Molecular mechanisms of statin-induced myotoxicity

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PhD thesis

Molecular mechanisms of statin-induced myotoxicity

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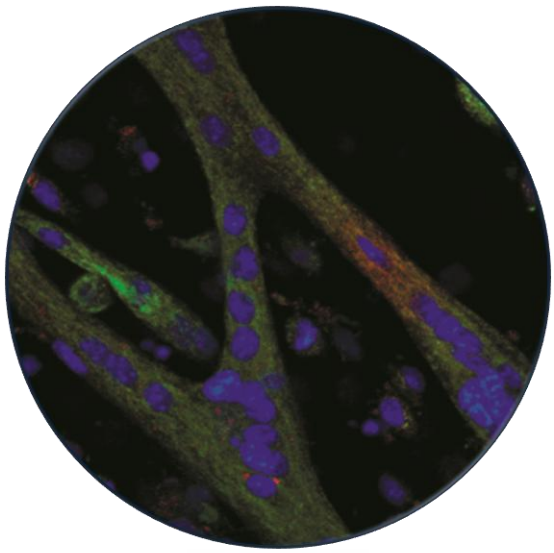

December 2010

This work was carried out in the laboratory of Stephan Krähenbühl

Clinical Pharmacology and Toxicology
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Dedication

To Dai, for Christmas.

Merci

MY THANKS must go first to Stephan for supporting my work in his lab. Besides the obvious financial support, his encouragement to develop my own ideas and directions for this thesis was invaluable. His checking of my wilder ideas also taught me to think more rigorously and I will always fondly remember our discussions.

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All those above have made my work in Basel both enjoyable and productive, but all of this would have been impossible without the help, understanding and genius poster designs of my husband, Dai. I can't wait to bag new things on a new continent with you (and dissuade you from driving my golf buggy there). I love you ■

Contents

The research in this thesis is presented in the form of three scientific papers that have either been published or are in preparation. Reference lists for each paper are presented at the end the relevant section. A reference list covering the general introduction and discussion is at the end of the thesis.

Important abbreviations

DMSO	Dimethyl sulphoxide
ECAR	Extracellular acidification rate
EGFP	Enhanced green fluorescent protein
EPAC	Exchange protein activated by cAMP
ETC	Electron transport chain
GGOH	Geranylgeraniol
HK	Hexokinase
HMG-CoA	Hydroxy-methylglutaryl-coenzyme A
HMGCR	HMG-CoA reductase
Igf-1	Insulin-like growth factor-1
NRVM	Neonatal rat cardiomyocyte
OCR	Oxygen consumption rate
SREBP	Sterol regulatory element binding protein
TMRE	Tetramethylrhodamine ethyl ester
VDAC	Voltage-dependent anion channel
ψm	Membrane potential

Summary

STATINS ARE among the most prescribed drugs in Western countries. They reduce morbidity and mortality from coronary heart disease and mitigate the risk of stroke. Their major site of action is the liver, where they inhibit HMG-CoA (hydroxyl-methyl-glutaryl-coenzyme A) reductase, the rate-limiting step in cholesterol biosynthesis. Inhibition of this pathway also inhibits various other processes, such as ubiquinone production and the isoprenylation and *N*-linked glycosylation of proteins. Altering these processes can reduce inflammation, oxidative stress and platelet adhesion – leading to the positive effects of statins. However, inhibition of these processes can also lead to negative side-effects, such as skeletal muscle myopathy, which is seen in 1–5% of patients. These side-effects can impact on quality of life and compliance, and in extreme cases lead to death. Uncovering the mechanism by which statins lead to these side-effects is therefore of great urgency.

This thesis includes three papers that have been published or submitted for publication.

Our first paper presents a comprehensive comparison of the effects of simvastatin on the cholesterol pathway and its intermediates in mouse skeletal muscle C2C12 myotubes and human liver HepG2 cells. C2C12 myotubes are susceptible to statin-induced toxicity, whereas HepG2 cells are not. Differences between the two could therefore point to possible toxic or protective mechanisms. We show that differences in ubiquinone and cholesterol content are not responsible for toxicity, and suggest that altered geranylgeranylation could cause toxicity in the C2C12 myotubes. We also show a decrease in the rate of *N*-linked glycosylation in the C2C12 myotubes. This, and the

need for geranylgeranylated proteins, suggests that an impairment in cell signalling pathways is responsible for statin-induced toxicity.

Our second paper expands on these results by showing that an impairment in Igf-1/Akt signalling leads to both mitochondrial toxicity and upregulation of the pro-atrophy atrogin-1. We show that Igf-1/Akt signalling is not impaired in the HepG2 cells, and that inhibition of this pathway makes the HepG2 cells sensitive to simvastatin. Finally, we provide evidence that a small GTPase, Rap1, is integral to C2C12 myotube mitochondrial integrity, and that mitochondrial respiration can be partially rescued by expressing constitutively active Rap1 in those cells. Rap1 is a geranylgeranylated protein that has been hypothesized to link cAMP/EPAC signalling to Igf-1/Akt signalling, and is therefore a prime candidate as a causative factor in statin-induced myotoxicity.

The final paper takes the work of the previous two papers and places it into a novel environment – cardiac muscle. Statins are primarily prescribed to prevent cardiovascular disease, and we present evidence that suggest that simvastatin can be toxic in cardiomyocytes. We start with an observation of a lighter heart in simvastatin-treated Wistar rats, and use *ex vivo* cardiomyocytes and *in vitro* H9C2 cardiomyocytes to confirm toxicity. We show that, similar to the C2C12 myotubes, simvastatin leads to mitochondrial dysfunction, inhibition of Igf-1/Akt signalling and upregulation of atrogin-1 expression. This is the first time that these effects have been seen in the heart, and warrant further investigation to ensure that these effects so not pose a risk in susceptible patients ■

Introduction

2.1. Cholesterol

CHOLESTEROL IS essential throughout the living world. It is used as a precursor to steroid hormones, vitamins and bile acids.[1-3] It is also an integral component of membranes, where it ensures correct structure and stability.[3] In animals, it is synthesized in the liver, with extra cholesterol consumed in the diet. Excess cholesterol is stored as cholesterylesters in cells.[4] Circulating cholesterol is present in the plasma as protein-lipid conjugates of varying densities, including: high density (HDL cholesterol); low density (LDL cholesterol); and very low density (vLDL cholesterol).[5] The importance of cholesterol is seen in the inherited disorder, hypobetalipoproteinaemia. In this disorder, circulating LDL cholesterol is reduced, and patients are susceptible to neurological problems, sensory disorders and blood clotting.[6] Conversely, high cholesterol levels are also pathological, with high circulating LDL cholesterol being a risk factor for atherosclerosis, cardiovascular disease, diabetes and stroke to name but a few.[7–11] The synthesis and regulation of cholesterol content is therefore vital for correct cell and body function.

2.1.1. Why study cholesterol?

Cardiovascular disease is the number one killer of adults in the Western world.[12–14] A combination of bad diet and little exercise, amongst other factors, leads to build-up of cholesterol-rich LDL-cholesterol in atherosclerotic plaques, and presentation of the disease. Considering the increasing number of elderly and obese people in Western populations, understanding and controlling how cholesterol is produced is of

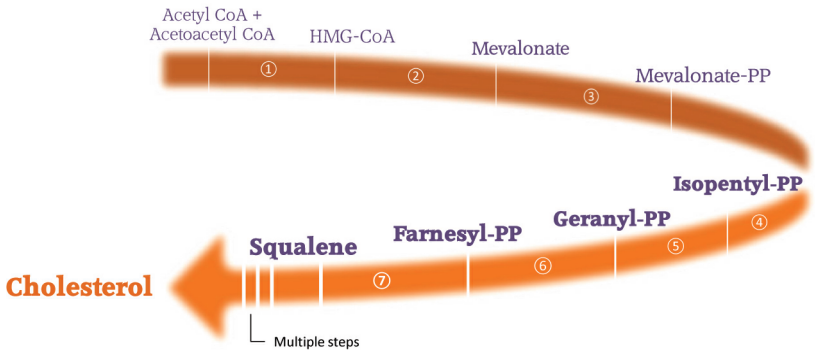


Figure 1

The mevalonate pathway. Cholesterol production from acetyl CoA, with the later stages in bold. The bold intermediates are important in other processes such as isoprenylation and N-linked glycosylation. ① HMG-CoA synthase ② HMG-CoA reductase ③ Mevalonate kinase & phosphomevalonate kinase ④ Mevalonate-5-pyrophosphate decarboxylase ⑤ Farnesyl-PP synthase ⑥ Geranyl-PP synthase ⑦ Squalene synthase

paramount concern. There is a need for better prevention of cardiovascular and related diseases, in order to save both lives and money, and increase quality of life in affected individuals. Cholesterol-lowering is the main preventative measure, and understanding the cholesterol pathway and how it can be inhibited has therefore been of great importance.[15]

2.1.2. Cholesterol synthesis: The mevalonate pathway

Endogenous cholesterol is produced in the cytoplasm and endoplasmic reticulum (ER) of liver cells via the mevalonate pathway (**Fig 1**). Acetyl-CoA is converted to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) via HMG-CoA synthase. The conversion of HMG-CoA to mevalonate is the rate-limiting step, and is performed by HMG-CoA reductase (HMGCR). From here, isopentenyl pyrophosphate (IPP) is formed and converted to geranylpyrophosphate (GPP) and farnesylpyrophosphate (FPP). FPP is converted to squalene, which leads through to cholesterol.[16]

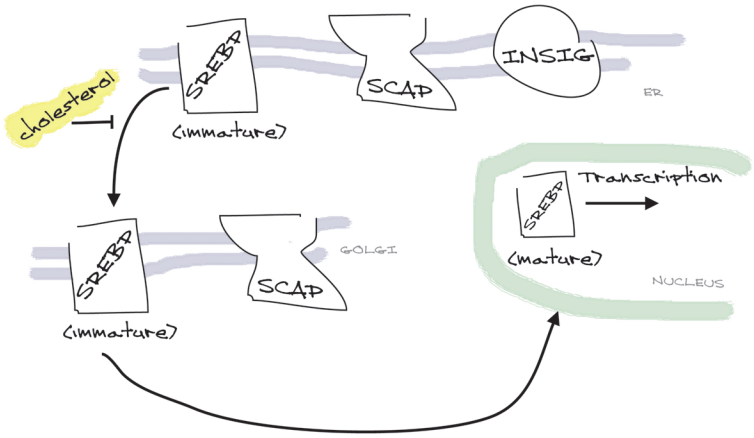


Figure 2

Regulation of SREBPS. These are kept in an inactive state in the presence of high cholesterol. Low cholesterol levels release SREBP from the inhibitory complexes, and lead to cleavage to the active transcription factor. SREBPs transcribe many genes, including those important for cholesterol production and uptake.

2.1.2.1. ***Regulation of cholesterol metabolism***

The pioneering work of Goldstein and Brown in the 1970s began to unravel the complex regulation of cholesterol metabolism. They first observed that familial hypercholesterolemia was associated with a lack of LDL receptors, and then vastly expanded upon this. They discovered a feedback loop linking external cholesterol and the number of LDL receptors, and also a feedback loop linking cholesterol uptake and cholesterol production.[17] All this work came to a climax in 1993, when Yokayama, supported by Goldstein and Brown, discovered the sterol regulatory element binding proteins (SREBPs).[18] These transcription factors are controlled by the amount of cholesterol in the cell (**Fig 2**). High cholesterol concentrations keep the SREBPs in an inactive state, whereas low cholesterol concentrations lead to their cleavage from inhibitory factors, activation and translocation to the nucleus. At the nucleus, they bind to sterol regulatory element DNA sequences, and upregulate transcription of genes associated with cholesterol synthesis or uptake (such as those for HMGCR and the LDL receptor).[18,19]

2.1.2.2. *Regulation of HMGCR*

HMGCR is one of the most highly regulated enzymes in the human body. HMGCR transcription is enhanced by SREBP binding, but the importance of the mevalonate pathway demands multiple levels of regulation.[20] Recently, alternative splice forms of HMGCR have been discovered.[21] Although not completely characterized, one splice variant (HMGCR-D13) lacks part of the catalytic domain that is important for binding the substrates. A functional SNP (rs3846662) that regulates the splicing of HMGCR has also been recently discovered.[22] Phosphorylation of HMGCR by an AMP-activated protein kinase leads to a reversible inhibition of the enzyme.[20] This inhibition ensures that acetyl-CoA is not used to produce cholesterol during times of low cellular energy (when AMP would be high, and induce HMGCR phosphorylation). A similar phosphorylation inactivates acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid metabolism, providing further control of acetyl-CoA levels during low energy status.[20]

2.2. Statins

At the same time as Goldstein and Brown were working on regulation of the mevalonate pathway, Endo derived two compounds from the fungus *Penicillium citrinum* that could competitively inhibit HMG-CoA reductase.[23,24] He named one of these compounds mevastatin, and Goldstein and Brown used this compound to inhibit HMG-CoA reductase in fibroblasts from hypercholesterolemia patients.[25] Endo then used a rat model to demonstrate *in vivo* the ability of mevastatin to reduce cholesterol synthesis. This work also shows that the inhibition is mostly found in hepatic HMG-CoA reductase, where most cholesterol is produced.[26]

The isolation of lovastatin by Merck heralded a new era in the treatment of cardiovascular disease.[27,28] Following extensive clinical trials, lovastatin received FDA

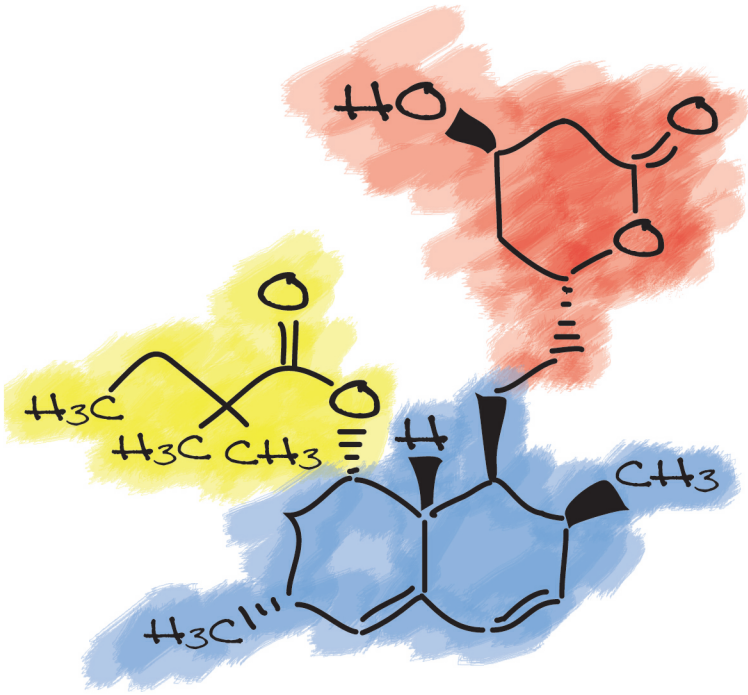


Figure 3

Structure of simvastatin. The three main structural regions of statins: (red) the pharmacophore, which is structurally similar to HMG-CoA; (yellow) a butyryl group for type 1 statins, or a flurophenyl group of type 2 statins; (blue) decalin ring structure of type 1 statins, type 2 statins have larger groups.

approval in 1987.[29] Evidence followed linking statin use with a reduction of mortality in people with high cholesterol.[30–32] Since that time, further statins have been derived and synthesized, including simvastatin, atorvastatin, pitavastatin and cerivastatin (**Fig 3**).[33,34]

2.2.1. Side-effects of statins

Statin use has been increasing since their release onto the market, a trend that is likely to continue as elderly and obese populations grow. Potential side-effects must therefore be understood and prevented. The most severe side-effect is rhabdomyolysis, which is characterized by destruction of skeletal muscle leading to the release of muscle proteins and compounds into the blood.[35–38] Release of potassium can lead to disruption in heart rhythm, and phosphates can cause hyocalcemia by precipitating with calcium in the blood.[39] The most severe consequence of rhabdomyolysis is the accumulation of myoglobin in kidney tubules, which can severely damage the kidney via acute tubular necrosis and eventually lead to kidney failure if left untreated. Rhabdomyolysis is usually associated with a sharp increase in serum creatine kinase (CK) levels to over 10

times normal levels. Statin toxicity became of public interest in 2001, when cerivastatin was withdrawn from the market.[40,41] Fifty-two deaths from rhabdomyolysis were caused by the drug.[42] The risk of rhabdomyolysis was 10 times higher than with other statins, and those on combination therapy with gemfibrozil were at particular risk.[43,44] Health organizations were prompted to reassure statin users of the safety and benefits of the taking the remaining statins. Rhabdomyolysis now occurs at a rate of 0.44 per 10,000 patient years.[38]

Although extreme, rhabdomyolysis is not the only side-effect associated with statin-use. Other myopathies occur in 1–5% of patients, and myalgias in 9–20%.[35–38,45–47] Less serious skeletal muscle effects may be even more frequent, and may lead to a reduced quality of life and lack of treatment compliance.

2.2.1.1. ***Risk factors***

Side-effects are usually dose-dependent, with Silva *et al.* showing a 10-fold increase in myopathy in patients taking a high dose of atorvastatin or simvastatin (80 mg/day) compared to patients on a lower dose.[48] The positive side to the high dose is a decreased risk of cardiovascular disease.[49]

Myotoxicity is associated with all statins, but to varying degrees.[38,50] A large variation in the pharmacokinetic properties of the different statins could be a factor in determining which statins lead to more side-effects.[50] Lipophilicity also varies between the statins (for example, simvastatin is lipophilic whereas pravastatin is not, meaning simvastatin can enter a cell more easily than pravastatin).[50]

Interactions with other drugs, as highlighted with cerivastatin and gemfibrozil, are also risk factors for skeletal muscle side-effects. Three commonly prescribed statins – simvastatin, lovastatin and atorvastatin – are metabolized by CYP3A4.[51–53] This

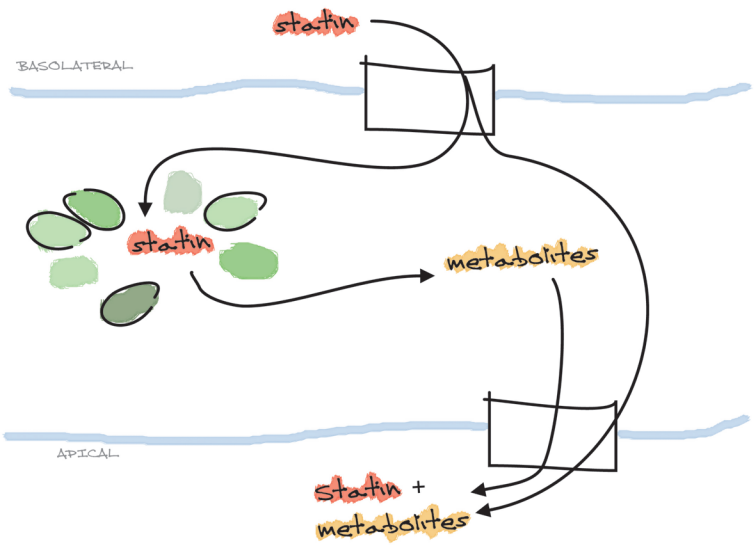


Figure 4

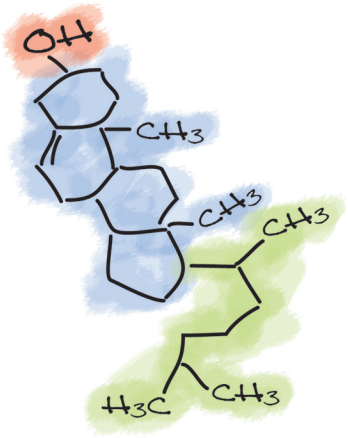
Statin metabolism in the liver. Statins enter liver cells via SLCO transporters (rectangle in baso-lateral membrane). Some statins are metabolized by CYP450 enzymes (green circles). Statins and their metabolites are then excreted at the apical membrane via ABC trans-porters (rectangle in apical mem-brane). The isoforms of all these enzymes vary from statin to statin.

isoenzyme metabolizes more than 50% of prescribed drugs, leading to a large risk of drug-drug interactions.[54,55] Co-treatment with inhibitors of CYP3A4 could also raise concentrations of statins, and therefore increase the risk of side-effects.[56,57] One study suggests that simvastatin-associated muscle disorders were six-fold higher when patients were taking CYP3A4 inhibitors at the same time.[58] They saw no change in patients taking CYP3A4 inhibitors with pravastatin (which is not metabolized by CYP3A4).[58] Fluvastatin is primarily metabolized by CYP2C9, and is therefore subject to possible interactions with CYP2C9 inhibitors, such as diclofenac and fluconazole.[59,60]

In addition to drug metabolism, drug uptake also has an effect on the risk of skeletal muscle side-effects.[61] The hepatic transporter OATP1B1 is the main transporter of statins into the liver (**Fig 4**). Inhibiting uptake may lead to increased plasma statin levels, which may be how gemfibrozil increases risk of myopathy.[61] The gene encoding OATP1B1, *SLCO1B1*, is also the only gene which has been associated with risk for statin-induced myopathy.[62] A genome-wide study in patients who suffered statin-induced myopathy identified only one associated polymorphism – over 60% carried the rs4363657 SNP in *SLCO1B1*.[62]

Figure 5

Structure of cholesterol. The three main regions are shown: (red) a polar hydroxyl group; (blue) four hydrocarbon rings, which forms the basis of all steroid hormones; (green) a non-polar hydrocarbon ring.



There are also reports of statins exacerbating or uncovering muscle conditions such as myasthenia gravis, McArdle's disease and myotonic dystrophy.[63–65] These studies suggest that care should be taken when prescribing statins to patients with signs, or a family history of, muscle disorders.

