Mechanical loading modulates heterotopic ossification in calcific tendinopathy through the mTORC1 signaling pathway

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Abstract. Excessive mechanical loading is a major factor affecting heterotopic ossification (HO), which is a major pathological alteration in calcific tendinopathy. However, physical therapies with mechanical loading as the functional element have exhibited promising results in the treatment of calcific tendinopathy. The dual effects that mechanical loading may have on the pathogenesis and rehabilitation of calcified tendinopathy remain unclear. The present study was designed to investigate the effects of mechanical loading on HO in calcific tendinopathy. In the present study, a tendon cell in vitro stretch model and an Achilles tenotomy rat model were used to simulate different elongation mechanical loading scenarios in order to investigate the effects of mechanical loading on HO of the tendon. In addition, rapamycin, a selective mammalian target of rapamycin complex-1 (mTORC1) signaling pathway inhibitor, was employed to determine whether mechanical loading modulates heterotopic ossification in calcific tendinopathy through the mTORC1 signaling pathway. The data indicate that mechanical loading modulated HO of the tendon through the mTORC1 signaling pathway. The data indicate that mechanical loading modulated HO of the tendon through the mTORC1 signaling pathway, and that low elongation mechanical loading attenuated HO, while high elongation mechanical loading accelerated HO in vivo. This study may improve the understanding of the effect of physical therapies used to treat calcific tendinopathy, so as to guide clinical treatment more effectively. Furthermore, rapamycin may be a potential drug for the treatment of calcific tendinopathy.

Introduction

The major pathological basis of calcific tendinopathy, the later stage of tendinopathy, is heterotopic ossification (HO) in the tendon, which severely affects health and quality of life, and can be career-ending for professional athletes (1). Treatment options for calcific tendinopathy are limited and tendon healing usually requires long-term rehabilitation (2). Physical therapies using mechanical loading as their functional element, such eccentric exercise, have shown promising results in the treatment of calcific tendinopathy (3,4). However, excessive mechanical loading is also a major factor in the pathogenesis of calcific tendinopathy and is associated with pain onset (5). The dual effects that mechanical loading may have in the pathogenesis and rehabilitation of calcified tendinopathy have not yet been fully clarified.

Mammalian target of rapamycin complex-1 (mTORC1), an evolutionarily-conserved serine-threonine kinase, controls protein synthesis, ribosome biogenesis and nutrient transport (6,7). Several studies have demonstrated that mechanical loading is sufficient for the activation of the mTORC1 signaling pathway because it can induce phosphorylation of p70 S6 kinase (P70S6K), a downstream protein in the mTORC1 signaling pathway (8-10). Previous studies using the mTORC1 inhibitor rapamycin or mTORC1 gene ablation demonstrated that mTORC1 signaling exerts either stimulatory or inhibitory effects on osteogenic differentiation, which leads to HO (11-14). Thus, mechanical loading may regulate osteogenic differentiation of tendon cells through the mTORC1 signaling pathway, however the mechanism remains unclear.

In the present study, an Achilles tenotomy rat model and a tendon cells stretch model were used to investigate the role mechanical loading may have in regulating HO of the tendon through the mTORC1 signaling pathway in calcific tendinopathy.

Materials and methods

Animals and treatment. A total of 50 male Sprague-Dawley rats [10 rats aged 2-3 weeks (50±5 g) and 40 rats aged 8 weeks (250±10 g)] were purchased from the Experimental Animal Research Center of Southern Medical University.
All assays were performed in triplicate and samples were annealing at 65˚C for 20 sec and extension at 72˚C for 30 sec. 2 min, followed by 40 cycles of denaturation at 94˚C for 20 sec, and extension at 72˚C for 30 sec. All assays were performed in triplicate and samples were normalized to GAPDH using the delta delta Cq method (15).

