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Susceptibility to simvastatin-induced toxicity is partly determined by mitochondrial respiration and phosphorylation state of Akt

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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 detect differences that could uncover why statins are toxic in skeletal muscle but less so in the liver. 10 μM simvastatin caused a decrease in mitochondrial respiration in the primary mouse myocytes and C2C12 myotubes, but had no effect in the HepG2 cells. Mitochondrial integrity is maintained by multiple signaling pathways. One of these pathways, Igf-1/Akt signaling, is also heavily implicated in causing statin-induced toxicity by upregulating atrogin-1. We found that 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 that disruption of Igf-1/Akt signaling is a causative factor in simvastatin-induced mitochondrial dysfunction in C2C12 myotubes, whereas HepG2 cells are protected by maintaining Igf-1/Akt signaling. We conclude that phosphorylation of Akt is a key indicator of susceptibility to statin-induced toxicity. How statins can disrupt Igf-1/Akt signaling 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 signaling. Transient transfection of constitutively active Rap1 into C2C12 myotubes led to a partial rescue of simvastatin-induced inhibition of mitochondrial respiration, providing a novel link between signaling and respiration.

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]. Their major site of action is the liver, where adverse effects are rare — elevated liver enzymes are sometimes observed, but are generally not considered to pose a risk [11,12]. We have previously shown 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 mechanisms of toxicity involve disrupting mitochondrial health, and upregulating atrophy-inducing proteins [13,14].

Studies in various tissues show that statins can affect the mitochondria directly, *via* inhibition of the respiratory chain complexes, or indirectly *via* lowering of ubiquinone levels [15,16]. We have already shown that statins can alter mitochondrial function in skeletal muscle [13]. 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 signaling pathways [17–19]. Some groups have suggested that impairment in cell signaling is a major cause of statin-induced myotoxicity [20–22]. 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 [23–26]. We therefore aim to discover whether any potential

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differences in the mitochondria of simvastatin-treated C2C12 myotubes and HepG2 cells can be explained by differences in Igf-1/Akt signaling.

Baviera et al. suggest that a geranylgeranylated protein, Rap1, could potentiate Igf-1/Akt signaling [27]. 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 signaling through the Igf-1/Akt pathway. Igf-1/Akt signaling is also vital in protecting skeletal muscle from atrophy, and statin-induced reductions in signaling lead to an increase in levels of the atrophy-inducing protein atrogin-1 in skeletal muscle [14]. This increase in atrogin-1 levels is reversible by the addition of compounds leading to geranylgeranylation (such as geranylgeranyl pyrophosphate, GGPP) [28]. We will therefore investigate if expression of constitutively active Rap1 can prevent the upregulation of atrogin-1 in C2C12 myotubes.

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

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. [29]. 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.

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 mouse myoblasts using a protocol modified from Springer et al. [30]. Briefly, we digested the skeletal muscle with sequential trypsinization 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 5000 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.

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.

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.

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 min and analyzed by flow cytometry, using a DAKO Cyan cytometer.

2.6. Measurement of mitochondrial membrane potential

We used tetramethylrhodamine, ethyl ester, perchlorate (TMRE; Invitrogen) to detect changes in mitochondrial membrane potential (ψm) [31]. Cells were incubated with simvastatin, then detached and incubated with 100 nM TMRE for 30 min 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 (10 μM) acted as a positive control.

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 labeled 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. The software quantified the amount of antibody staining that co-localized with the DAPI nuclear stain.

2.8. Measurement of oxygen consumption

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 min at 37 °C in a CO_2-free incubator, before being transferred to the XF24 analyzer. We measured basal oxygen consumption (OCR), and then sequentially injected 1 μM oligomycin, FCCP (1 μM for the HepG2 cells, and 10 μM

for the C2C12 myotubes and isolated mouse leg myotubes), and 1 μM rotenone to assess maximal oxidative capacity.

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 commercialized by Miltenyi Biotec (Germany) [32]. 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.

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-labeled anti-rabbit antibody, and chemiluninescence substrate (GE Healthcare) were used for analysis.

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′-CCTGCTTCAC-CACCTTCTTGA-3′), and no-template and no-reverse-transcription controls ensured non-specific amplification could be excluded.