2.3. Linking the mevalonate pathway to statin-induced myopathy

The mechanisms of statin-induced myopathy are not fully elucidated. The fact that the mevalonate pathway not only produces cholesterol, but a myriad of other essential compounds, enables statins to affect many cellular processes – any or all of which might contribute to myopathy.

2.3.1. Cholesterol

Cholesterol is produced via the mevalonate pathway (**Fig 5**). Previous *in vitro* studies in rat skeletal muscle show that squalene, the direct precursor to cholesterol, cannot rescue statin-induced myotoxicity.[66] Inhibitors of squalene synthase do not cause muscle toxicity either.[67] This is strong evidence that it is not cholesterol-lowering that leads to toxicity. Further evidence is provided by our studies showing that simvastatin is toxic to C2C12 myotubes even when cellular cholesterol levels are not decreased.[68]

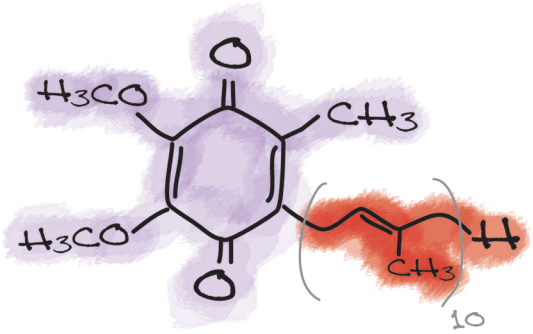


Figure 6

Structure of ubiquinone. Ubiquinones have two region: (purple) the quinine structue; (red) the isoprenoid side-chain, the number of which varies depending on the ubiquinone, here it represents CoQ10.

2.3.2. Ubiquinone

Ubiquinines are also produced via the mevalonate pathway (**Fig 6**). Ubiquinones are used as electron carriers between complex I or II and complex III in the mitochondrial electron transport chain (ETC).[69] It is hypothesized that 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.[70,71] Further to this, ubiquinone is also an important antioxidant in its reduced form.[72] Ubiquinones are also involved in regulating the mitochondrial permeability transition pore.[73] Depletion of ubiquinone plays a role in some mitochondrial myopathic diseases, although its role in statin-induced myopathy is more controversial. Numerous *in vitro* studies show no reduction in ubiquinone levels after statin treatment.[68,74,75] Studies in humans also show ubiquinone supplementation does not reverse myopathy during statin treatment.[76,77] Despite this, numerous clinicians suggest ubiquinone supplements to patients on statin treatment.

2.3.3. *N*-linked glycosylation

N-linked glycosylation requires dolichol, a polyprenol downstream from farnesyl- and isopentyl-pyrophosphate (**Fig 7**). Oligosaccharides need to be linked to dolichol before they are added to asparagine residues of target proteins, and dolichol is then cleaved during the linkage to asparagine.[78] *N*-linked glycosylation is required for the correct function of many proteins. It increases protein stability and facilitates

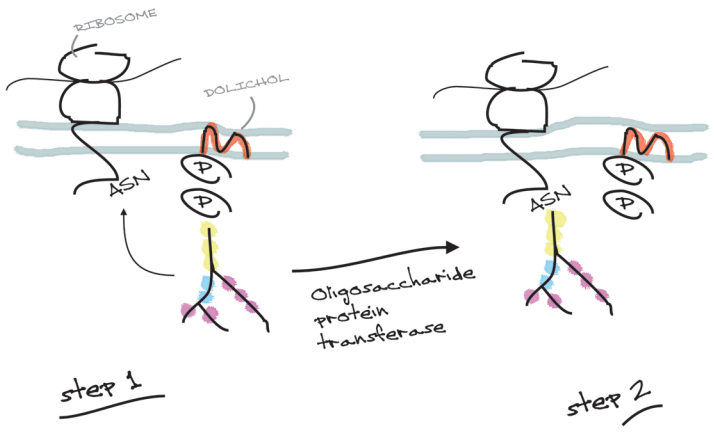


Figure 7

Importance of dolichol in *N*-linked glycosylation. The process is shown at the ER, with dolichol phosphate anchoring oligosaccharide chain (red) composed of *N*-acetylglucosamine (yellow) mannose (blue), and glucose (purple) to the ER membrane. The oligosaccharides are transferred to an asparagine residue on the translated protein, with dolichol phosphate remaining in the ER membrane.

interactions between proteins and ligands.[79] An example is the Igf-1 receptor (Igf-1r). This receptor requires correct *N*-linked glycosylation before it can be cleaved from the proreceptor to the mature receptor.[80–82] Statins are known to increase levels of the proreceptor, decrease expression of the mature Igf-1r at the cell surface and promote apoptosis in Ewing's sarcoma, and other, cells.[80,82,83] Insufficient *N*-linked glycosylation may also contribute to congenital muscular dystrophies, suggesting that statin-induced disruption of *N*-linked glycosylation in muscle could present as a myopathy.[84]

2.3.4. ***O*-mannosylation**

Dolichol is also required for the addition of *O*-mannosyl groups to proteins.[85,86] Proteins that are *O*-mannosylated are important in muscle and brain development, and are therefore candidates for causative factors in statin-induced myopathy.[87] One protein that is *O*-mannosylated is α-dystroglycan (α-DG), one of many proteins that form the dystrophin-glyoprotein complex in skeletal muscle.[88] The dystrophin-glycoprotein complex acts as a transmembrane bridge between the intracellular cytoskeleton and extracellular matrix; disruption of this complex could therefore have a negative effect on the structure of skeletal muscle fibres (**Fig 8**).[88–90] Walker-Warburg syndrome, a congenital disorder, shows the importance of correct *O*-mannosylation.[91–93] This

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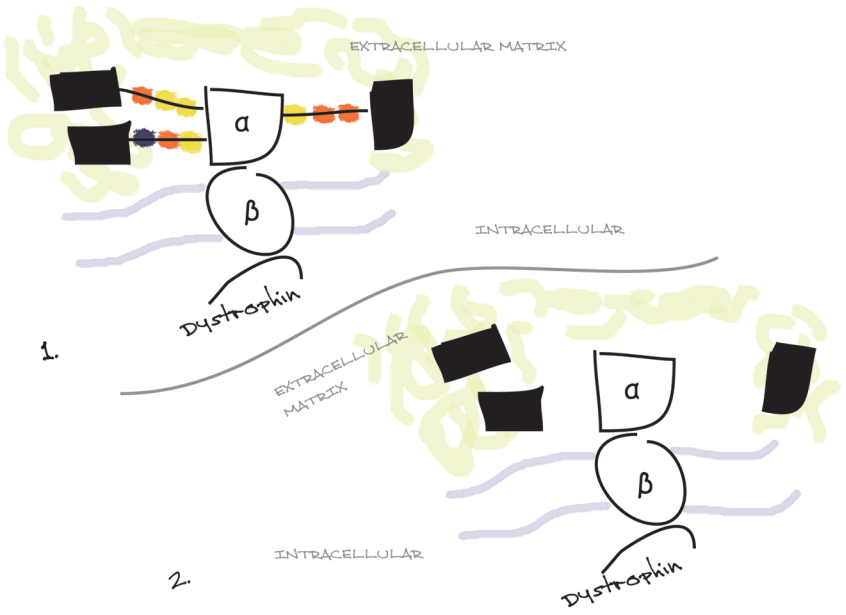


Figure 8

Disruption of the dystrophin-glycoprotein complex.

1. Normal muscle. α-dystroglygan is heavily *O*-mannosylated (yellow, red and blue groups). *O*-mannosylation links α-dystroglycan to extracellular matirix proteins (shown in black). α and β dystroglycan can then act as a link between the intracellular cytoskeleton and the extracellular matrix.

2. Walker-Warburg muscle. A lack of *O*-mannosylation prevents the link between α-dystroglycan and the extracellular matrix.

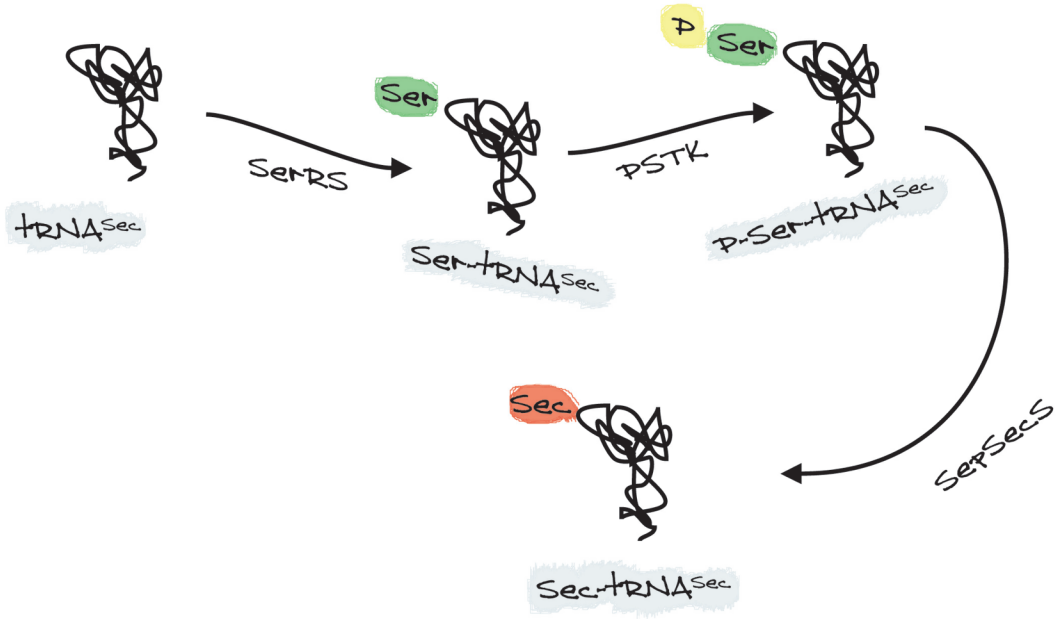


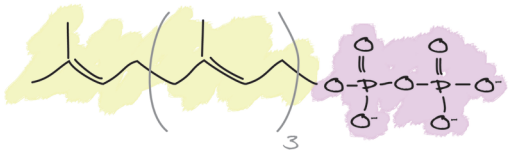
Figure 9

Incorporation of selenium into sec-tRNA^{sec}. Naked tRNA incorporates a serine (green), which is then phosphorylated (yellow) and replaced by a selenocysteine (red). **SerRS:** Seryl-tRNA synthetase, **PSTK:** O-phosphoteryl-tRNA kinase, **SepSecs:** Sep-tRNA:Sec-tRNA synthase.

Continued from page 12

disorder is caused by mutations in the enzymes that link the *O*-mannosyl moiety to proteins, and leads to congenital muscular dystrophy. A statin-induced lowering of dolichol levels in skeletal muscle could therefore lead to a breakdown in muscle structure.

Figure 10



Structure of geranylgeranylpyrophosphate. There are two main regions: an isoprenoid region (yellow) containing 20 carbons, (note that farnesylpyrophosphate contains only 15 carbons) a pyrophosphate group (purple).

2.3.5. Selenocysteine tRNA

Isopentyl pyrophosphate is required for the correct production of selenocysteine tRNA. It is added to an adenosine, without which there is an 80–90% reduction in selenoprotein translation (**Fig 9**).[94] Selenocysteine tRNA is required for the production of selenoproteins, a small group of proteins that have not been greatly studied yet.[95,96] Selenoproteins are important in numerous cellular functions, one being the antioxidant glutathione pathways that provide protection from reactive oxygen species.[97] Selenium deficiency is known to cause numerous skeletal muscle disorders, including myotonic dystrophy, multiminicore disease and white muscle disease.[98–100] Multiminicore disease can be caused by mutations in the selenoprotein N gene, *SelN*, leading to a loss of organization of muscle fibres.[101] Selenoproteins are also important in maintaining correct cardiac muscle function, and low dietary selenium is associated with two cardiomyopathies: Keshan disease and Chagas' disease.[102,103]

A statin-induced reduction of isopentyl pyrophosphate has been shown to lead to a reduction in selenoprotein translation, which could therefore impact both skeletal and cardiac muscle function.[94]

2.3.6. Prenylation

Prenylation involves the addition of a farnesyl or geranylgeranyl moiety to a protein at a C-terminus cysteine. Farnesyl and geranylgeranyl moieties are derived from intermediates of the mevalonate pathway (**Fig 10**).[104] They are added to numerous proteins, but particularly small GTPases, and contribute to correct localization.[105,106] Small GTPases play myriad critical roles in multiple signalling pathways controlling cell growth, repair, differentiation and adhesion.[107–110] One small GTPase that is farnesylated is Ras. Ras has received a lot of attention due to it being hyperactive in many types of cancer.[111] The importance of its farnesyl group in correct localization led to much research into the use of farnesyltransferase inhibitors (FTIs) to prevent cancer development.[112–113] These studies met with mixed success, probably due to the ability of Ras to compensate for loss of a farnesyl group by addition of a geranylgeranyl group.[114]

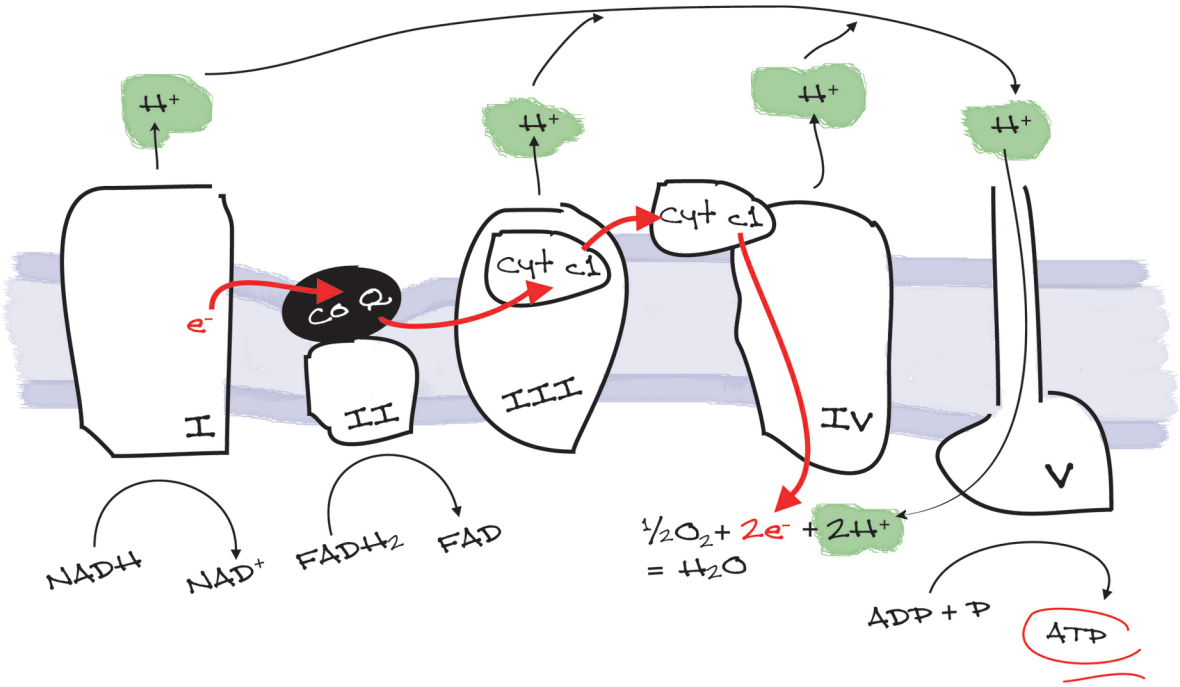


Figure 11

Mitochondrial electron transport chain and oxidative phosphorylation. The complexes are in the inner mitochondrial membrane. Substrates can be reduced at complex I or complex II. The electrons flow through the first four complexes, facilitating the efflux of protons into the intermembrane space. A proton gradient is built, and protons flow back through complex V (ATP synthase), with the energy released enabling the production of ATP.

Geranylgeranylation is a promising area of research into the toxicity of statins. Addition of geranylgeraniol (GGOH) to statin-treated cells rescues them from apoptosis, and treating cells with geranylgeranyltransferase inhibitors (GTIs) increases apoptosis, suggesting that geranylgeranylated proteins play a major role in statin-induced myotoxicity.[68,74,115] This is given further credence by a study by Itagaki *et al*. showing that simvastatin-induced cell death in L6 myoblasts is accompanied by a redistribution of the small GTPase RhoA from the plasma membrane to the cytoplasm.[116]

2.4. Potential mechanisms of myopathy

2.4.1. Direct effects on the mitochondrial ETC

The mitochondria ETC is an essential energy-producing process in cells (**Fig 11**). Statins have been shown to directly inhibit complexes of the mitochondrial ETC. One study shows that complex IV of the ETC is impaired, whereas Nadanaciva *et al.* showed all complexes except complex II in rat livers are inhibited by both simvastatin and lovatstatin.[117,118] The inhibition cannot be due to a change in prenylation or *N*-linked glycosylation of the proteins involved, as the inhibition occurs both immediately and on isolated mitochondria. A direct inhibition of the ETC would have drastic effects on the cells, as energy levels would be depleted, mitochondrial integrity compromised, and apoptosis triggered.[119] It is worth pointing out that many studies of mitochondrial ETC complex activities use very high concentrations of statins.[118,120] Whether localized concentrations of statins could reach high enough levels in the mitochondria of patients is debatable, but the mitochondria may already be compromised in patients or genetic variations in the complexes or transporters may make more people susceptible.[121]

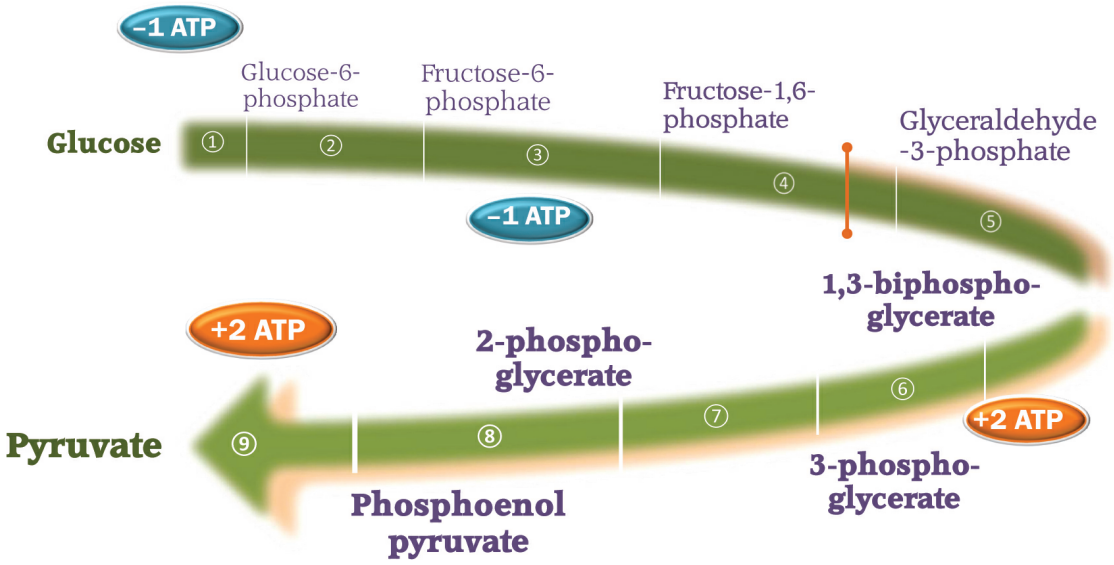


Figure 12

Glycolysis. Two ATPs are used to convert one glucose to two glyceraldehyde-3-phosphates. The splitting of the glucose is shown by an orange and green arrow after glycerahdehyde-3-phosphate. The two glyceraldehyde-3-phosphates are converted to two pyruvates, with the production of four ATPs, leaving a net gain of two ATPs. ① Glucokinase / hexokinase ② Phosphoglucoisomerase ③ Phosphofructose kinase ④ Aldolase ⑤ Glyceraldehyde-3-phosphate dehydrogenase ⑥ Phosphoglucokinase ⑦ Phosphoclucomutase ⑧ Enolase ⑨ Pyruvate kinase

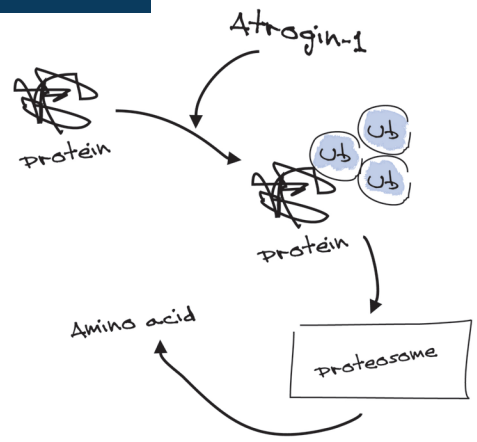
Further evidence of a direct effect of statins on the mitochondrial ETC is provided by Sirvent *et al.*[122] They present data showing that simvastatin induces an efflux of Ca^{2+} from the mitochondria of isolated human muscle fibres. They suggest that the efflux is caused by a disruption in mitochondrial function, as a result of a direct inhibition of one or more of the complexes of the ETC. Altered Ca^{2+} homeostasis in the muscles could lead to muscle dysfunction and dysregulation.[122]

2.4.2. Effects on glycolysis

Cells can also produce energy via glycolysis (**Fig 12**). The glycolytic pathway converts glucose into pyruvate with a net production of two ATP molecules and two NADH molecules.[123] Previous studies show that glycolysis can also be affected by statins.[124,125] One possible mechanism is via direct or indirect inhibition of one of the enzymes involved in the pathway by altering post-translational modifications or interfering in

activation or regulation of activity. A second mechanism has been postulated by Klawitter *et al.* who show that lovastatin can reduce the expression of enzymes involved in glycolysis (such as triosephosphate isomerise 1, α-enolase and dihydrolipoamide S-acetyltransferase).[125] A reduction in glycolysis could compromise the ability of a cell to produce energy and, especially if combined with inhibition of mitochondrial ETC, lead to apoptosis.