Western blot analyses. Following the stretching procedure, cells were rinsed with cold PBS and lysed in cold RIPA buffer [1xPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology)]. Lysates were incubated on ice for 20 min with frequent vortexing and cleared twice by centrifugation (11,000 x g, 10 min, 4˚C). Protein was subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked for 60 min at room temperature in 5% non-fat milk/Tris-buffered saline/0.1% Tween-20 (TBST). Following washing with TBST, the membranes were incubated overnight at 4˚C in primary antibodies, which were phospho-P70S6K (Thr389; diluted 1:1,000 in 5% BSA; #9234; Cell Signaling Technology Inc., Danvers, MA, USA), runt-related transcription factor 2 (RUNX2; 1:1,000; #8486; Cell Signaling Technology, Inc.), osterix (OSX; 1:1,000; ab22552; Abcam, Cambridge, UK) or GAPDH (1:1,000; #2118; Cell Signaling Technology, Inc.). Membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:2,000; #7047; Cell Signaling Technology, Inc.) at room temperature for 1 h, and subsequently washed. Image detection was performed according to the manufacturer's instructions using SignalFire enhanced chemiluminescence reagent (#6883; Cell Signaling Technology, Inc.). Data are representative of at least three experiments with similar results performed in triplicate. Images were analyzed using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Establishment of animal model and drug treatment. A total of 40 male Sprague-Dawley rats aged 8 weeks and weighing 250±10 g were randomly distributed into 8 groups of 5 animals per group: Sham; Sham + rapamycin (Rapa); Control (Ctrl); Ctrl + Rapa; low elongation (LE); LE + Rapa; high elongation (HE); HE + Rapa. Rats were anesthetized with 10% chloral hydrate [300 mg/kg, intraperitoneally (i.p.)]. The rats in groups Ctrl, Ctrl + Rapa, LE, LE + Rapa, HE and HE + Rapa underwent midpoint Achilles tenotomy on the right leg through a posterior approach under aseptic conditions; rats in the Sham and Sham + Rapa groups received an incision only as a sham operation. Incisions were routinely closed with an interrupted 4-0 silk suture. Subsequently, the operated hind limbs of rats in Ctrl and Ctrl + Rapa groups and the non-operated hind limbs of the rats in the HE and HE + Rapa groups were fixed in plaster casts from the toes up to ~2.5 cm above the knee. The operated hind limbs of the rats in the LE and LE + Rapa groups were fixed in a plaster cast from ~1 cm below the ankle up to ~2.5 cm above the knee, allowing the toes to touch the ground directly. The ankle and knee were fixed at functional positions. The outer surface of the cast was coated with black pepper powder for protection (16). Thus, the animal models were established with three tendon stretch levels: Totally fixed (groups Ctrl and Ctrl + Rapa), low elongation stretch (groups LE and LE + Rapa) and high elongation stretch (groups HE and HE + Rapa). The rats in groups Sham + Rapa, Ctrl + Rapa, LE + Rapa and HE + Rapa received rapamycin (20 mg/kg/day, i.p.) every day for 6 weeks after operation (Table II).
Histological analyses. Following the operation, the tendon samples were immediately fixed in 4% buffered paraformaldehyde at 4°C for 24 h. The samples were then decalcified, embedded in paraffin, cut into 5 μm sections and placed on poly-L-lysine-coated slides for further procedures. For hematoxylin and eosin staining, the sections were stained with hematoxylin and eosin carried out according to the protocol in the product manual. For immunohistochemical staining, antigen retrieval of the sections was performed using the microwave method (17). Sections were incubated in 3% H2O2 at room temperature for 30 min to quench endogenous peroxidase activity, the sections were subsequently incubated with 10% normal goat serum (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 30 min to block nonspecific binding. The sections were then incubated with antibody against RUNX2 (diluted 1:100 in 5% BSA; ab23981; Abcam) and OSX (diluted 1:100; ab22552; Abcam), followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (diluted 1:100; #7074; Cell Signaling Technology, Inc.). The protein of interest was visualized using 3,3'-diaminobenzidine using a light microscope and the images were analyzed by applying Image Pro-Plus 6.0 software (Media Cybernetics, Inc.).

