Simvastatin reduces fibrosis and protects against muscle weakness after massive rotator cuff tear

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Background: Chronic rotator cuff tears are a common source of shoulder pain and disability, and patients with chronic cuff tears often have substantial weakness, fibrosis, inflammation, and fat accumulation. Identifying therapies to prevent the development of these pathologic processes will likely have a positive impact on clinical outcomes. Simvastatin is a drug with demonstrated anti-inflammatory and antifibrotic effects in many tissues but had not previously been studied in the context of rotator cuff tears. We hypothesized that after the induction of a massive supraspinatus tear, simvastatin would protect muscles from a loss of force production and fibrosis.

Methods: We measured changes in muscle fiber contractility, histology, and biochemical markers of fibrosis and fatty infiltration in rats that received a full-thickness supraspinatus tear and were treated with either carrier alone or simvastatin.

Results: Compared with vehicle-treated controls, simvastatin did not have an appreciable effect on muscle fiber size, but treatment did increase muscle fiber specific force by 20%. Simvastatin also reduced collagen accumulation by 50% but did not affect triglyceride content of muscles. Several favorable changes in the expression of genes and other markers of inflammation, fibrosis, and regeneration were also observed.

Conclusions: Simvastatin partially protected muscles from the weakness that occurs as a result of chronic rotator cuff tear. Fibrosis was also markedly reduced in simvastatin-treated animals. Whereas further studies are necessary, statin medication could potentially help improve outcomes for patients with rotator cuff tears.

Level of evidence: Basic Science, In Vivo Animal Study.

Keywords: Rotator cuff; fatty degeneration; muscle atrophy; statin; myosteatosis; fibrosis; HMG-CoA reductase inhibitor

Tears to the rotator cuff are among the most common and devastating upper extremity injuries, with more than a quarter of a million surgical repairs performed in the United States each year. The ability to successfully repair the torn cuff and to promote the return of patients to normal strength and function is often complicated by fibrosis, atrophy, and fatty infiltration of the rotator cuff muscles. These changes, termed myosteatosis or fatty degeneration, increase with time and are a
limiting factor for adequate repair as well as for postoperative rehabilitation and recovery.\textsuperscript{13,25} The extent of fatty degeneration can be quantified with magnetic resonance imaging or computed tomography techniques, and there is a positive correlation between the amount of fatty degeneration present in a muscle and poor functional outcomes as well as an increased risk for structural failure after repair.\textsuperscript{15} Therapies that reverse or halt the progression of fatty degeneration may therefore lead to an improvement in function and greater patient satisfaction after rotator cuff tear.

Hydroxymethylglutaryl (HMG) coenzyme A (CoA) reductase inhibitors, or “statins,” are among the most frequently prescribed medications in the United States.\textsuperscript{31} These medications are most commonly used in the treatment of hypercholesterolemia as they are effective at lowering low-density lipoprotein cholesterol and improving clinical outcomes of patients with coronary artery disease and other cardiovascular conditions.\textsuperscript{6,31} In addition to promoting cardiovascular disease, hypercholesterolemia is associated with a greater risk for rotator cuff tendon tear and impaired tendon-bone regeneration.\textsuperscript{1,4} Aside from their efficacy in treating hypercholesterolemia, there are emerging roles for statins in the treatment of inflammatory diseases.\textsuperscript{6,31} Statins work by inhibiting the activity of the HMG-CoA reductase enzyme, which catalyzes the conversion of HMG-CoA into mevalonate, a precursor for cholesterol and other isoprenoids that either directly or indirectly activate proinflammatory signaling pathways.\textsuperscript{6} Numerous studies have identified the ability of statins to prevent fibrosis and inflammation in several diseased or injured tissues, including the heart, blood vessels, lungs, kidneys, skin, and articular cartilage.\textsuperscript{2,6,23,32} To our knowledge, the ability of statins to prevent fibrosis, atrophy, inflammation, and fat accumulation in skeletal muscle tissue, and specifically the rotator cuff, has not been explored to date.

