

Original Article

Investigation of gene Expression in C₂C₁₂ Myotubes Following simvastatin Application and Mechanical Strain

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Aim: The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are the most effective prescribed drugs for lowering serum cholesterol; however, although statins are extremely safe medications and have brought significant benefits to patients with hypercholesterolemia, they have been shown to produce myalgia, cramps, exercise intolerance and fatigue. The aim of the study was to investigate the molecular mechanisms that may mediate statin myopathy.

Methods: We used DNA microarray analysis to examine the changes in gene expression profiles induced by 1 hour and 6 hours of statin treatment on differentiated C₂C₁₂ myotubes. Four genes were selected for analysis at the protein level using Western blot analysis on myotubes treated with statin with or without additional mechanical stretching.

Results: Eighty-five genes exhibited more than a 2-fold up- or down-regulation in expression, of which 46 have known biological functions related primarily to transmembrane transport, signal transduction, cell growth/maintenance, protein metabolism, or apoptosis. At protein level, three of the four proteins were induced (Adrb1, Socs4 and Cflar) and one was repressed (Birc4). Changes in protein expression largely mirrored the changes in their corresponding transcripts, although the fold-change was less dramatic. The addition of imposed muscle fiber stretching did not exacerbate the expression of these genes at the protein level with the exception of Cflar, a pro-apoptotic protein.

Conclusion: These data suggested that alterations in the expressions of some statin-regulated genes could be causative factors for statin toxicity in muscle. Repression of the anti-apoptosis gene (Birc4) and activation of the pro-apoptosis gene (Cflar) indicated that cell death may play an important role in statin-induced myopathy.

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Key words; Statin, C₂C₁₂ cells, Microarray analysis, Mechanical strain, Myopathy

Introduction

Statins are a class of drugs that attenuate cholesterol biosynthesis by inhibiting the rate-limiting enzyme HMG-CoA reductase, which leads to reduced

cholesterol biosynthesis. Statins are widely prescribed to reduce the risk of cardiovascular disease-related mortality¹). Although statins are extremely safe medications and have brought significant benefits to patients with hypercholesterolemia, they have been shown to produce myalgia, cramps, exercise intolerance, and fatigue¹⁻⁴), and on rare occasions, rhabdomyolysis⁵). Regular exercise is effective in reducing cholesterol and is usually recommended for patients with hypercholesterolemia⁶); however, one study demonstrated that as many as 25% of statin users who exercise intensely

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may experience muscle fatigue, weakness, aches, and cramping associated with statin therapy⁷. One explanation offered to account for this undesired side effect is that exercise may affect the absorption, distribution, metabolism, and excretion of a variety of pharmaceuticals, leading to altered pharmacokinetics⁸; however, the specific mechanisms underlying potential exercise-exacerbated statin-induced myopathy remain unclear⁹.

Exposure to statins may interfere with the expression of exercise-regulated genes, and therefore contribute to myopathy³. Statin-induced apoptosis has been considered a contributing factor causing myopathy and has been demonstrated in a variety of cell types, such as smooth muscle cells, cardiac myocytes, and several types of cancer cells¹⁰⁻¹². Several statins have been shown to induce a concentration- and time-dependent increase in apoptosis on cultured myotubes at pharmacological concentrations¹³. A study of humans treated with statins did not reveal a change in the expression of apoptosis-related genes, although it was observed that exercise, especially including eccentric (muscle lengthening) contractions, induced increases in genes encoding components of the ubiquitin proteasome pathway¹⁴.

To examine the molecular mechanisms that mediate statin-associated changes in muscle function in striated skeletal muscles, we exploited the well-characterized mouse satellite cell line C₂C₁₂. Cycling C₂C₁₂ myoblasts can be induced to fuse and form multinucleated striated skeletal myotubes when transferred to a growth factor-deficient differentiation medium (DM)¹⁵. We treated C₂C₁₂ myotubes with simvastatin and performed microarray analysis to examine the changes in gene expression. To simulate the effects of exercise, we also subjected statin-treated myotubes to mechanical stretching. The two purposes of this study were: 1) to identify global changes in myotube gene expression following statin exposure; and 2) to determine if some of these changes were expressed at the protein level following statin exposure with/without mechanical strain.