Micro-computed tomography (μCT) analyses. μCT analysis of the right Achilles tendons was performed using a high-resolution μCT (Skyscan 1172; Bruker Corporation, Ettlingen, Germany). Hind limb samples were collected and fixed overnight at 4°C in 4% paraformaldehyde, then scanned using the settings 60 kV, 150 μA with a mean 20 μm slice thickness. The reconstructed tendon images were selected for quantification of bone mass. Bone volume density [bone volume/total volume (BV/TV)%], trabecular number (Tb.N; l/mm) and trabecular thickness (Tb.Th; mm) were calculated.

Statistical analyses. Data were analyzed using one-way analysis of variance while homogeneity of variance tests were used to evaluate data homogeneity (SPSS 21.0 software; IBM SPSS, Armonk, NY, USA). If the variances were equal, least-significant difference tests were employed; otherwise, Dunnett’s tests were used. Results are presented as the mean ± standard deviation unless otherwise indicated. P<0.05 was considered to indicate a statistically significant difference.

Results

Low elongation mechanical loading reduces osteogenic differentiation and high elongation mechanical loading enhances osteogenic differentiation in vitro. Tendon cells in OIM were subjected to 4% elongation mechanical loading and protein and mRNA expression of RUNX2 and OSX were measured using western blot and RT-qPCR analyses, respectively. Protein and mRNA expression of RUNX2 and OSX were significantly decreased in the 4% stretch group compared with the NS group (P<0.05; Fig. 2A and B) while the 12% stretch group exhibited the opposite result (P<0.05; Fig. 2A and B). These data suggested that osteogenic differentiation in tendon cells was reduced by low elongation mechanical loading and enhanced by high elongation mechanical loading.

mTORC1 signaling pathway was modulated by different levels of elongation mechanical loading in tendon cells. To examine the effects of mechanical loading on the mTORC1 signaling pathway in rat tendon cells, a downstream protein in the mTORC1 signaling pathway, p-P70S6K, was investigated using western blot analyses. Mechanical loading at 4% elongation decreased the protein expression of p-P70S6K, while mechanical loading at 12% elongation significantly increased p-P70S6K levels in rat tendon cells compared with the NS group (P<0.05; Fig. 3). These data suggested that low elongation mechanical loading suppressed the mTORC1 signaling pathway, while high elongation had the opposite effect on tendon cells.

mTORC1 inhibition blocks the effect of mechanical loading on osteogenic differentiation of tendon cells. In the in vitro tendon cells stretch model, the presence of rapamycin decreased protein expression of RUNX2 and OSX in the NS + Rapa group compared with the NS group (P<0.05; Fig. 4). Rapamycin-treated cells at 4% elongation mechanical loading decreased protein expression of RUNX2 and OSX compared with the untreated 4% group (P<0.05; Fig. 4). Additionally, the presence of rapamycin decreased 12% elongation mechanical loading-induced protein expression of RUNX2 compared with the untreated 12% group (P<0.05; Fig. 4). These data suggested that the osteogenic differentiation of tendon cells was mTORC1-dependent and that mechanical loading regulated the osteogenic differentiation of rat tendon cells through the mTORC1 signaling pathway.

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

| Gene     | Primer sequence                  |
|----------|----------------------------------|
| RUNX2    | F 5'-CACAAAACACCACAGACAC-3'     |
|          | R 5'-TTGCTGTCTCTCTGAGAAA-3'     |
| OSX      | F 5'-CAAAGAGTTCGATCTGAGATTG-3'  |
|          | R 5'-GATCAAACTTGCTGACCGCT-3'    |
| GAPDH    | F 5'-GAGAGGCTCTCTGCTGACTA-3'    |
|          | R 5'-TAGGTAGGTGGCGCTCAA-3'      |

RUNX, runt-related transcription factor 2; OSX, osterix.
In order to investigate the roles the mTORC1 signaling pathway plays in mechanical loading‑induced HO of the tendon, the mTORC1 selective inhibitor rapamycin was employed. The presence of rapamycin decreased expression of RUNX2 and OSX in rats in the Ctrl + Rapa group compared with the Ctrl group (Fig. 5). Rapamycin treated rats in the LE + Rapa group had decreased expression of RUNX2 and OSX compared with the untreated LE group (Fig. 5). Additionally, rapamycin decreased the HE mechanical loading‑induced expression of RUNX2 and OSX in the HE + Rapa group compared with the untreated HE group (Fig. 5). These data suggested that HO in tendon was mTORC1‑dependent and that mechanical loading regulated HO in rat tendon through the mTORC1 signaling pathway.