As therapeutic interventions to prevent muscle scar tissue formation and inflammation may enhance the treatment of chronic rotator cuff disease, our objective was to evaluate the ability of a commonly used statin medication, simvastatin (Zocor), to prevent atrophy and fibrosis after rotator cuff tear. We hypothesized that after an induction of a massive supraspinatus tear, simvastatin would enhance muscle fiber force production and prevent fibrosis and fat accumulation. To test this hypothesis, we used a well-described rat model of full-thickness chronic rotator cuff tear,\textsuperscript{16,26,36} treated rats with either vehicle or simvastatin, and measured changes in muscle fiber type and contractility and biochemical and molecular markers of fibrosis and fatty degeneration 28 days after induction of tear.

\textbf{Methods}

\textbf{Animals and surgical procedures}

This study used 6-month-old male retired breeder Sprague-Dawley rats. Animals were housed in specific pathogen-free conditions and randomly assigned to either the control group (N = 8 rats) or the simvastatin treatment group (N = 8 rats). A bilateral full-thickness supraspinatus tenectomy was performed in each rat as previously described.\textsuperscript{16,17} Rats were anesthetized with 2% isoflurane and placed in a lateral decubitus position, and the skin above the shoulder was shaved and scrubbed with chlorhexidine gluconate. A deltoïd-splitting transacromial approach was used to visualize the supraspinatus tendon, which was then clamped and sharply detached from its insertion on the humerus. A full-thickness incision was made just distal to the myotendinous junction, and the tendon was removed to prevent healing and scarring into the surrounding connective tissue. A splash block of 1% lidocaine was administered for analgesia, and the deltoid was closed with 4-0 chronic gut (Johnson & Johnson, New Brunswick, NJ, USA). The skin was closed with a running subcutaneous suture of 5-0 Vicryl (Johnson & Johnson) that was reinforced with GLUte (Abbott Laboratories, Abbott Park, IL, USA). Rats also received subcutaneous buprenorphine (0.05 mg/kg) as analgesia postoperatively. After 28 days of recovery, the animals were anesthetized with sodium pentobarbital (50 mg/kg), and the supraspinatus muscles on both sides were harvested and weighed. The distal ends of all muscles were mobile and showed no sign of scar or lateral adhesions of the muscle. The rats were then humanely euthanized by overdose of sodium pentobarbital, which was followed by creation of a bilateral pneumothorax. The left supraspinatus from each rat was used for histology and single-fiber contractility, and the right supraspinatus was finely minced and used for gene expression and biochemical analysis.

\textbf{Simvastatin administration}

Pharmaceutical-grade simvastatin tablets (80-mg tablets; Cadila Pharmaceuticals, Ahmedabad, India) were finely ground with a mortar and pestle and extensively mixed with vehicle (1% hydroxypropyl methylcellulose) fresh daily. Rats received once-daily simvastatin at a dose of 20 mg/kg or vehicle (1% hydroxypropyl methylcellulose) administered by oral gavage. This dosage was selected on the basis of results from previous studies.\textsuperscript{2,39} Treatment began 2 hours before the surgery to induce rotator cuff tear and continued each day until the rats were euthanized.

\textbf{Muscle fiber contractility}

The proximal portion of the left supraspinatus muscle was used for muscle fiber contractility analysis. Tissue was prepared, and the cross-sectional area (CSA), maximum isometric force (F\textsubscript{m}), and specific force (S\textsubscript{Fm}, which is calculated by dividing F\textsubscript{m} by CSA) were determined as described at a sarcomere length of 2.5 μm.\textsuperscript{16,17,27} Ten to 12 type II fibers were tested from each supraspinatus muscle.