Material and Methods

C₂C₁₂ Cell Cultures

Mouse C₂C₁₂ cells (ATCC, Manassas, USA) were seeded at 0.5×10^4 cells/cm² onto 35-mm culture dish (Nunc, Roskilde, Denmark) or on Matrigel-coated silicone membranes held in custom-designed stretcher cassettes as described previously¹⁶. The cells were cultured in growth medium (GM) (Dulbecco's modified Eagle's medium, DMEM) (Gibco BRL, Grand Island, USA) containing 10% fetal bovine serum (Hyclone,

Logan, USA) and 100 IU/mL each of penicillin and streptomycin in a humidified 10% CO₂ atmosphere at 37°C. Cultures were fed daily with fresh medium until cells reached 75–85% confluency and then were switched to DMEM supplemented with antibiotics and 2% horse serum (Hyclone, Logan, USA) (differentiation medium, DM) to promote myotube formation. After 2–3 days in DM, 15 μM of Simvastatin (Merck & Co., Inc., West Point, USA) was added to the cultures and the cells were harvested 1 and 6 hours later.

Microarray Analysis

Total RNA was collected 1 and 6 hours after Simvastatin application using Qiagen RNeasy kits (Qiagen, Valencia, USA). Five micrograms of total RNA was used for cDNA synthesis, and biotinylated cRNA was generated using a 1 Cycle Amplification kit (Affymetrix, Santa Clara, USA). Ectopic-type RNA was added as an internal prior to cDNA generation and hybridization for quality control. Fifteen micrograms of fragmented cRNA were hybridized at high stringency to each Affymetrix MOE430A microarray (Affymetrix, Santa Clara, CA) for 16h. All samples were run in duplicate on independent microarrays and the results were averaged across duplicates.

Stringent quality control criteria for array analysis were used as described previously^{17, 18}. Briefly, all arrays fulfilled the following quality control measures: cRNA fold changes between 5 to 10; scaling factor from 0.5–1.5; percentage of “present” (P) calls from 45–55%; and GAPDH spike in probe set controls with a 5'/3' ratio between 0.8–3.0. Total RNA integrity was assessed using spectrophotometry, and cRNA integrity and fragmentation were assessed using 1% agarose gel electrophoresis.

Time series analysis provided data for differing expression levels of all genes in the mouse genome across the three experimental time points. Expression values were generated using MAS5 software (Affymetrix, Santa Clara, CA) and analyzed using GeneSpring software (Silicon Genetics, Santa Clara, CA). Only probe sets with “present” or “marginal” results in all replicates were retained for further analysis. The differential gene expression data were filtered for genes that exhibited at least a 2-fold up-regulation (200% increase in expression) or a 2-fold down-regulation (50% decrease in expression) across both time-points as compared to the baseline. Gene ontology (biological functions and cellular locations associated with these genes) was dissected using bioinformatic databases such as Affymetrix NetAffx (<https://www.affymetrix.com/analysis/netaffx/index.affx>), the Gene Ontology

database (<http://www.geneontology.org/>) and NCBI (<http://www.ncbi.nlm.nih.gov/>).

Mechanical Cyclic Strain

Stretch chambers were constructed by suspending a silastic membrane between concave clamps as previously described¹⁶. These membranes were coated with an atomized solution of collagen (type I from rat tail, BD) and laminin (from EHS tumor, Sigma) in 1% acetic acid to a surface density of 10 $\mu\text{g}/\text{cm}^2$ collagen and 1 $\mu\text{g}/\text{cm}^2$ laminin. Prior to cell seeding, the coated membranes were subjected to and maintained at 50% stretch. This treatment results in oriented surface coating and encourages the alignment of myotubes near the stretch axis. The cell stretcher was attached to each end of the silicon culture chamber and tension was applied by a stepper motor-driven device controlled by Labview software as described previously¹⁹. Stretch was applied cyclically at 10% strain at 1 Hz for 1 hour with 15-second intervals initiated every 45 seconds throughout the study. Differentiated myotubes were treated with 15 μM statin for 1 or 6 hours at 37°C. Mechanical strain was applied coincident with the cells treated with statin for 1 hour. For cells treated with statin for 6 hours, mechanical strain was applied to the cells during the last hour. Cells treated with statin but without stretching served as controls for the effects of mechanical strain.

