same manner as in the experiments for LTB<sub>4</sub>. The 5-LO expression level of the cells at 4% stretching was moderately decreased, apparently unchanged at 8%, but slightly increased at 12%. Four separate experiments were performed, and similar results were obtained.



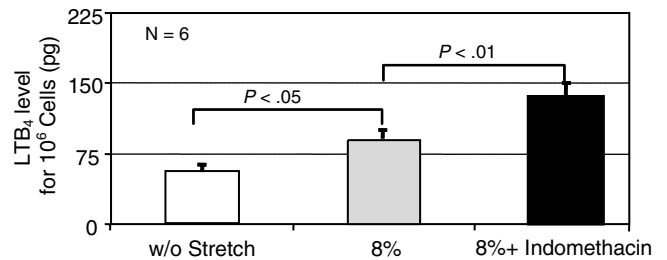
**Figure 4.** The application of MK-886 (10μM, an inhibitor of FLAP, significantly decreased leukotriene B<sub>4</sub> production by the stretched tendon fibroblasts compared to the untreated, stretched cells.

(*P* = .34, Figure 2). For all 4 conditions, compared to the nonstretched control, the expression level of 5-LO tended to be lower at 4% stretching, unchanged at 8% stretching, and higher at 12% stretching, although these differences were not statistically significant (*P* > .05) (Figure 3).

In the presence of indomethacin the level of prostaglandin E<sub>2</sub> produced by the stretched tendon fibroblasts was significantly decreased (*P* < .05) compared to the stretched cells without the indomethacin treatment.<sup>25</sup> When the FLAP-inhibitor MK-886 was present during stretching of the tendon fibroblasts, the level of LTB<sub>4</sub> produced by the cells was significantly decreased (*P* < .01) compared to the cells subjected to the same stretching magnitude in the absence of MK-886 (Figure 4). Also, in the presence of MK-886, the level of PGE<sub>2</sub> production by the stretched tendon fibroblasts was, on average, 2.7 times higher than cells not treated with MK-886 (Figure 5). Finally, decreasing the production of PGE<sub>2</sub> by tendon



**Figure 5.** Decreasing leukotriene B<sub>4</sub> production by tendon fibroblasts with MK-886 significantly increased the production of prostaglandin E<sub>2</sub> compared to the untreated, stretched cells. Cyclic stretching (8%) was applied in the presence of the MK-886 (10 μM).



**Figure 6.** Inhibiting cyclooxygenase with indomethacin (25 μM) increased the production of leukotriene B<sub>4</sub> by the tendon fibroblasts. Note that the leukotriene B<sub>4</sub> levels here are lower than those in Figure 4, possibly because frozen medium instead of fresh one was used.

fibroblasts with indomethacin increased LTB<sub>4</sub> production (Figure 6).

## DISCUSSION

Using an in vitro model system, the inflammatory responses of HPTFs to cyclic mechanical stretching were investigated. A significant advantage of this system over many others is that it can control the alignment, shape, and mechanical stretching conditions of the tendon fibroblasts.<sup>25</sup> With such a system, this study showed that cyclic stretching of HPTFs increases LTB<sub>4</sub> production in a stretch-magnitude-dependent manner. Under the stretching protocol used in this study (ie, stretching 4 hours, followed by 4 hours of rest), increased 5-LO activity, instead of increased expression, appears to be responsible for increased LTB<sub>4</sub> production because cyclic stretching only slightly altered 5-LO expression within the range of stretching magnitudes applied (ie, 0% to 12%). Together, these results confirmed our first hypothesis that cyclic stretching of HPTFs affects LTB<sub>4</sub> production and 5-LO activity. This study also showed that inhibition of PGE<sub>2</sub> synthesis leads to an increased LTB<sub>4</sub> level and vice versa, thus confirming our second hypothesis.

The results of our experiments agree with previous *in vitro* studies, which have shown that cyclic stretching of fibroblasts increases production of PGE<sub>2</sub> and LTB<sub>4</sub>.<sup>2,3</sup> However, our *in vitro* model system differs from others in several ways. First, it allows the shape and alignment of tendon fibroblasts to be controlled, both of which are important factors influencing cell metabolism and responsiveness to mechanical loading.<sup>10</sup> Second, uniaxial stretching, instead of biaxial stretching and/or mixed types of stretching,<sup>2,3</sup> was applied to the tendon fibroblasts. Finally, the maximum substrate deformation (12%) was much smaller than that of the previous study (up to 25%).<sup>2</sup>