\textbf{Histology}

Histology was performed as previously described.\textsuperscript{16,17} The distal portion of the left supraspinatus muscle was placed in Tissue-Tek (Sakura, Torrance, CA, USA) and frozen in isopentane cooled to approximately −160°C. Muscles were sectioned at a thickness of 10 μm and labeled with monoclonal antibodies against myosin heavy chain (Developmental Studies HybriDoma Bank, Iowa City, IA, USA). Primary antibodies were detected with Alexa Fluor conjugated secondary antibodies (Invitrogen, Grand Island, NY,
USA), and the extracellular matrix (ECM) was identified with wheat germ agglutinin lectin conjugated to Alexa Fluor 488 (Invitrogen). High-resolution images of slides were obtained with an Axioplan 2 (Zeiss, Jena, Germany) microscope equipped with AxioCam (Zeiss) cameras. Quantitative histomorphometry was performed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Gene expression**

RNA isolation and gene expression were performed as previously described.16,17 RNA was isolated from right supraspinatus muscles with an miRNeasy kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Qiagen) to eliminate genomic DNA. RNA was reverse transcribed with the RT² First Strand Kit (Qiagen) to eliminate genomic DNA. RNA was reverse transcribed with the RT² First Strand Kit (Qiagen), and cDNA was amplified in a CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA, USA) with RT² SYBR Green qPCR mix (Qiagen) and primers for specific mRNA species (Qiagen). Expression of mRNA transcripts (Supplementary Table I) was normalized to the stable housekeeping gene β-actin, and the simvastatin samples were further normalized to the control samples by the 2-ΔΔCt approach.35

**Lipid analysis**

Muscle tissue was weighed, homogenized, and suspended in a 0.9% NaCl solution at a concentration of 20 μg/mL. Lipid was extracted according to the methods of Bligh and Dyer7 in a 2:2:1.8 chloroform:methanol:water solution to separate phospholipids and then dried and further developed in an 80:20:1.5 hexane:diethyl ether:acetic acid solution to separate apolar lipids. To visualize lipid species, plates were stained with rhodamine 6G (Sigma, Saint Louis, MO, USA) and imaged in a ChemiDoc XRS system (Bio-Rad). Standards of known lipid species were used as an internal control across different plates. Densitometry of triglyceride and phospholipid bands was performed with ImageJ.

**Matrix metalloproteinase activity assay**

Matrix metalloproteinase (MMP) activity was measured in samples with a SensoLyte Colorimetric Assay kit (AnaSpec, Fremont, CA, USA) by techniques modified from Kumar.24 Fifty milligrams of minced muscle was homogenized and sonicated in 1 mL of ice-cold T-PER (Pierce, Rockford, IL, USA), which was subsequently centrifuged at 12,000 × g for 10 minutes. The supernatant was collected, and the concentration of protein in samples was measured with a BCA assay (Pierce). Fifty micrograms of protein was loaded into a 96-well plate and incubated with the MMP chromogenic substrate for 1 hour at 37°C. The absorbance of samples was then measured at 412 nm in a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Hydroxyproline assay**

Hydroxyproline is an amino acid that makes up approximately 14% of the dry mass of fibrillar collagens and is commonly used...
as a marker of the collagen content of tissues.\textsuperscript{30} Measurement of hydroxyproline was performed as previously described.\textsuperscript{28} Briefly, 25-mg portions of finely minced supraspinatus muscles were desiccated for 4 hours at 90°C, and the dry mass of samples was then recorded. Samples were then digested into free amino acids in 6.0 N HCl overnight at 110°C and neutralized in an equal volume of 6.0 N NaOH. The hydroxyproline content was then determined by a colorimetric assay\textsuperscript{41} that was measured in a SpectraMax microplate reader and normalized to the dry mass of the muscle tissue.

\textbf{Statistical analysis}

Data are presented as mean ± standard deviation. Differences between vehicle-treated control samples and simvastatin-treated samples were tested by unpaired t tests (\( \alpha = .05 \)) in Prism 6.0 (GraphPad, La Jolla, CA, USA).

