Inhibition of p38 mitogen-activated protein kinase signaling reduces fibrosis and lipid accumulation after rotator cuff repair

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### Background:
The repair of rotator cuff tears is often complicated by fatty degeneration, which is the combination of lipid accumulation, fibrosis, inflammation, and muscle weakness. A signaling molecule that plays a central role in these processes is p38 mitogen-activated protein kinase (MAPK). The purpose of this study was to evaluate the ability of a small molecule inhibitor of p38 MAPK, SB203580, to reduce fatty degeneration in a preclinical model of rotator cuff injury and repair.

### Materials and Methods:
Adult rats underwent a bilateral supraspinatus tenotomy that was repaired 30 days later. Rats were treated with SB203580 or vehicle every 2 days, with injections beginning 3 days before surgery and continuing until 7 days after surgery. Two weeks after surgical repair, muscles were analyzed using histology, lipid profiling, gene expression, and permeabilized muscle fiber contractility.

### Results:
Inhibition of p38 MAPK resulted in a nearly 49% reduction in fat accumulation and a 29% reduction in collagen content, along with changes in corresponding genes regulating adipogenesis and matrix accumulation. There was also a marked 40% to 80% decrease in the expression of several proinflammatory genes, including \textit{IL1B}, \textit{IL6}, and \textit{COX2}, and a 360% increase in the anti-inflammatory gene \textit{IL10}. No differences were observed for muscle fiber force production.

### Conclusion:
Inhibition of p38 MAPK was found to result in a significant decrease in intramuscular lipid accumulation and fibrosis that is usually seen in the degenerative cascade of rotator cuff tears, without having negative effects on the contractile properties of the rotator cuff muscle tissue.

### Level of evidence:
Basic Science Study; In Vivo Animal Model

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### Keywords:
Fatty degeneration; myosteatosis; rotator cuff; fibrosis; atrophy; inflammation; p38 MAPK

Rotator cuff disease is one of the most frequently occurring musculoskeletal conditions treated by orthopedic surgeons, with nearly 250,000 surgical repairs performed in the United States on an annual basis.\textsuperscript{5} Currently, the ability to repair the cuff and allow for normal strength and function is often complicated by atrophy, retraction, and fatty infiltration of the diseased muscle.\textsuperscript{2,15,17} The degree of these changes, termed...
“fatty degeneration,” increases with time and is a limiting factor for adequate repair as well as postoperative functioning and recovery. Restoring muscle size and strength is essential in the postoperative period; however, for unclear reasons, the rates of failure of repair and overall failure remain high. Fatty degeneration is a common pathologic change that occurs in torn rotator cuff muscles; however, little is known behind the pathophysiological pathway of this phenomenon. It has been shown that even after surgical repair, fatty degeneration does not improve and in most cases continues to worsen over time.

Fat accumulation and atrophy are correlated with poor functional outcomes and increased risk of retear, and identifying new treatments that can reverse or prevent fatty degeneration will likely improve clinical outcomes for patients with chronic rotator cuff tears.

The protein p38 mitogen-activated protein kinase (MAPK) is a signaling molecule within cells that is involved in many different cellular signaling processes associated with inflammation, muscle atrophy, fibrosis, and adipogenesis. Activation of p38 MAPK occurs in response to the treatment of cells with transforming growth factor β (TGF-β) and tumor necrosis factor α (TNF-α) signaling pathways, among others. The central role of p38 MAPK in inducing inflammatory responses in many different tissues has led to the development of specific inhibitors of this molecule, and several clinical trials are under way to test the ability of small molecule inhibitors of p38 MAPK to block inflammation and promote regeneration in different disease states. For skeletal muscle, p38 MAPK signaling has been associated with an accumulation of extracellular matrix, inflammation, atrophy, and an overall general loss of muscle function with aging, and the targeted inhibition of p38 MAPK can markedly reverse degenerative changes and restore muscle function.