Western Blotting Analysis

Protein isolation and Western blotting and analysis were performed as previously described²⁰. Briefly, the total protein content of the cell lysate was measured by the method of Lowry using commercially available reagents (Sigma, St. Louis, USA). Samples were heated for 5 minutes at 95°C and then subjected to SDS-PAGE using 7–15% gradient gels (Bio-Rad Laboratories, Hercules, USA) before proteins were electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, USA). Membranes were then incubated in a solution of Tris-buffered saline (PH 7.6) containing 5% non-fat dry milk (Carnation, Nestle, Solon, USA) and 0.1% Tween-20. Precision Plus Kaleidoscope Protein Standards (Bio-Rad Laboratories, Hercules, USA) were used as molecular weight markers on each gel.

A subset of genes displaying the highest up- or down-regulation was selected for Western blot analysis. Membranes were incubated with commercially available primary antibodies against the following proteins: Anti-beta 1 adrenergic receptor (Ab3546, rabbit polyclonal antibody; Abcam Inc., Cambridge, USA); anti-Hilp/XIAP baculoviral IAP repeat-containing 4

(clone 28, monoclonal antibody, BD Biosciences, San Jose, USA); anti-SOCS4 (suppressor of cytokine signaling 4) (rabbit polyclonal antibody; Abcam Inc., Cambridge, USA); and anti-Flip (CASPER, N-terminal) against protein CASP8 and FADD-like apoptosis regulator (rabbit polyclonal antibody; Chemicon International Inc., Temecula, USA). All antibodies were diluted in a solution of TBS/0.1% Tween-20 containing 5–7% bovine serum albumin (BSA) (Sigma) and used at their optimal dilution according to the manufacturer's recommendations. Membranes were then incubated in horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, USA) for 1h at room temperature and washed five times in 0.1% Tween-20 in TBS before antibody binding was detected using enhanced chemiluminescence (ECL) kits (GE Healthcare, Piscataway, USA). Protein expression levels were measured using a Fotodyne Image Analysis System (Fotodyne, Hartland, USA) and TotalLab software (Nonlinear Dynamics, Durham, USA).

Statistical Analysis

For each selected protein, its expression level was the mean value obtained from six separate samples. Using 2-way ANOVA we compared the protein expression between the control group (no statin treatment, no mechanical strain), statin-only groups (1 and 6 hours) and the statin plus mechanical strain (1 hour) group. The SPSS 14.0 computer program (SPSS, Inc., Chicago, USA) was used for statistical analysis. Significance was set at $p < 0.05$.

Results

Effects of Statin on Gene Expression

To determine the effects of statins on gene expression in striated skeletal muscle fibers, we treated differentiated C2C12 myotubes with 15 μM simvastatin for 1 or 6 hours and then isolated RNA for gene expression profiling analysis. Sixty-one of 435 differentially expressed genes exhibited a ≥ 2 -fold up-regulation at both 1- and 6-hour time-points compared to baseline (data not shown). Of these genes, only 37 have known biological functions and/or localizations. Twenty-four of the 435 differentially expressed genes exhibited a ≥ 2 -fold decrease in expression at both time-points, and biological functions and/or localizations are known for only 9 of the encoded proteins. **Table 1** lists differentially expressed transcripts known to be associated with the following major biological functions: transport or signal transduction, cell growth/maintenance, protein metabolism, and apop-

Table 1. Effects of statin treatment for 1 and 6 hours on gene expression (*genes further analyzed for protein levels)

