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Effect of simvastatin on cholesterol metabolism in C2C12 myotubes and HepG2 cells, and consequences for statin-induced myopathy

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ABSTRACT

The mechanism of statin-induced skeletal muscle myopathy is poorly understood. We investigated how simvastatin affects cholesterol metabolism, ubiquinone levels, and the prenylation and *N*-linked glycosylation of proteins in C2C12 myotubes. We used liver HepG2 cells for comparison, as their responses to statins are well-characterized in terms of their cholesterol metabolism (in contrast to muscle cells), and statins are well-tolerated in the liver. Differences between the two cell lines could indicate the mechanism behind statin-induced myopathy. Simvastatin reduced *de novo* cholesterol production in C2C12 myotubes by 95% after 18 h treatment. The reduction was 82% in the HepG2 cells. Total cholesterol pools, however, remained constant in both cell lines. Simvastatin treatment similarly did not affect total ubiquinone levels in the myotubes, unlike in HepG2 cells (22% reduction in CoQ10). Statin treatment reduced levels of Ras and Rap1 prenylation in both cell lines, whereas *N*-linked glycosylation was only affected in C2C12 myotubes (21% reduction in rate). From these observations, we conclude that total cholesterol and ubiquinone levels are unlikely to be involved in statin-mediated myopathy, but reductions in protein prenylation and especially *N*-linked glycosylation may play a role. This first comparison of the responses to simvastatin between liver and skeletal muscle cell lines may be important for future research directions concerning statin-induced myopathy.

1. Introduction

Statins, hydroxyl-methyl-glutaryl-coenzyme A reductase (HMG-CoA) inhibitors, are among the most prescribed drugs in Western countries. They reduce morbidity and mortality from coronary heart disease and mitigate the risk of stroke [1,2]. Their major site of action is the liver. Statins inhibit HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis. This reduces hepatic cholesterol production, leading to increased LDL receptor expression, enhanced uptake of circulating LDL particles, and a reduction in peripheral LDL levels [3,4]. They are generally well-tolerated but there are dose-dependent side effects, particularly in skeletal muscle. Myopathy occurs in 1–5% of patients, and can lead to fatal rhabdomyolysis if not recognized [5–8]. The mechanisms of statin-induced myopathy are not fully elucidated.

Statins are thought likely to induce myopathy by disrupting isoprenoid intermediates in the cholesterol synthesis pathway [9]. Ubiquinones, for instance, are produced from the isoprenoid geranylgeranyl pyrophosphate [10]. A reduction in geranylgeranyl pyrophosphate production under statin therapy has been implicated in the reduction of the production of ubiquinones, which are used as electron carriers in the electron transport chain [6,11]. Therefore, disruption of ubiquinone production may lead to dysfunction of the electron transport chain, which could reduce muscle cell ATP levels, increase radical production and lead to apoptosis [6,11].

The post-translational modifications of isoprenylation and *N*-linked glycosylation are also dependent on the cholesterol synthesis pathway. Many small GTPases, such as Ras and Rap1, are isoprenylated via the addition of farnesyl or geranylgeranyl moieties. Altered isoprenylation affects the localization and activity of such proteins. This may alter normal cell growth and differentiation as they are involved in the control of the cell cycle and entry into apoptosis [12–14]. The isoprenoid dolichol is required in *N*-linked glycosylation to link sugars to proteins [15]. Many proteins, such as α-dystroglycan and the IGF-1 receptor, require correct *N*-linked glycosylation [16,17]. *N*-glycosylated proteins have various roles within cells: the IGF-1 receptor is important in regulating cell growth and differentiation, while α-dystroglycan forms part of a complex that links the cytoskeleton to the extracellular matrix. Disrupting these processes leads to cell death, and skeletal muscle damage [18,19].

Previous work shows how statins affect cholesterol metabolism in liver cells [20,21]. No studies have so far investigated the effect

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of statins on cholesterol metabolism in skeletal muscle cells. To address this, we used mouse C2C12 myotubes to model skeletal muscle. C2C12 myotubes are a well-established *in vitro* model for skeletal muscle studies. This is the first study to characterize the effects of statins on skeletal muscle cholesterol metabolism *in vitro*. For comparison we used HepG2 cells to model the hepatic system, as they are a well-characterized hepatic model. This allowed us to elucidate differences between the effects of simvastatin on liver and skeletal muscle cholesterol metabolism. Such differences could suggest causes of statin-induced myotoxicity. We also investigated the effect of statins on ubiquinone levels, and the prenylation and *N*-linked glycosylation of proteins to fully determine differences between C2C12 myotube and HepG2 cell responses to statin treatment. This has not been compared previously, and would allow a more detailed understanding of the mevalonate pathway and how statins may lead to skeletal muscle damage.

2. Materials and methods

2.1. Chemicals

Simvastatin (Sigma–Aldrich, St. Louis, MO, USA) was converted into the active acid following the protocol of Bogman et al. [22]. Stock solutions of 10 mM simvastatin in DMSO were stored at −20 °C. Radioactive compounds were supplied by GE Healthcare (Amersham, UK). We bought the ToxiLight® assay kit LT07-117 from Lonza (Basel, Switzerland), the Pierce BCA protein assay kit from Merck (Darmstadt, Germany) and the Amplex® Red cholesterol assay kit from Gibco (Paisley, UK). All other chemicals were supplied by Sigma–Aldrich (St. Louis, MO, USA), except where indicated.

2.2. Cell culture

C2C12 myoblasts were from the American Type Culture Collection. We grew the myoblasts in Dubecco's modified Eagle's medium (DMEM) high glucose medium (4.5 g/l) containing 10% foetal bovine serum (FBS). The myoblasts were seeded at 80,000 cells per well in a 6-well plate, and grown for 2 days. We induced the myoblasts to differentiate into myotubes using a medium containing 2% horse serum. We let the myoblasts differentiate for 8 days, and used a medium with no horse serum or FBS for the final 24 h (to induce the cholesterol synthesis pathway). We added simvastatin at a concentration of 10 μM. DMSO was used as a control; its concentration was always 0.1%.

We chose the human liver HepG2 cell line as a control. Prof. Dietrich von Schweinitz (University Hospital Basel, Switzerland) kindly provided the HepG2 cells. We grew the HepG2 cells in DMEM low glucose (1 g/l) containing 10% FBS, 1% HEPES and 1% non-essential amino acids. We seeded 500,000 cells per well in a 6-well plate. Cells were grown for 1 day, and then the medium was changed to contain no FBS. The cells were grown in the FBS-deficient medium for one further day, and simvastatin treatment was as per the HepG2 cells.

Both cell lines were grown in a humidified incubator with 5% CO_2 at 37 °C.

2.3. Cytotoxicity assay

We used the ToxiLight assay to determine the toxicity of simvastatin on HepG2 cells and C2C12 myotubes after 1.5, 6 and 18 h. Co-incubation of simvastatin with 100 μM mevalonate, 10 μM farnesol, 10 μM geranylgeraniol or 10 μM squalene, was used to investigate which branches of the cholesterol synthesis pathway are important in simvastatin-induced myotube cytotoxicity. Using luminescence, the kit detected the release of adenylate kinase from dying cells. Briefly, 20 μl medium was removed after and mixed with 100 μl ToxiLight reaction buffer. The mixture was left for 5 min in the dark. Luminescence was measured with a HTS 700 Plus Bio Assay reader and data analyzed with PerkinElmer HTSoft 2.0 software.