\textbf{Results}

Twenty-eight days after a tear was induced, no differences in body mass (643 ± 84.8 g for control rats and 673 ± 69.2 g for simvastatin rats; \( P = .22 \)) or wet mass of supraspinatus muscles (402 ± 41.4 mg for control rats and 444 ± 120 mg for simvastatin rats; \( P = .19 \)) were observed. The CSA of muscle fibers was generally similar, with a slight increase in the size of pathologic type IIB muscle fibers in simvastatin-treated rats (\( P = .04 \); Fig. 1, A), although the percentage of type IIB fibers decreased by 38\% (\( P = .02 \); Fig. 1, B). For muscle fiber contractility, no differences in fiber CSA (\( P = .27 \); Fig. 2, A) or \( F_0 \) were observed (\( P = .10 \); Fig. 2, B), but simvastatin treatment resulted in an approximately 20\% increase in \( sF_0 \) compared with controls (\( P = .04 \); Fig. 2, C).
Differences in molecular and biochemical markers of fatty degeneration and fibrosis were then measured. For genes related to adipogenesis and lipid accumulation, simvastatin treatment reduced the expression of peroxisome proliferator-activated receptor \( \gamma \) (PPAR-\( \gamma \); \( P = .03 \)) and CCAAT/enhancer binding protein \( \alpha \) (c/EBP-\( \alpha \); \( P = .01 \)) and also increased the expression of ACAT1 (\( P = .04 \); Fig. 3, A). Although there was a downregulation in PPAR-\( \gamma \) and c/EBP-\( \alpha \), no differences in total triglyceride content were observed (\( P = .32 \); Fig. 4, A). Phospholipids, which are lipid species that are mainly found in the plasma membranes and were therefore not anticipated to change on the basis of the drug treatment, were present in similar levels between control and treatment groups (\( P = .47 \); Fig. 4, B).

Simvastatin also substantially reduced the expression of the early muscle regeneration marker embryonic myosin heavy chain (\( P = .03 \)) and decreased the expression of the M1 macrophage marker CD68 (\( P = .04 \)), with a modest increase in the expression of the proinflammatory cytokine interleukin-1\( \beta \) (\( P = .03 \); Fig. 3, B). Simvastatin treatment reduced the expression of most ECM synthesis, fibrosis, and fibroblast proliferation genes measured (Fig. 3, C), including type I collagen (\( P = .01 \)), tenomodulin (\( P = .02 \)), platelet-derived growth factor receptor \( \alpha \) (\( P = .04 \)), MMP-2 (\( P = .01 \)), MMP-14 (\( P = .01 \)), tissue inhibitor of metalloproteinase (TIMP)-1 (\( P = .01 \)), and TIMP-2 (\( P = .02 \)). Whereas MMP and TIMP expression levels changed, on the whole no change in overall MMP activity was detected by a broad-spectrum MMP assay (\( P = .11 \); Fig. 5, A). However, consistent with the decrease in type I collagen gene expression, there was a nearly 50% decrease in hydroxyproline content of simvastatin-treated muscles (\( P = .01 \); Fig. 5, B).

**Discussion**

Statin medications are commonly used for the treatment of hypercholesterolemia, and emerging studies have suggested that statins can be efficacious in the treatment of chronic inflammatory conditions and in the acceleration of wound healing.\(^2\)\(^,\)\(^3\)\(^,\)\(^23\)\(^,\)\(^32\) The current study is the first to date to evaluate the use of statin medication in the prevention of myosteatosis after rotator cuff tear. We hypothesized that simvastatin would enhance muscle fiber force production and prevent fibrosis and fat accumulation after rotator cuff tear. The combined results from this study partially support our hypothesis in that simvastatin protected against a loss in muscle fiber s\( F_o \) production and markedly reduced the accumulation of ECM after chronic rotator cuff tear; however, no impact of simvastatin on total triglyceride levels was observed.