Because there are no known pharmacologic treatments available to effectively prevent degeneration or cause regeneration of torn rotator cuff muscles after repair, using a preclinical rat model of rotator cuff injuries, we sought to determine the ability of a small molecule inhibitor of p38 MAPK, SB203580, to reduce muscle tissue damage from inflammation, fatty degeneration, and muscle atrophy after rotator cuff repair. We hypothesized that the postoperative treatment of torn rotator cuff muscles with SB203580 would reduce tissue inflammation and muscle fatty degeneration while enhancing the formation of a stable enthesis after rotator cuff repair.

Materials and Methods

Overview

Six-month-old male Sprague-Dawley rats (14 rats in the vehicle control group and 14 rats in the drug group, with 4 rats in each group used for pilot studies and 10 rats in each group used for the main tear-and-repair experiments), maintained in specific pathogen-free conditions, were used for this study. On the basis of anatomic similarities with humans and previous studies, a rat model was selected to study rotator cuff healing. Although some rat models of rotator cuff tear do not fully reproduce fatty degenerative changes because the free end of the torn tendon will randomly reattach to surrounding tissue, substantial fatty degeneration will occur in the rat rotator cuff by ensuring the tendon cannot spontaneously reattach. The general techniques and methods in this study have been previously published and are provided with brevity in this article.

Animal surgery

A bilateral tear-and-repair approach was used in this study. To induce a supraspinatus tear, rats were anesthetized with 2% isoflurane and placed in a lateral recumbent position, and the surgical area was shaved and scrubbed with ChloraPrep (CareFusion, El Paso, TX, USA). The supraspinatus tendon was visualized using a deltoid-splitting incision and transacromial approach, and once clearly visualized and isolated, the tendon was sharply detached from the greater tuberosity with a No. 15 blade and secured using a modified Mason-Allen stitch within sterile nonpyrogenic surgical tubing (Pharmed BPT; Saint-Gobain, Valley Forge, PA, USA) to prevent the tendon from forming adhesions to the surrounding tissue, allowing the supraspinatus to freely retract. Animals were allowed to recover for 30 days. This time point was selected because many of the pathologic changes in rats 1 month after a tear mimic the early chronic changes seen in patients with rotator cuff tears. To repair the tear, rats were anesthetized and prepared as described earlier. The tendon was identified and silicone tube removed. A modified Mason-Allen stitch using No. 5-0 Ethibond sutures (Johnson & Johnson, New Brunswick, NJ, USA) was placed in the tendon stump. After removal of small amounts of scar tissue and complete debridement of the native enthesis on the greater tuberosity, crossed bone tunnels were drilled at the anterior and posterior margins of the cuff footprint and 2 mm lateral to the articular surface using a 0.7-mm K-wire (gSource, Emerson, NJ, USA). Suture ends were passed through the bone tunnels and tied over the humeral metaphyseal cortex to anatomic repair the supraspinatus to the native footprint. In all surgical procedures, a splash block of 1% lidocaine was administered, the deltoid was closed using No. 4-0 chromic gut sutures (Johnson & Johnson), and the skin was closed using a subcutaneous running suture of No. 4-0 Vicryl (Johnson & Johnson) with GLUture (Abbott Laboratories, Abbott Park, IL, USA) applied over the incision. Subcutaneous buprenorphine at a dose of 0.05 mg/kg was administered for analgesia during postoperative recovery. Weight bearing and cage activity were allowed postoperatively, and rats were monitored for signs of distress or infection. All rats were grossly ambulatory to the same extent and showed signs of adequate food and water intake.
For the pilot study to determine the efficacy of SB203580 treatment in the inhibition of p38 MAPK, rats were anesthetized 1 day after the repair and supraspinatus muscles were removed for analysis. The muscles were finely homogenized and prepared for the p38 MAPK phosphorylation assay. In the main study, rats were allowed to recover for 14 days, at which time after repair, rats were anesthetized and the supraspinatus muscle was removed for analysis. The proximal two-thirds of the right supraspinatus was finely minced and used for biochemical and molecular biology, whereas the distal one-third of the muscle was used for histology. The proximal one-third of the left supraspinatus was used for muscle fiber contractility testing. After removal of tissue, rats were euthanized by an overdose of pentobarbital sodium followed by induction of a bilateral pneumothorax.