2.4. HMG-CoA reductase activity assay

We followed the protocol of Scharnagl et al. with some modifications [21]. We added the drug to the cultured cells for 1.5, 6 and 18 h. Culture medium was removed after incubation and the cells washed twice with 1 ml ice-cold wash buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.4). Cells were suspended in 1 ml of wash buffer and centrifuged for 5 min at 2000 rpm at 4 °C. Supernatant was discarded and cell pellets stored in liquid nitrogen until use. Simvastatin was therefore no longer present, and enzyme induction could be measured.

After defrosting on ice, cells were resuspended in 125 μl lysis buffer (50 mM K_2HPO_4, 5 mM EDTA Na_2, 0.2 mM KCl, 1% Triton X-100, 5 mM dithiothreitol, pH 7.4) and incubated for 10 min at 37 °C and 300 rpm. We centrifuged the lysate for 2 min at 13,000 rpm, and transferred the supernatant to new tubes. We adjusted protein levels to 2 mg/ml with lysis buffer and added 624 μM [^{14}C]-HMG-CoA (4 μCi/ml). The reaction mixture was as described by Scharnagl et al. [20].

We incubated the samples for 90 min at 37 °C, and then added 20 μl HCl to stop the reaction. We added 20 μl [^{3}H]-mevalonolactone (2.27 nCi/μl) as an internal standard, and 50 μl mevalonoactone (0.1 mg/μl) to enable visualisation during thin-layer chromatography (TLC).

Each sample had 1 g of dried sodium sulphate added and was extracted 3 times for 10 min with diethylether. The ether phases were collected and evaporated under N_2 at 37 °C. We suspended the residue in 100 μl ice-cold chloroform:methanol (2:1 by volume).

We separated the samples using TLC with a mobile phase of toluene:acetone (1:1 by volume). Plates were developed with iodine and the mevalonoactone spots scraped and dissolved in 1.2 ml H_2O. Radioactivity was measured using a liquid scintillation counter. Data were expressed as nmol of [^{14}C]-mevalonoactone produced per hour and per milligram of total cell protein.

2.5. Production of esterified and unesterified cholesterol

We incubated the cells with simvastatin for 6 and 18 h. After the first 30 min of incubation, we added 10 μl of 2-[^{14}C]-acetate (2 μCi/ml medium) to the cells. After incubation, we removed the medium and washed the cells twice with buffer A (150 mM NaCl, 50 mM Tris–HCl, 2 mg/ml bovine serum albumin, pH 7.4) and once with buffer B (150 mM NaCl, 50 mM Tris–HCl, pH 7.4). Cells were harvested with isopropanol:hexane (2:3 by volume), and 10 μl of [^{3}H]-cholesterol (1 μCi/ul in toluene) added as an internal standard. We extracted the lipids for 15 min. The samples were centrifuged for 10 min at 4000 × *g*, and the supernatant evaporated to dryness under N_2. The protein pellet was dissolved in 1 ml of 0.1N NaOH and 2% SDS, and used for protein determination. We resuspended the residue in 100 μl chloroform:methanol (1:1 by volume) and separated the lipids via TLC with a solvent of hexane:diethylether:formic acid (40:15:1 by volume). Cholesterol and cholesterol ester standards were run concurrently, to enable identification of the correct spots. We developed the plates with iodine, cut out the spots containing esterified and unesterified cholesterol, and dissolved them in 1 ml H_2O. A liquid scintillation counter determined radioactivity and results were expressed as nmol of [^{14}C]-acetate incorporated per hour and per milligram of total cell protein.

2.6. Measurement of total cell cholesterol

Cells were incubated with simvastatin for 6 and 18 h. We removed cell medium and washed 3 times with 500 μl PBS. We used hexane:isopropanol (3:2 by volume) to extract lipids. Extraction was for 15 min. We then added 500 μl chloroform (containing 2% Triton X-100) to enhance extraction. We centrifuged the samples for 5 min at 3000 rpm, and dried the organic layer under N_2. We resuspended the extracted lipids in 300 μl H_2O.

We used the Amplex Red kit to determine levels of free and total cholesterol. Plates were incubated at 37 °C in the dark, and fluorescence measured on a Spectra Max Gemini at 530–560 nm and an emission of 590 nm. We ran the samples with and without esterases to allow quantification of cholesterol esters and free cholesterol.

2.7. LDL receptor expression

Simvastatin incubation was for 1.5, 6 and 18 h. We removed cell medium and lysed the cells with 350 μl RLT buffer (Qiagen, Valencia, CA, USA). We transferred the lysate to Qiashredder columns and centrifuged for 2 min at 13,000 rpm. The flow-through was purified using the Qiagen RNeasy mini extraction kit, with a DNA digest step to ensure pure RNA. We synthesized cDNA using the Qiagen omniscript system, and used 10 ng of the cDNA for quantitative RT-PCR. We used a primer–probe assay on demand for LDL receptor from Applied Biosystems, Foster City, CA (Mm01177349_m1 and Hs0018192_m1). We calculated relative quantities of specifically amplified cDNA with the comparitive-threshold cycle method. GAPDH acted as endogenous reference (Eurogentec, Seraing, Belgium). No-template and no-reverse-transcription controls ensured nonspecific amplification could be excluded.

2.8. Total ubiquinone quantification

We used the method of Cordoba-Pegrosa et al. to quantify total ubiquinone levels [23]. We grew the cells in 175 cm^2 flasks. We incubated the cells with simvastatin for 1.5, 6 or 18 h. We then removed the medium and washed the cells twice with 10 ml ice-cold 0.9% NaCl. We used 2 ml 1% SDS to solubilize the cells, and added 4 ml ethanol:isopropanol (95:5 by volume). We added 25 μl menaquinone (diluted 1:10 in methanol) as an internal standard. We mixed the samples with 10 ml of hexane, vortexed 5 times for 1 min, and centrifuged at 1000 rpm for 5 min. We recovered the upper organic phase and repeated the extraction twice. The hexane fractions were evaporated to dryness under N_2 and resuspended in 250 μl methanol.

We quantified total ubiquinone levels with high-performance liquid chromatography using a reverse phase C-18 column. The mobile phase was methanol:isopropanol (2:1 by volume), and we used a flow rate of 0.7 ml/ml, an injection volume of 20 μl and UV detection at 275 nm. Data were analyzed with EZChrom Elite software version 3.1.5. We ran a standard curve with the samples to allow quantification.