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.

3. Results

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 [6]. 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% ($p<0.05$, 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.

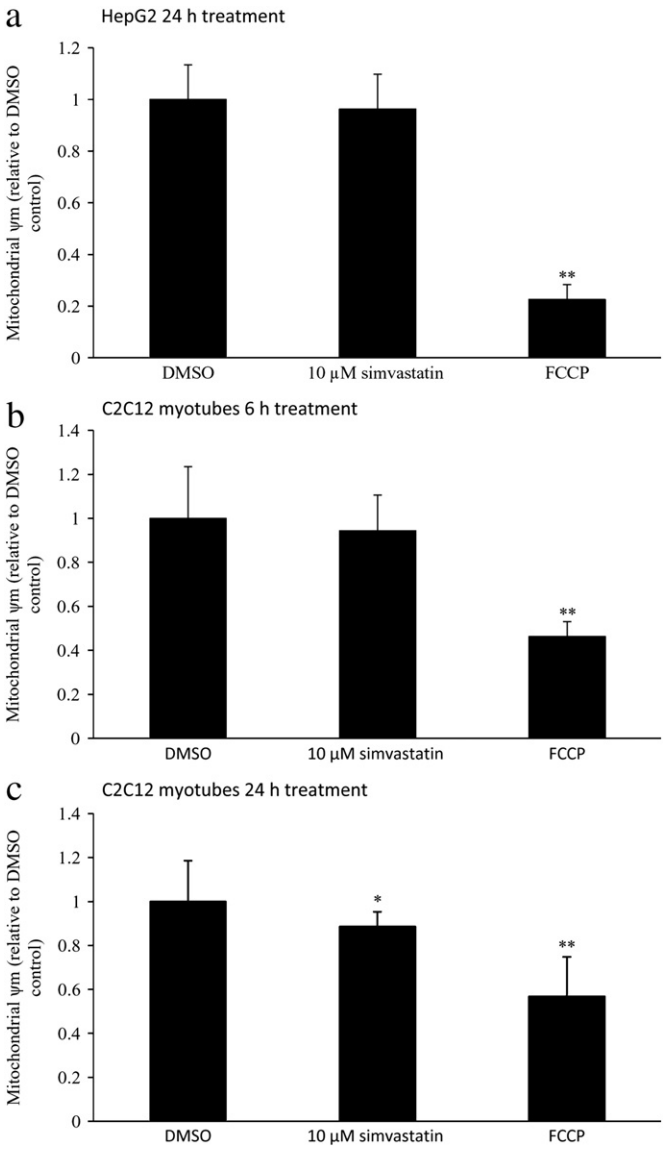


Fig. 1. Effect of simvastatin on mitochondrial ψm in HepG2 cells and C2C12 myotubes. Cells were incubated with DMSO or 10 μM simvastatin. (a) HepG2 cells were treated for 24 h. C2C12 myotubes were treated for (b) 6 or (c) 24 h. TMRE was used to determine mitochondrial membrane potential, and Near-IR 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.

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 in simvastatin-treated intact C2C12 myotubes and HepG2 cells. OCRs provided a measurement of the activity of the electron transport chain (ETC). 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% ($p<0.01$ 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.