Muscle weakness is a common complaint for patients with chronic rotator cuff tears.\(^5\) In rats, there is a reduction in both \( F_o \) and s\( F_o \) of infraspinatus muscle fibers 1 month after rotator cuff tear.\(^16\)\(^,\)\(^17\) In the current study, both groups had s\( F_o \) values lower than the values for healthy, adult rats, which is around 130 kPa.\(^16\) However, simvastatin treatment increased s\( F_o \) by approximately 20% over that of controls. The accretion of type IIB muscle fibers is a common indicator of chronic muscle injuries or diseases,\(^34\) and type
IIB muscle fibers accumulate after chronic rotator cuff tear. Whereas simvastatin treatment slightly increased the size of type IIB fibers, it also dramatically decreased the percentage of type IIB fibers after chronic tear. No other changes in fiber CSA or fiber type distribution were noted. Consistent with these findings, no differences in the muscle-specific E3 ubiquitin ligases atrogin-1 and MuRF-1, which are the major rate-limiting steps in skeletal muscle protein degradation, and no changes in the expression of autophagy-related genes beclin 1, ATG16L1, ATG5, or Vps34 were observed. Although simvastatin treatment resulted in a slight increase in interleukin-1β expression, no differences in the expression of other proinflammatory genes, such as cyclooxygenase 1, cyclooxygenase 2, 5-lipoxygenase, or interleukin-6, were noted. Simvastatin, however, did decrease the expression of embryonic myosin heavy chain, suggesting an acceleration of regeneration after tear. These results suggest that simvastatin is able to enhance sFo production after chronic rotator cuff tear, likely through an acceleration of regeneration as opposed to an inhibition of inflammation.

Patients with chronic rotator cuff tears often have substantial fibrosis, and this accumulation of fibrotic connective tissue is believed to decrease the elasticity and reparational of chronically torn rotator cuff muscles. Fibroblasts are thought to be the predominant cell type in muscle that secretes type I collagen, and we therefore evaluated changes in markers of fibroblasts and their precursors. Whereas no differences in the expression of the fibroblast markers fibroblast-specific protein 1 or scleraxis were observed, we did note a robust decrease in the fibroblast proliferation marker tenomodulin as well as a slight decrease in the expression of the fibroblast precursor marker platelet-derived growth factor receptor α. To quantitatively measure ECM abundance, we used hydroxyproline as a marker for fibrillar collagen content and observed a nearly 2-fold reduction in hydroxyproline with simvastatin treatment. Consistent with this finding, there was a decrease in type I collagen expression of similar magnitude. In addition to directly downregulating type I collagen expression, simvastatin can also indirectly regulate the ECM content of tissues by modulating MMP expression and activity levels. Aktas et al. reported that simvastatin reduced the levels of MMP-3 in articular chondrocytes after induction of an anterior cruciate ligament tear, and Yao et al. reported that simvastatin decreased MMP-9 expression in the vasculature of rats with experimentally induced pulmonary hypertension. Although we anticipated a similar finding, no differences in MMP-3 or MMP-9 expression were observed in the current study. As we did observe changes in MMP-2 and MMP-14 expression along with changes in the expression of TIMP-1 and TIMP-2, which regulate the activity of MMP enzymes, we performed an MMP assay to evaluate functional changes in MMP activity. No differences were observed in functional MMP activity between control and simvastatin-treated animals. Together, these results suggest that simvastatin is able to reduce fibrosis after rotator cuff tear primarily through the downregulation of type I collagen production.

An accumulation of ectopic lipid is also commonly observed after rotator cuff tear. PPAR-γ and c/EBP-α are two transcription factors with well-established roles in promoting adipogenesis, and our group and others have observed increases in PPAR-γ and c/EBP-α expression after rotator cuff tear. In the current study, a downregulation in PPAR-γ and c/EBP-α expression was observed in the simvastatin-treated group. Whereas we did observe slight increases in other markers of lipid accumulation, such as ATGL and ACAT1, other markers of lipid synthesis and storage, such as DGAT1, CD36, GPAT4, perilipin 1, perilipin 5, FITT-1, FITT-2, CGI58, CIDEC, PLD1, and ApoE, were not different between the 2 groups. Despite the changes in the expression of genes related to adipogenesis and lipid storage, surprisingly no differences in total triglyceride levels were observed. These results indicate that although some molecular markers of fatty infiltration were different, simvastatin treatment did not have an effect on the fat content of torn rotator cuff muscles.