2.9. Western blot of SREBP-2, Ras and Rap1

After simvastatin incubation for 1.5, 6 and 18 h, we removed the medium and washed twice with 1 ml PBS. We lysed the cells, for 15 min on ice, with 200 μl NET lysis buffer (0.05 M Tris–HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% NP-40 and protease inhibitor tablet). The samples were vortexed and then centrifuged for 10 min at 13,000 rpm at 4 °C. We collected the supernatant and determined protein levels. This represented the whole cell protein fraction. We separated the proteins (50 μg for SREBP-2 and 20 μg for Ras and Rap1) on a denaturing SDS polyacrylamide gel (4% stacking, 10% separating for SREBP-2, and a 4–12% gradient for Ras and Rap1). We blotted the proteins to either nitrocellulose membranes (SREBP-2) or polyvinylidendifluoride membranes (Ras and Rap1). We used an antibody against SREBP-2 that recognizes both the mature and immature proteins, thereby removing the need to fractionate the protein lysate (1:1000 dilution, BD Biosciences, Franklin Lakes, NJ). The Ras, Rap1 and Rap1A antibodies were at a 1:250 dilution (Ras from BD Biosciences, Rap1 and Rap1A from Santa Cruz Biotechnology, USA). Peroxidase-labelled anti-mouse, anti-goat and anti-rabbit antibodies, and chemiluminescence substrate (GE Healthcare) were used for analysis. Rap1, Rap1A and Ras antibodies were also used on protein lysates from 18 h treatment with FTI-277 and GGTI-2133.

2.10. N-linked glycosylation

We followed the protocol of Larsson et al. to determine the rate of *N*-linked glycosylation [24]. Briefly, we added 5 μl [^{3}H]-glucosamine to the cell medium for the final 4 h of simvastatin incubation. After simvastatin incubation for 6 or 18 h, we removed the medium and washed twice with 1 ml PBS. We lysed the cells, for 15 min on ice, with 200 μl NET lysis buffer (0.05 M Tris–HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% NP-40 and protease inhibitor tablet). The samples were vortexed and then centrifuged for 10 min at 13,000 rpm at 4 °C. We collected the supernatant and added 50 μl 100% TCA to precipitate the proteins. The precipitate was washed twice with 10% TCA, collected with 1.2 ml H_2O and dissolved in scintillation fluid. Radioactivity was measured on a liquid scintillation counter.

2.11. Statistical evaluation

All results are expressed as mean ± SD and evaluated with Student's *t*-test, where p values of <0.05 considered significant.

3. Results

3.1. Cytotoxicity

We measured the release of AK from cells to determine the cytotoxicity of simvastatin (Fig. 1). Prior work in our lab showed that 10 μM simvastatin was the lowest concentration that led to widespread cell death of C2C12 myotubes after 48 h. A subsequent

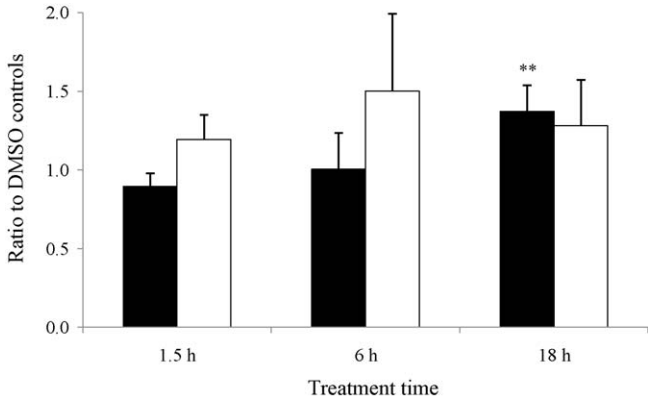

Fig. 1. Toxicity of 10 μM simvastatin on C2C12 myotubes and HepG2 cells. C2C12 myotubes (black bars) and HepG2 cells (white bars) were incubated with 10 μM simvastatin for the times indicated. We measured the release of AK into the medium. DMSO-treated cells were used as a control. Results are expressed as ratios to the DMSO control. Each C2C12 bar represents the mean of five independent experiments carried out in duplicate. Each HepG2 bar represents the mean of three independent experiments carried out in duplicate. $^{**}p < 0.01$ versus control.

Table 1
Rescue of simvastatin toxicity in C2C12 myotubes by co-incubation with cholesterol pathway intermediates.

Co-incubation	Rescue (mean% ± SD)
Mevalonate (100 μM)	89.66 ± 10.39*
Farnesol (10 μM)	57.75 ± 6.39*
Geranylgeraniol (10 μM)	92.37 ± 20.06*
Squalene (10 μM)	21.22 ± 23.09

Cells were incubated with 10 μM simvastatin plus the treatments shown, and AK release into medium was measured. 0% = no rescue when compared to cells treated with simvastatin only; 100% = complete rescue (back to values of DMSO-treated controls). Results are means of four independent experiments in triplicate.
* $p < 0.01$ versus control.

time course experiment observed the first significant increase in toxicity at 18 h (data not shown). We have now observed no significant toxicity after 1.5 or 6 h. C2C12 myotubes treated with simvastatin for 18 h had a significant increase in AK release of 1.37-fold compared to control cells. We did not observe any significant toxicity on the HepG2 cells at any timepoint. We then added intermediates of the cholesterol synthesis pathway and measured cytotoxicity. This would determine the relative importance of each branch of the cholesterol synthesis pathway in simvastatin-induced cytotoxicity. Co-incubation of simvastatin with mevalonate or geranylgeraniol rescued C2C12 myotubes from cytotoxicity, whereas farnesol rescued to a lesser extent. Squalene did not rescue the C2C12 myotubes (Table 1).

3.2. HMG-CoA reductase activity

Inhibition of the cholesterol synthesis pathway with simvastatin has been previously shown to increase levels of HMG-CoA reductase in HepG2 cells [25]. This has not been investigated in C2C12 myotubes. We used protein lysates from simvastatin-treated cells to determine HMG-CoA reductase activity. This removed simvastatin from the system, and allowed us to determine if levels of HMG-CoA reductase changed with treatment time. Addition of [^{14}C] HMG-CoA, followed by quantification of [^{14}C] mevalonate production, enabled determination of enzyme activity. C2C12 myotubes showed an initial reduction in enzyme activity to 50% of control myotubes after 1.5 h (Fig. 2a). The level of inhibition reduced over time so that after 18 h enzyme activity was 71% of control. HepG2 cells showed an inhibition to 64% of control after 1.5 h treatment with simvastatin (Fig. 2b). Treatment for 18 h resulted in an increase of enzyme activity to 356% of control, representing a strong induction in HMG-CoA reductase expression or activity.

3.3. De novo synthesis of cholesterol and cholesterol esters, and total cellular cholesterol pool

We examined the effect of simvastatin on the biosynthesis of unesterified cholesterol and cholesterol esters in C2C12 myotubes and HepG2 cells. Incorporation of [^{14}C] acetate determined the rates of synthesis. Simvastatin reduced free cholesterol production in C2C12 myotubes (to 6% after 6 h and 5% after 18 h; Fig. 3a). We observed a weaker reduction in the HepG2 cells (to 19% at 6 h and 18% at 18 h; Fig. 3b). HepG2 cells produced nearly 10-fold more cholesterol than C2C12 myotubes. Cholesterol ester synthesis dropped slightly in C2C12 myotubes after 6 h treatment (Fig. 3c). After 18 h, cholesterol ester synthesis dropped to only one third of control cells. In contrast, we observed almost complete inhibition of HepG2 cell cholesterol ester synthesis after 6 and 18 h (Fig. 3d).