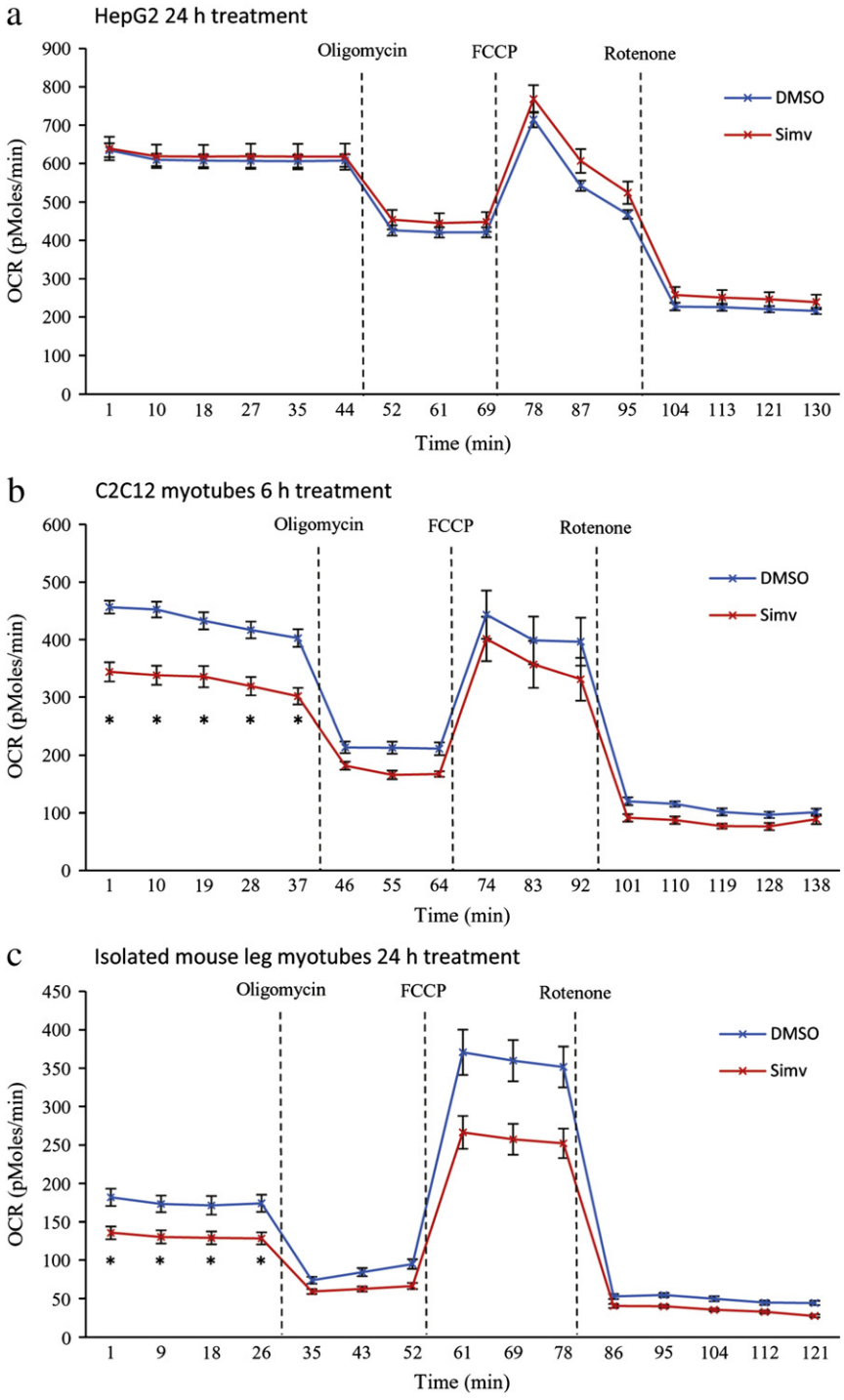


Fig. 2. Effect of simvastatin on oxygen consumption in intact HepG2 cells, C2C12 myotubes and isolated mouse leg myotubes. Cells were incubated with DMSO (blues lines) or 10 μM (red lines) simvastatin. The XF24 analyzer was used to take measurements. OCRs are shown for (a) 24 h treatment in HepG2 cells, (b) 6 h treatment in C2C12 myotubes and (c) 24 h treatment in isolated mouse leg myotubes. 1 μM oligomycin, FCCP (1 μM for the HepG2 cells, and 10 μM for the C2C12 myotubes and isolated mouse leg myotubes), and 1 μM rotenone were added at the indicated points. Results are indicative of three independent experiments. *$p<0.05$ versus control.

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%, $p<0.01$; Fig. 2c). This offers further evidence that simvastatin impairs skeletal muscle mitochondrial function.

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 13.4% $p<0.01$ and 12.2% $p<0.01$, respectively; Fig. 3).