Location	Probe Set ID	Gene Symbol	Gene Title	Category	Fold Change	
					1h	6h
Cell membrane	1423420_at	Adrb1*	adrenergic receptor, beta 1	Signal Transduction	18.5	9.3
	1428111_at	Slc38a4	solute carrier family 38, member 4	Cell Growth and Maintenance	10.6	8.0
	1421424_a_at	Anpep	alanyl (membrane) aminopeptidase	Protein Metabolism	5.6	5.4
	1424284_at	Pomt1	protein-O-mannosyl transferase 1	Protein Metabolism	2.9	3.5
	1420653_at	Tgfb1	transforming growth factor, beta 1	Signal Transduction, Cell Growth & Maint., Protein Met.	2.6	2.5
	1423341_at	Cspg4	chondroitin sulfate proteoglycan 4	Signal Transduction, Cell Growth and Maintenance	2.1	5.2
	1424190_at	Pigc	phosphatidylinositol glycan, class C	Protein Metabolism	-2.9	-2.5
Cytoplasm	1449711_at	Atp6v1e1	ATPase, H ⁺ transporting, VI subunit E isoform 1	Cell Growth and Maintenance	3.4	3.7
	1417091_at	Chuk	conserved helix-loop-helix ubiquitous kinase	Signal Transduction, Protein Metabolism	2.1	2.4
Cytoskeleton	1417729_at	Myh6	myosin, heavy polypeptide 6, cardiac muscle, alpha	Cell Growth and Maintenance	2.5	2.1
Extracellular Space	1438694_at	Csn1s2a	casein alpha s2-like A	Cell Growth and Maintenance	3.4	3.4
	1417130_s_at	Angptl4	angiopoietin-like 4	Apoptosis	3.4	2.3
	1420380_at	Ccl2	chemokine (C-C motif) ligand 2	Signal Transduction	-3.2	-2.5
Intracellular	1420284_at	Bat4	HLA-B-associated transcript 4	Transcription	-2.7	-2.3
	1426636_a_at	Birc4*	baculoviral IAP repeat-containing 4	Apoptosis	-2.8	-27.8
	1450840_a_at	Rpl39	ribosomal protein L39	Protein Metabolism	2.4	2.2
Mitochondrion	1417273_at	Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	Signal Transduction	2.3	2.2
Nucleus	1425208_at	Lbh	limb bud and heart	Transcription	27.8	2.3
	1425895_a_at	Idb1	inhibitor of DNA binding 1	Transcription	6.1	2.8
	1418175_at	Vdr	vitamin D receptor	Cell Growth and Maintenance, Transcription	3.5	2.2
	1416350_at	Klf16	Kruppel-like factor 16	Transcription, Signal Transduction	2.8	2.5
	1425139_at	Sesn2	sestrin 2	Cell Growth and Maintenance	2.0	2.1
	1439374_x_at	Rps10	ribosomal protein S10	Transcription	2.0	4.6
	1416363_at	Fkbp4	FK506 binding protein 4	Protein Metabolism	2.0	2.3
	1451566_at	BC005471	cDNA sequence BC005471	Transcription	-2.5	-2.1
Unknown	1450482_a_at	Pitx2	paired-like homeodomain transcription factor 2	Signal Transduction, Cell Growth and Maintenance	-2.3	-2.2
	1421274_at	Socs4*	suppressor of cytokine signaling 4	Cell Growth and Maintenance, Signal Transduction	15.0	4.3
	1425686_at	Cflar*	CASP8 and FADD-like apoptosis regulator	Apoptosis, Protein Metabolism	12.9	3.3
	1427005_at	Plk2	polo-like kinase 2 (Drosophila)	Cell Growth and Maintenance, Protein Metabolism	-2.4	-2.1