We also measured the total cellular cholesterol concentrations to determine whether the strong inhibition of cholesterol synthesis influences the overall cholesterol pool in both cell lines. Both cell

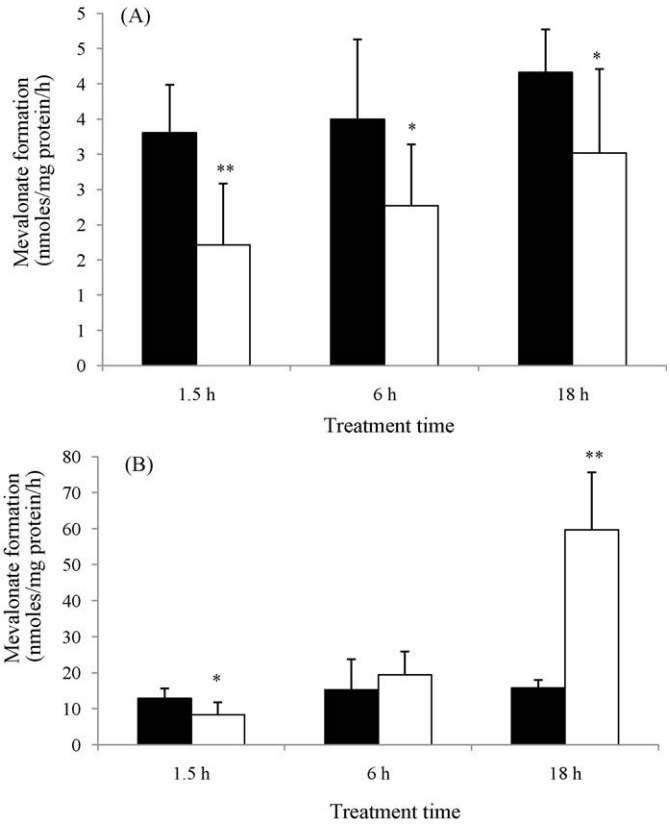

Fig. 2. Effect of 10 μM simvastatin on HMG-CoA reductase activity. Cells were incubated with DMSO (black bars) or simvastatin (white bars) for the indicated times. Protein lysate was collected and enzymatic activity assay performed. Results are for (A) C2C12 myotubes and (B) HepG2 cells. The data represents the mean of four experiments carried out in duplicate. *$p < 0.05$ and **$p < 0.01$ versus control.

lines showed no variation in free or esterified cholesterol content after treatment with simvastatin (Fig. 4). The composition of the cholesterol pool was different in the two cell lines, C2C12 myotubes contained more free cholesterol than the HepG2 cells (by around 1.5-fold).

3.4. LDL receptor expression

In the body, the liver compensates for a reduction in cholesterol synthesis by up-regulating the expression of the LDL receptor, scavenging circulating LDL particles. It is not known if this occurs in skeletal muscle. Quantitative RT-PCR determined the impact of simvastatin treatment on LDL receptor mRNA expression levels. The C2C12 myotubes showed no increase in LDL receptor mRNA expression (Fig. 5a). LDL receptor mRNA expression in HepG2 cells increased 1.8-fold after 6 h treatment, and 2.7-fold after 18 h treatment (Fig. 5b).

3.5. SREBP-2 activation

Cleaved mature SREBP-2 relocates to the nucleus to act as a transcription factor. It regulates the transcription of genes involved in cholesterol synthesis and uptake, such as *hmgr* and *ldlr*. We used Western blotting to detect the mature and immature forms of the SREBP-2 transcription factor. The antibody detected both the immature protein and the cleaved active mature form. Mature SREBP-2 substantially increased in HepG2 cells treated with simvastatin (Fig. 6c). This correlates with the corresponding increase in LDL receptor expression and HMG-CoA reductase activity. SREBP-2 was not expressed in the C2C12 myotubes. Both

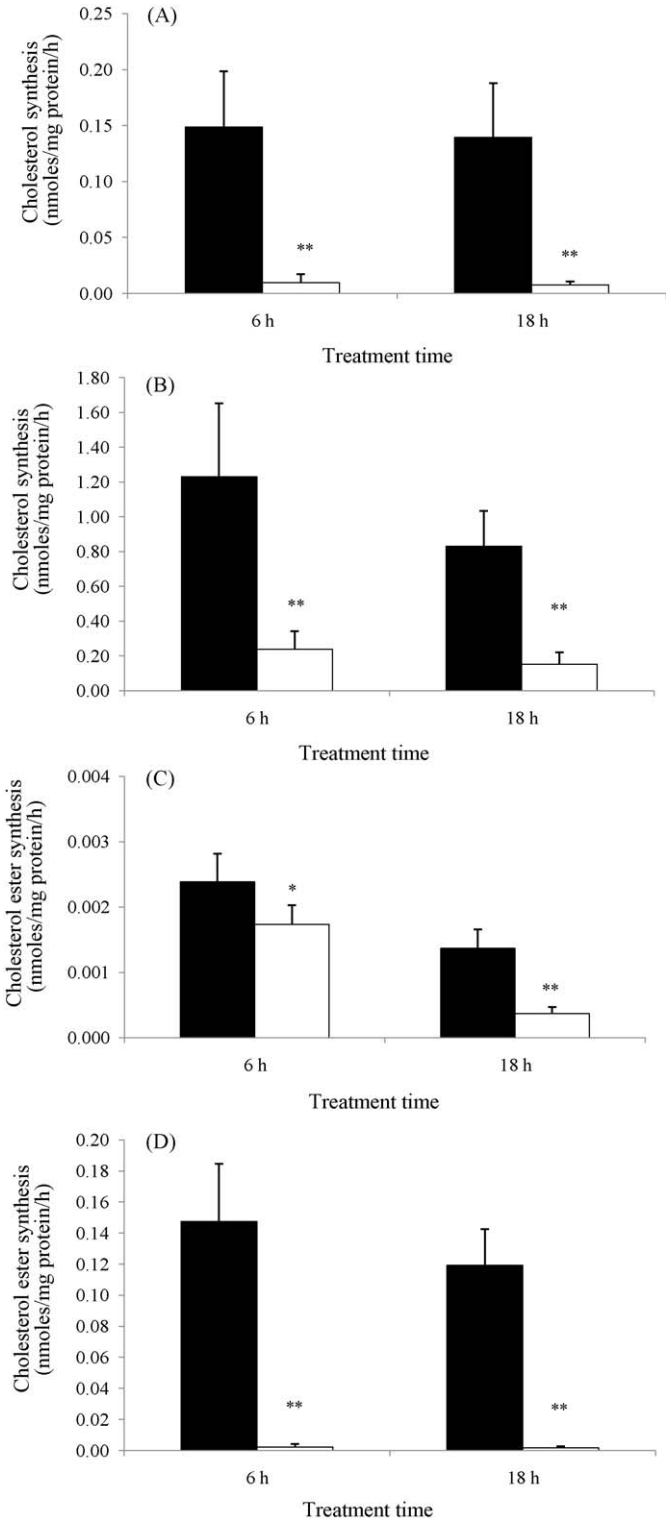

Fig. 3. Effect of 10 μM simvastatin on production of cholesterol in C2C12 myotubes and HepG2 cells. Cells were incubated with DMSO (black bars) or simvastatin (white bars) for the times indicated. 2-[^{14}C]-acetate was added 30 min after start of drug incubation. The graphs show production of cholesterol in (A) C2C12 myotubes and (B) HepG2 cells. Cholesterol ester production is shown for (C) C2C12 myotubes and (D) HepG2 cells. The HepG2 results are means of four independent experiments carried out in duplicate. The C2C12 results are means of five independent experiments in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control.