Administration of drug

SB203580 (Alfa Aesar, Ward Hill, MA, USA) was dissolved in 1:100 dimethyl sulfoxide in phosphate-buffered saline solution (PBS) and administered via intraperitoneal (IP) injection at a dose of 1 mg/kg every 2 days. SB203580 is not directly soluble in water or PBS and must first be dissolved in an organic solvent before dilution in a physiological buffer. To mimic a potential clinical scenario in which patients who undergo rotator cuff repair would begin treatment before repair surgery and continue treatment in the acute postsurgical recovery phase, injections began 3 days before surgery and continued until 7 days after surgery. Rats in the control group received IP injections of vehicle only (1:100 dimethyl sulfoxide in PBS). This dose and timing regimen was selected based on pilot experiments, the interest in minimizing the stress animals experience with IP injections, and previous studies evaluating p38 MAPK inhibition in muscle and connective tissue. An IP route was selected to precisely control the delivered dose, and the total volume of solution injected IP was less than 1 mL per dose.

Activation of p38

A MAGPix Luminex–based system (Luminex, Austin, TX, USA) was used to measure p38 phosphorylation as described. In brief, 100 mg of muscle was homogenized in Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific), homogenized, and spun at 12,000g for 10 minutes, and the supernatant was collected and stored at −80°C until use. A BCA protein assay (Thermo Scientific) was used to determine protein content, and the relative abundance of phosphorylated p38 MAPK (phosphorylation at Thr180 and Tyr182 residues) from 50 μg of total protein was analyzed using a Milliplex-p38 phospho-protein magnetic bead assay (EMD Millipore, Billerica, MA, USA).

Histology

The distal third of the supraspinatus muscles was snap frozen in TissueTek (Sakura Finetek, Torrance, CA, USA) using isopentane-cooled liquid nitrogen and stored at −80°C until use. Muscles were cryosectioned and stained with hematoxylin and oil red O to measure lipid content and with hematoxylin and eosin to measure fiber cross-sectional area (CSA) using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Gene expression

Gene expression analysis was performed as previously described. RNA was isolated from 100-mg portions of the distal third of the supraspinatus muscles using a Qiagen miRNeasy kit (Qiagen, Valencia, CA, USA) and treated with deoxyribonuclease I (Qiagen) to eliminate genomic DNA. RNA integrity was verified using a Bioanalyzer RNA system (Agilent Technologies, Santa Clara, CA, USA). After reverse transcription of RNA with an RT² First Strand Kit (Qiagen), quantitative polymerase chain reaction was conducted using RT² SYBR Green reagents and commercial primers (Qiagen). Target gene expression was normalized to the stable housekeeping gene β-actin and then to the vehicle control group using the 2ΔΔCT technique.

Hydroxyproline assay

Hydroxyproline measurements of muscle tissue were performed from 25-mg portions of muscle using a colorimetric assay as previously described. The hydroxyproline content was normalized to the dry mass of the muscle tissue.

Muscle fiber contractility measurements

Bundles from the proximal third of the supraspinatus muscle were prepared for muscle fiber contractility measurements as previously described. In brief, fibers were placed in a chamber filled with relaxing solution and were secured at one end to a servomotor (model 322C; Aurora Scientific, Aurora, Ontario, Canada) and at the other end to a force transducer (model 403A; Aurora Scientific). The length of the fiber was adjusted until an average sarcomere length of 2.5 μm was achieved. The CSA of fibers was then measured from top- and side-view images, and the fiber was exposed to a high Ca²⁺ solution to develop maximum isometric force. Maximum isometric force was then normalized by the CSA to determine specific force. Ten fast fibers from each muscle were tested.