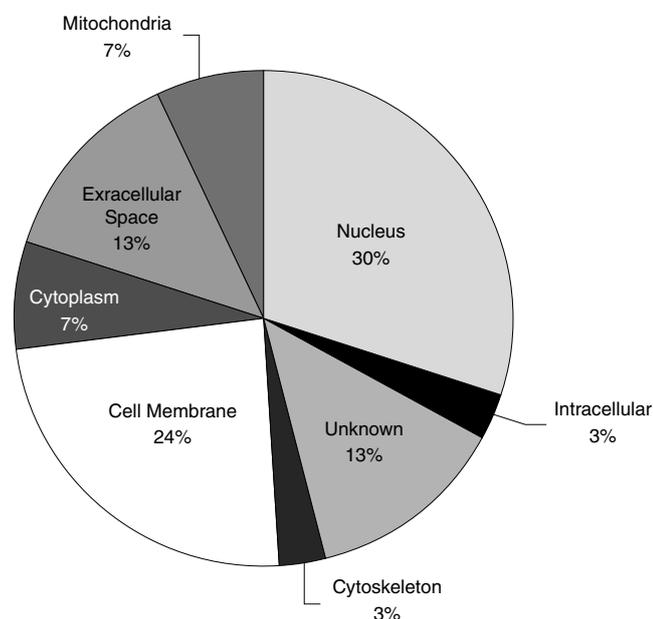


Fig. 1. General subcellular distribution for proteins encoded by differentially-expressed transcripts in C₂C₁₂ myotubes treated with statins for 1 or 6 hours.

Data were generated via microarray analysis.

toxis. Subsets of the protein products from this list can be found in the extracellular space, cell membrane, nucleus, mitochondria, cytoplasm, and cytoskeleton (Fig. 1). Thirty percent of the differentially expressed genes are located in the nucleus and the majority functions as general transcription factors. The largest fold changes in gene expression observed at 1 and 6 hours encode proteins associated with signal transduction and cell growth/maintenance.

Effects of Mechanical Strain on Statin-Induced Protein Expression

We extended our analysis to the protein level for encoded proteins that changed more than 2-fold and for which commercial antiserum was available. Four candidate genes that met these selection criteria included ADRB1 (adrenergic receptors beta 1), BIRC4 (baculoviral IAP repeat-containing 4), SOCS4 (suppressor of cytokine signaling) and CFLAR (Caspase 8 and FADD-like apoptosis regulator). Western blot analysis was performed with proteins from cells treated with statin alone (1 and 6 hours) and statin plus mechanical strain (1 hour). Changes in expression at the protein level largely mirrored those observed at the message level, although the fold change was less marked (Fig. 2-5).

The expression of ADRB1 protein increased sig-

nificantly 1 and 6 hours after statin treatment (2.4- and 1.6-fold, $p < 0.001$) (Fig. 2). The change in expression was even more marked at the mRNA level, where ADRB1 transcripts were elevated 18.5- and 9.3-fold at 1 and 6 hours, respectively. Mechanical stretching for 1 hour after statin treatment did not significantly change the statin-associated increase in ADRB1 protein accumulation relative to the non-stretched controls (2.1- and 1.4-fold at 1 and 6 hours, respectively) and these values were still significantly higher than the untreated controls ($p < 0.001$ and $p < 0.05$, respectively); therefore, statins increased ADRB1 expression at both the RNA and protein levels and this effect was not altered by the induced mechanical stretching of the myotubes.

Statin-induced increases in SOCS4 protein expression were transient and only observed in the 1-hour treatment group (Fig. 3) (1.8-fold, $p < 0.005$). The expression of SOCS4 protein had returned to the baseline by 6 hours (1.39-fold, $p > 0.05$). SOCS4 expression appeared to be unresponsive to mechanical stretching since there was no further significant change in its protein abundance in any treatment groups (Fig. 3).

A similar expression pattern was observed for CFLAR protein. There was a significant increase in expression in the 1-hour treatment group (1.8-fold, $p < 0.005$) but not in the 6-hours group (1.2-fold, $p > 0.05$) (Fig. 4). Mechanical strain induced only a statistically significant change in CFLAR expression in the 6-hour group (1.5-fold, $p < 0.05$) (Fig. 4).

Expression of the anti-apoptosis protein BIRC4 was significantly decreased in cells treated with statin for 6 hours (0.4-fold, $p < 0.001$), but this decline was modest relative to the fold change observed at the mRNA level (27.8-fold) (Fig. 5). Mechanical strain did not induce further change in the protein product for BIRC4 at either time point.