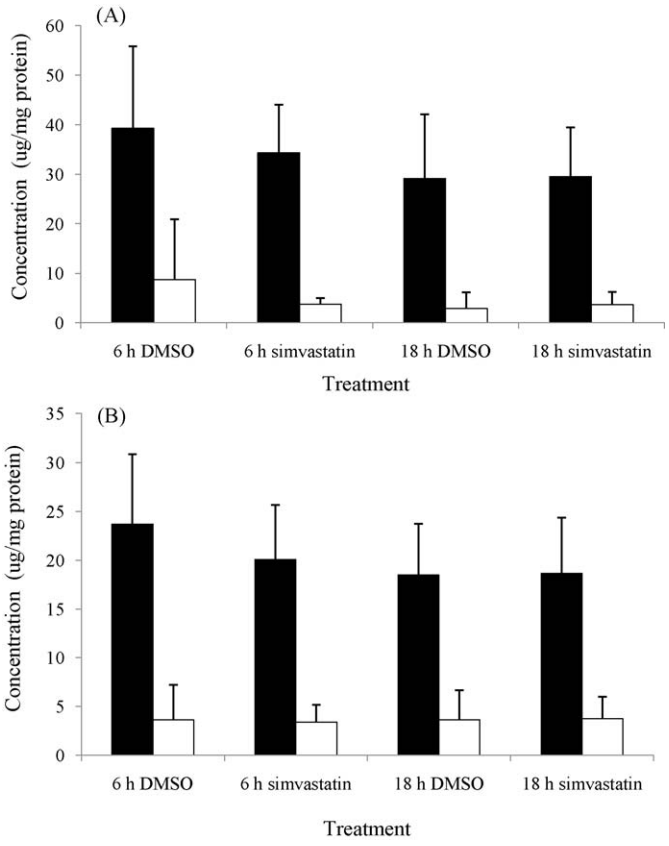

Fig. 4. Effect of 10 μM simvastatin on cellular lipid content. Cells were incubated with DMSO or simvastatin for the times indicated. The graphs show free cholesterol (black bars) and cholesterol ester concentrations (white bars) in (A) C2C12 myotubes and (B) HepG2 cells. Data are means of four independent experiments carried out in duplicate.

cell lines expressed SREBP-1, but simvastatin treatment did not alter the levels of mature SREBP-1 (data not shown).

3.6. Total cellular ubiquinone pool

Ubiquinone synthesis is also dependent upon the cholesterol synthesis pathway. The effect of simvastatin treatment on total ubiquinone levels was determined using HPLC. The major ubiquinone in HepG2 cells was CoQ10, whereas C2C12 myotubes contained a majority of CoQ9. This represents the species difference between humans and mice. Total ubiquinone levels did not alter in simvastatin-treated C2C12 myotubes (Fig. 7a). Simvastatin-treated HepG2 cells exhibited reduced CoQ10 levels in a time-dependent manner. A significant reduction to 73% of control occurred after 18 h (Fig. 7b).

3.7. Protein prenylation

Post-translational prenylation of proteins requires the cholesterol synthesis pathway intermediates geranylgeranyl pyrophosphate and farnesyl pyrophosphate. We used Ras as a representative of farnesylated proteins and Rap1 to represent the geranylgeranylated proteins. We used two antibodies to determine the geranylgeranylation state of Rap1, one detected all Rap proteins and the other only ungeranylgeranylated Rap1. Overall levels of Rap1 remained constant in both cell lines during simvastatin treatment (Fig. 8a and c). Ungeranylgeranylated Rap1 was only present in C2C12 myotubes and HepG2 cells treated with simvastatin (Fig. 8b and d). The proportion of ungeranylgeranylated Rap1 increased as treatment time increased. This effect was

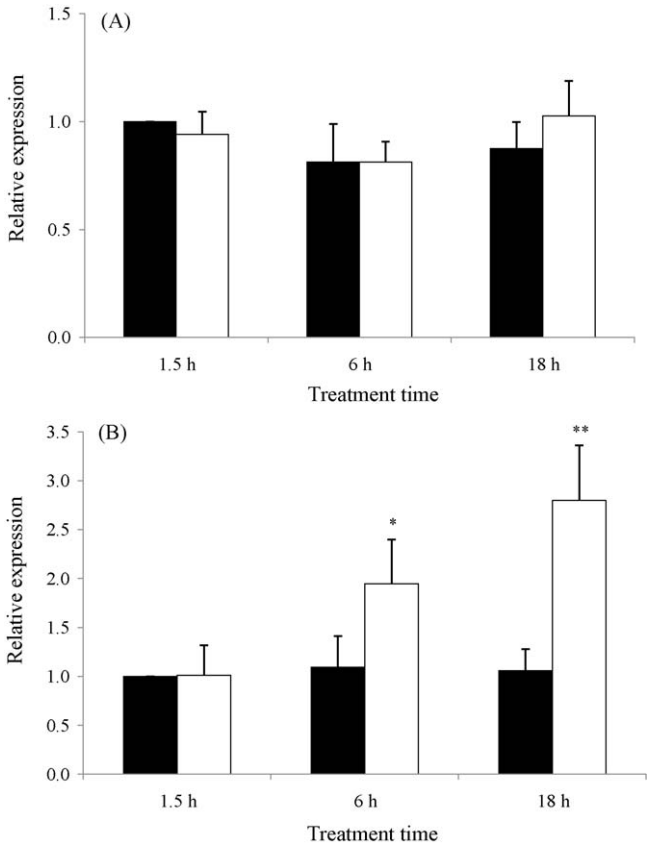

Fig. 5. Effect of 10 μM simvastatin on LDL receptor mRNA expression. Total RNA was extracted from cells after treatment with DMSO (black bars) or simvastatin (white bars). Expression was measured by quantitative RT-PCR with GAPDH as an endogenous control. Expression after simvastatin treatment is shown as a ratio of expression in DMSO-treated control cells. Ratios are for (A) C2C12 myotubes and (B) HepG2 cells. The results are the mean of four experiments carried out in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control.