Inflammatory mediators such as leukotrienes play an important role in the pathophysiology of inflammatory processes,<sup>11,14</sup> which is part of the reason why LTB<sub>4</sub> was the subject of our study. LTB<sub>4</sub> has been shown to be able to activate neutrophils and lead to the production of several cytokines.<sup>13</sup> Also, injection of LTB<sub>4</sub> into tissues results in the accumulation of neutrophils to the site.<sup>9</sup> Many inflammatory conditions, such as rheumatoid arthritis, gout, inflammatory bowel disease, and chronic bronchitis, have been associated with elevated levels of LTB<sub>4</sub>.<sup>12</sup> Because tendinopathy is also a condition preceded by inflammation and often followed by degenerative changes in the tendon, the possible role of increased LTB<sub>4</sub> levels due to mechanical stretching is intriguing and can be further addressed with an animal model. Indeed, some *in vivo* microdialysis studies have shown that elevated levels of inflammatory mediators (such as PGE<sub>2</sub>) are increased in the peritendinous space after exercise in normal subjects.<sup>15</sup> But other microdialysis studies failed to find elevated levels of PGE<sub>2</sub> in tendons with chronic tendinopathy.<sup>1</sup> However, there are important differences between the 2 aforementioned studies because the first study examined the response to exercise in normal tendons, whereas the second dealt with abnormal tendons with known tendinopathy.

When compared to the control (no stretch) group, there was a trend of decreased 5-LO expression at 4% stretching and slightly increased 5-LO expression at 12% stretching, although statistical significance was not achieved. It is known that both 5-LO and FLAP translocate to the nuclear membrane during leukotriene synthesis.<sup>17,26</sup> For example, studies have shown that 5-LO translocates to the nuclear membrane in response to extracellular signals.<sup>8,23</sup> Therefore, it is plausible that lower stretching magnitudes, such as 4% and 8% in this study, cause 5-LO to translocate to the nuclear membrane, whereas a higher stretch magnitude (eg, 12%) may actually lead to increased expression levels of the enzyme. As a result, the 5-LO levels determined from the Western blot analysis of total proteins collected mainly from cytosol were decreased or unchanged at 4% and 8% but increased at 12%. The proposed mechanism, however, needs to be further investigated by measuring nuclear 5-LO and nuclear membrane-associated 5-LO.

Finally, our results also point to the delicate interplay between the 5-LO and COX pathways, which are responsible for the cellular production of LTB<sub>4</sub> and PGE<sub>2</sub>, respectively. Inhibition of the 5-LO pathway by MK-886 led to an

increase in the production of PGE<sub>2</sub>. Similarly, blocking the PGE<sub>2</sub> pathway by indomethacin, a NSAID, resulted in increased production of LTB<sub>4</sub>. This finding is of interest because NSAIDs are routinely used in clinical practice for the symptomatic treatment of tendinopathy, such as inflammation and pain. However, our study raises the possibility that although treatment with a COX inhibitor decreases PGE<sub>2</sub> levels thereby relieving pain, it might also cause increased LTB<sub>4</sub> production. If this phenomenon is shown to occur *in vivo*, the increased LTB<sub>4</sub> level due to treatment with NSAIDs could potentially exacerbate the situation by leading to neutrophilic infiltration and lymphocytic activation,<sup>6</sup> paradoxically causing further inflammation and degenerative changes in the tendon. Therefore, our results add valuable new information to the study of tendinopathy by demonstrating that the leukotriene pathway is activated when fibroblasts are cyclically stretched and that the treatment of NSAIDs may potentially have unintended consequences.

There are a few limitations in this study. First, cyclic stretching was relatively brief (4 hours), and only 1 stretching frequency (0.5 Hz) was applied. Also, we used only 3 stretching magnitudes (4%, 8%, and 12%) in this study because our previous work has shown that PGE<sub>2</sub> production and COX expression are markedly increased at these stretching magnitudes.<sup>25</sup> Of note, because of the incomplete transfer of dish deformation to dish culture surface, and also the dish substrate strains to the individual cells, the actual strain transmitted to the cell is much smaller, for example, about 77.2% in endothelial cells.<sup>24</sup> Therefore, longer stretching durations and various frequencies should be used in future experiments to evaluate how these factors influence the levels of inflammatory mediators. Also, the biological effects (eg, cell proliferation and protein synthesis) of these inflammatory mediators on the tendon fibroblasts must also be investigated.

In conclusion, this study shows that cyclic stretching of HPTFs increases LTB<sub>4</sub> production and alters the expression level of 5-LO. Also, the COX and 5-LO pathways interplay in a balanced manner, such that inhibition of the COX pathway leads to markedly increased production of LTB<sub>4</sub> via the 5-LO pathway. Even though leukotrienes and prostaglandins have important physiological roles, their abnormally increased production by the tendon fibroblast might represent a pathological condition. Although there is no clear clinical evidence to suggest this, the results of this study suggest that the routine use of COX inhibitors for the symptomatic relief of inflammatory tendon conditions might inadvertently worsen the processes responsible for the development of tendinopathy. Further studies, however, are required to investigate this very intriguing possibility.

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