Discussion

Myopathy is one of the key factors in the failure of statin users to continue treatment¹⁾. This statin-induced myopathy is exacerbated by exercise, but the synergistic mechanisms that mediate this adverse effect are unknown^{1, 4, 7)}. To help gain insight into the molecular basis of this process, we treated C₂C₁₂ myotubes with Simvastatin and then analyzed transcript levels of over 20,000 genes in an unbiased manner. Our results showed that the expression of 85 genes (about 0.2% of the differentially expressed genes in our arrays) changed more than 2-fold at both 1 and 6 hours following statin treatment. Of these genes, 61 (71.8%) exhibited ≥ 2 -fold up-regulation and 24

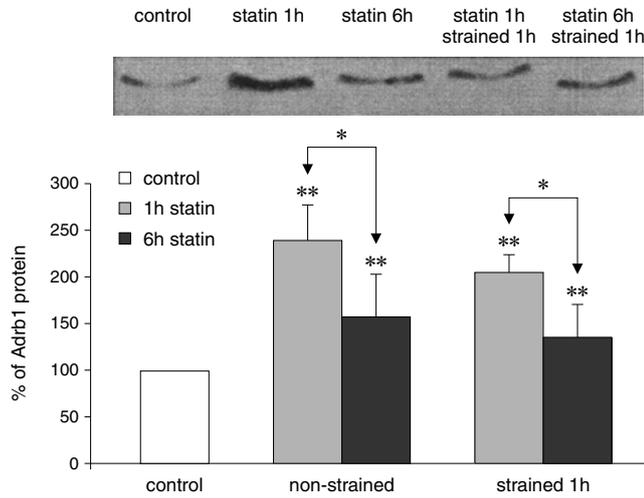


Fig. 2. Effects of statin treatment alone, or in the presence of mechanical stimulation (strained) on ADRB1 levels in C₂C₁₂ myotubes.

*significant difference between the two statin treatments; **significant difference between the treatment and control groups.

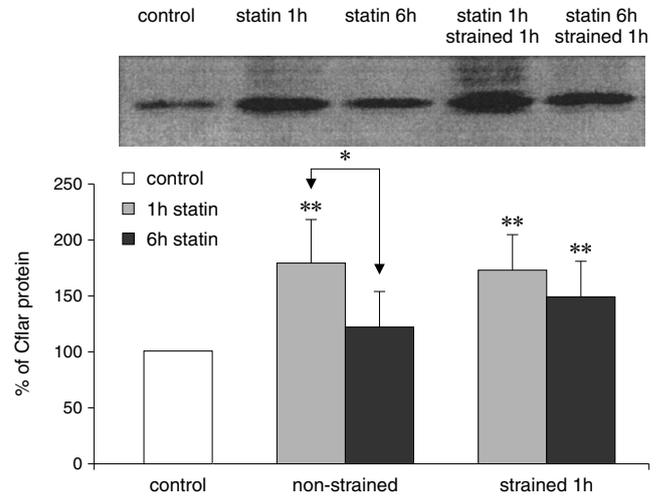


Fig. 4. Effects of statin treatment alone, or in the presence of mechanical stimulation (strained) on CFLAR levels in C₂C₁₂ myotubes.

*significant difference between the two statin treatments; **significant difference between treatment and control groups.

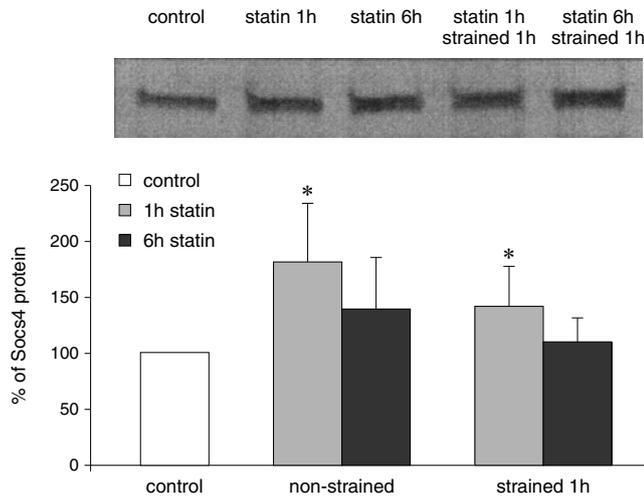


Fig. 3. Effects of statin treatment alone, or in the presence of mechanical stimulation (strained) on SOCS4 levels in C₂C₁₂ myotubes.