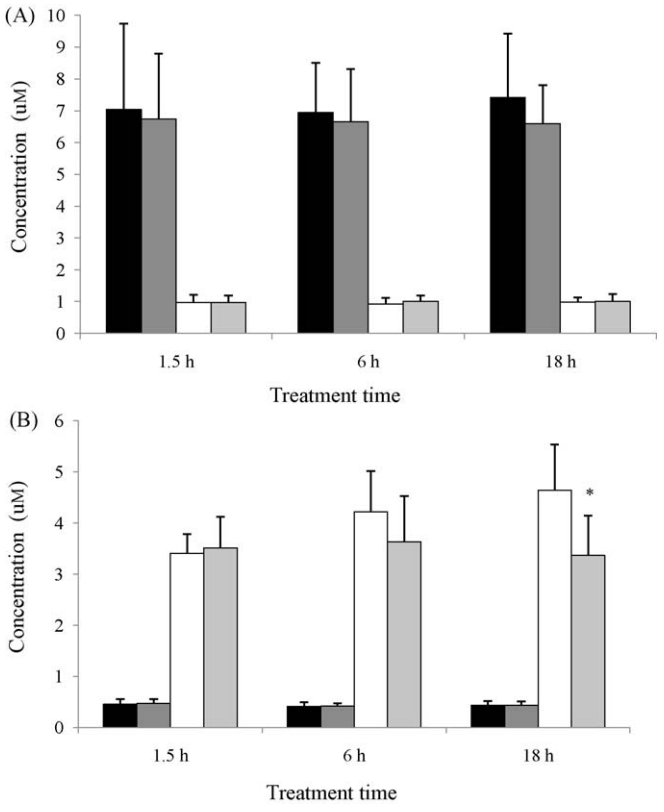

Fig. 7. Effect of 10 μM simvastatin on cellular ubiquinone concentration. Cells were incubated with DMSO or simvastatin for 1.5, 6 or 18 h. Total cellular ubiquinone was extracted and measured by HPLC. Total CoQ9 (black bars for DMSO and dark grey bars for simvastatin) and CoQ10 levels (white bars for DMSO and light grey bars for simvastatin) are shown for (A) C2C12 myotubes and (B) HepG2 cells. Results are expressed as the ratio to DMSO control, and represent the mean of three independent experiments. $^{*}p < 0.05$ versus control.

reversed upon co-incubation with GGOH (data not shown). Incubation with the geranylgeranylation inhibitor GGTI-2133 also led to an expected increase in ungeranylgeranylated Rap1 in C2C12 myotubes (Fig. 10a and b). Similar results were observed with GGTI-treated HepG2 cells (data not shown).

We determined alterations in farnesylation by comparison of Ras protein size on a Western blot. Unfarnesylated Ras has a higher molecular weight than farnesylated Ras due to cleavage of the last three carboxy terminal residues. This difference can be detected using one antibody [26]. Both cell lines showed higher weight Ras protein after 18 h treatment with simvastatin,

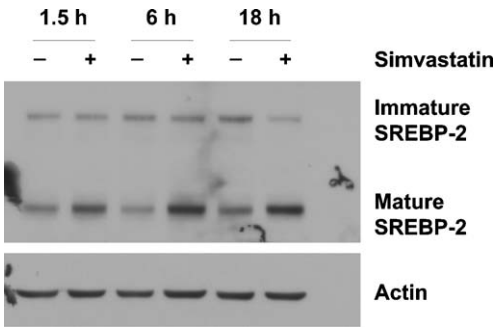

Fig. 6. Effect of 10 μM simvastatin on SREBP-2 transcription factor expression and processing. Total protein was extracted after treatment with DMSO or simvastatin. Actin was used to confirm equal loading. SREBP-2 expression is only shown for HepG2 cells. Results are indicative of three independent experiments.

indicating a reduction in farnesylation (Fig. 9). Co-incubation with FOH increased the farnesylation of Ras in both cell lines treated with simvastatin (data not shown). Incubation with the farnesylation inhibitor FTI-277 also reduced the level of farnesylated Ras in C2C12 myotubes (Fig. 10c). We saw similar results with HepG2 cells treated with the FTI (data not shown).

3.8. N-linked glycosylation

Dolichol is produced from the cholesterol synthesis pathway, and is vital in anchoring *N*-linked sugars to proteins and ensuring correct protein function. We determined the rate of *N*-linked glycosylation in simvastatin-treated cells via addition of [^{3}H]-glucosamine. Treatment of HepG2 cells with simvastatin did not alter incorporation of [^{3}H]-glucosamine into proteins. C2C12 myotubes showed a significant reduction in [^{3}H] glucosamine-labelled proteins after 18 h treatment to 79% of control, and therefore a reduction in *N*-linked glycosylation of proteins (Fig. 11).

4. Discussion

Statins inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [3,4]. Statins exert their effects primarily in the liver, where they are well-tolerated and not toxic, whereas skeletal muscle is the site of the majority of side effects observed with statins [5–8]. We aimed to compare responses to simvastatin in liver HepG2 cells and skeletal muscle C2C12 myotubes. Differences between the two cell lines could indicate the

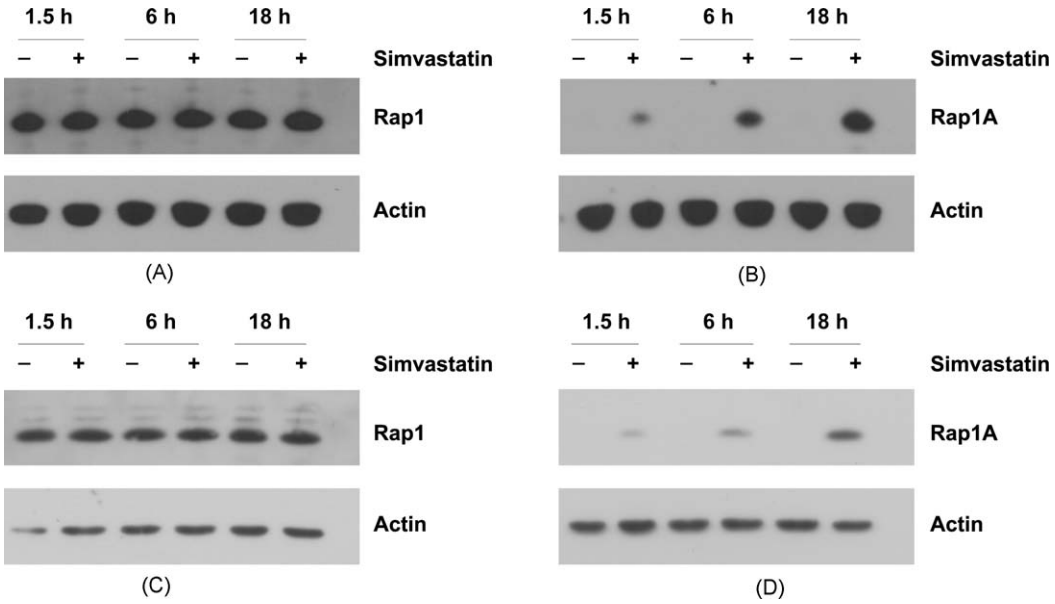

Fig. 8. Effect of 10 μM simvastatin on geranylgeranylation of Rap1. Total protein was extracted after treatment with DMSO or simvastatin. Actin was used to confirm equal loading. Total Rap1 expression is shown for (A) C2C12 myotubes and (C) HepG2 cells. Ungeranylgeranylated Rap1 expression is shown in (B) C2C12 myotubes and (D) HepG2 cells. Results are indicative of three independent experiments.