Lipid analysis

Lipids were extracted from 50 mg of muscle tissue and analyzed with thin-layer chromatography as previously described. Thin-layer chromatography plates were stained...
with a rhodamine 6G solution (Sigma, St Louis, MO, USA) and imaged in a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Densitometry of triglyceride bands was performed using ImageJ software.

**Statistical analysis**

Differences between the control and SB203580 groups were tested using unpaired t tests with $\alpha = .05$ in GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA, USA). We powered this study based on contractility values from a previous study. To detect a 30% difference in specific force, using a power of 0.80 and $\alpha = .05$, we determined that 8 rats were required for each group. We included an additional 2 rats per group to account for any rats that were lost to infection or other complications.

**Results**

All rats survived to analysis. All rotator cuff repairs in both groups were intact postoperatively at the time of sacrifice, with no signs of humeral fractures, damage to transosseous tunnels, or failed repairs. Values for control rats for many of the experimental techniques used in this study have been previously published. In pilot studies, treatment with SB203580 resulted in a 68% reduction in p38 phosphorylation 1 day after repair of a chronically torn rotator cuff ($P < .01$, Fig. 1).

After establishing that the dosing was effective at reducing p38 MAPK activation, we conducted the main study. No differences in body mass ($636 \pm 69$ g for vehicle group vs $612 \pm 40$ g for p38 MAPK inhibitor group, $P = .17$) were detected. Treatment with the p38 MAPK inhibitor SB203580 resulted in no change in muscle mass ($P = .17$; Fig. 2, A) or muscle fiber CSA measured using histology ($P = .33$; Fig. 2, B). Treatment with p38 MAPK inhibitor also resulted in a 29% decrease in the content of the collagen biomarker hydroxyproline ($P = .01$; Fig. 2, C) and a 48% decrease in the levels of triglyceride ($P < .01$; Fig. 3, A and B), which is the major lipid species present in skeletal muscle. This reduction in triglyceride levels was also observed grossly in oil red O–stained muscle cross sections (Fig. 3, C).

To assess muscle function, we measured the contractility of permeabilized muscle fiber cells. The permeabilization process does result in an increase in CSA compared with measurements performed using histologic techniques, but similar to histology, no difference in permeabilized fiber CSA was observed between the 2 groups ($P = .19$; Fig. 4, A). No differences were observed for maximum isometric force ($P = .48$; Fig. 4, B) or specific force ($P = .08$; Fig. 4, C), which is maximum isometric force normalized to CSA.

Finally, we measured the expression of several genes that are involved in the response of muscle to injury and regeneration (Fig. 5). For genes associated with lipid accumulation and adipogenesis, the p38 MAPK inhibitor group had a 38% reduction in peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) ($P = .01$), a 27% reduction in CCAAT/enhancer binding protein $\alpha$ (cEBP$\alpha$) ($P < .01$), and a 24% reduction in CD36 ($P = .04$). Inhibition of p38 MAPK also had an effect on several genes associated with extracellular matrix production and fibrosis, resulting in a 27% reduction in type I collagen $\alpha2$ ($P = .02$), a 44% reduction in type III collagen $\alpha1$ ($P = .04$), a 170% increase in matrix metalloproteinase 8 ($P < .01$), a 134% increase in tissue inhibitor of matrix metalloproteinase 2 ($P < .01$), and a 27% reduction in the expression of fibroblast specific protein 1 ($P = .01$). No differences in matrix metalloproteinase 3 ($P = .06$) or tissue inhibitor of matrix metalloproteinase 1 ($P = .18$) were observed. Inhibition of p38 MAPK altered the expression of several genes associated with inflammation as well, including a 64% reduction in cyclooxygenase 2 ($P < .01$), a 79% reduction in interleukin (IL) 1$\beta$ ($P < .01$), a 44% reduction in IL-6 ($P < .01$), and a 360% increase in the expression of IL-10 ($P < .01$), although no differences were observed for cyclooxygenase 1 ($P = .30$).