Figure 13



E3-ligase action of atrogin-1. Atrogin-1 adds ubiquitin groups (blue) to proteins, targeting them for degradation by the proteasome.

A statin-induced inhibition of glycolysis could also explain the beneficial effects of statin treatment in some cancers. Cancer cells generally have an increase in ATP production via glycolysis, so that they can counteract the anaerobic environment found in the centre of tumour tissues (the Warburg effect).[126] Numerous studies show that cancer cells are sensitive to the inhibition of glycolysis, and that drugs that target this process may form an effective strategy in overcoming the Warburg effect.[127–129] This could also overcome drug resistance, lead to cancer cell apoptosis and have a large clinical impact in cancer care. Statins may form part of this strategy.

2.4.3. Atrogin-1: A prime candidate

2.4.3.1. Evidence of a role in statin-induced myopathy

Much work has focussed on linking upregulation of an atrophy-inducing protein, atrogin-1, during statin-induced myopathy.[115,130] Atrogin-1 is an E3 ubiquitin ligase,

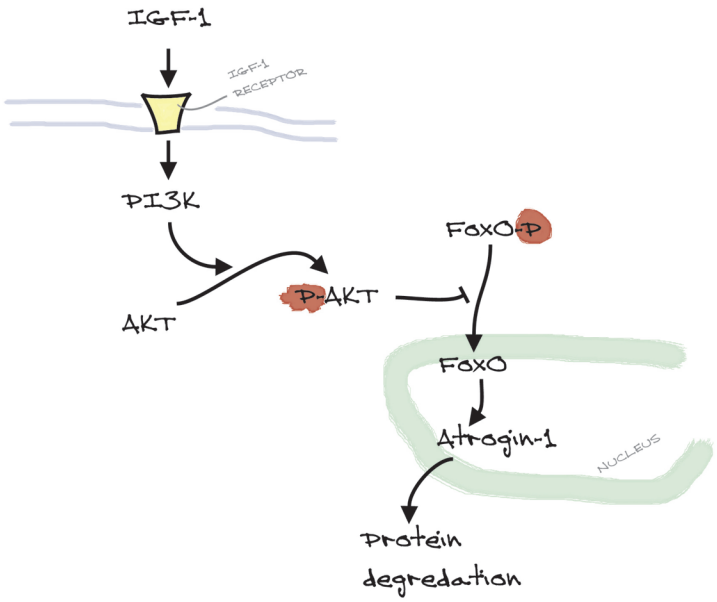


Figure 14

Control of *atrogin-1* transcription by Igf-1/Akt/Foxo signalling. Signalling through the IGF-1 receptor (yellow) leads to phosphorylated Akt. Phosphorylated Akt can, in turn, phosphorylate Foxo transcription factors, leading to their exclusion from the nucleus. Reduced Akt phosphorylation leads to an increase in unphorsphorylated Foxos, which can translocate to the nucleus and transcribe *atrogin-1*.

which is involved in the ubiquitylation and degradation of proteins (**Fig 13**).[131–133] Atrogin-1 is tightly regulated at the transcriptional level by the Foxo transcription factors, Foxo1 and Foxo3a.[133] Nuclear localization of the Foxo transcription factors is determined, in part, by their phosphorylation state.[134] Phosphorylated Foxo1 and Foxo3a are excluded from the nucleus, and therefore unable to transcribe the atrogin-1 gene. Phosphorylation of Foxo1 and Foxo3a is controlled, in part, by signalling through the Igf-1/Akt pathway.[134] Igf-1 signalling leads to phosphorylated, active Akt, which can then phosphorylate Foxo1 and Foxo3a. The integral role of the Igf-1/Akt pathway in preventing atrophy is shown by Igf-1 treatment reducing transcription of atrogin-1, and rescuing the cells from atrophy (**Fig 14**).[135,136]

Hanai *et al.* presented stunning evidence of the integral role atrogin-1 plays in statin-induced myopathy. They found that atrogin-1 knockout mice and knockdown zebrafish are resistant to statin-induced myopathy.[130] Cao *et al.* add to this by showing that

Figure 15 **Postulated link between cAMP/Epac and Igf-1/Akt signalling.** cAMP is produced after signalling through G-protein coupled receptors. cAMP, binds to Epac, enabling the conversion of inactive GDP-Rap1 to the active GTP-Rap1 (yellow). Rap1 might stimulate PI3K (red), an integral component of Igf-1/Akt signalling.

addition of GGOH to statin-treated mice and zebrafish preventrs atrogin-1 expression and statin-induced muscle damage.[115]

How statins can lead to a dysregulation of Igf-1/Akt signalling, and the corresponding increase in atrogin-1 levels, is unknown. It could be that statins directly affect the Igf-1r. As mentioned previously, the Igf-1r requires *N*-glycosylation for correct cleavage from its proreceptor form to the mature, plasma membrane localized, receptor.[80–83] A decrease in mature Igf-1r could lead to reduced Akt phosphylation, increased nuclear translocation of Foxo1 and Foxo3a, and an upregulation in atrogin-1 synthesis. A second possibility is that statins alter the prenylation of various small GTPases such as Ras, Rap1, Rac or Rho. Small GTPases are integral in many signalling pathways, and their incorrect processing could also reduce Igf-1/Akt signalling.[137–138]

2.4.3.2. *Rap1: Linking statins to atrogin-1?*

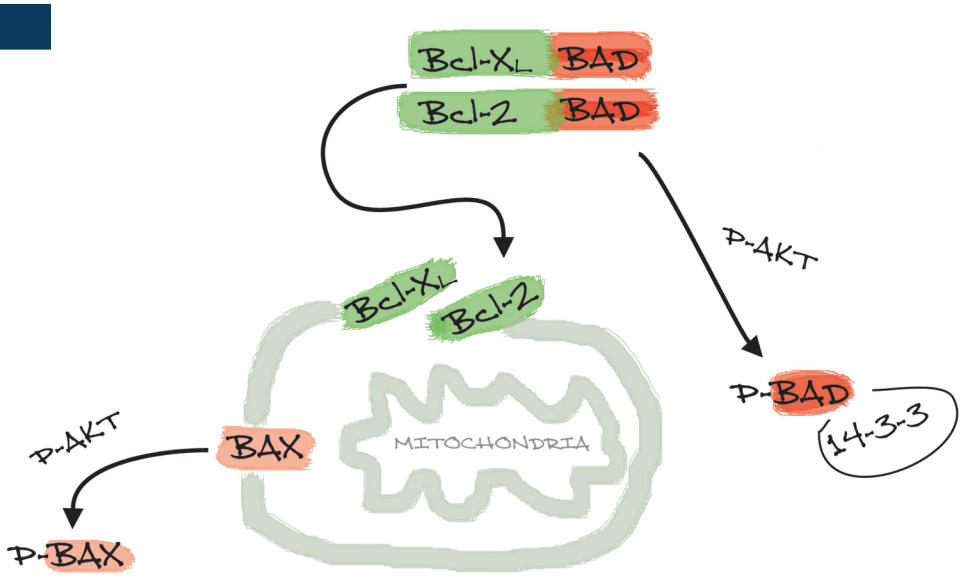
Rap1 is a small GTPase that is involved in numerous cell processes, most notably cell adhesion.[139–141] As it is a small GTPase, it is regulated by guanine nuclear exchange factors. One family of such exchange factors are the exchange proteins directly activated by cAMP (EPACs). EPACs are activated by cAMP signalling, leading to an activation of Rap1.[142,143] Numerous studies link cAMP/EPAC/Rap1 signalling to Igf-1/Akt signalling, although it is not completely known where the two pathways interact (**Fig 15**).[44,146] Work by Baviera *et al.* in extensor digitorum longus muscles from rats, points to Rap1 interacting with PI3K, downstream of the Igf-1r but upstream of Akt.[147] Gonçalves *et al.* linked cAMP signalling with atrophy in a paper in 2009.[148] They showed that increasing cAMP levels, via the cAMP phosphodiesterase inhibitor isobutylnethylxanthine (IBMX), negated the increase in atrogin-1 expression induced by dexamethasone. This negation was via an increase in Akt phosphorylation, and concomitant increase in Foxo phosphorylation and nuclear exclusion.

The above work offers tantalizing evidence that statins could inhibit Igf-1r/Akt signalling via inhibition of cAMP signalling through Rap1.

2.5. Can dysregulation of Igf-1/Akt signalling also explain the effects on mitochondria?

Since cell energy status, survival and apoptosis are dependent upon the mitochondria, it is important to integrate cell survival signalling and mitochondria. It is therefore of no surprise to discover that mitochondria are also controlled by cellular signalling pathways. Numerous studies report the influence of all the major cell signalling pathways upon mitochondrial function, from PKA to ERK and JNK.[149–151] Of particular interest is the influence of Igf-1/Akt signalling upon the mitochondria, as this is the pathway most associated with dysfunction in statin-induced myopathy.[115]

Figure 16



Multiple roles of Akt at the mitochondria. Phosphorylated Akt can inhibit pro-apoptotic proteins (red), and also prevent them sequestering anti-apoptotic proteins (green).

Akt is known to inhibit the pro-apoptotic protein Bad (**Fig 16**). Active Akt directly phosphorylates Bad, which causes Bad to dissociate from anti-apoptotic proteins, and then bind to the adaptor protein 14-3-3.[152–154] The anti-apoptotic proteins, such as members of the Bcl-2 family, are then free from inhibition and cell survival is encouraged. Akt is also able to phosphorylate Bax, inhibiting its ability to enhance mitochondrial pore formation and prevent the release of cytochrome *c*.[155–157]

Akt can also regulate metabolism via regulating the activities of hexokinases (HKs) and voltage dependent anion channels (VDACs) (**Fig 17**). HKs control the first step of glycolysis, the conversion of glucose to glucose-6-phosphate.[158] Two isoforms, HK I and HKII, are also known to bind to the outer mitochondrial membrane, and become dissociated from there during apoptosis.[159] Overexpression of HKI or HKII can protect cardiomyocytes from H_2O_2-induced cell death, and this protection is lowered when the mitochondrial binding motifs are deleted.[160] HKII has been particularly linked with being a downstream effector by which Akt can inhibit cell death.[161,162] The fact that both

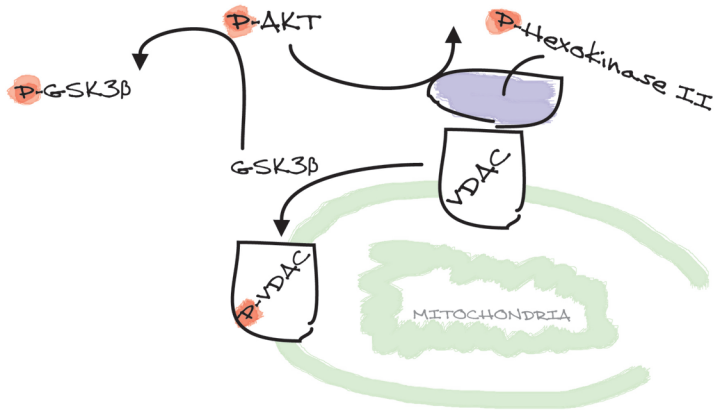


Figure 17

The importance of Akt in maintaining HK/VDAC interactions. Phosphorylated Akt phosphorylates hexokinase II, enabling its localization to the outer mitochondrial membrane. Phosphorylated Akt also inhibits GSK3β via phosphorylation, preventing it from phosphorylating VDAC. This keeps the VDAC-hexokinase II complex together.

Akt and HKs require glucose to promote cell survival suggests a link.[157,163] Further evidence is provided by human HKII containing an Akt consensus sequence important in enabling Akt to phosphorylate its targets.[164] Akt is able to phosphorylate HKs and block their dissociation of HKs from the outer mitochondrial membrane, and therefore prevent apoptosis.[163] The importance of HK localization at the outer mitochondrial membrane is highlighted by HK-dissociation impairing the anti-apoptotic effects of Akt, and also its ability to promote mitochondrial integrity.[165] Akt therefore acts as a common mediator of cell survival and energy metabolism.

VDACs are important pores on the outer mitochondrial membrane, and are also regulated by Akt signalling. They maintain the polarization of the outer mitochondrial membrane.[166–168] When unphorsphorylated, VDACs bind to HKs, the VDAC pore is kept open and HKs have access to mitochondrial ATP.[166–168] Dissociation is caused by phosphorylation by GSK3β.[169] Active GSK3β is known to promote apoptosis, and GSK3β is inactivated by Akt.[170–172] Akt can therefore enhance HK binding to VDAC by both phosphorylation of HKs and preventing the phosphorylation of VDACs.

It is therefore clear that Igf-1/Akt signalling is integral to mitochondrial stability, energy metabolism and prevention of cell death. The fact that statins are known to disrupt Igf-1/Akt signalling offers a potential explanation as to how they can inhibit mitochondrial function as well.

2.6. Linking atrogin-1 to the mitochondria

Atrophic muscle fibres also have disruptions in their mitochondria, and stimulation of mitochondria biogenesis by overexpression of PGC1α can inhibit muscle atrophy.[173] A recent study by Romanello *et al.* highlights the important role the mitochondria network plays in muscle atrophy.[174] They present evidence that mitochondrial fission is an integral part of atrophy, and that preventing fission also prevents atrophy. They suggest that mitochondrial fission enhances the activation of Foxo3a independent of its phosphorylation state, probably via AMPK signalling. The activated Foxo3a is then able to translocate to the nucleus and enhance transcription of pro-atrophic genes such as atrogin-1. Whether mitochondrial fission and fusion are affected by statins are unknown, but other drugs such as berberine are known to affect both mitochondrial function and upregulate atrogin-1 expression.[175]

2.7. Statins and cardiac muscle

Statins are mainly taken to combat cardiovascular disease, yet side-effects of statins in cardiac muscle have been much less studied than in skeletal muscle. There are only a few papers on the subject, which show that lovastatin can reduce cardiomyocyte viability, in part via an increase in apoptotic pathways.[176,177] This suggests an effect of statins on cardiac mitochondria. Atrogin-1 is also present in cardiac muscle, and is known to be upregulated during experimental heart failure.[178,179] It is therefore possible that statins could induce increase in atrogin-1 expression in the heart as they do in

skeletal muscle. These side-effects may be missed by falsely attributing them to the underlying cardiovascular disease ■

Aims

THIS THESIS has three main aims. Firstly, we aimed to uncover differences between the effects of simvastatin on skeletal muscle and liver. As simvastatin is not toxic in liver, we reasoned that determining these differences would point to possible mechanisms for simvastatin-induced skeletal muscle toxicity. We chose the well-established skeletal muscle cell line C2C12, and induced differentiation to myotubes, to represent skeletal muscle. Simvastatin does not induce toxicity in the human liver HepG2 cell line, and hence we chose that to represent the liver.

We initially performed a comprehensive analysis of the different components of the cholesterol synthesis pathway, and looked for differences in these components. This was be the first time such an analysis would have been performed, and would offer suggestions into how simvastatin leads to skeletal muscle toxicity.

The second aim of this thesis was to expand these differences by investigating effects on the mitochondria and the Igf-1/Akt signalling pathway in C2C12 myotubes and HepG2 cells. Statins have been reported to inhibit mitochondrial function in various cell lines, and we aimed to uncover why simvastatin is only inhibitory in the C2C12 myotubes and not the HepG2 cells. As mitochondrial integrity and function are controlled by various signalling cascades, we looked for differences in the Igf-1/Akt pathway – a pathway implicated in statin-induced toxicity.

The final aim of this thesis was to look for toxicity in cardiac muscle. This has not been investigated before, but is of great importance as preventing cardiovascular disease is a major reason for prescribing statins. We used our results from our C2C12 myotube

studies as a guide, and investigated how simvastatin affects cardiac mitochondria and atrophic pathways. We used *in vivo*, *ex vivo* and *in vitro* techniques to present, for the first time, evidence of a toxic effect of simvastatin in cardiomyocytes ■

Paper One

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

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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

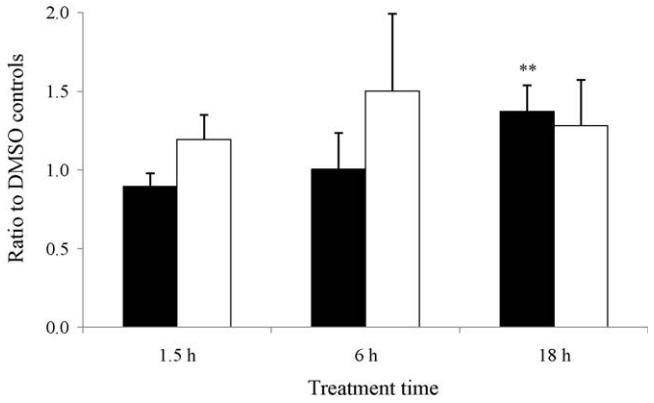

Fig. 1. Toxicity of 10 μM simvastatin on C2C12 myotubes and HepG2 cells. C2C12 myotubes (black bars) and HepG2 cells (white bars) were incubated with 10 μM simvastatin for the times indicated. We measured the release of AK into the medium. DMSO-treated cells were used as a control. Results are expressed 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

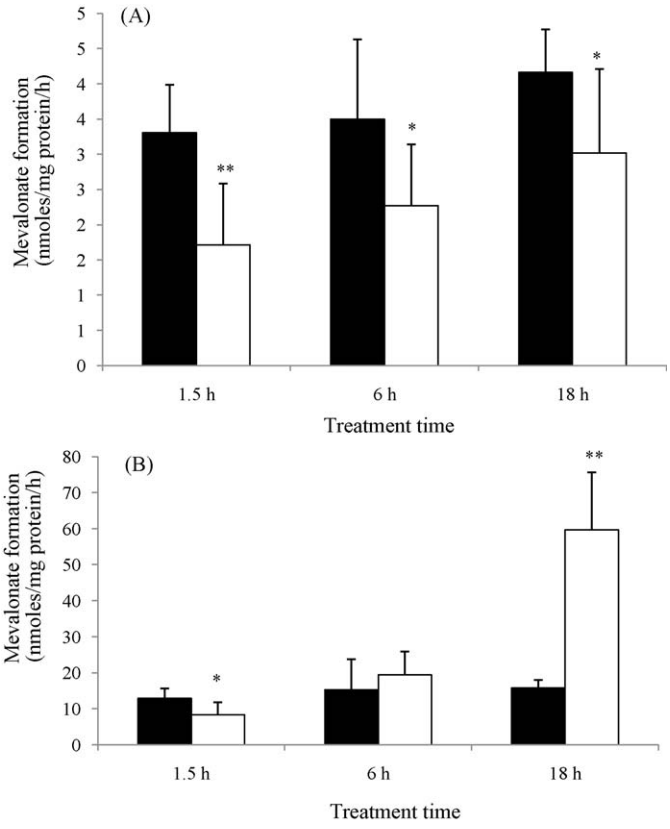

Fig. 2. Effect of 10 μM simvastatin on HMG-CoA reductase activity. Cells were incubated with DMSO (black bars) or simvastatin (white bars) for the indicated times. Protein lysate was collected and enzymatic activity assay performed. Results are for (A) C2C12 myotubes and (B) HepG2 cells. The data represents 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

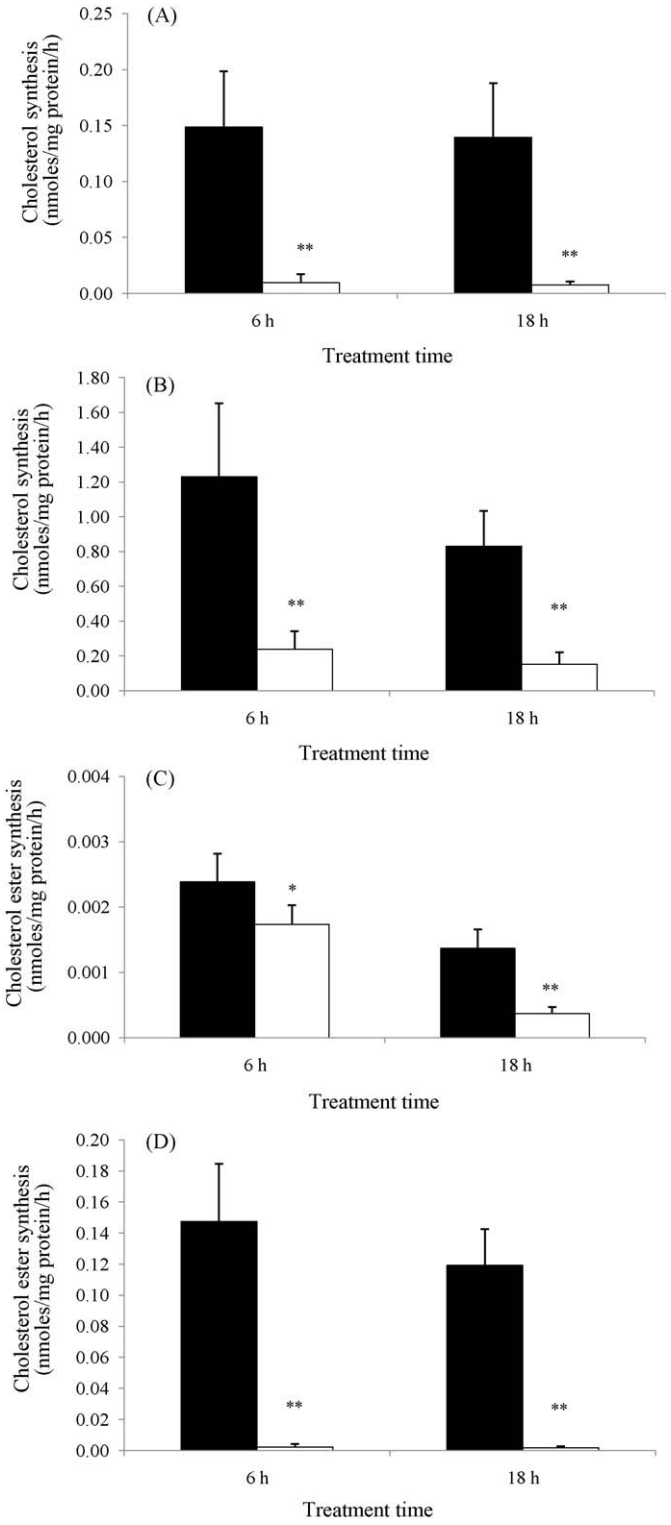

Fig. 3. Effect of 10 μM simvastatin on production of cholesterol in C2C12 myotubes and HepG2 cells. Cells were incubated with DMSO (black bars) or simvastatin (white bars) for the times indicated. 2-[^{14}C]-acetate was added 30 min after start of drug incubation. The graphs show production of cholesterol 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.