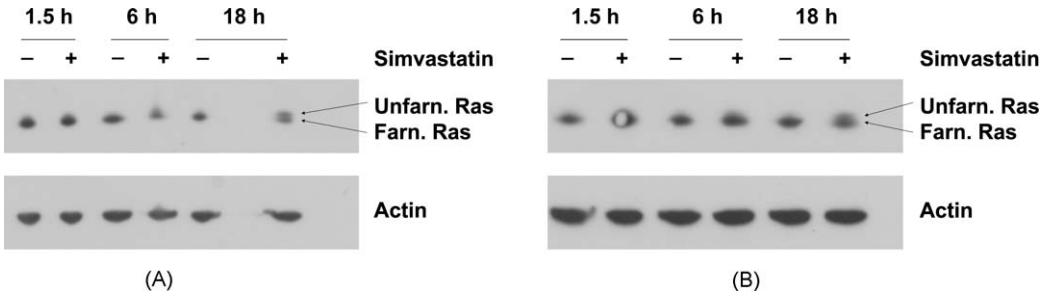

Fig. 9. Effect of 10 μM simvastatin on farnesylation of Ras. Total protein was extracted after treatment with DMSO or simvastatin. Actin was used to confirm equal loading. Ras expression is shown for (A) C2C12 myotubes and (B) HepG2 cells. The upper bands represent unfarnesylated Ras protein. Results are indicative of three independent experiments.

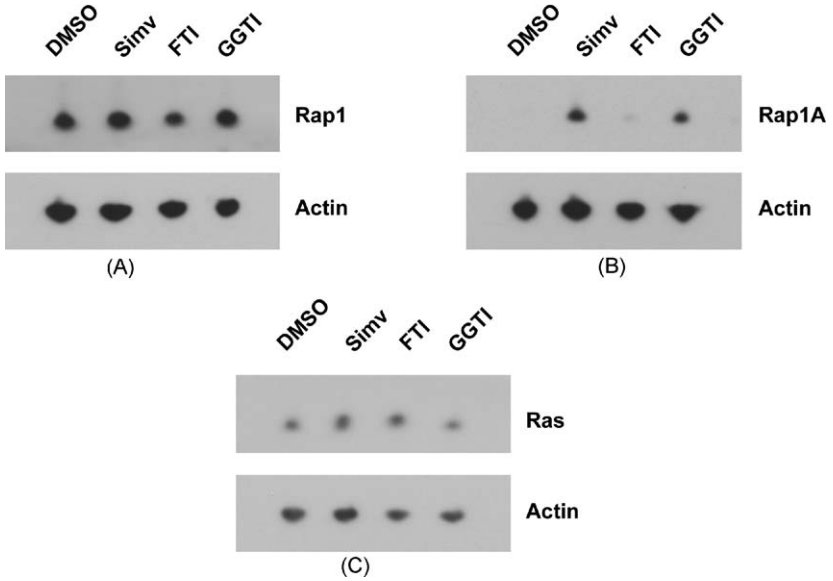

Fig. 10. Effect of FTI and GGTI incubation on prenylation of Rap1 and Ras in C2C12 myotubes. Total protein was extracted from C2C12 myotubes treated for 18 h with DMSO, simvastatin, FTI or GGTI. Expression of (A) Rap1, (B) Rap1A and (C) Ras is shown.

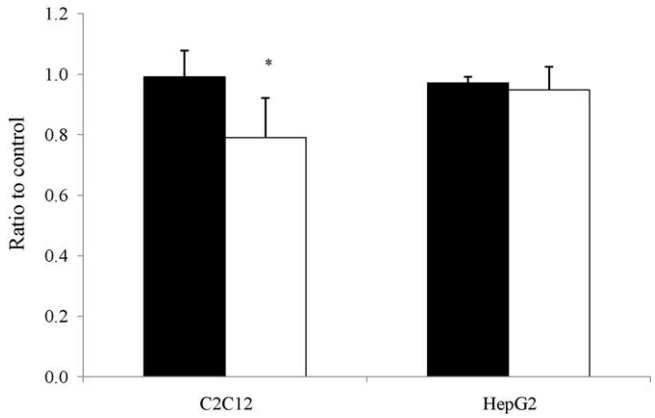

Fig. 11. Effect of 10 μM simvastatin on *N*-linked glycosylation of proteins. Cells were incubated with DMSO (black bars) or simvastatin (white bars) for 6 or 18 h, and pulse-chased with [^{3}H]-glucosamine for the final 4 h. Protein was extracted and the incorporated radioactivity was measured. Results are expressed as the ratio to DMSO control, and represent the mean of four independent experiments. $^*p < 0.05$ versus control.

mechanism behind statin-induced myopathy. We confirmed that 10 μM simvastatin has a significant toxic effect in the C2C12 cell line [27]. This toxicity was not observed in the HepG2 cell line, which suggests that our system adequately represents the *in vivo* situation. In contrast, Tavintharan et al. found that treatment for 18 h with 10 μM simvastatin is toxic to HepG2 cells [28]. The 10 μM simvastatin we used is higher than would be expected in the plasma of patients, where values of up to 1 μM are observed. However, localized concentrations in skeletal muscle could be higher than the observed plasma levels, especially when combined with fibrates or cytochrome P450 inhibitors [29]. The rescue of the C2C12 myotubes by mevalonate confirms that statin-induced toxicity is a direct result of inhibition of the cholesterol synthesis pathway, and not an off-target effect.

Statins exert their therapeutic effects via a reduction of circulating LDL levels. Statins reduce the production of cholesterol in the liver, leading to a SREBP-2-dependent increase in LDL receptor expression and scavenging of circulating LDL [3,4]. How statins affect cholesterol metabolism in skeletal muscle has not been previously investigated. We addressed this using C2C12 myotubes.