Table II. Establishment of animal model and drug treatments.

| Group       | Achilles tenotomy | Right leg total fixation | Right leg partial fixation | Left leg total fixation | Rapa injection |
|-------------|-------------------|--------------------------|---------------------------|-------------------------|----------------|
| Sham        | -                 | -                        | -                         | -                       | -              |
| Sham + Rapa | -                 | -                        | -                         | -                       | +              |
| Ctrl        | +                 | +                        | -                         | -                       | +              |
| Ctrl + Rapa | +                 | +                        | -                         | -                       | -              |
| LE          | +                 | -                        | +                         | -                       | -              |
| LE + Rapa   | +                 | -                        | +                         | -                       | +              |
| HE          | +                 | -                        | +                         | -                       | -              |
| HE + Rapa   | +                 | -                        | +                         | +                       | +              |

Rapa, rapamycin; Ctrl, control; LE, low elongation; HE, high elongation.

Figure 1. Effects of mechanical loading on HO of Achilles tendon in rats. The rats in Ctrl, LE and HE groups underwent midpoint Achilles tenotomy on the right hind limbs; operated hind limbs of rats in the Ctrl group and the non‑operated hind limbs of the rats in the HE group were fixed in plaster casts from the toes up to ~2.5 cm above the knee. The operated hind limbs of the rats in the LE group were fixed in a plaster cast from ~1 cm below the ankle up to ~2.5 cm above the knee, allowing the toes to touch the ground directly. The ankle and knee were fixed at functional positions. At 6 weeks after operation, the heterotopic ossification of the operated tendons were detected by using (A) µCT analysis. Scale bar, 100 µm. (B) BV/TV (bone volume density), Tb.N and Tb.Th were measured for each group. (C) Hematoxylin and eosin staining was performed on tendon sections from each group. Data represent the mean ± standard error, n=5. *P<0.05 vs. ctrl group. Ctrl, control; LE, low elongation; HE, high elongation; µCT, micro‑computed tomography; BV/TV, bone volume/total volume; Tb.N, trabecular number; Tb.Th, trabecular thickness.
Discussion

It was recently demonstrated that excessive mechanical loading may be a major factor involved in HO (5), which is the main pathological alteration of calcific tendinopathy; however, few studies have investigated the underlying mechanism. Therefore, the present study was established to investigate the underlying molecular mechanism of mechanical loading on heterotopic ossification in tendons.

Tendon stem cells or mesenchymal stem cells (MSCs) can be induced to differentiate into the osteogenic lineage following mechanical loading treatment, as evidenced by increased protein and mRNA expression levels of RUNX2 and OSX (18-20). In contrast, a previous study demonstrated that MSCs subjected to mechanical loading applied using an FX-4000T system had significantly decreased RUNX2 protein and mRNA expression (21). These contradictory results may have resulted from the different cell types and different parameters of the stretch equipment used in each experiment.

Figure 2. Effects of mechanical loading on osteogenic differentiation of tendon cells. Tendon cells were subjected to 4 or 12% elongation mechanical stimulation in vitro, which was applied for 8 h daily for 1 week with the osteogenic induction medium replaced every 3rd day. NS control cells were cultured under the same conditions without mechanical stimulation. The level of RUNX2 and OSX were evaluated (A) at the translational level using western blot or (B) at the transcriptional level using RT-qPCR. Data represent the mean ± standard error, n=5. *P<0.05 vs. NS group. NS, no stimulation; RUNX, runt-related transcription factor 2; OSX, osterix.