*significant difference between the two statin treatments; **significant difference between the treatment and control groups.

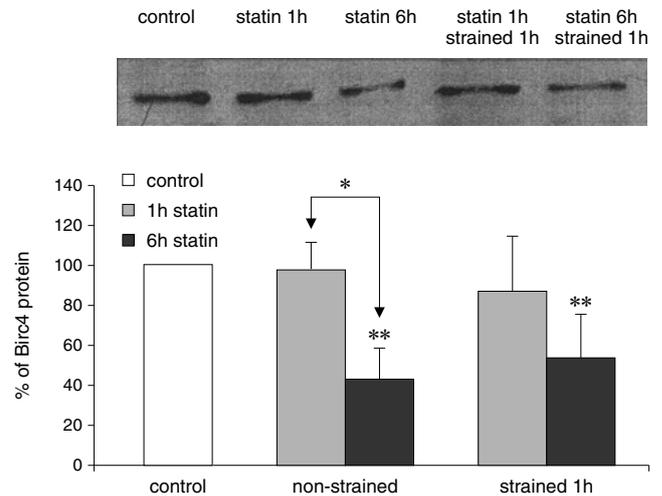


Fig. 5. Effects of statin treatment alone, or in the presence of mechanical stimulation (strained) on BIRC4 levels in C₂C₁₂ myotubes.

*significant difference between the two statin treatments; **significant difference between treatment and control groups.

(28.2%) exhibited ≥ 2 -fold down-regulation compared to the baseline, suggesting that statin treatment is more associated with gene induction than repression. Among the 85 differentially expressed genes, 46 have known biological functions and/or localizations and those that are associated with major cellular processes, signal transduction, transcription, cell growth/maintenance, pro-

tein metabolism, and apoptosis, are listed in **Table 1**.

We selected four of these genes for further examination at the protein level (ADRB1, BIRC4, SOCS4 and CFLAR). In general, changes in the abundance of these proteins changed in a similar manner to their corresponding transcripts, except that the magnitude of this change was lower. This is not surprising since the

latency between the time of drug treatment and analysis was so brief that the proteins may not have had sufficient time to either accumulate or turn over.

Of the genes that were differentially expressed in response to statin treatment, 27% encode proteins that are associated with signal transduction. Among these, *Adrb1* (adrenergic receptors beta 1) was the most affected gene with 18.5-fold induction at 1 hour and 9.3-fold at 6 hours. Expression of *Adrb1* protein was also significantly increased in the myotubes at both times. The expression of *Adrb1* was not altered by mechanical stretching, suggesting that the effects of exercise on statin-induced myopathies are unlikely to be mediated by this protein. In an early *in vivo* study on human skeletal muscle, exercise training also failed to induce a detectable change in beta-adrenoceptor density in both type I and type II muscle fibers²¹.

A large proportion (27%) of statin-induced changes in gene expression was associated with cell growth and maintenance. In particular, two of the genes, *Socs4* and *Slc38a4*, were induced more than 10-fold at 1 hour after statin treatment (10.6-fold and 15.0-fold, respectively). *Socs* proteins (suppressors of cytokine signaling) help the recruitment of an E3 ubiquitin protein ligase complex, which in turn helps target *Socs*-associated proteins for proteasomal degradation²². E3 ubiquitin protein ligases are an important regulatory component in the ubiquitin proteasome pathway. In a recent study of human skeletal muscles we observed substantial changes in genes related to the ubiquitin proteasome pathway in statin users after exercise but not in individuals just taking statins alone¹⁴. Mechanical strain did not result in a further increase in protein expression of *Socs4* in stretched myotubes, which was not parallel to our *in vivo* results with human subjects¹⁴.