Simvastatin inhibited HMG-CoA reductase in cultured cells, as seen by the drop in the rate of unesterified and esterified cholesterol synthesis in both HepG2 cells and C2C12 myotubes. This confirms the findings of a study by Scharnagl et al. on HepG2 cells [20]. We showed that HepG2 cells compensated for this inhibition by up-regulating the HMG-CoA reductase enzyme, but the high concentration of simvastatin meant that inhibition still occurred. This can be explained by the observed increase in mature SREBP-2, leading to an increase in transcription of HMG-CoA reductase. No up-regulation of HMG-CoA reductase occurred in the C2C12 myotubes, probably because of the lack of expression of the SREBP-2 transcription factor in this cell line. Despite the drop in cholesterol synthesis, total cellular cholesterol levels were not reduced in either cell line. Cholesterol turnover may, however, need longer than the 18 h incubation we used until changes are detected. Two other mechanisms might also explain our observation that total cholesterol levels do not change in statin-treated cells: (1) increased receptor-mediated uptake of LDL particles from the medium, and (2) a reduced efflux of cholesterol-containing particles. Since we used starvation medium lacking FBS it seems unlikely that the cells keep their cholesterol levels constant via uptake of cholesterol, thus the second hypothesis seems more plausible. Previous studies also show that statin treatment leads to reduced secretion of cholesterol-containing lipoproteins from HepG2 cells [20]. Reduced efflux of cholesterol could help maintain total cellular cholesterol levels during *in vitro* statin treatment. DMSO-treated cells would still maintain the regular lipid efflux. This could explain why total cholesterol levels are unchanged between statin-treated cells and those treated with DMSO, even though cholesterol synthesis is greatly reduced under simvastatin treatment. This means that the myotubes are not dying as a result of cholesterol depletion, and adds weight to the hypothesis that suggests myopathy is not primarily caused by lowering of cholesterol. We did not observe any increase in LDL receptor mRNA expression in the C2C12 myotubes. This can again be explained by the lack of expression of the transcription factor SREBP-2 in this cell line. This is in stark contrast to HepG2 cells, in which we confirm earlier work which shows statins up-regulate LDL receptor mRNA expression [20,30]. Others have shown statin toxicity to be a cholesterol-independent event [31,32]. Flint et al. inhibited squalene synthase, which is downstream from HMG-CoA reductase and is only involved in cholesterol synthesis. They did not observe toxicity in rat muscle cultured and treated with squalene synthase inhibitors *in vitro* [31]. We confirm this observation by showing that co-incubation of simvastatin with squalene does not prevent toxicity. The compounds produced from the squalene synthase-independent branches of the cholesterol synthesis pathway are involved in many diverse cellular processes, and therefore are attractive candidates for causing myopathies.

One candidate compound is ubiquinone, which is an electron carrier in the electron transport chain. Depletion of ubiquinone plays a role in some mitochondrial myopathic diseases [33,34]. Folkers et al. amongst others, hypothesized that statins could deplete ubiquinone levels, leading to mitochondrial dysfunction, disruption of cellular energy supplies and apoptosis [35,36]. Previous work in our lab shows simvastatin to only affect the electron transport chain at high concentrations (50 μM) [37]. This study has expanded on that observation and found no evidence that simvastatin reduces total cellular ubiquinone content in C2C12 myotubes. This is the first such study in C2C12 myotubes and is consistent with previous studies in other systems [14,38]. This result also complements studies in humans, which show ubiquinone supplementation does not reverse myopathy during statin treatment [39,40]. We did observe a decrease in HepG2 CoQ10 levels, but this did not lead to any observable toxicity, suggesting that the electron transport chain can still function with a 27% decrease in cellular CoQ10 levels. In comparison, Tavintharan et al. show that larger drops in cellular CoQ10 is associated with toxicity in HepG2 cells [28]. Taken together, ubiquinone levels do not appear to be relevant to statin-induced myopathy.

Post-translational prenylation of proteins is also dependent on the cholesterol synthesis pathway [9]. The addition of a farnesyl or geranylgeranyl moiety is important for correct localization of proteins, particularly of small GTPases such as Ras and Rap1 [41]. These proteins play critical roles in multiple signalling pathways controlling cell growth, repair, differentiation and cellular adhesion. Statins are known to inhibit both farnesylation and geranygeranylation in a variety of cell lines [31,41]. We have shown that simvastatin increased levels of unfarnesylated Ras and ungeranylgeranylated Rap1 in both cell lines. We did not, however, observe any HepG2 toxicity, indicating that impaired prenylation of Ras and Rap1 has no impact on cell survival in these liver cells. We hypothesize that, in contrast to HepG2 cells, incorrect localization of small GTPases could lead to altered signalling and cell death in C2C12 myotubes. The importance of prenylation was further seen by geranylgeraniol reducing the toxicity of simvastatin in C2C12 myotubes, a finding that correlates with previous work [14,42]. Intriguingly, addition of farnesol does not

rescue the cells to the same extent as geranylgeraniol. Cao et al. also show that specific inhibitors of geranylgeranyltransferase increase apoptosis, unlike farnesyltransferase inhibitors [42]. This evidence points to geranylgeranylated proteins playing a large part in statin-induced myopathy. The large number of prenylated proteins warrants further investigation into the effects of dysprenylation caused by statins. Future work in our lab will determine the actual proteins that, when ungeranylgeranylated, are associated with myopathy.

N-linked glycosylation is a post-translational modification that is also dependent upon the cholesterol synthesis pathway [15]. This process requires dolichol, a polyprenol downstream from farnesyl- and isopentenyl-pyrophosphate. Oligosaccharides need to be linked to dolichol before they are added to asparagine residues of target proteins, and dolichol is cleaved during the linkage to asparagine [15]. No prior studies have looked at how statins affect *N*-linked glycosylation in C2C12 myotubes. We showed that simvastatin reduced the rate of *N*-linked glycosylation in C2C12 myotubes to 80% of control. In contrast, we observed no reduction in *N*-linked glycosylation in HepG2 cells. This suggests that aberrant *N*-linked glycosylation may have a role in statin-induced toxicity in C2C12 myotubes, although which proteins are affected is not yet known.

Glycosylation of proteins is crucial for correct function of many proteins. It increases protein stability and facilitates interactions between proteins and ligands [43]. Previous studies implicate statins in aberrant processing of *N*-linked glycoproteins [44–49]. One such protein is the Igf1 receptor. This receptor requires correct *N*-linked glycosylation before it can be cleaved from the proreceptor to the mature receptor [47–49]. Statins increase levels of proreceptor, decrease the expression of mature Igf1 receptor at the cell surface and promote apoptosis in Ewing's sarcoma cells [50]. We aim to investigate whether this also occurs in skeletal muscle cells.

The glycoprotein α-dystroglycan, which is part of a complex that anchors the cytoskeleton to the extracellular matrix, is also heavily glycosylated [16,19]. It is also implicated in many of the congenital muscular dystrophies, which can be caused by aberrant glycosylation of proteins [18]. Although there is more *O*-linked than *N*-linked glycosylation on α-dystroglycan, insufficient *N*-linked glycosylation may contribute to congenital muscular dystrophies. A reduction in *N*-linked glycosylation of α-dystroglycan, caused by statins, could potentially affect the skeletal muscle and lead to myopathy.

Further work is required to fully determine the proteins that are affected by aberrant *N*-linked glycosylation under statin treatment. Linking these future studies to those investigating altered small GTPase function could lead to breakthroughs in understanding statin-induced myopathies, and is a direction we aim to undertake.

Our data showed for the first time that total cholesterol and ubiquinone contents are not altered by simvastatin in C2C12 myotubes, and are therefore not likely to associate with statin-induced myotoxicity. Overall, the inhibition of HMG-CoA reductase by statins can also affect the production of numerous compounds other than cholesterol and ubiquinone. Our comparison between C2C12 myotubes and HepG2 cells allows us to suggest that the skeletal muscle side effects of statins may be related, at least in part, to cholesterol-independent effects, particularly reductions in prenylation or *N*-linked glycosylation of proteins.

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