Figure 3. mTORC1 signaling pathway was influenced by different degrees of elongation mechanical loading in tendon cells. Tendon cells were subjected to 4 or 12% elongation mechanical stimulation in vitro, which was applied for 8 h daily for 1 week with the osteogenic induction medium replaced every 3rd day. NS control cells were cultured under the same conditions without mechanical stimulation. (A) Level of p-P70S6K was detected by western blotting and (B) relative band density was calculated. Data represent the mean ± standard error, n=5. *P<0.05 vs. NS group. NS, no stimulation; p-P70S6K, phospho-p70 S6 kinase.

Figure 4. Rapa blocks the effect of mechanical loading on osteogenic differentiation of tendon cells. Tendon cells were subjected to 4 or 12% elongation mechanical stimulation, which was applied for 8 h daily for 1 week with the osteogenic induction medium, with or without 10 nM Rapa, replaced every 3rd day. NS control cells were cultured under the same conditions without mechanical stimulation. Level of RUNX2 and OSX were detected by western blotting and relative band density was calculated. Data represent the mean ± standard error, n=5. *P<0.05 vs. NS group; †P<0.05 vs. 4% group; ‡P<0.05 vs. 12% group. NS, no stimulation; Rapa, rapamycin; RUNX, runt-related transcription factor 2; OSX, osterix.
In order to investigate the effects of relatively low and high elongation mechanical loading on osteogenic differentiation in tendon cells, 4 and 12% elongation were chosen to represent low and high elongation of mechanical loading, respectively. Runx2 and OSX, the primary regulators of osteogenic differentiation (22), were measured. The results indicated that low elongation mechanical loading promoted osteogenic differentiation of rat tendon cells. By contrast, high elongation mechanical loading suppressed osteogenic differentiation of rat tendon cells.

The Achilles tenotomy rat model has been used to investigate HO prophylaxis and treatment based on its relative simplicity and predictability (23-25). In the present study, the Achilles tenotomy rat model was employed to investigate how mechanical loading modulates HO in the tendon. The model was established with certain modifications by using plaster casts to simulate different levels of mechanical loading. Micro CT analysis of the BT/VT, Tb.N and Tb.Th parameters of tendon tissues suggested that HO in rats was attenuated by low elongation mechanical loading, but enhanced by high elongation mechanical loading. Through immunohistochemical and histological staining, the present study also revealed that low elongation mechanical loading downregulated RUNX2 and OSX by weakening mineralization, whereas high elongation mechanical loading exhibited the opposite effect. These results may illustrate that low elongation mechanical loading prohibited HO, while high elongation mechanical loading accelerated HO in the animal model.

Eccentric exercise, with mechanical loading as its functional element, has exhibited promising results in the treatment of calcific tendinopathy (3,4); however in other studies,
eccentric exercise demonstrated relatively poor therapeutic effects (26,27). The present findings may explain how eccentric exercise has a therapeutic role through mechanical loading. The healing of tendons is a physiological process involving the dynamic equilibrium of micro-damage and micro-repair. This physiological process of healing may be changed into a pathological process when the balance is broken. Thus, low elongation mechanical loading is preferred and excessive training should be avoided when receiving physical therapy.

In the present study, rapamycin, a selective mTORC1 signaling pathway inhibitor, exhibited capacity to inhibit osteogenic differentiation in vitro and attenuate HO in vivo. These results suggested that the mTORC1 signaling pathway may have a crucial role in the occurrence and development of HO. Previous studies have shown that mTORC1 signaling has an important role in modulating protein metabolism and energy metabolism (5,7,28). Therefore, the mTORC1 signaling pathway may modulate HO through the regulation of protein metabolism and energy metabolism. Thus, rapamycin may be a potential drug treatment for calcific tendinopathy.

In conclusion, the present study provided novel insights into the effects of mechanical loading on heterotropic ossification. The results demonstrated that low elongation mechanical loading attenuated HO while high elongation mechanical loading accelerated HO. Furthermore, mTORC1 signaling may be involved (Fig. 6) as an underlying mechanism in this process.

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