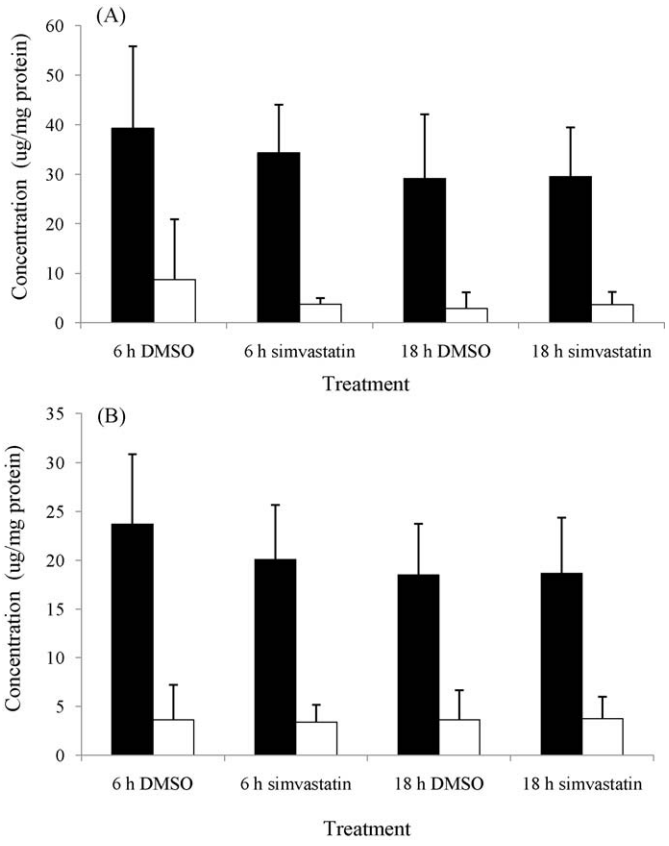

Fig. 4. Effect of 10 μM simvastatin on cellular lipid content. Cells were incubated with DMSO or simvastatin for the times indicated. The graphs show free cholesterol (black bars) and cholesterol ester concentrations (white bars) in (A) C2C12 myotubes and (B) HepG2 cells. Data are means of four independent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

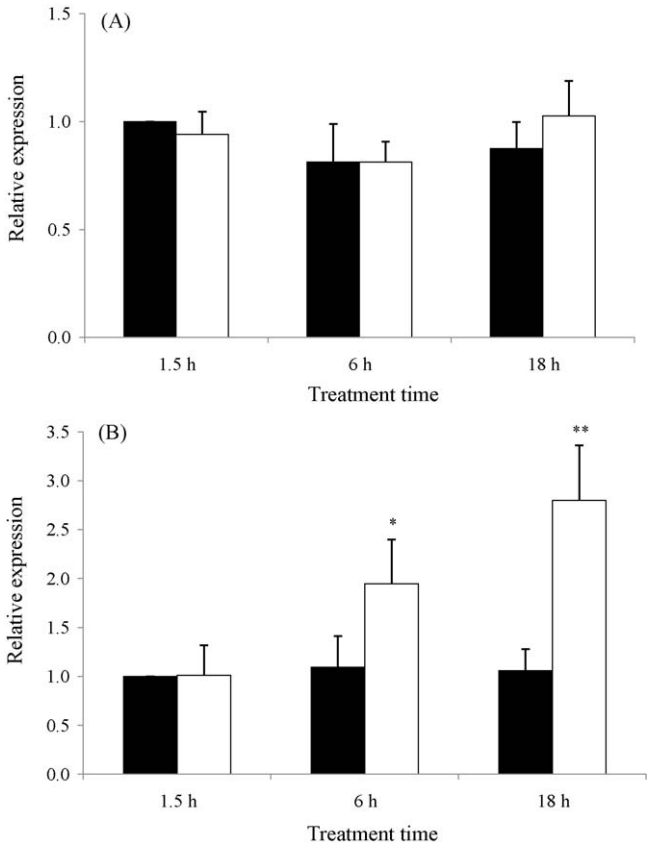

Fig. 5. Effect of 10 μM simvastatin on LDL receptor mRNA expression. Total RNA was extracted from cells after treatment with DMSO (black bars) or simvastatin (white bars). Expression was measured by quantitative RT-PCR with GAPDH as an endogenous control. Expression after simvastatin treatment is shown 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.

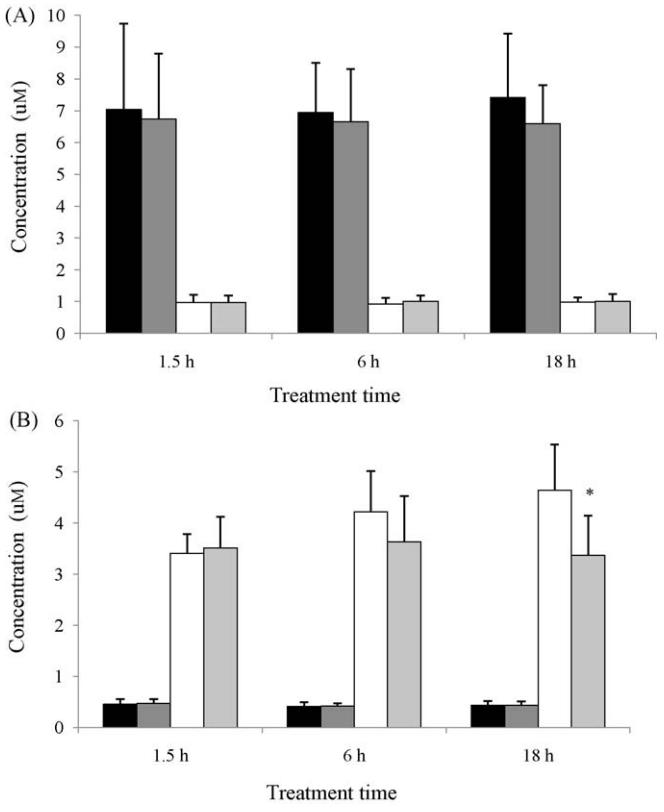

Fig. 7. Effect of 10 μM simvastatin on cellular ubiquinone concentration. Cells were incubated with DMSO or simvastatin for 1.5, 6 or 18 h. Total cellular ubiquinone was extracted and measured by HPLC. Total CoQ9 (black bars for DMSO and dark grey bars for simvastatin) and CoQ10 levels (white bars for 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,

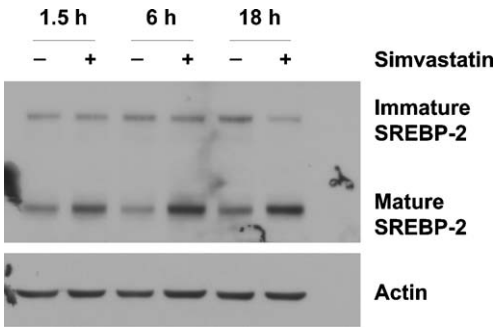

Fig. 6. Effect of 10 μM simvastatin on SREBP-2 transcription factor expression and processing. Total protein was extracted after treatment with DMSO or simvastatin. Actin was used to confirm equal loading. SREBP-2 expression is only shown for HepG2 cells. Results are indicative of three independent experiments.

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

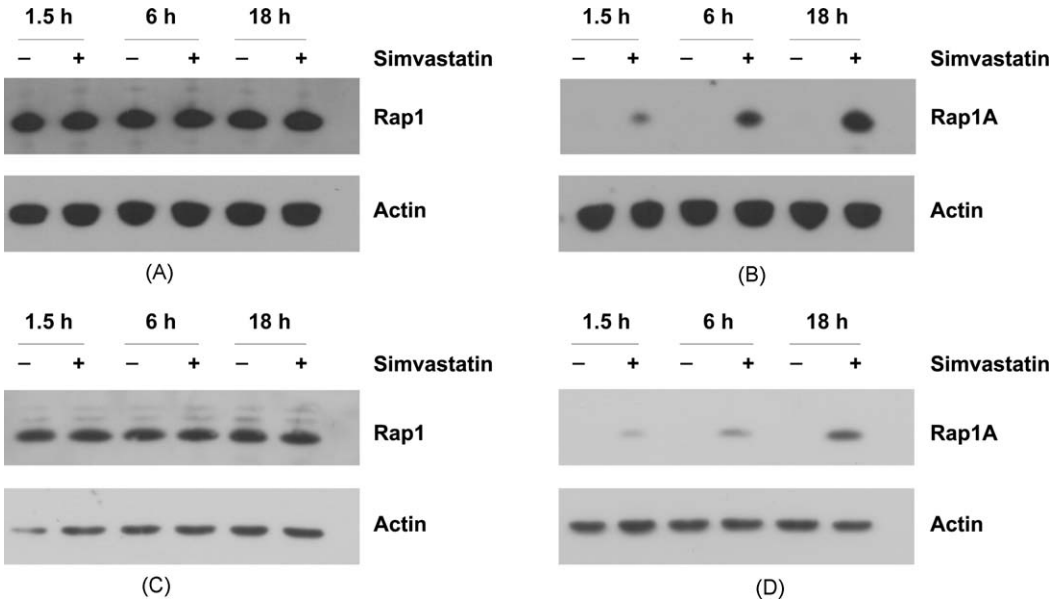

Fig. 8. Effect of 10 μM simvastatin on geranylgeranylation of Rap1. Total protein was extracted after treatment with DMSO or simvastatin. Actin was used to confirm equal loading. Total Rap1 expression is shown for (A) C2C12 myotubes and (C) HepG2 cells. Ungeranylgeranylated Rap1 expression is shown in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

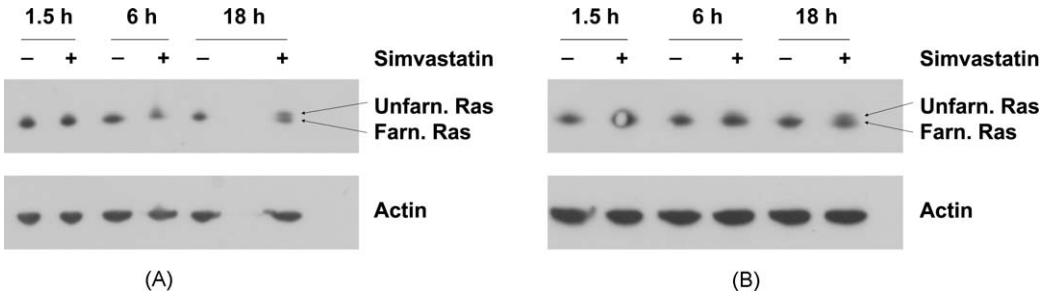

Fig. 9. Effect of 10 μM simvastatin on farnesylation of Ras. Total protein was extracted after treatment with DMSO or simvastatin. Actin was used to confirm equal loading. Ras expression is shown for (A) C2C12 myotubes and (B) HepG2 cells. The upper bands represent unfarnesylated Ras protein. Results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

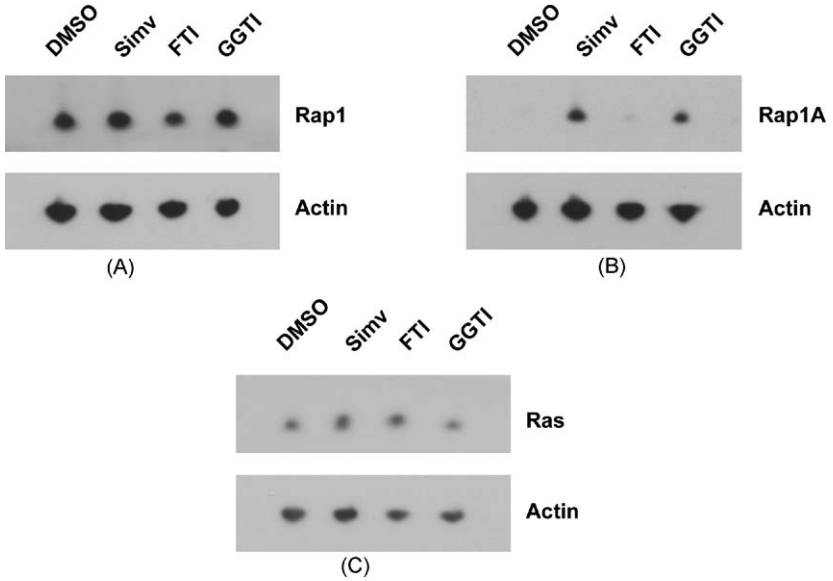

Fig. 10. Effect of FTI and GGTI incubation on prenylation of Rap1 and Ras in C2C12 myotubes. Total protein was extracted from C2C12 myotubes treated for 18 h with DMSO, simvastatin, FTI or GGTI. Expression of (A) Rap1, (B) Rap1A and (C) Ras is shown.

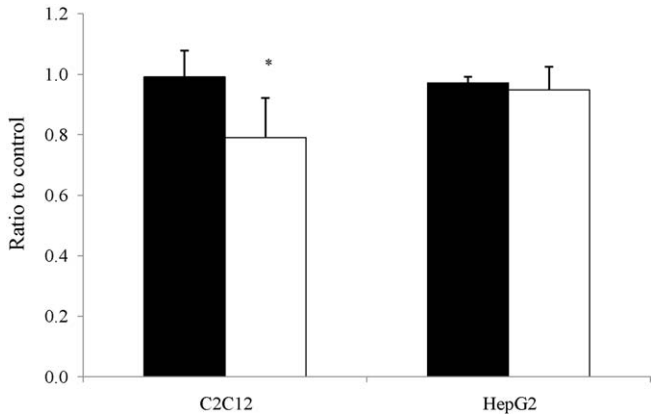

Fig. 11. Effect of 10 μM simvastatin on *N*-linked glycosylation of proteins. Cells were incubated with DMSO (black bars) or simvastatin (white bars) for 6 or 18 h, and pulse-chased with [^{3}H]-glucosamine for the final 4 h. Protein was extracted and the incorporated radioactivity was measured. Results 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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Paper two

Susceptibility to simvastatin-induced toxicity is determined by phosphorylation state of Akt

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5. Abstract

Statins are widely used to prevent cardiovascular diseases. They are well-tolerated, with side-effects mainly seen in skeletal muscle. How these side-effects are caused is unknown. We compared isolated primary mouse skeletal muscle myocytes, C2C12 myotubes and liver HepG2 cells to uncover differences that could uncover why statins are toxic in skeletal muscle but not the liver. 10 μM simvastatin caused a decrease in mitochondrial respiration and glycolysis in the primary mouse myocytes and C2C12 myotubes, but had no effect in the HepG2 cells.

Mitochondrial integrity is maintained by multiple signalling pathways. One of these pathways, Igf-1/Akt signalling, is also heavily implicated in causing statin-induced toxicity by upregulating atrogin-1. We found phosphorylated Akt was reduced in C2C12 myotubes but not in HepG2 cells. HepG2 mitochondrial respiration became susceptible to simvastatin-treatment after Akt inhibition, and mitochondrial respiration was rescued in Igf-1-treated C2C12 myotubes. These results suggest disruption of Igf-1/Akt signalling is a causative factor in simvastatin-induced mitochondrial dysfunction in C2C12 myotubes, whereas HepG2 are protected by maintaining Igf-1/Akt signalling. How statins can disrupt Igf-1/Akt signalling is unknown. Statins reduce geranylgeranylation of small GTPases, such as Rap1. Previous studies implicate Rap1 as a link between cAMP/Epac and Igf-1/Akt signalling. Transient transfection of constitutively active Rap1 into C2C12 myotubes led to a partial rescue of simvastatin-induced inhibition of mitochondrial respiration. There was however, no reduction in atrogin-1 levels, although nuclear translocation of Foxo3a was reduced. We conclude that phosphorylation of Akt is a key indicator of susceptibility to statin-induced toxicity.

5.1. Introduction

Statins 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, where they inhibit HMG-CoA (hydroxyl-methyl-glutaryl-coenzyme A) reductase, the rate-limiting step in cholesterol biosynthesis.[3] Inhibition of this pathway also inhibits various other processes, such as ubiquinone production and the isoprenylation and N-linked glycosylation of proteins.[4–6] Altering

these processes can reduce inflammation, oxidative stress and platelet adhesion – leading to the positive effects of statins.[7,8]

Statins are generally well tolerated but there are dose-dependent side-effects, particularly in skeletal muscle where myopathy is seen in 1–5% of patients.[9,10] Statin-induced side effects are rarely observed at their site of action in the liver. We have previously showed that the human liver HepG2 cell line is not susceptible to simvastatin toxicity, and consider this a viable model to uncover mechanisms of resistance to simvastatin-induced toxicity.[6] Two possible mechanism of toxicity involve disrupting mitochondrial health, and upregulating atrophy-inducing proteins.[11,12]

Studies in various tissues show statins can affect the mitochondria directly, via inhibition of the respiratory chain complexes, or indirectly via lowering of ubiquinone levels.[13,14] We have already showed that statins can alter mitochondrial function in skeletal muscle.[11] It is not known whether statins can affect mitochondrial respiration in liver cells. We aim to check for any differences between mitochondrial respiration in C2C12 myotubes and liver HepG2 cells. Mouse C2C12 myotubes are a well-established in vitro model of skeletal muscle.[6] Uncovering differences could provide key insights into what makes certain cell lines resistant to statin toxicity whereas other cell lines are susceptible.

Mitochondrial health is maintained, part, by multiple signalling pathways.[15–17] Some groups have suggested that impairment in cell signalling is a major cause of statin-induced myotoxicity.[18-20] One pathway of great interest is the Igf-1/Akt pathway. It is already known that this pathway is integral to mitochondrial health, and its impairment has also been implicated as a contributing factor in statin toxicity.[21–24] We therefore aim to discover whether any potential differences in the mitochondria of simvastatin-treated

C2C12 myotubes and HepG2 cells can be explained by differences in Igf-1/Akt signalling.

Baviera et al. suggest that a geranylgeranylated protein, Rap1, could potentiate Igf-1/Akt signalling.[25] We therefore aim to express constitutively active Rap1 in C2C12 myotubes to discover whether it can rescue the myotubes from simvastatin-induced mitochondrial toxicity. This would increase our knowledge of how simvastatin leads to mitochondrial toxicity, and how it can affect signalling through the Igf-1/Akt pathway.

Igf-1/Akt signalling is also vital in protecting skeletal muscle from atrophy, and statin-induced reductions in signalling lead to an increase in levels of the atrophy-inducing protein atrogin-1 in skeletal muscle.[12] This increase in atrogin-1 levels is reversible by the addition of compounds leading to geranylgeranylation (such as geranylgeranyl pyrophosphate, GGPP).[26] We will therefore investigate if expression constitutively active Akt can prevent the upregulation of atrogin-1 in C2C12.

These experiments will increase our understanding of how statins lead to mitochondrial toxicity, the role that Igf-1/Akt signalling plays in that, and whether Rap1 mediates the change in Igf-1/Akt signalling.

5.2. Materials and methods

5.2.1. Chemicals

Simvastatin (Sigma-Aldrich, St. Louis, MO, USA) was converted into the active acid following the protocol of Bogman *et al.*[27] Stock solutions of 10 and 100 mM simvastatin in DMSO were stored at –20°C. The ToxiLight® assay kit LT07-117 was from Lonza (Basel, Switzerland) and the Pierce BCA protein assay kit from Merck (Darmstadt, Germany). Antibodies to FoxO1, FoxO3a, Akt and P-Akt were provided by Cell Signaling Technology (Danvers, MA, USA). The Alexa Fluor® secondary antibodies

were from Invitrogen (Basel, Switzerland). All other chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), except where indicated.

5.2.2. Primary neonatal mouse myoblast culture

We took skeletal muscle from the hind limbs of neonatal BL6 mice (kindly provided by Prof. Daniel Bodmer, University Hospital Basel), and isolated mouse myoblasts using a protocol modified from Springer *et al.*[28] Briefly, we digested the skeletal muscle with sequential trypsinisation and trituration. We plated the cells for an hour, during which the non-myocytes adhered to the plate, and collected the enriched suspension of myocytes. Myocytes were then suspended in Ham's F10 medium supplemented with 20% FBS, 2% L-glutamine, 1% bicarbonate and 1% pen/strep. We added 2 ng/ml bFGF to prevent the proliferation of non-myocytes. The myoblasts were seeded at a density of 5,000 cells per well of a 96-well plate coated with collagen I. After 24 h, the concentration of bFGF was increased to 5 ng/ml. Differentiation was induced by changing to Ham's F10 medium containing 5% horse serum.

5.2.3. Cell culture

C2C12 myoblasts were from the American Type Culture Collection. We grew the myoblasts in Dulbecco modified Eagle's medium (DMEM) high-glucose medium (4.5 g/l) containing 10% FBS and 5 mM HEPES. The myoblasts were seeded at 150,000 cells per well in a 6-well plate (or equivalent), and grown for 3 days to achieve confluence. We induced the myoblasts to differentiate into myotubes using a medium containing 2% horse serum. The myotubes were fully differentiated after 3 days, after which treatment occurred for 6 h. We added simvastatin at a concentration of 10 μM and other compounds at the concentrations indicated. DMSO was used as a control; its concentration was always 0.1%.