**Discussion**

Fibrosis and fatty infiltration into the muscle tissue are common pathologic changes that occur in rotator cuff tears; however, our current understanding of the pathophysiology of this phenomenon is limited. Unfortunately, even after surgical repair, this fatty degeneration does not improve and in most cases continues to worsen over time. Because the muscle in patients with chronic rotator cuff tears is shortened by 30% or more, repairing a chronically torn rotator cuff by affixing the free tendon end back to the native footprint results in a secondary stretch-induced muscle injury that likely further exacerbates the inflammatory state of the muscle. Given that fat accumulation and atrophy have been correlated with poor functional outcomes and increased risk of retear, reversing or preventing the fatty degeneration cascade has the potential to improve clinical outcomes for patients with chronic rotator cuff tears. The p38 MAPK pathway appears to play a central role in the development of fatty degeneration and inflammation in rotator cuff tears.
Rotator cuff tears and p38 MAPK signaling

As p38 MAPK is widely regarded as one of the key regulators of adipogenesis, the critical role of this kinase in regulating adipocyte activity and lipid storage was the primary rationale for conducting this study. Primarily p38 MAPK functions in adipogenesis by activating various transcription factors such as Activating Transcription Factor 2 (ATF2) that bind to DNA and induce the expression of the transcription factors PPARγ and CEBPα, which then direct the expression of several genes required for preadipocyte proliferation, adipocyte differentiation, and lipid uptake and storage. Consistent with this, in our study, we observed that inhibiting p38 MAPK resulted in a marked reduction in PPARγ and CEBPα, as well as CD36, which functions to transport fatty acids into adipocytes and muscle fibers. The reduction in PPARγ, CEBPα, and CD36 corresponded with a decrease in the content of triglyceride, which is the major lipid species found in muscle fibers and adipocytes. These results indicate that blocking p38 MAPK activity is effective at reducing functional lipid accumulation in this preclinical model of rotator cuff disease.

Elevated inflammation is frequently observed in patients with chronic rotator cuff tears. Because p38 MAPK plays a critical role in inducing inflammation in multiple tissue types, we sought to evaluate whether inhibition of p38 MAPK could regulate the expression of several genes known to be regulated by p38 MAPK that are involved in inflammation in skeletal muscle tissue. IL-1β and IL-6 are potent proinflammatory cytokines known to direct atrophy and collagen production, and IL-10 is an anti-inflammatory gene that can protect against atrophy and fibrosis in skeletal muscle tissue. In the current study, inhibition of p38 MAPK was effective at reducing the expression of IL-1β and IL-6 and inducing IL-10. The generally favorable effects of p38 MAPK inhibition are further supported by the observed reduction in the expression of COX2, which is a gene that produces proinflammatory prostaglandins that can inhibit muscle regeneration. Combined, these results indicate that the targeted inhibition of p38 MAPK is able to effectively reduce the expression of proinflammatory biomarkers in injured rotator cuff muscles.

Muscle fibrosis is commonly observed in torn rotator cuff muscles and likely contributes to poor functional outcomes. Activation of p38 MAPK by TNF-α, TGF-β, and myostatin, which are cytokines that are induced and activated after muscle

Figure 2  (A) Muscle mass, (B) muscle fiber cross-sectional area (CSA), and (C) hydroxyproline content (micrograms of hydroxyproline per milligram of dry muscle mass) of vehicle and p38 mitogen-activated protein kinase (MAPK) inhibitor–treated (p38 Inhib) muscles. Values are given as mean ± standard deviation. There were 10 rats in each group. An asterisk indicates a significant difference compared with the vehicle group (P < .05).