Whereas the current study identified a positive role for simvastatin in the prevention of muscle weakness and fibrosis, statin medication can also have a detrimental effect on skeletal muscle function. Between 5% and 10% of patients who take statins develop a myopathy, and a small number of these patients will go on to develop frank rhabdomyolysis. These conditions are thought to be brought on by a decrease in cholesterol levels as a marked reduction in cholesterol can destabilize muscle fiber plasma membranes. Another mechanism of statin-induced myopathy involves a dramatic upregulation in atrogin-1 expression, which then triggers widespread proteolysis in the muscle. On the basis of the results of Hanai et al., we anticipated a potential upregulation in atrogin-1 in simvastatin-treated muscles in the current study but found no difference. The discrepancies between our 2 studies may be the choice of statin medication, as Hanai et al. used pravastatin (Pravachol) in their work. Indeed, although statin medications are often grouped together, they do have somewhat different mechanisms of action and pharmacokinetics and appear to have different safety profiles. There also appears to be genetic variation in the way in which different statins are metabolized by different patients, and different medical comorbidities also appear to influence statin sensitivity, which may further explain the nonuniform response of patients to statin therapy.

There are several limitations to this study. Although the rat is widely used as an animal model for the study of cuff tears, rats do not develop the severity of fatty degeneration that is observed in humans. We did not measure the contractility of type I muscle fibers because of their relatively low abundance. We used only a single time point to evaluate early
chronic changes in torn cuff muscles and did not evaluate acute or long-term changes in muscle function and morphology. The effect of simvastatin on preexisting chronic rotator cuff tears was not studied, and the ability of simvastatin to reverse fatty degeneration that was already present was not determined. Whereas we measured the expression of numerous mRNA molecules, we did not directly measure protein levels, and changes in gene expression may not reflect changes in protein abundance. On the basis of previous studies in rodents, we chose a single dose of drug and did not determine whether there were dose-dependent effects of simvastatin treatment. Despite these limitations, however, this work provided new insight into the pathophysiologic mechanism of rotator cuff tears and identified simvastatin as a potential therapy to limit the development of weakness and fibrosis after rotator cuff tear.

Recent work has eloquently explored the epidemiologic relationships between statin medication and the development of a rotator cuff tear as well as the mechanistic impact of hypercholesterolemia on tendon material properties and susceptibility to tendon tears. Patients with rotator cuff tears had an increase in circulating triglycerides, low-density lipoprotein, and total cholesterol and no difference in high-density lipoprotein levels.1 In mice, chronic hypercholesterolemia decreased the elastic modulus of patellar tendons, which can increase the susceptibility of tendons to rupture.2 For rats that received a rotator cuff tear and repair, a high-cholesterol diet was associated with decreased healing stiffness 1 month after repair.3 Whereas Abboud et al1 did not directly explore statins in their studies, they postulated that these medications may have a positive impact on tendon-bone healing. Combined with the results from the current study, it is possible that statin medication would improve muscle regeneration after repair of a chronically torn rotator cuff to its original anatomic footprint. Despite these optimistic results, further studies that evaluate statin medications in the context of acute and chronic rotator cuff injury and repair models that assess the muscle, tendon, and enthesis and provide further information on the molecular mechanisms of action of these medications will help inform potential clinical studies that may be conducted down the road.

Conclusions

Identifying new therapies to prevent muscle weakness and fibrosis formation is likely to improve the treatment of patients with chronic rotator cuff tears. The results of the current study demonstrated that simvastatin partially protected muscles against the loss in active force production that occurs after rotator cuff tear and dramatically reduced fibrosis as well. Whereas these findings are encouraging, future studies that explore statin medication in the context of acute and chronic rotator cuff repair should be conducted to determine whether a clinical trial to evaluate statin medication in patients with rotator cuff tears is warranted.

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