The human liver HepG2 cell line was kindly provided by Prof. Dietrich von Schweinitz (University Hospital Basel, Switzerland). 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 (or equivalent). Cells were grown for 1 day, and then treated with the relevant compounds for 24 h.

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

5.2.4. Transfection of C2C12 myoblasts

The pEGFP-C3 plasmid (Clontech, CA, U.S.A.) containing constitutively active Rap1, and the empty plasmid, were kindly provided by Prof. Mark R. Philips (New York University School of Medicine). C2C12 myoblasts were seeded as before, and grown for 1 day, before transfection using Lipofectamine 2000 (Invitrogen, CA, U.S.A.). Cells were then allowed to grow as normal, and differentiate into myotubes. Approximately equal numbers of GFP-expressing myotubes were obtained with both plasmids.

5.2.5. Detection of apoptosis after simvastatin treatment

We used an *in situ* apoptosis detection kit for Annexin V binding and propidium iodide (PI) staining (Vybrant™ Apoptosis Assay Kit #2, from Invitrogen). After incubation with simvastatin, we stained detached cells with 5 μl Annexin V-AlexaFluor 488 and 1 μl PI (final concentration 1 μg/ml) in Annexin V buffer as per the kit protocol. The samples were incubated for 15 minutes and analysed by flow cytometry, using a DAKO Cyan cytometer.

5.2.6. Measurement of mitochondrial membrane potential

We used tetramethylrhodamine, ethyl ester, perchlorate (TMRE; Invitrogen) to detect changes in mitochondrial membrane potential (ψm).[29] Cells were incubated with simvastatin, then detached and incubated with 100 nM TMRE for 30 minutes at 37°C and 5% CO_2. We used a Live/Dead® Near-IR Dead Cell stain kit from Invitrogen to

exclude dead cells. Cells were analyzed via flow cytometry with a DAKO Cyan cytometer. The mitochondrial uncoupler FCCP acted as a positive control.

5.2.7. Confocal microscopy

C2C12 myotubes were grown in Lab-Tek microscopy chambers (Nunc, NY, USA) and treated with simvastatin for 6 h. The cells were fixed with 4% PFA, permeabilized with 0.2% Triton-X and then labelled with the appropriate antibody. We used DAPI to stain nuclei. We used a Zeiss LSM 710 confocal microscope to take the images, and Zen software to process them.

5.2.8. Measurement of oxygen consumption and extracellular acidification rates

We measured intact cellular respiration with a Seahorse XF24 analyzer (Seahorse Biosciences, North Billerica, MA, USA). HepG2 cells and C2C12 myotubes were grown in Seahorse XF 24-well cell culture microplates 250 μl growth medium. We treated the cells with simvastatin and other relevant compounds for the times indicated, and then replaced the medium with 750 μl unbuffered medium. Cells were equilibrated to the unbuffered medium for 45 minutes at 37°C in a CO_2-free incubator, before being transferred to the XF24 analyzer. We measured basal oxygen consumption (OCR) and extracellular acidification rates (ECAR), and then sequentially injected 1 μM rotenone, 10 μM FCCP and 1 μM oligomycin to assess maximal oxidative capacity.

5.2.9. Isolation of mitochondria

We seeded 2.7 million C2C12 myoblasts in a T175 flask, and allowed them to differentiate. We isolated mitochondria with the technique of Hornig-Do *et al.* that has been commercialised by Miltenyi Biotec (Germany).[30] After enrichment, the mitochondria were washed and resuspended in Miltenyi storage buffer, and kept on ice until use. We measured protein concentration with a BCA kit, and averaged a yield of 300 μg mitochondrial protein per T175 flask of differentiated myotubes.

5.2.10. Immunoblotting

After treatment, we lysed the cells and collected total cell protein as described previously. We separated 15 μg protein on a 4-12% gradient gel (Invitrogen, CA, U.S.A.), transferred them to polyvinylidendifluoride membranes, and probed with the relevant antibody (all used at a 1:1000 dilution). Peroxidase-labelled anti-rabbit antibody, and chemiluninescence substrate (GE Healthcare) were used for analysis.

5.2.11. Atrogin-1 mRNA expression

C2C12 myotubes were treated as described. RNA was extracted and 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 cDNA for quantitative RT-PCR. We used SYBR green with primers specific for atrogin-1(forward primer: 5′-GAAGACCGCTACTGTGGAA-3′; reverse primer: 5′-ATCAATCGCTTGCGGATCT-3′). Relative quantities of specifically amplified cDNA were calculated with the comparative-threshold cycle method. GAPDH acted as endogenous reference (forward primer: 5′-CATGGCCTTCCGTGTTCCTA-3′; reverse primer: 5′-CCTGCTTCACCACCTTCTTGA-3′), and no-template and no-reverse-transcription controls ensured non-specific amplification could be excluded.

5.2.12. Statistical analysis

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

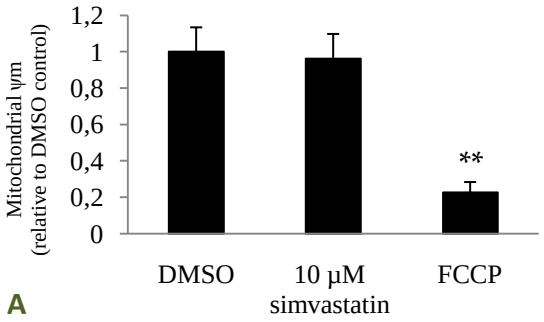
5.3. Results

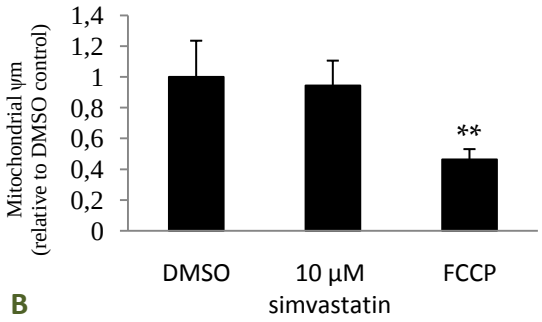
5.3.1. Mitochondrial membrane potential (ψm) is impaired by simvastatin in C2C12 myotubes but not in HepG2 cells

We previously showed that 10 μM simvastatin is toxic to C2C12 myotubes, but not to HepG2 cells. We now determined whether there is a difference in the effects on mitochondria. The mitochondrial ψm is a reliable indicator of mitochondrial health, and it was not altered in HepG2 cells treated with 10 μM simvastatin for up to 24 h (**Fig 1A**). C2C12 mitochondrial ψm was not reduced after 6 h treatment with 10 μM simvastatin (**Fig 1B**), but had significantly reduced by 11.5% (p=0.04, compared to control) by 24 h treatment (**Fig 1C**). We therefore only treated HepG2 cells for 24 h for the following experiments, and used both a 24 h treatment and earlier 6 h treatment to assess toxic mechanisms in C2C12 myotubes.

5.3.2. Simvastatin reduces oxygen consumption in intact C2C12 myotubes, but not in HepG2 cells

To delve deeper into how simvastatin affects C2C12 mitochondria, we used a XF24 analyzer to determine changes in OCR and ECAR in simvastatin-treated intact C2C12 myotubes and HepG2 cells. OCRs provided a measurement of the activity of the electron transport chain (ETC), and ECARs an indication of the rate of glycolysis. Oxygen consumption was unaltered after 24 h treatment with 10 μM simvastatin in HepG2 cells (**Fig 2A**). After only 6 h treatment with 10 μM simvastatin in C2C12 myotubes, baseline OCR values were already significantly reduced by 24.2% (p=0.00006





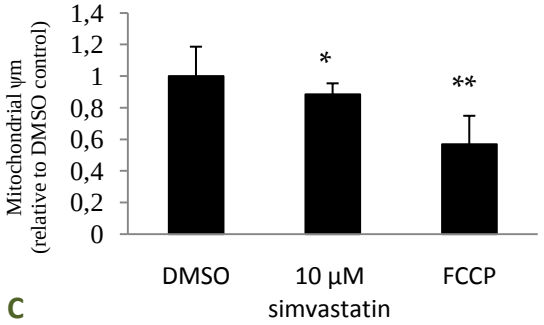


Figure 1

Effect of simvastatin on mitochondrial ψm in HepG2 cells and C2C12 myotubes. Cells were incubated with DMSO or 10 µM simvastatin. **A** HepG2 cells were treated for 24 h. C2C12 myotubes were treated for 6 h (**B**) or 24 h (**C**). TMRE was used to determine mitochondrial membrane potential, and Near-IR dye from Invitrogen to exclude dead cells. FCCP was added to one of the DMSO samples to act as a positive control. The results are means of four independent experiments carried out in triplicate.

* $p < 0.05$ and ** $p<0.01$ versus control.

compared to control; **Fig 2B**). The OCR reduction with 10 µM simvastatin was still evident after 24 h treatment (data not shown). FCCP was added to the cells to determine maximum respiration. The maximum respiration was not reduced in C2C12 myotubes treated with 10 µM simvastatin for 6 h, or HepG2 cells treated for 24 h.

5.3.3. Oxygen consumption is also reduced in primary mouse myotubes

We also determined the OCRs in primary mouse myotubes treated with 10 µM simvastatin to confirm our *in vitro* studies. The primary myotubes showed a similar reduction in OCR to the C2C12 myotubes, but only after 24 h treatment (by 25.4%, p=0.00001; **Fig 2C**). This offers further evidence that simvastatin impairs skeletal muscle mitochondrial function.

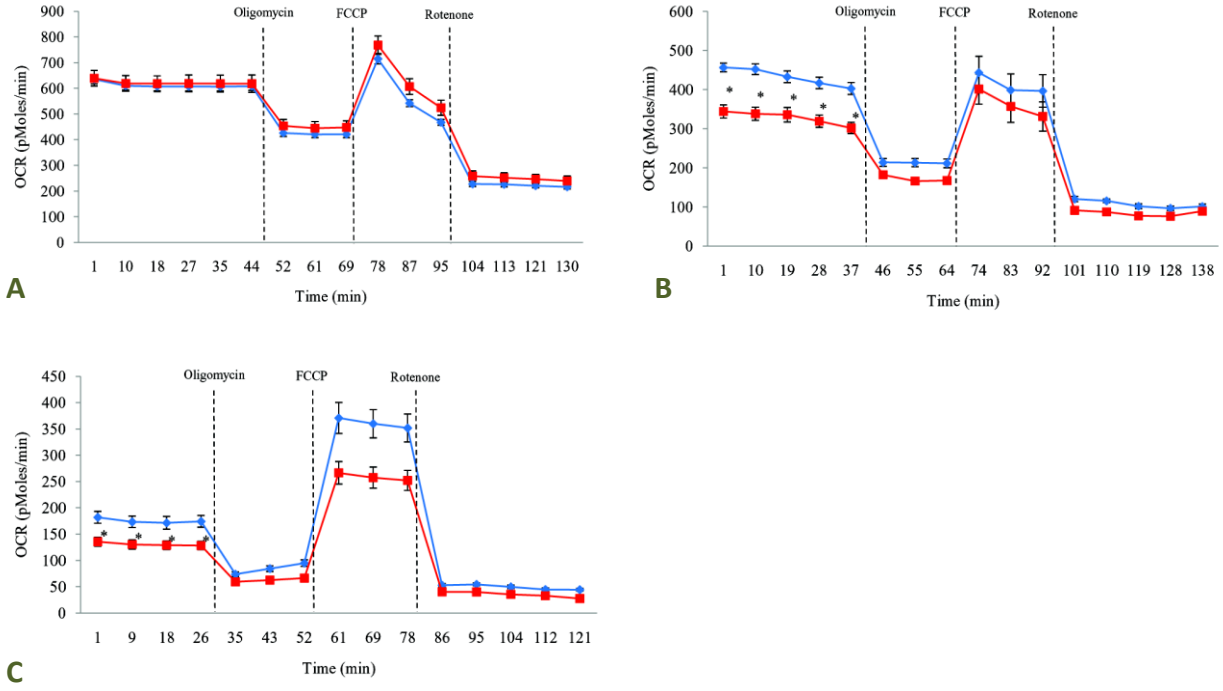


Figure 2

Effect of simvastatin on oxygen consumption in intact HepG2 cells, C2C12 myotubes and isolated mouse leg myotubes. Cells were incubated with DMSO (blues lines)or 10 μM simvastatin (red lines). The XF24 analyzer was used to take measurements. OCRs are shown for 24 h treatment in HepG2 (**A**) cells, 6 h treatment in C2C12 myotubes (**B**) and 24 h treatment in isolated mouse leg myotubes (**C**). 1 μM oligomycin, 10 μM FCCP and 1 μM rotenone were added at the indicated points. Results are indicative of three independent experiments.

5.3.4. Oxygen consumption is also reduced in mitochondria isolated from C2C12 myotubes

We isolated mitochondria from C2C12 myotubes treated with 10 μM simvastatin, to fully confirm our results. State II (absence of exogenous ADP), and state III (presence of exogenous ADP) respiration was measured on the XF-24 analyzer. No changes in respiration were observed using glutamate/malate/pyruvate as substrates (data not shown). With succinate as the substrate, both state II and state III respiration were significantly reduced in mitochondria isolated from C2C12 myotubes treated with 10 μM simvastatin for 6 h (by 23.4% $p<0.01$ and 22.2% $p<0.01$, respectively; **Fig 3**).

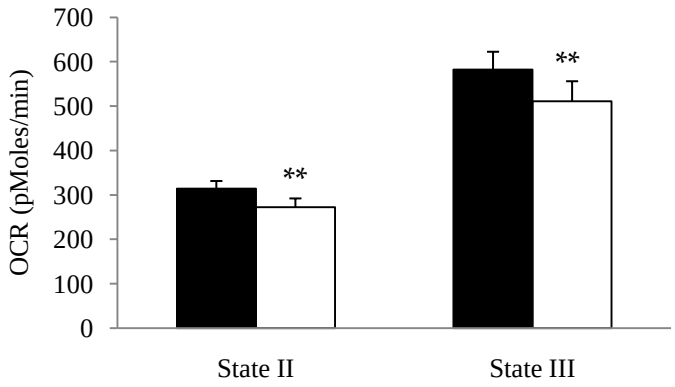


Figure 3

Effect of simvastatin on oxygen consumption in mitochondria isolated from C2C12 myotubes. Cells were incubated with DMSO (black bars) or 10 μM simvastatin (white bars) for 6 h, mitochondria isolated, and the XF24 analyzer used to measure oxygen consumption at state II and state III. Results are indicative of four independent experiments carried out in quintuplicate.

** $p < 0.01$ versus DMSO control.

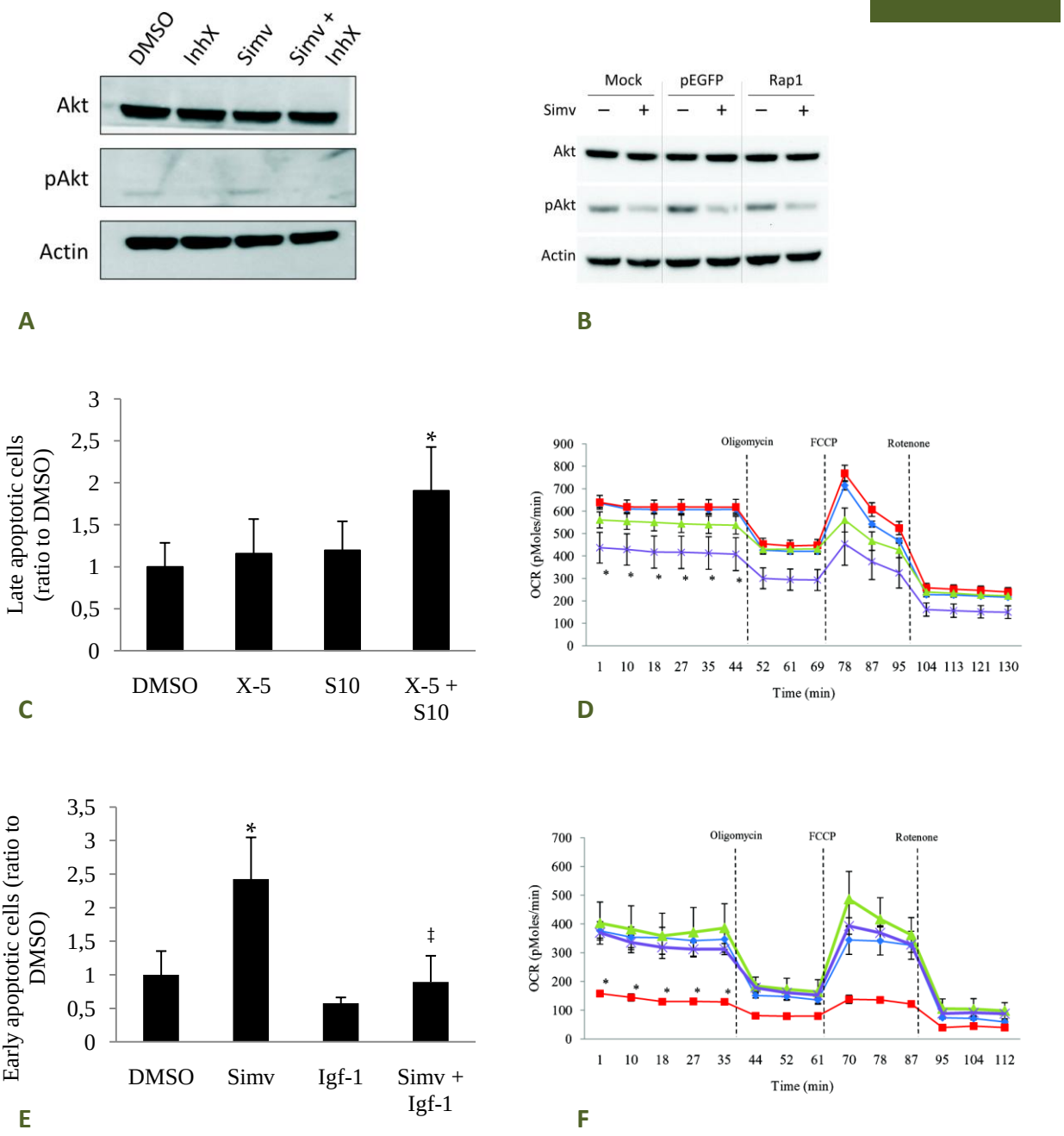
5.3.6. Inhibiting Akt leads to toxicity and inhibition of the ETC in simvastatin-treated HepG2 cells

We inhibited Akt in HepG2 cells using the specific inhibitor X from Sigma (5 μM).We confirmed inhibition of Akt phosphorylation by immunoblotting (**Fig 4A**), and also showed that Akt remains unphosphorylated when HepG2 cells are treated with 10 μM simvastatin and 5 μM inhibitor X (**Fig 4B**). Coincubation of 10 μM simvastatin with 5 μM inhibitor X also increased the number of apoptotic HepG2 cells (**Fig 4C**), and reduced basal mitochondrial oxygen consumption (**Fig 4D**).

5.3.7. Activation of the Igf-1/Akt pathway rescues C2C12 myotubes from simvastatin-induced mitochondrial impairment

It is well recorded that Igf-1 can rescue skeletal muscle from statin-induced damage, so we investigated whether Igf-1 could also rescue mitochondrial respiration in C2C12 myotubes. We used a 24 h treatment time, as there was no change in the number of apoptotic cells after 6 h treatment with 10 μM simvastatin. Myotubes treated with10 μM simvastatin showed a significant increase in the number of early apoptotic cells when compared to DMSO control (by 2.43-fold, p=0.04; **Fig 4E**). Co-incubation of Igf-1 with 10 μM simvastatin led to the expected reduction in the number of apoptotic C2C12 myotubes (reduced 2.72-fold compared to 10 μM simvastatin treatment, p=0.03; **Fig 4E**).

Figure 4



The influence of Igf-1/Akt signalling on simvastatin-induced mitochondrial toxicity in HepG2 cells and C2C12 myotubes. Immunoblots for total and phosphorylated Akt in HepG2 cells treated for 24 h with the indicated drugs (**A**), and transfected C2C12 myotubes treated for 6 h with 10 μM simvastatin (**B**). Actin was used as a loading control. The results are indicative of three independent experiments. **C** Annexin V/PI analysis of HepG2 cells treated with simvastatin and/or Akt inhibitor X, and analyzed by flow cytometry. The bars show the ratio of late apoptotic cells relative to DMSO, and are averages of four independent experiments carried out in triplicate. **D** Comparison of OCRs in HepG2 cells treated with simvastatin and/or Akt inhibitor X. The results are the means of four independent experiments carried out in quintuplicate. **E** Annexin V/PI analysis of C2C12 myotubes treated with simvastatin and/or Igf-1, and analyzed by flow cytometry. The bars show the ratio of early apoptotic cells relative to DMSO, and are averages of four independent experiments carried out in triplicate. **F** Comparison of OCRs in C2C12 myotubes treated with simvastatin and/or Igf-1. The results are the means of four independent experiments carried out in quintuplicate. * $p < 0.05$ versus DMSO control, and ‡ $p < 0.05$ versus 10 μM simvastatin.

The coincubation of Igf-1 with 10 μM simvastatin also increased mitochondrial respiration in C2C12 myotubes after 24 h treatment (**Fig 4F**).

5.3.8. Constitutively active Rap1 also partially rescues C2C12 myotubes from simvastatin-induced mitochondrial toxicity

We decided to further investigate how simvastatin could lead to inhibition of the Igf-1/Akt pathway, and lead to the reduction in mitochondrial respiration. Previous work by Baviera *et al.* suggests that Rap1 could link cAMP signalling to Igf-1/Akt signalling.[25] Our own lab has also already shown that simvastatin reduces Rap1 geranylgeranylation.[6] We hypothesized that reduction in Rap1 activity could therefore lead to a reduction in signalling through the Igf-1/Akt pathway. We therefore transiently expressed constitutively active Rap1in C2C12 myotubes (C2C12-Rap1) to discover whether it could rescue mitochondrial respiration after treatment with 10 μM simvastatin. Mock-transfected (C2C12-mock) and pEGFP-transfected (C2C12-pEGFP) myotubes acted as controls. C2C12-mock and C2C12-pEGFP myotubes showed the expected decrease in OCR after treatment with 10 μM simvastatin (by 37.1% and 33% respectively; **Fig 5**). C2C12-Rap1 myotubes had partially rescued mitochondrial respiration when treated with 10 μM simvastatin for 6 h (a decrease of only 16.1%; **Fig 5**). This suggests that Rap1 could play a role in mitochondrial health, and that its dysregulation may be an important stage in simvastatin-induced myotoxicity.