Among the differentially expressed genes identified in our study, two (*Birc4* and *Cflar*) have been shown to encode direct regulators of apoptosis. Apoptosis is a form of cellular suicide. Misregulation of apoptosis is associated with numerous human diseases, ranging from cancer to neurodegenerative disorders. Most cells become committed to die when they activate one or more executioner caspases, such as caspase-3²³. Caspase activation can be inhibited by members of the inhibitor-of-apoptosis (IAP) family of proteins, and *Birc4* is a potent inhibitor of both the initiator caspase-9 and the executioner caspases-3 and -7^{24, 25}. In the present study, we observed significant repression of *Birc4* at mRNA and protein levels, which is consistent with the hypothesis that statin treatment sensitizes cells to death through apoptosis.

The other apoptosis-related gene isolated in our

screen encodes *Cflar*, which we found was significantly up-regulated at both 1 and 6 hours after statin treatment. *Cflar* is a key signal transducer for death receptor-induced apoptosis. Statin-induced apoptosis has been observed in a variety of cell types, including smooth muscle cells, cardiac myocytes, several types of cancer cells, skeletal myoblasts and myotubes, and differentiated primary human skeletal muscle cells^{7, 10-12}. Our data on the significant repression of anti-apoptosis protein (*Birc4*) and significant activation of pro-apoptosis protein (*Cflar*) support the hypothesis that statin-induced apoptosis may play an important role in statin-induced myopathy; however, while treating rats for 14 days with cervistatin resulted in muscle damage, there was no detectable increase in apoptosis²⁶. To fully conclude that *Birc4* and *Cflar* play a mechanistic role in the induction of myopathy by statins, further experiments are necessary to show that statins do indeed induce apoptosis in C₂C₁₂ cells under the conditions used in this study.

Our current results of global RNA expression profiles were different from a recent study by Morikawa *et al.*²⁷. In that study, treatment with atorvastatin, cerivastatin and pitavastatin of rat and human skeletal muscle cell lines induced the expression of four genes related to cholesterol metabolism. They suggested that muscle cells are sensitive to the inhibition of HMG-CoA reductase, which may be related to the pathogenesis of muscle damage associated with statin therapy. A previous study has shown that cells *in vitro* treated with statins are concentration- and time-dependent¹³. In our study, the statin treatment time was rather short (1 and 6 hours) compared with other studies^{13, 27}. The higher concentration of Simvastatin (15 micromole/L) used in our study was intended to provide a good sense of what pathways may be involved when inducing cell toxicity in cell culture. Further studies are needed to determine the role of cholesterol metabolism and apoptosis in statin-induced myopathy, especially to examine the long-term effects of simvastatin at lower concentrations on gene expression in muscle cells.

One-hour mechanical strain induced a significant increase in *Cflar* protein expression in cells treated with statin for 6 hours, suggesting that activity could possibly exacerbate apoptosis in statin-treated cells. This result is consistent with our recent results demonstrating enhanced apoptosis in human skeletal muscles in response to statins¹⁴. Using microarray analysis of skeletal muscles we observed that 2% of the differentially expressed genes were associated with apoptosis in statin users following eccentric exercise; however, in contrast to the results of statin plus exer-

cise, exercise alone produced a marked increase in the number of genes involved in apoptotic and inflammatory processes. With limited data on studies of exercise and statin treatment, it is still unclear if exercise exacerbates statin-induced apoptosis in skeletal muscles. A follow-up *in vitro* study of the change in gene expression induced by cyclic strain in the absence of statins is needed to distinguish the molecular pathway of muscular pathogenesis induced by statins and physical activity.

In conclusion, our study found that treatment of myotubes with statins resulted in significant changes in mRNA abundance that was paralleled by changes in gene expression at the protein level. Our results suggest that alterations in the expressions of some of these candidate genes could be causative factors for statin toxicity in myotubes. Apoptosis-associated genes were markedly affected by statin treatment, consistent with the hypothesis that cell death may play an important role in statin-induced myopathy. Mechanical strain also contributed to changes in the protein expression of apoptosis-associated genes, suggesting that muscle strain could synergize with statin toxicity.

Acknowledgments

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