Figure 3 Lipid content: (A) levels of triglyceride measured in relative units (RU), (B) representative rhodamine 6G–stained thin-layer chromatography triglyceride bands, and (C) representative oil red O histology of vehicle and p38 mitogen-activated protein kinase inhibitor–treated (p38 Inhib) muscles. Values are given as mean ± standard deviation. There were 10 rats in each group. An asterisk indicates a significant difference compared with the vehicle group (P < .05).
injury, leads to the induction of muscle fibrosis, type I and III collagen expression, and connective tissue accumulation. In this study, in rats treated with the p38 MAPK inhibitor, we observed a decrease in the expression of the major muscle extracellular matrix components, type I and type III collagen, as well as the biomarker of collagen content, hydroxyproline. The TNF-α, TGF-β, and myostatin pathways can also induce muscle atrophy and a loss in muscle fiber force production through p38 MAPK, as well as other downstream pathways. Although we anticipated that blocking p38 MAPK would improve muscle fiber size and force production, no such effects were observed, and the specific force values of both groups were 30% lower than in uninjured muscles. These collective results suggest that in the context of rotator cuff injuries, the p38 MAPK pathway plays an important role in regulating fibrosis but not muscle atrophy and force production.

Although we have provided important insights into the potential therapeutic role of p38 MAPK inhibition in the treatment of rotator cuff tears, there are several limitations to this study. Rats are a frequently used preclinical model in the study of rotator cuff healing, but compared with humans, they show a much better rate of structural regeneration and do not have the same extent of fatty infiltration and atrophy development. There is a gradient of degenerative changes seen throughout injured rotator cuff muscles, with the more pronounced changes observed at the distal end and decreasing in intensity moving proximally. The quantitative measures of triglyceride levels in the proximal regions of the muscle there may have underestimated the changes that occurred in the distal region of the muscle. We measured the expression of several genes, and although we do not anticipate substantial post-translational regulation of these transcripts, it is possible that changes in RNA levels would not predict subsequent changes in protein abundance. On the basis of our previous work and the work of other authors, we selected a single time point for the tear duration and repair duration that we think was predictive of long-term outcomes after the repair of chronically torn rotator cuff muscles. Despite these limitations, this study provided important insight into the biology of rotator cuff regeneration and identified a promising pharmacologic target worth further investigation.

Figure 4  Permeabilized muscle fiber contractility: (A) permeabilized muscle fiber cross-sectional area (CSA), (B) maximum isometric force, and (C) specific force (maximum isometric force normalized to CSA) of vehicle and p38 mitogen-activated protein kinase inhibitor–treated (p38 Inhib) muscles. Values are given as mean ± standard deviation. There were 10 rats in each group. No significant differences were found compared with the vehicle group (P < .05).

Figure 5  Expression of genes associated with lipid accumulation, extracellular matrix synthesis and fibrosis, and inflammation. The expression of each gene was normalized to β-actin and further normalized to the vehicle group. Values are given as mean ± standard deviation. There were 10 rats in each group. An asterisk indicates a significant difference compared with the vehicle group. Rats are a frequently used preclinical model in the study of rotator cuff regeneration and identified a promising pharmacologic target worth further investigation.
Conclusion

Several studies have shown that p38 MAPK signaling plays an important role in regulating the adaptation of tissues to mechanical loading, as well as the regeneration of tissue from injury. In this study, inhibition of p38 MAPK at the time of rotator cuff repair resulted in a clinically favorable decrease in lipid accumulation, which is a hallmark in the rotator cuff degenerative cascade and predictive of clinical outcomes. Inhibition of p38 MAPK was also effective at reducing collagen content and inflammatory biomarkers. In addition, we found that it is possible to markedly reduce fat accumulation and fibrosis without affecting muscle fiber force production. There are no approved p38 MAPK inhibitors currently on the market, but several of these compounds are being evaluated in clinical trials for a wide variety of conditions. Targeting p38 MAPK may be particularly helpful in improving outcomes at the time of surgical repair of chronic rotator cuff tears, given that the process of reattaching the chronically shortened muscle-tendon unit leads to a secondary stretch-injured injury to the muscle, and in this study we were able to show that inhibition of p38 MAPK limits some of the fibrosis and fat accumulation that occur after the repair. Although further studies are necessary, given the encouraging results from this preclinical model of rotator cuff repair, the targeted inhibition of p38 MAPK may be able to improve the outcomes of patients with chronic rotator cuff tears.

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