5.3.9. Constitutively active Rap does not prevent the transcription of atrogin-1

Dysregulation of the Igf-1/Akt pathway is known to be a key step in the induction of atrophy via reduction of Akt phosphorylation, translocation of the Foxo transcription factors to the nucleus and transcription of the E3 ubiquitin ligase atrogin-1. Previous studies show that atrogin-1 transcription is increased after statin treatment, and that atrogin-1 knockout zebrafish are resistant to statin-induced myopathy – offering strong evidence that atrogin-1 is a key regulator of statin-induced myopathy. We therefore

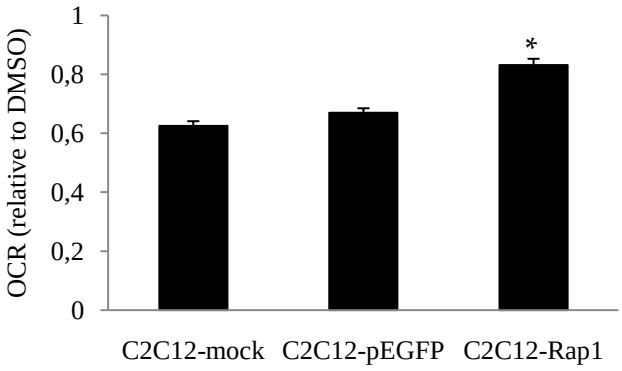
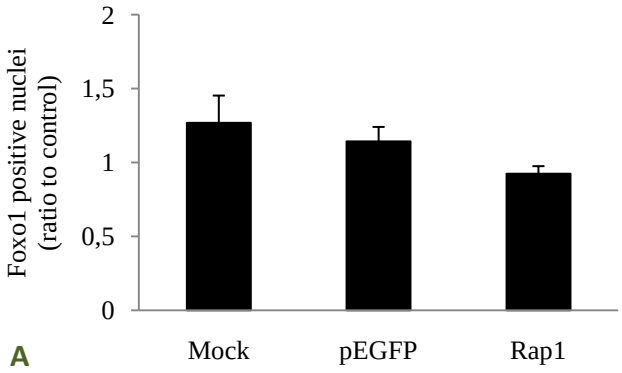


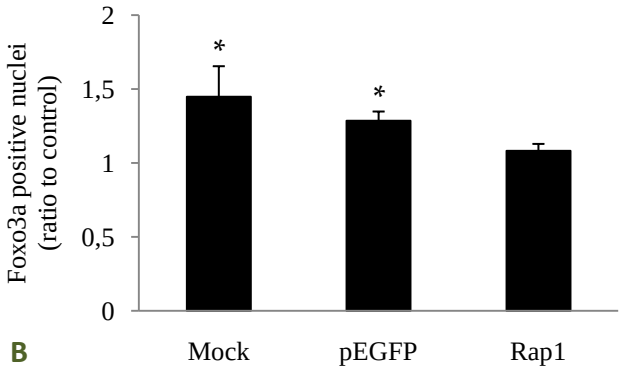
Figure 5

The effect of expressing constitutively active Rap1 on mitochondrial toxicity in C2C12 myotubes. C2C12-mock, C2C12-pEGFP and C2C12-Rap1 myotubes were treated with DMSO or 10 μM simvastatin for 6 h. OCR was measured on a XF24 analyzer. Results are indicative of three independent experiments carried out in quintuplicate.

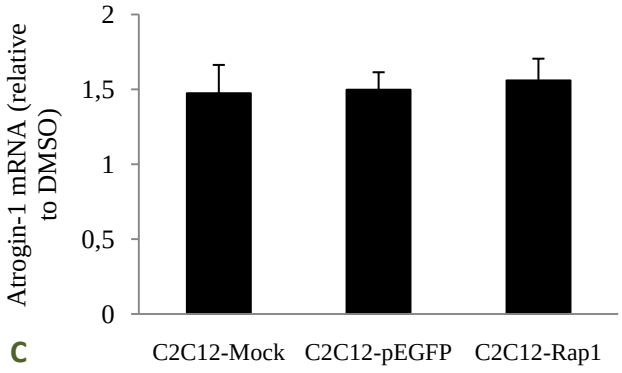
* $p < 0.05$ versus C2C12-mock.



A



B



C

Figure 6

Effect of expressing constitutively active Rap1 on atrogin-1 induction in C2C12 myotubes. **A** Co-localization of Foxo1 with nuclei. Results are the mean of 3 independent experiments. **B** Co-localization of Foxo3a with nuclei. **C** Atrogin-1 mRNA levels were also measured in transfected C2C12 myotubes. The results are means of three independent experiments.

* $p < 0.05$ versus DMSO control.

investigated whether Akt phosphorylation, Foxo nuclear translocation and atrogin-1 expression are altered in C2C12-Rap1 myotubes.

C2C12-mock, C2C12-pEGFP and C2C12-Rap1 myotubes all showed reduced levels of phosphorylated Akt after simvastatin treatment (**Fig 4B**). C2C12-mock, C2C12-pEGFP and C2C12-Rap1 myotubes showed no increase in Foxo1 nuclear translocation when treated with 10 μM simvastatin (**Fig 6A**). An increase in Foxo3a nuclear translocation was observed in 10 μM simvastatin-treated C2C12-mock and C2C12-pEGFP myotubes (**Fig 6B**). This increase in Foxo3a nuclear translocation was not observed in C2C12-Rap1 myotubes treated with 10 μM simvastatin (**Fig 6B**).

C2C12-mock and C2C12-pEGFP myotubes treated with 10 μM simvastatin showed the expected increase in atrogin-1 transcription (**Fig 6C**). The reduction in nuclear Foxo3a in 10 μM simvastatin-treated C2C12-Rap1 myotubes did not lead to a decrease in atrogin-1 transcription.

5.4. Discussion

Statins are widely reported to cause side-effects in skeletal muscle, ranging from mild weakness to severe rhabdomyolysis.[31–33] There major site of action is the liver, where they are well-tolerated and not toxic. We have previously shown differences in cholesterol metabolism between liver HepG2 cells and skeletal muscle C2C12 myotubes.[6] Other studies show that statins are mitochondrial toxins, and that reduction in mitochondrial respiration may be a contributing factor to skeletal muscle myopathy.[11,34,35] We have expanded on these studies by showing that simvastatin significantly reduces oxygen consumption in C2C12 myotubes, but has no effect on oxygen consumption in simvastatin-treated HepG2 cells. This result complements the

previous studies, and suggests that mitochondrial health is an important indicator of susceptibility to simvastatin in skeletal muscle.

Numerous signalling pathways are known to be important in maintaining mitochondrial health, and statins are also known to dysregulate a wide range of signalling pathways such as AMPK, JNK and Igf-1 signalling.[36–38] We therefore decided to investigate whether differences in cell signalling could explain the difference in mitochondrial respiration in simvastatin-treated HepG2 cells and C2C12 myotubes. We chose the Igf-1/Akt signalling pathway as it is both well-described to be altered by statin treatment in muscle and play a role in mitochondrial maintenance.[21–24]

We observed a key difference in phosphorylation state of Akt between the two cell lines: Akt was dephosphorylated in simvastatin-treated C2C12 myotubes, whereas simvastatin-treated HepG2 cells showed no change in Akt phosphorylation. This is the first study to show no change in Akt phosphorylation after simvastatin treatment, although a previous study does show reduced Akt phosphorylation in pravastatin-treated HepG2 cells.[39] The decrease in phosphorylated Akt in simvastatin-treated C2C12 myotubes complements the results of Hanai *et al.* that show lovastatin decreases phosphorylated Akt in C2C12 myotubes.[26] Further weight is added by the study of Mallinson *et al*., who saw similar reductions in Akt phosphorylation in simvastatin-treated Wistar rats.[40]

This difference could reflect a difference in the activity of the Igf-1/Akt signalling pathway in the two cell lines, as Akt phosphorylation is essential to its activity, and points to a potential explanation to why simvastatin is toxic to C2C12 myotubes but not to HepG2 cells: simvastatin-induced toxicity is dependent on Akt maintaining mitochondrial function

To test our theory, we inhibited Akt activity in HepG2 cells, and activated Akt in C2C12 myotubes. Coincubation of simvastatin with Akt inhibitor X in HepG2 cells led to a lowering of mitochondrial respiration and cell viability. This suggests that the ability of HepG2 cells to increase Akt phosphorylation upon simvastatin-treatment is one mechanism by which they are protected from toxicity. It also points to the maintenance of mitochondrial respiration playing an important role in protecting HepG2 cells after simvastatin-treatment. Coincubation of C2C12 myotubes with simvastatin and Igf-1 had the expected effect of rescuing the cells from apoptosis, and also reinstated mitochondrial respiration. This confirms the previous studies describing the importance of Igf-1/Akt signalling in statin-induced myotoxicity, and adds to this knowledge by showing that Igf-1/Akt signalling can rescue mitochondrial function in simvastatin-treated cells.[12,26] Although we did not perform activity assays on the complexes of the mitochondrial respiratory chain, the fact that Igf-1 rescued mitochondrial respiration in C2C12 myotubes implies that direct inhibition of the complexes may not be a relevant concern.

It is so far unknown how statins can reduce Igf-1/Akt signalling and lead to toxicity. Previous work, including studies from our own lab, show that statins can decrease the production of various intermediates in the cholesterol production pathway.[4–6] These intermediates, such as ubiquinone, geranylgeranyl pyrophosphate (GGPP) and dolichol, are important in a wide-range of cellular processes.[41–43] GGPP is important for the geranylgeranylation of proteins, and many geranylgeranylated proteins are required for cell signalling. Our previous work shows that Rap1, a small GTPase, has reduced geranylgeranylation after simvastatin treatment in C2C12 myotubes.[6] Other groups show Rap1 potentiates Igf-1 signalling, and Baviera *et al.* have postulated that Rap1 links cAMP signalling to Igf-1/Akt signalling.[25] On this evidence, we queried

whether it is the disruption of Rap1 in C2C12 myotubes that leads to a dysregulation of mitochondrial respiration after simvastatin-treatment.

We transfected constitutively active Rap 1 into C2C12 myotubes (C2C12-Rap1), and witnessed a partial rescue of mitochondrial respiration after simvastatin treatment compared to C2C12-mock and C2C12-pEGFP myotubes. This implies that Rap1 activity is important in maintaining mitochondrial function. This complements previous work by Qiao *et al.* showing that Epac, an activator of Rap1, is partially located in the mitochondria.[44] Others also show Rap1 can be found in mitochondria.[45,46] cAMP is also located in mitochondria, and thought to regulate mitochondrial function.[47,48] Our data adds to this knowledge by offering further evidence of the importance of Rap1 to mitochondrial health. Our data also provides evidence that simvastatin exerts its deleterious effects on C2C12 myotube mitochondria via impairment of Rap1.

Our results do not, however, point to Rap1 activating the Igf-1/Akt pathway. Although we saw a decrease in nuclear Foxo3a, we saw no increase in Akt phosphorylation or decrease in atrogin-1 transcription in simvastatin-treated C2C12-Rap1 when compared to C2C12-mock or C2C12-pEGFP myotubes. This suggests that Rap1 protects the mitochondria via a non-Igf-1/Akt mechanism. When taken with our data showing that Igf-1 can rescue simvastatin-induced mitochondrial impairment, our results point to multiple pathways having a role in both inducing, and protecting from, toxicity.

How simvastatin can impair the Igf-1/Akt pathway is still unknown, but Rap1 may still be a key player. It may be that the level of constitutively active Rap1 was not high enough to activate the pathway. Our transfection efficiency was only 30%, which may be high enough to rescue mitochondrial respiration but not rescue Akt signalling. We aim to further investigate the role of Rap1 in the Igf-1/Akt signalling pathway by increasing this efficiency.

Our data has, for the first time, provided an explanation for the difference in susceptibility to toxicity between liver HepG2 cells and skeletal muscle C2C12 myotubes. The effect of statins on Akt phosphorylation are well-reported, but this is the first study providing evidence for differing Akt phosphorylation states predisposing cells to simvastatin-induced toxicity. Also, although preliminary, the ability of Rap1 to restore mitochondrial respiration in C2C12 myotubes provides an important link between cellular metabolism and signalling, and a potential partial explanation for simvastatin-induced toxicity.

5.5 Acknowledgements

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5.6 Conflicts of interest

The authors have no conflicts of interest.

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Paper three

Simvastatin induces mitochondrial dysfunction and signs of atrophy in cardiomyocytes

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6. Abstract

Aims: Statins are widely used to prevent cardiovascular diseases. They are well-tolerated, with adverse reactions mainly seen in skeletal muscle. We studied whether they also have adverse effects in cardiomyocytes. **Methods and results:** We treated Wistar rats, isolated rat cardiomyocytes and H9C2 cells with simvastatin to thoroughly investigate any toxic effects. We assayed for signs of apoptosis, mitochondrial dysfunction and cellular atrophy. Lighter hearts were observed in simvastatin-treated rats (80 mg/kg/day for 13 days). Both isolated

cardiomyocytes and H9C2 cells showed toxicity and reduction in mitochondrial oxygen consumption after exposure to 10 μM simvastatin. The mitochondrial membrane potential was also compromised in the H9C2 cells. Signs of atrophy – Foxo3a nuclear translocation and atrogin-1 transcription – were increased in H9C2 cardiomyocytes. **Conclusion:** Simvastatin reduces heart weight in Wistar rats and us toxic to rat cardiomyocytes and H9C2 cells. This could be explained by compromised mitochondrial function and induction of atrophy by increased expression of atrogin-1. Further work is required to discover if this is also the case in humans.

6.1. Introduction

Statins are among the most prescribed drugs in Western countries. They reduce morbidity and mortality from coronary heart disease (CHD) and mitigate the risk of stroke.[1,2] Their major site of action is the liver, where they inhibit HMG-CoA (hydroxyl-methyl-glutaryl-coenzyme A) reductase, the rate-limiting step in cholesterol biosynthesis. Inhibition of this pathway also impairs other processes, such as ubiquinone production and the isoprenylation and *N*-linked glycosylation of proteins.[3] Altering these processes can reduce inflammation, oxidative stress and platelet adhesion.[4] This helps prevention of, and recovery from, CHD, the major cause of death in industrialized countries.[5]

Statins are generally well tolerated but there are dose-dependent adverse reactions, particularly in skeletal muscle where myopathy is seen in 1–5% of patients.[6,7] These adverse reactions are also thought to be linked to the pleiotropic effects of statins. We have demonstrated previously that a reduction in geranylgeranylation and *N*-linked glycosylation in C2C12 myotubes could be the cause of myotoxicity.[8]

Adverse reactions of statins in cardiac muscle have been much less studied, but could be of great clinical relevance. To our knowledge there are only a few previous papers on

the subject, which show that lovastatin reduces cardiomyocyte viability, in part via an increase in apoptotic pathways.[9,10] Studies in other tissues show statins can affect the mitochondria directly, via inhibition of the complexes of the respiratory chain, or indirectly via lowering of ubiquinone levels.[11,12] Previous work in our lab showed that statins can alter mitochondrial function in skeletal muscle, but no studies have so far addressed how statins affect cardiac mitochondria.[13] Statins are also known to increase levels of the atrophy-inducing protein atrogin-1 in skeletal muscle.[14] This increase in atrogin-1 levels is thought to be a contributing factor in statin-induced skeletal muscle toxicity. Previous studies show atrogin-1 is present in cardiac muscle, and is upregulated during experimental heart failure.[15,16] Whether this occurs in statin-treated cardiomyocytes has not been addressed.

We aimed to investigate how simvastatin affects cardiomyocyte mitochondria and the atrophy pathway. Male Wistar rats provided a model to observe any initial adverse effects of simvastatin on the heart *in vivo*. We then used isolated primary rat cardiomyocytes *ex vivo*, and the rat cardiomyocyte cell line H9C2, which is a well-established *in vitro* model for cardiac cell studies, for a more in-depth analysis.

This is the first study elucidating how simvastatin adversely affects cardiomyocytes and induces signs of atrophy. It allows both a deeper understanding of the pleiotropic effects of statins in cardiomyocytes and the risk they might pose to cardiovascular patients.

6.2. Materials and methods

6.2.1. Chemicals

Simvastatin (Sigma-Aldrich, St. Louis, MO, USA) was converted into the active acid following the protocol of Bogman *et al*.[17] Stock solutions of 10 and 100 mM simvastatin

in DMSO were stored at –20°C. The ToxiLight® assay kit LT07-117 was from Lonza (Basel, Switzerland) and the Pierce BCA protein assay kit from Merck (Darmstadt, Germany). Antibodies to Foxo1 and Foxo3a were provided by Cell Signaling Technology (Danvers, MA, USA). The Alexa Fluor® secondary antibodies were from Invitrogen (Basel, Switzerland). All other chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), except where indicated.

5.2.2. Animals

Male Wistar rats (8-weeks old) were divided into two groups: control and simvastatin-treated. We dissolved simvastatin in water and intragastrically administered 80 mg/kg body weight/day for 13 days. Control animals received an equivalent amount of water. The rats were killed after 14 days, and hearts harvested according to the 'Ethical Principles in Animal Research' adopted by the Brazilian College of Animal Experimentation (COBEA). The experiments were approved by the Ethical Commission of Ethics in Animal Research (157/2008), School of Medicine of Ribeirão, University of São Paulo, Brazil.

6.2.3. Isolation of neonatal rat cardiomyocytes

We isolated neonatal rat ventricular myocytes (NRVMs) from Wistar rats using a protocol modified from Bursac *et al.*[18] Briefly, we digested the hearts with sequential collagenase incubations. We plated the cells for an hour, allowing non-myocytes to adhere to the plate, and collected the enriched suspension of myocytes. Myocytes were seeded at a density of 100,000 cells/cm^2 and 100 μM bromodeoxyuridine (BrdU) added to prevent non-myocyte proliferation.

6.2.4. Cell culture

H9C2 cardiomyocytes were provided by Dr Pfister (University Hospital Basel, Switzerland), and grown in Dulbecco modified Eagle's medium (DMEM) high-glucose

medium (4.5 g/l) containing GlutaMAX™-1 (Gibco 61965), supplemented with 10% FBS, 5 mM HEPES, 1 mM sodium pyruvate and 500 µg/ml penicillin-streptomycin. Myoblasts were seeded at 150,000 cells/well of a 6-well plate (or equivalent), and grown for 2 days before drug treatment. We added simvastatin at a concentration of 10 and 100 µM. DMSO was used as a control; its concentration was 0.1%. Cells were grown in a humidified incubator with 5% CO_2 at 37°C.

6.2.5. Cytotoxicity assay

The loss of cell membrane integrity results in the release of adenylate kinase (AK), which can be quantified by the ToxiLight assay kit. After incubation with simvastatin for 6 and 24 h, we used the protocol outlined in Mullen *et al*.[8]

6.2.6. Detection of apoptosis after simvastatin treatment

We used an *in situ* apoptosis detection kit for Annexin V binding and propidium iodide (PI) staining (Vybrant™ Apoptosis Assay Kit #2, from Invitrogen). After 24 h incubation with simvastatin, we stained detached cells with 5 µl Annexin V-AlexaFluor 488 and 1 µl PI (final concentration 1 µg/ml) in Annexin V buffer as per the kit protocol. The samples were incubated for 15 minutes and analysed using a DAKO Cyan cytometer.

6.2.7. Measurement of mitochondrial membrane potential

We used tetramethylrhodamine ethyl ester (TMRE) to detect changes in mitochondrial membrane potential (ψm).[19] Cells were incubated with simvastatin for 24 h, then detached and incubated with 100 nM TMRE for 30 minutes at 37°C and 5% CO_2. We used a Live/Dead® Near-IR Dead Cell stain kit from Invitrogen to exclude dead cells. Cells were analyzed with a DAKO Cyan cytometer. The mitochondrial uncoupler FCCP acted as a positive control.

6.2.8. Confocal microscopy

H9C2 cardiomyocytes were grown in Lab-Tek microscopy chambers (Nunc, NY, USA) and treated with simvastatin for 24 h. The cells were fixed with 4% PFA, permeabilized with 0.2% triton-X and then labelled with the appropriate antibody. We used DAPI to stain nuclei. We used a Zeiss LSM 710 confocal microscope to take the images, and Zen software to process them (Carl Zeiss, Switzerland).

6.2.9. Measurement of oxygen consumption and extracellular acidification rates

We measured intact cellular respiration with a Seahorse XF24 analyzer (Seahorse Biosciences, North Billerica, MA, USA). H9C2 cells were seeded in Seahorse XF 24-well cell culture microplates at 5000 cells per well in 250 μl growth medium. We treated the cells with simvastatin for 24 h, and then replaced the medium with 750 μl unbuffered medium. Cells were equilibrated to the unbuffered medium for 45 minutes at 37°C in a CO_2-free incubator, then transferred to the XF24 analyzer. We measured basal oxygen consumption (OCR) and extracellular acidification rates (ECAR), and then sequentially injected 1 μM rotenone, 15 μM FCCP and 1 μM oligomycin to assess maximal oxidative capacity.

6.2.10. Measurement of reactive oxygen species

We used a fluorescent-based microplate assay to evaluate the oxidative stress in simvastatin-treated H9C2 cardiomyocytes. The cardiomyocytes were co-incubated with DCFH-DA. DCFH-DA is hydrolysed in the cytoplasm by intracellular esterases to the non-fluorescent DCFH, which can be oxidized in the presence of reactive oxygen species (ROS) to the fluorescent (DCF). We measured fluorescence, at an excitation wavelength of 490 nm and an emission wavelength of 535 nm, with a HTS 700 Plus Bio Assay reader (Perkin Elmer, Switzerland).

6.2.11. Atrogin-1 mRNA expression

We treated H9C2 cardiomyocytes with simvastatin for 24 h. RNA was extracted and 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 cDNA for quantitative RT-PCR. We used SYBR green with primers specific for atrogin-1(forward primer: 5′-GAAGACCGCTACTGTGGAA-3′; reverse primer: 5′-ATCAATCGCTTGCGGATCT-3′). Relative quantities of specifically amplified cDNA were calculated with the comparative-threshold cycle method. GAPDH acted as endogenous reference (forward primer: 5′-CATGGCCTTCCGTGTTCCTA-3′; reverse primer: 5′-CCTGCTTCACCACCTTCTTGA-3′), and no-template and no-reverse-transcription controls ensured non-specific amplification could be excluded.

6.2.12. Statistical analysis

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

6.3. Results

6.3.1. Simvastatin reduces growth and heart weight in Wistar rats

We treated Wistar rats with 80 mg/kg/day simvastatin for 13 days so that we could observe any effects of the drug on heart size and weight. Animals were sacrificed on day 14. The overall growth rate was significantly reduced after 7 days in the treated animals compared to control rats (**Fig 1A**). The simvastatin-treated rats also had significantly lighter hearts (a reduction of 21% compared to control, $p<0.05$; **Fig 1B**). The ratio of heart weight to body weight was also reduced at the end of the study (from 0.33% in control rats to 0.29% in treated rats), indicating that the reduced heart weight was not due to a generic reduction throughout the body. This suggested that

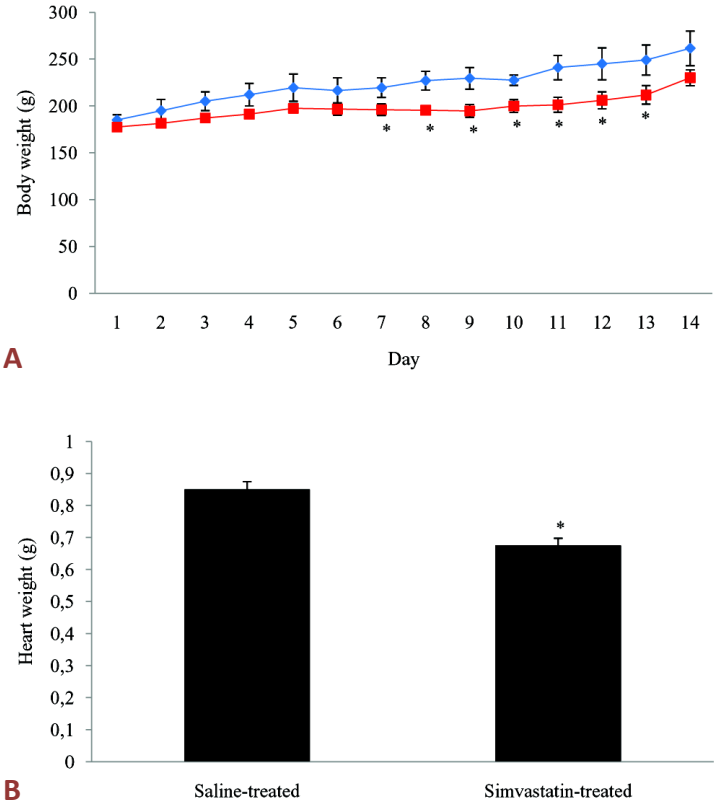

Figure 1

Effect of simvastatin on growth rate and cardiac atrophy in Wistar rats. Male Wistar rats received water (blue line) or simvastatin (80 mg/kg/day, s.c.; red line) for 13 days. **A** Body weight was measured each day. **B** Heart weight was measured for rats treated with water or simvastatin. Each data point represents the mean of 8 or 9 muscles.

* $p < 0.05$ in relation to control group.

simvastatin is associated with cardiac atrophy, and led us to investigate the toxicity and induction of atrophy in *ex vivo* and *in vitro* models.

6.3.2. Simvastatin induces cytotoxicity in primary NRVMs and H9C2 cardiomyocytes

We treated isolated NRVMs with simvastatin to discover if simvastatin is directly toxic to cardiomyocytes. We measured the release of AK from cells to determine the cytotoxicity. NRVMs showed significant toxicity with 10 µM simvastatin (an increase of 1.43 fold compared to control, $p<0.05$; **Fig 2A**). The rat cell line H9C2 then provided an *in vitro* model to further investigate whether simvastatin could be toxic to cardiomyocytes. We used a range of concentrations from 0.1 to 100 µM simvastatin (**Fig 2B**). We observed

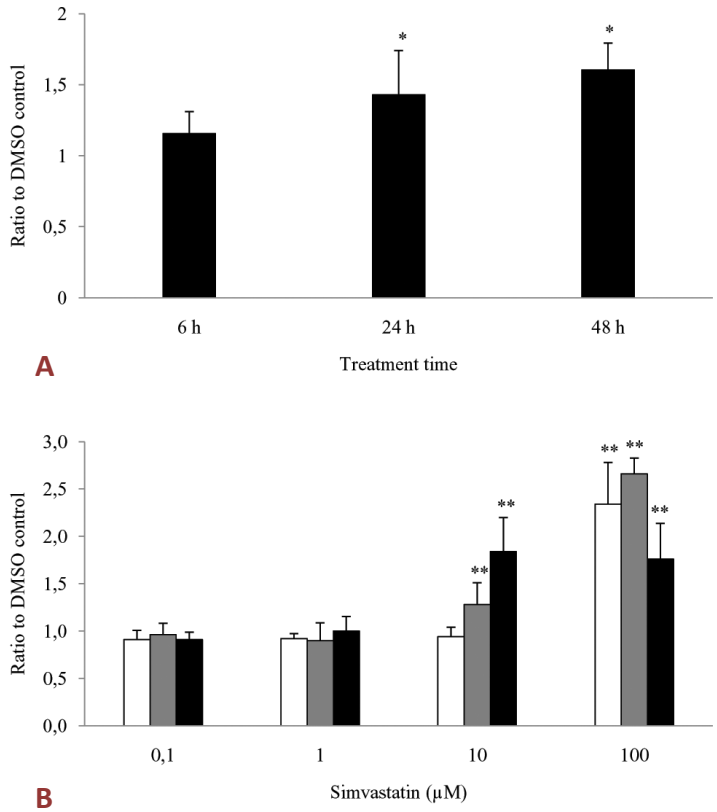

Figure 2

Toxicity of simvastatin on cardiomyocytes. **A** Isolated primary rat cardiomyocytes were treated with DMSO or 10 µM simvastatin for 6 h, 24 h or 48 h. **B** H9C2 cardiomyocytes were incubated for 6 h (white bars), 24 h (grey bars) or 48 h (black bars) with the simvastatin concentrations 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 bar represents the mean of five independent experiments carried out in duplicate.

* $p < 0.05$ and ** $p < 0.01$, versus control.

no significant toxicity with 0.1 or 1 µM at any timepoint. 10 µM was the lowest concentration that led to cell death of H9C2 cardiomyocytes after 24 h – they had a significant increase in AK release of 1.28 fold compared to control cells. 100 µM was already toxic at 6 h. These results allowed us to consider H9C2 cardiomyocytes a suitable model for simvastatin-induced cardiomyocyte toxicity. We used 24 h treatment with 10 and 100 µM simvastatin for the remaining experiments.

6.3.3. Apoptosis is the major form of cell death

We used Annexin V/PI staining to measure the type of cell death seen in H9C2 cardiomyocytes treated with simvastatin for 24 h. H9C2 cardiomyocytes treated with 10 µM simvastatin showed a significant increase in early apoptotic cells when compared to control (6.33 versus 2.97%; **Fig 3**). This increase was concentration dependent – treatment with 100 µM simvastatin showed a larger increase in early apoptotic cells

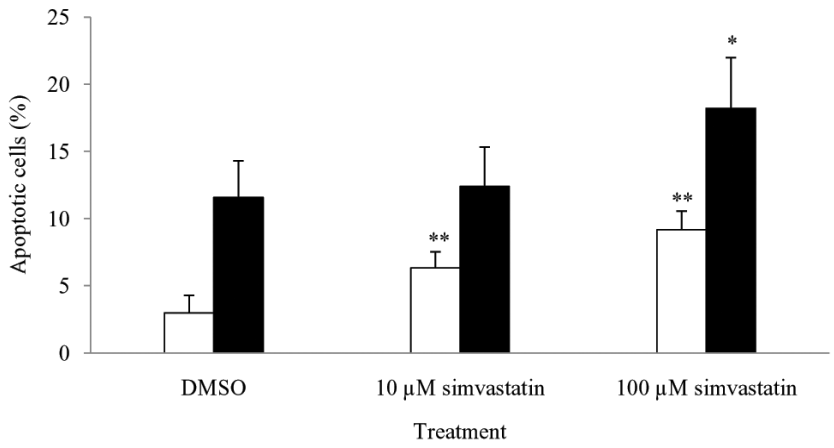

Figure 3

Annexin V/PI analysis after simvastatin treatment. Cells were incubated with DMSO, 10 µM or 100 µM simvastatin for 24 h. The cells were then labelled with Annexin V and PI, and analysed via flow cytometry. Early (white bars) and late (black bars) apoptotic cells were quantified. The data represents the mean of four experiments carried out in triplicate.

* $p < 0.05$ and ** $p < 0.01$, versus control.

(9.18%; **Fig 3**). An increase in the late apoptotic fraction was only seen in cells treated with 100 µM simvastatin (18.21 versus 11.58%; **Fig 3**).

6.3.4. Mitochondrial ψm is reduced

Previous work by our lab and others points to statins impairing the mitochondria in other cell types. To see if this was the case in cardiomyocytes, we assayed for changes in the mitochondrial ψm with the dye TMRE. TMRE localization to mitochondria is dependent upon the mitochondrial ψm – a reduction in mitochondrial ψm leads to a reduction in the fluorescent signal.[19] A compromised mitochondrial ψm is an indicator of impaired mitochondrial function. We pre-treated the cells for 24 h with DMSO, 10 µM and 100 µM simvastatin (**Fig 4**). H9C2 mitochondrial membrane potential was significantly reduced after treatment with both 10 µM and 100 µM simvastatin (by 18.4 and 34.7%, respectively). The positive control, FCCP, showed the expected reduction in mitochondrial membrane potential (by 78.2%).

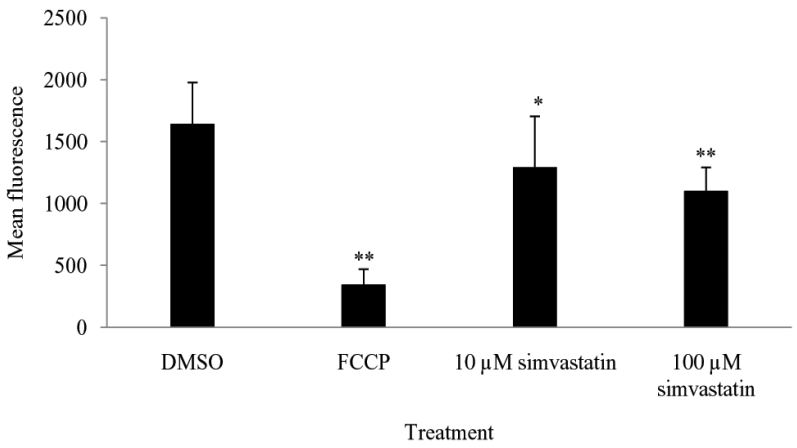

Figure 4

Effect of simvastatin on mitochondrial membrane potential in H9C2 cardiomyocytes. Cells were incubated with DMSO, 10 µM or 100 µM simvastatin for 24 h. TMRE was used to determine mitochondrial membrane potential, and Near-IR dye from Invitrogen to exclude dead cells. FCCP was added to one of the DMSO samples to act as a positive control. The results are means of four independent experiments carried out in triplicate. * $p < 0.05$ and ** $p < 0.01$, versus control.

6.3.5. Simvastatin reduces oxygen consumption in intact H9C2 myocytes

To delve deeper into how simvastatin affects H9C2 mitochondria, we used a XF24 analyzer to determine changes in OCR and ECAR in simvastatin-treated intact H9C2 cardiomyocytes. OCRs provided a measurement of the activity of the electron transport chain (ETC), and ECARs an indication of the rate of glycolysis. DMSO-treated control cells showed very low ECAR values, suggesting that H9C2 cardiomyocytes do not rely on glycolysis for energy. OCR rates suggested a functional ETC in control cells. After 6 h treatment with 10 µM simvastatin, baseline OCR values were already significantly reduced by 16% (**Fig 5A**). The OCR reduction with 10 µM simvastatin was time dependent, with a larger drop of 50% observed after 24 h treatment (**Fig 5B**). FCCP was added to the cells to determine maximum respiration. The maximum respiration was lower in simvastatin-treated cells at both timepoints (reductions of 9% at 6 h, and 47% at 24 h). We saw no change in ECAR values after 6 h treatment with 10 µM simvastatin (**Fig 5C**). ECAR values did, however, drop significantly by 51 % after 24 h treatment with 10 µM simvastatin (**Fig 5D**). Treatment for only 6 h with 100 µM simvastatin resulted in a complete abolition of oxygen consumption and extracellular acidification, suggesting a

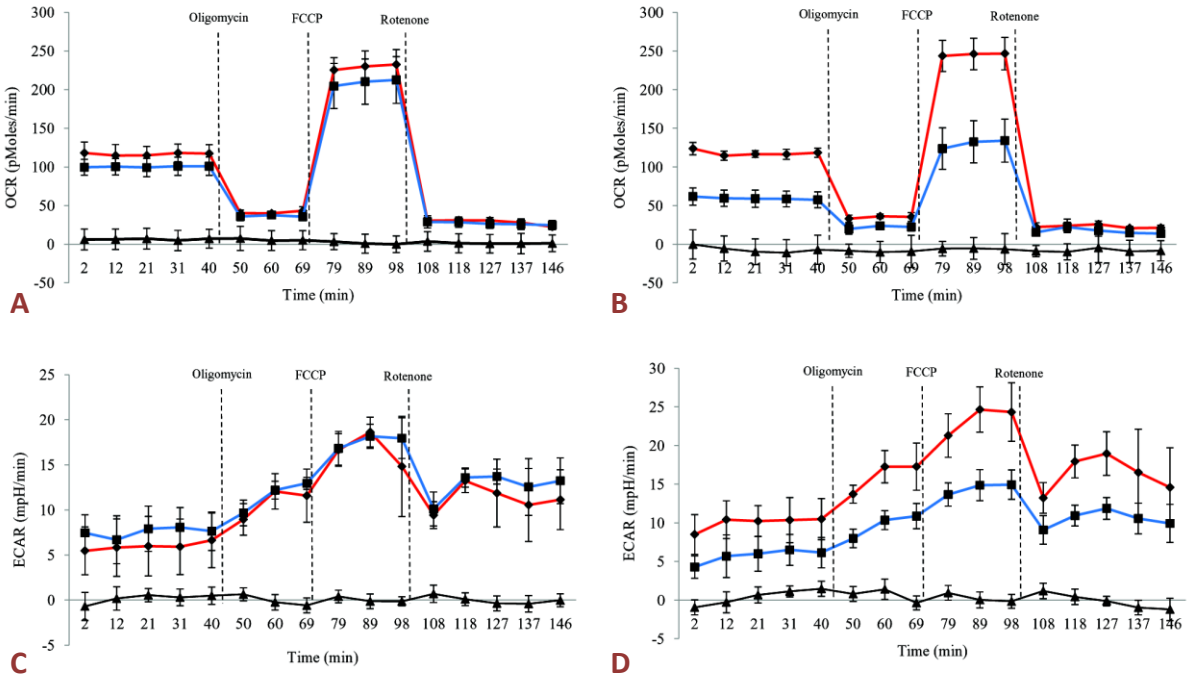

Figure 5

Effect of simvastatin on oxygen consumption and extracellular acidification in intact H9C2 cardiomyocytes. Cells were incubated with DMSO (red lines), 10 µM (blue lines) or 100 µM (black lines) simvastatin. The XF24 analyzer was used to take measurements. OCRs are shown for 6 h (**A**) and 24 h (**B**) incubation. ECARs are shown for 6 h (**C**) and 24 h (**D**) incubation. 1 µM oligomycin, 15 µM FCCP and 1 µM rotenone were added at the indicated points. Results are indicative of three independent experiments.

complete inhibition of the ETC and glycolysis at this concentration (**Fig 5A** and **5C**). Mitochondrial mass did not change after treatment (data not shown).

6.3.6. Oxygen consumption is also reduced in primary cardiomyocytes

We also determined the OCRs in primary cardiomyocytes treated with 10 µM simvastatin to confirm our *in vitro* studies. The primary cardiomyocytes also showed a reduction in OCR (of 18%), offering further evidence that simvastatin impairs cardiac mitochondrial function (**Fig 6A**). Baseline ECAR was also reduced by 18% (**Fig 6B**).

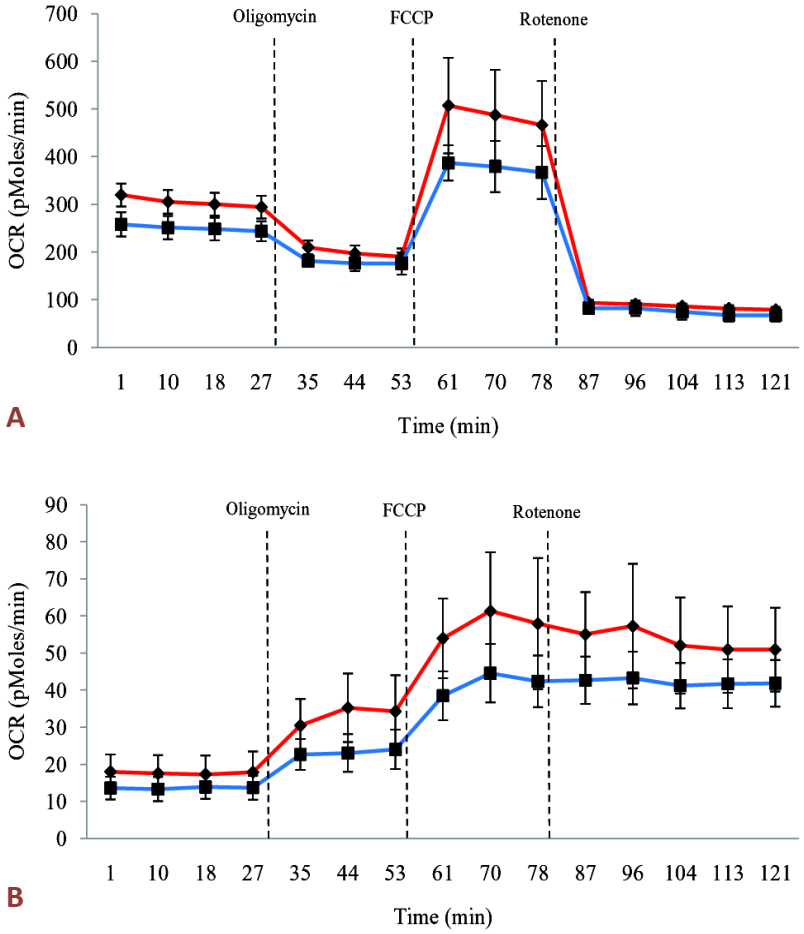

Figure 6

Effect of simvastatin on oxygen consumption in isolated primary rat cardiomyocytes. Cells were incubated with DMSO (red line) or 10 μM simvastatin (blue line) for 24 h. The XF24 analyzer was used to take measurements of (*A*) OCR and (*B*) ECAR. Results are indicative of three independent experiments.

6.3.7. ROS levels are not altered by simvastatin

We measured the ROS levels to investigate whether the decrease in OCR is associated with an increase in ROS production. ROS levels in H9C2 cardiomyocytes did not change after 24 h treatment with 10 μM simvastatin (**Fig 7**). Benzbromarone acted as a positive control, and showed the expected increase in ROS levels.[20]

6.3.8. Simvastatin also leads to Foxo nuclear translocation and transcription of atrogin-1

The decrease in heart weight in the simvastatin-treated Wistar rats suggested that atrophy could be induced by simvastatin. One inducer of atrophy, atrogin-1, is thought

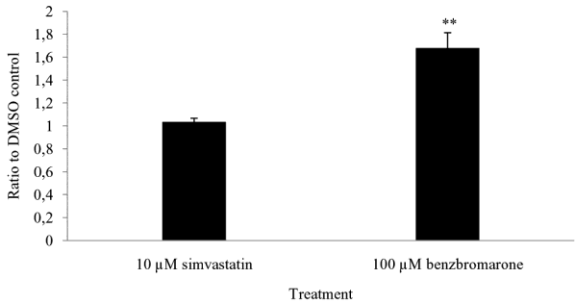

Figure 7

ROS levels in simvastatin-treated H9C2 cardiomyocytes. We incubated the cells with DMSO or 10 µM simvastatin for 24 h. We used 100 µM benzbromarone as a positive control. Results are shown as relative to DMSO-treated control cells. The results represent the mean of three independent experiments carried out in triplicate.

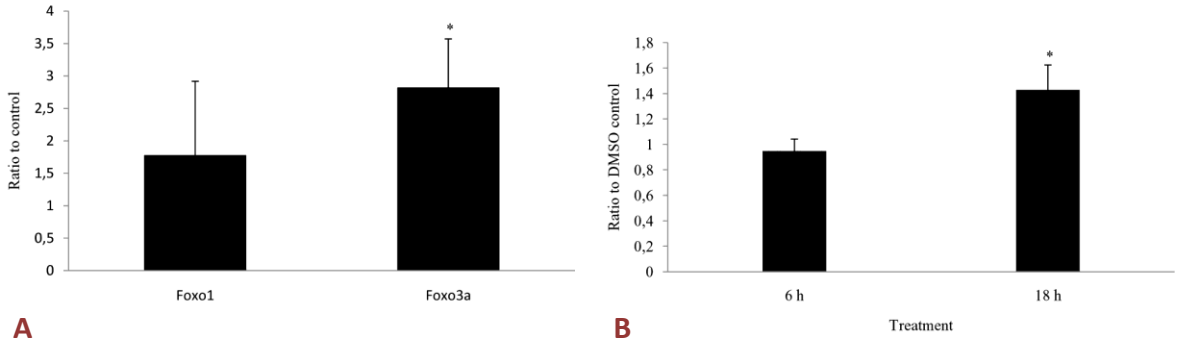

Figure 8

Simvastatin-induced atrogin-1 upregulation in H9C2 cardiomyocytes. The cardiomyocytes were incubated with DMSO or 10 µM simvastatin for 24 h. **A** We used confocal microscopy to quantify the number of nuclei positive for FoxO1 and FoxO3a. Results are expressed as ratios to DMSO, and represent three independent experiments carried out in triplicate. **B** Atrogin-1 mRNA levels in 10 µM simvastatin-treated cells relative to DMSO control. The results are means of three independent experiments carried out in triplicate. ** $p < 0.01$ versus control.

to play a key role in statin-induced skeletal muscle myopathy, and its transcription is controlled by the Foxo transcription factors. We therefore assayed for induction of atrogin-1 in the H9C2 cardiomyocytes. Using confocal microscopy, we observed no change in the location of Foxo1 (**Fig 8A**). Nuclear translocation of Foxo3a was, however, increased after 24 h treatment with simvastatin (2.82-fold, $p<0.05$; **Fig 8A**). Consequently, atrogin-1 mRNA levels were increased by 1.43 fold after 24 h simvastatin treatment ($p<0.05$; **Fig 8B**).

6.4. Discussion

The myopathic side-effects of statins are frequently reported in skeletal muscle, however, little is currently known of their effects on cardiac muscle.[21,22] Statins affect a multitude of processes, so investigation into whether they have toxic effects in cardiac muscle is warranted. This is particularly important as statins are often prescribed to patients with cardiovascular disease, so cardiac side-effects may be masked by falsely attributing them to the underlying disease.

We observed that simvastatin-treated rats had hearts of lower weight than controls, which pointed to a possible toxic effect associated with cardiac atrophy. We used *ex vivo* and *in vitro* models to test this hypothesis, with particular focus on how simvastatin affects cardiomyocyte death and atrophy pathways. We discovered that 10 μM simvastatin has a significant toxic effect in both primary NRVMs and H9C2 cardiomyocytes after 24 h incubation. The 10 μM concentration of simvastatin that we used is higher than would be expected in the plasma of patients, where values do not exceed 1 μM. However, localized concentrations could be higher than the observed plasma levels, especially when combined with inhibitors of cytochrome P450 or OATP1B1.[23]

Simvastatin is known to cause apoptosis in a variety of cell types.[24–27] We have added to this knowledge by showing that apoptosis also occurs in H9C2 cardiomyocytes treated with 10 μM simvastatin. This novel finding led us to further investigate signs and mechanisms of apoptosis. We initially observed a decrease in the mitochondrial ψm. ψm is an indicator of mitochondrial function, so its decrease points to statins having an effect on this organelle. We believe that this change is not a direct result of cholesterol lowering, as we and others show in other systems that the cholesterol precursor squalene does not reverse the effects of statins in other systems.[8,28] This

hypothesis is given more credence by studies showing statins inhibit mitochondrial function – either through direct inhibition of complexes of the electron transport chain or indirectly by reducing ubiquinone levels.[11,12] To investigate whether this was the case in H9C2 cardiomyocytes, we comprehensively assayed for changes in oxygen consumption in both intact primary NRVMs and H9C2 cells. We used a new technique to achieve this: the XF24 analyzer to measure OCR and ECAR. OCR indicates activity of the mitochondrial ETC, and ECAR the level of glycolysis. A major observation was that oxygen consumption was significantly reduced in intact cardiomyocytes after 6 h treatment with 10 μM simvastatin (a pre-toxic timepoint). This suggests that changes in the mitochondria are a cause, rather than a consequence, of simvastatin-induced toxicity in H9C2 cardiomyocytes. This complements work in other cell lines, and is the first time a direct effect on oxygen consumption in mitochondria has been seen in a cardiac cell line.[13,29,30] We confirmed this data in the isolated NRVMs, adding further credence to our hypothesis that mitochondria are impaired in cardiomyocytes. Maximum respiration was also reduced after simvastatin treatment, again suggesting an impairment in the electron transport chain. Only one previous study, in the liver, shows statins inhibit glycolysis.[31] We have expanded on this observation, by showing a reduction in ECAR values in the H9C2 cardiomyocytes, suggesting an inhibition of glycolysis. The cells are therefore unable to use glycolysis to compensate for the loss of mitochondrial energy production. Inhibition of both mitochondrial function and glycolysis explains the susceptibility of cardiomyocytes.

It is important to discern the mechanism by which simvastatin lowers oxygen consumption in cardiomyocytes. This decrease cannot be explained by a reduction in mitochondrial mass (we saw no change) or a reduction in ROS production (also unaltered, contradicting one of the perceived benefits of statin treatment in cardiovascular disease). Statin-induced reductions in oxidative stress occur in other

cells, such as erythrocytes and vascular smooth muscle cells.[32,33] Previous studies suggest that in skeletal muscle, simvastatin inhibits the complexes of the electron transport chain, but further work is required to determine whether this also occurs in cardiac muscle.[13]

Our work also uncovered a second major effect of simvastatin on cardiomyocytes: the induction of signs of atrophy. Our initial observation of the lighter atrophic heart was reinforced by our *in vitro* data. The statin-induced upregulation of atrogin-1 production is well-studied in skeletal muscle, and known to be controlled by Igf-1/Akt/Foxo signalling.[34,35] Atrogin-1 is hypothesized to be a major contributing factor to statin-induced skeletal muscle myopathy.[14,36] Our study is the first to show that simvastatin induces atrogin-1 in cardiomyocytes. This observation could have great impact on two fronts. (1) Positive: the atrophy-inducing effects of simvastatin may account for some of its cardiovascular benefits – they could counterbalance the tendency towards hypertrophic heart in some patients,[37,38] and (2) negative: statin-associated cardiac atrophy could negatively impact cardiac contractility, which may be relevant in patients with pre-existing heart disease.

Although preliminary, our data show for the first time that simvastatin adversely alters mitochondrial function and induces signs of atrophy in cardiomyocytes. Since mitochondrial damage and increased atrogin-1 expression are associated with apoptosis, cardiac atrophy can be explained by either event. The precise toxic mechanisms remain to be uncovered, so further work is required to fully determine any detrimental effects of statins on cardiac tissue. Furthermore, it is important to perform similar studies in cardiac tissue obtained from statin-treated humans.

6.5. Acknowledgements

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6.6. Conflicts of interest

The authors have no conflicts of interest.

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Final discussion

STATINS COMPETITIVELY inhibit HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis.[16] Although they exert their effects primarily in the liver, where they are well-tolerated and not toxic, the majority of their side-effects occur in skeletal muscle.[35–38] These side-effects range from mild muscle ache to potentially fatal rhabdomyolysis. It is of paramount importance to uncover the cause(s) of these side-effects for three reasons:

1. The increasingly elderly and obese population in the Western world suggests that the use of statins will increase year on year, meaning side-effects will be more and more relevant.
2. Side-effects decrease compliance, impacting negatively on the benefit of prescribed statins.
3. Statins have a vast number of pleiotropic effects and uses, from treating cancer to Alzheimer's disease, and therefore a better understanding of side-effects would lead to better care.

7.1. Comparing cholesterol metabolism in C2C12 myotubes and HepG2 cells highlights differences in responses to simvastatin

The first aim of this thesis is therefore to uncover possible mechanisms behind statin-induced side-effects in skeletal muscle. We initially chose an *in vitro* mouse skeletal muscle model, C2C12 myotubes, to represent a system that is susceptible to statin-induced myopathy. We compared responses in this cell line to those in the human

hepatic HepG2 cell line, with the aim of uncovering differences between the two that might point to why statins are toxic in skeletal muscle but not in the liver.

The first responses that we compared were direct effects on cholesterol metabolism. We thoroughly measured changes not only in cholesterol content and production, but also those in ubiquinone production, farnesylation and geranylgeranylation of small GTPases, and the rate of *N*-linked glycosylation. We found that simvastatin was toxic to C2C12 myotubes before there was any change in cellular cholesterol ubiquinone content, suggesting that toxicity is not related to these two factors. When we investigated how simvastatin affects the mitochondrial ETC, we saw that oxygen consumption was reduced after treatment, despite there being no change in ubiquinone content. This reduction occurred at a pre-toxic timepoint, and is therefore a potential candidate for a cause of the toxicity.

Interestingly, ubiquinone content was reduced in the HepG2 cells, but toxicity was not observed. The reduction in ubiquinone levels in the HepG2 cells did not lead to a reduction in mitochondrial oxygen consumption, raising the possibility that the ETC in HepG2 cells can still function with a reduced ubiquinone content. A study by Tavintharan *et al*. shows a larger decrease in HepG2 ubiquinone content, and that this reduction is associated with statin-induced toxicity.[180] This suggests that there is a critical amount of ubiquinone that HepG2 cells can compensate for.

When comparing farnesylation and geranylgeranylation, we observed an increase in the amount of unfarnesylated Ras and ungeranylgeranylated Rap1 in both the C2C12 myotubes and the HepG2 cells. At first glance, this would seem to suggest that disruption of these processes would therefore not lead to toxicity, but addition of geranylgeraniol completely rescued the C2C12 myotubes from simvastatin-induced toxicity. This rescue is in line with previous work, and shows the importance that

geranylgeranylated proteins play in maintaining cell viability.[68,74,115] The addition of farnesol did not rescue the C2C12 myotubes to the same extent, suggesting that farnseylated proteins, on their own, are not integral in leading to the observed toxicity.

We saw a difference between the cell lines in the rate of *N*-linked glycosylation. Simvastatin-treated C2C12 myotubes showed a reduction in *N*-linked glycosylation whereas HepG2 cells did not. This offers the possibility that alterations in *N*-glycosylated proteins could be a cause of the toxicity seen in the C2C12 myotubes. Previous studies implicate statins in incorrect processing of the Igf-1r, and our study also hints at this possibility.[80,82,83] It is important to point out that the addition of farnesol did not rescue the C2C12 myotubes from simvastatin-induced toxicity. Farnesol is essential in the formation of dolichol, and the lack of rescue with farnesol points to *N*-glycosylated proteins not having too prominent a role in leading to toxicity.

7.2. Igf-1/Akt signalling determines the response to simvastatin in C2C12 myotubes and HepG2 cells

The Igf-1/Akt pathway has been implicated in the progression of statin-mediated toxicity, and we investigated whether this was true with the C2C12 myotubes. The pathway was down-regulated at a pre-toxic timepoint, as shown by reduced phosphorylation of Akt, increased nuclear Foxo3a, and an upregulation of atrogin-1 transcription. How statins can disrupt this pathway is still unknown, and we used our knowledge from fully characterizing the mevalonate pathway to guide us.

Previous work by Baviera *et al.* shows a possible link between cAMP/EPAC signalling and the Igf-1/Akt pathway, and that the small GTPase Rap1 could be the link.[147] They showed that increasing cAMP levels using IBMX, a cAMP-PDE inhibitor, increases Akt phosphorylation and rescues dexamethasone-induced upregulation of atrogin-1. We postulated that the increase in ungeranylgeranylated Rap1 caused by simvastatin

would remove the link between cAMP/EPAC and Igf-1/Akt signalling. To add weight to our hypothesis, we noted that Akt signalling was not altered in HepG2 cells. Expressing constitutively active Rap1 in the C2C12 myotubes did not, however, reverse the simvastatin-induced decrease in Akt phosphorylation or prevent atrogin-1 transcription. Constitutively active Rap1 did, however, partially rescue mitochondrial oxygen consumption in the C2C12 myotubes. This result suggests that Rap1 is important in mitochondrial function, and hints again at the many effects statins can have in a cell.

Mitochondrial function and integrity is reliant upon multiple signalling pathways, one of which is the Igf-1/Akt pathway.[115,149–151] To test whether this pathway protects the HepG2 cells from statin-induced toxicity, we co-incubated these cells with an Akt inhibitor. Neither the Akt inhibitor nor simvastatin alone were toxic to the HepG2 cells, but co-incubation led to increased cell death, and also a decrease in mitochondrial oxygen consumption. This demonstrates both the importance of Igf-1/Akt signalling in maintaining correct mitochondrial function and also in protecting from simvastatin-induced toxicity.

To conclude, this part of the thesis has fully characterized the differing responses to simvastatin in skeletal muscle C2C12 myotubes and liver HepG2 cells. These differences in cholesterol metabolism, mitochondrial function and Igf-1/Akt signalling offer clues as to why statins can lead to toxicity in skeletal muscle and not in liver.

7.3. Cardiomyocytes are also susceptible to simvastatin-induced toxicity

The second part of the thesis investigated the effects of simvastatin-treatment on cardiac tissue and cells. This work is of vital importance as the majority of statins are prescribed to prevent cardiovascular diseases. Something that could have an adverse effect on the

very organ it is trying to protect could be disastrous, especially as cardiac side-effects could be masked by falsely attributing them to the underlying disease.

We observed a reduction in heart weight in Wistar rats treated with simvastatin, and used *in vivo* and *ex vivo* models for further investigation. Simvastatin-toxicity in H9C2 cardiomyocytes was seen at both a mitochondrial and atrophic level. Similar to the C2C12 myotubes, mitochondrial oxygen consumption was reduced and atrogin-1 expression increased in the treated cardiomyocytes. A major observation was that the reduction in oxygen consumption occurred after only 6 h treatment with 10 μM simvastatin (a timepoint at which we saw no cytotoxicity). This suggests that changes in the mitochondria are a cause, rather than a consequence, of simvastatin-induced toxicity in H9C2 cardiomyocytes. This complements work in other cell lines, and is the first time a direct effect on oxygen consumption in mitochondria has been seen in a cardiac cell line.[118,120,122] The reduction in mitochondrial respiration was confirmed in isolated neonatal rat cardiomyocytes. The cardiomyocytes also showed a reduction in glycolysis. It is tempting to link these reductions in oxygen consumption and glycolysis to a disruption in Igf-1/Akt signalling. An inhibition in Igf-1/Akt signalling is indirectly pointed to by our results showing an increase in Foxo3a translocation to the nucleus and atrogin-1 transcription.

Although preliminary, our data show for the first time that simvastatin could adversely alter mitochondrial function and induce signs of atrophy in cardiomyocytes. The exact toxic mechanisms remain to be uncovered, so further work is required to expand on these results and fully determine any detrimental effects of statins on cardiac tissue. This will be particularly important if similar statin-induced cardiotoxicity is found in human subjects. With demographic changes resulting in an aging population in Western countries, it can be expected that statin use will further increase in the future. It is therefore of great importance to ensure that these drugs are used safely.

7.4. Important points

In summary, we provide important differences in the response to simvastatin between C2C12 myotubes and HepG2 cells. These differences centre on the response of the Igf-1/Akt signalling pathway, and open up new areas of research into the exact mechanism of statin-induced toxicity – particularly involving the importance of the small GTPase, Rap1. In addition, we have become the first group to uncover adverse effects of simvastatin on cardiac muscle. As with skeletal muscle, we show inhibition of mitochondrial respiration and upregulation of atrogin-1 synthesis. Overall, this thesis contributes to our knowledge of how simvastatin causes toxicity, provides novel mechanisms for linking mitochondrial toxicity to cell signalling, and opens up a new area of research into toxic effects in cardiac muscle ■

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Nationality: British
Languages: English (native), French (intermediate), German (basic)

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Peter James Mullen

Key Skills

- **Track record of developing and implementing innovative projects**
- **Skilled in wide range of techniques, and eager to learn more**
- **Accomplished supervisor of students and project teams in the laboratory**
- **History of producing high-impact, talked-about presentations**

Laboratory Experience

Postdoctoral Fellow *University of Toronto 01.2011 – present*

I currently work in the laboratory of Dr Linda Penn. I am investigating how statins could be used as an anti-cancer therapy. My work involves uncovering the molecular basis behind statin-induced cancer cell death, developing a genetic signature to determine which patients will respond best to this therapy, and also how to improve the therapy.

PhD *University of Basel 08.2007 – 12.2010*

I obtained my PhD in the laboratory of Prof. Stephan Krähenbühl, investigating the molecular basis of statin-induced myopathy. I showed that statins affect post-translational modification of proteins, and uncovered potential side-effects in cardiomyocytes. I also looked at the effects of statins on the mitochondria. The techniques I used included immunoblotting, creation of stable cell lines, the XF24 Seahorse analyzer, radioactive assays and flow cytometry.

Asst Research Technician *University College London 08.2005 – 07.2007*

Based in the Centre for Cardiology in the Young and working under Prof. Bill McKenna, I screened arrhythmogenic right ventricular cardiomyopathy patients and their family members for mutations in genes such as plectin and desmin. Techniques used included PCR, DNA sequencing and dHPLC.

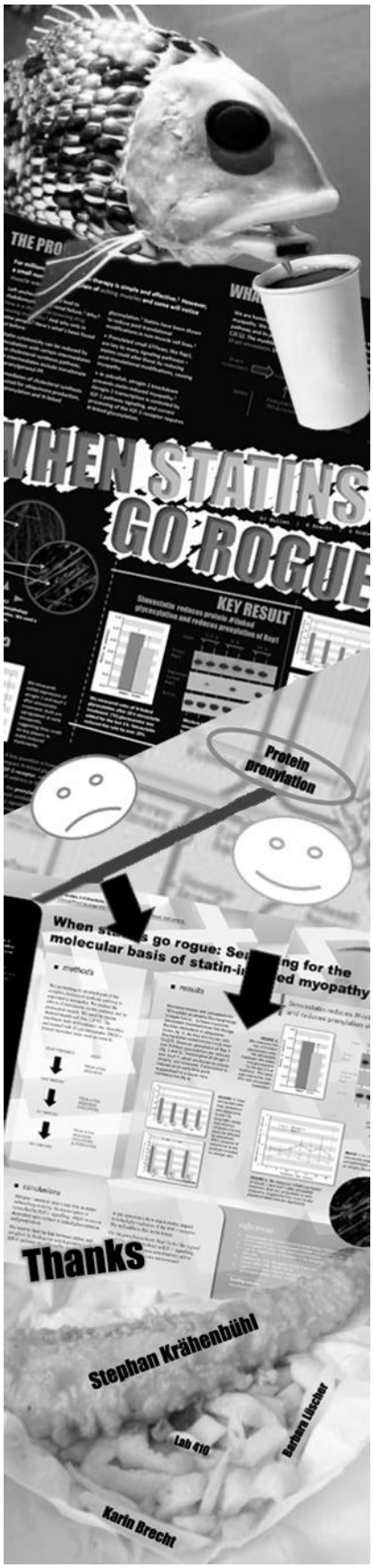
Master's Project (Distinction) *University of Manchester 03.2005 – 09.2005*

Working under the supervision of Prof. Bill Ollier, I identified polymorphisms in HLA and TNF-α that influenced longevity in a sample population from northern Spain. Techniques used included SnaPshot, HLA typing and patient database creation.

Education

University of Manchester 2004 – 2005
MSc Immunology and Immunogenetics

The Queen's College, Oxford University 1998 – 2002
MBiochem (2.2)



Peter James Mullen (cont.)

Publications, Meeting Abstracts, Awards

■ Mullen PJ, Lüscher B, Scharnagl H, Krähenbühl S, Brecht K: Effect of simvastatin on cholesterol metabolism in C2C12 myotubes and HepG2 cells, and consequences for statin-induced myopathy. *Biochem Pharmacol* 2010; **79:** 1200–1209.

■ Mullen PJ, Lüscher B, Scharnagl H, Krähenbühl S, Brecht K: Simvastatin reduces protein prenylation and *N*-linked glycosylation but has no effect on cellular cholesterol or ubiquionone content in C2C12 myotubes. Glycobiology 2009; **19:** 143. Annual Meeting of the Society for Glycobiology, San Diego, 2009.

■ Mullen PJ, Krähenbühl S, Brecht K: When statins go rogue. Annual Meeting of the Swiss Center for Applied Human Toxicology, Geneva, 2009.

■ Mullen PJ, Zahno A, Maseneni S, Krähenbühl S, Lindinger P: Simvastatin: The enemy within? European Muscle Conference, Padova, 2010. Received travel award from Seahorse Biosciences.

Manuscripts in Preparation

Provisional Titles

■ Mullen PJ, Zahno A, Maseneni S, Cassolla P, Lira E, Navegantes L, Kettelhut I, Brecht K, Krähenbühl S, Lindinger P: Simvastatin induces mitochondrial dysfunction and signs of atrophy in cardiomyocytes. Submitted to European Heart Journal

■ Mullen PJ, Zahno A, Lindinger P, Maseneni S, Krähenbühl S, Brecht K: Susceptibility to simvastatin-induced toxicity is determined by phosphorylation state of Akt.

■ Morand R, Todesco L, Donzelli M, Fischer-Barnicol D, Mullen PJ, Krähenbühl S: Effect of short- and long-term treatment with valproate on carnitine homeostasis in humans.

■ Setz C, Brand Y, Radojevic V, Hanusek C, Mullen PJ, Levano S, Bodmer D: Matrix metalloproteinases 2 and 9: Implications for aminoglycoside-induced auditory hair cell death.

I realize the importance of communicating well in science and have a track record of devising innovate and engaging posters and multimedia presentations. The images here have all been used by me in the last year.