3.5. Statin-treatment leads to a decrease in Akt phosphorylation in C2C12 myotubes, but not in HepG2 cells

Statins are widely reported to affect the Igf-1/Akt signaling pathway. This pathway is also important in maintaining mitochondrial health — we therefore investigated if differences in this pathway could explain the differing susceptibility to simvastatin in HepG2 cells and C2C12 myotubes. HepG2 treated with 10 μM simvastatin for 24 h saw no change in Akt phosphorylation (Fig. 4a). C2C12 myotubes had a reduced level of Akt phosphorylation after 6 h treatment with 10 μM simvastatin (C2C12-mock; Fig. 4b).

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. 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. 4a). 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).

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 with 10 μM simvastatin showed a significant increase in the number of early apoptotic cells when compared to DMSO control (by 2.43-fold, $p<0.05$; Fig. 4e). Co-incubation of 10 ng/ml 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, $p<0.05$; Fig. 4e). The coincubation of 10 ng/ml Igf-1 with 10 μM simvastatin also increased mitochondrial respiration in C2C12 myotubes after 24 h treatment (Fig. 4f).

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 signaling to Igf-1/Akt signaling [27]. Our own lab has also already shown that simvastatin reduces Rap1 prenylation. We hypothesized that reduction in Rap1 activity could therefore lead to a reduction in signaling 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.

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 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

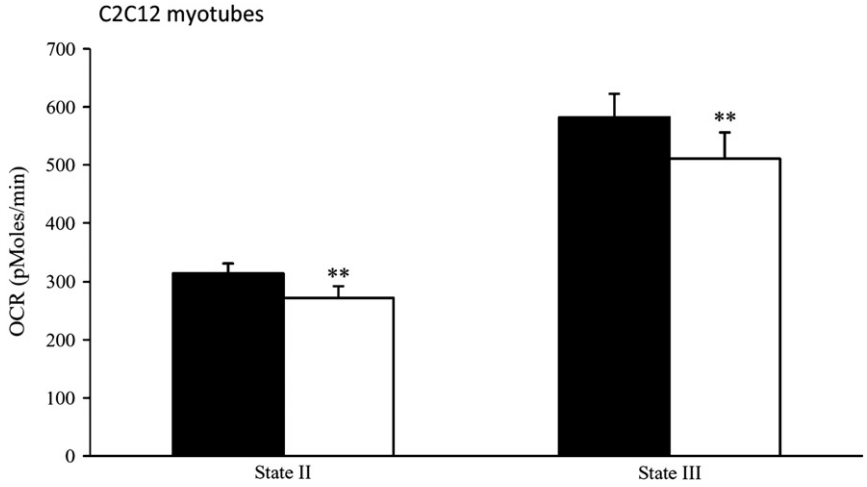


Fig. 3. Effect of simvastatin on oxygen consumption in mitochondria isolated from C2C12 myotubes. Cells were incubated with DMSO (black bars) or 10 μM simvastatin (white bars) for 6 h, mitochondria isolated, and the XF24 analyzer was used to measure oxygen consumption at state II and state III with succinate as the substrate. Results are indicative of four independent experiments carried out in quintuplicate. **$p<0.01$ versus DMSO control.

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.

4. Discussion

Statins are widely reported to cause side-effects in skeletal muscle, ranging from mild weakness to severe rhabdomyolysis [33–35]. Their major site of action is the liver, where they are well-tolerated and not toxic. Raised liver enzyme levels are sometimes observed, but they are not thought to be a risk [11,12]. 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 [13,36,37]. 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. 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, as explained above, especially when combined with inhibitors of cytochrome P450 or OATP1B1 [38].

Numerous signaling pathways are known to be important in maintaining mitochondrial health, and statins are also known to dysregulate a wide range of signaling pathways such as AMPK, JNK and Igf-1 signaling [39–41]. We therefore decided to investigate whether differences in cell signaling could explain the difference in mitochondrial respiration in simvastatin-treated HepG2 cells and C2C12 myotubes. We chose the Igf-1/Akt signaling pathway as it is both well-described to be altered by statin treatment in muscle and play a role in mitochondrial maintenance [23–26].

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 [42]. 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 [28]. Further weight is added by the study of Mallinson et al., who saw similar reductions in Akt phosphorylation in simvastatin-treated Wistar rats [43].

This difference could reflect a difference in the activity of the Igf-1/Akt signaling 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.

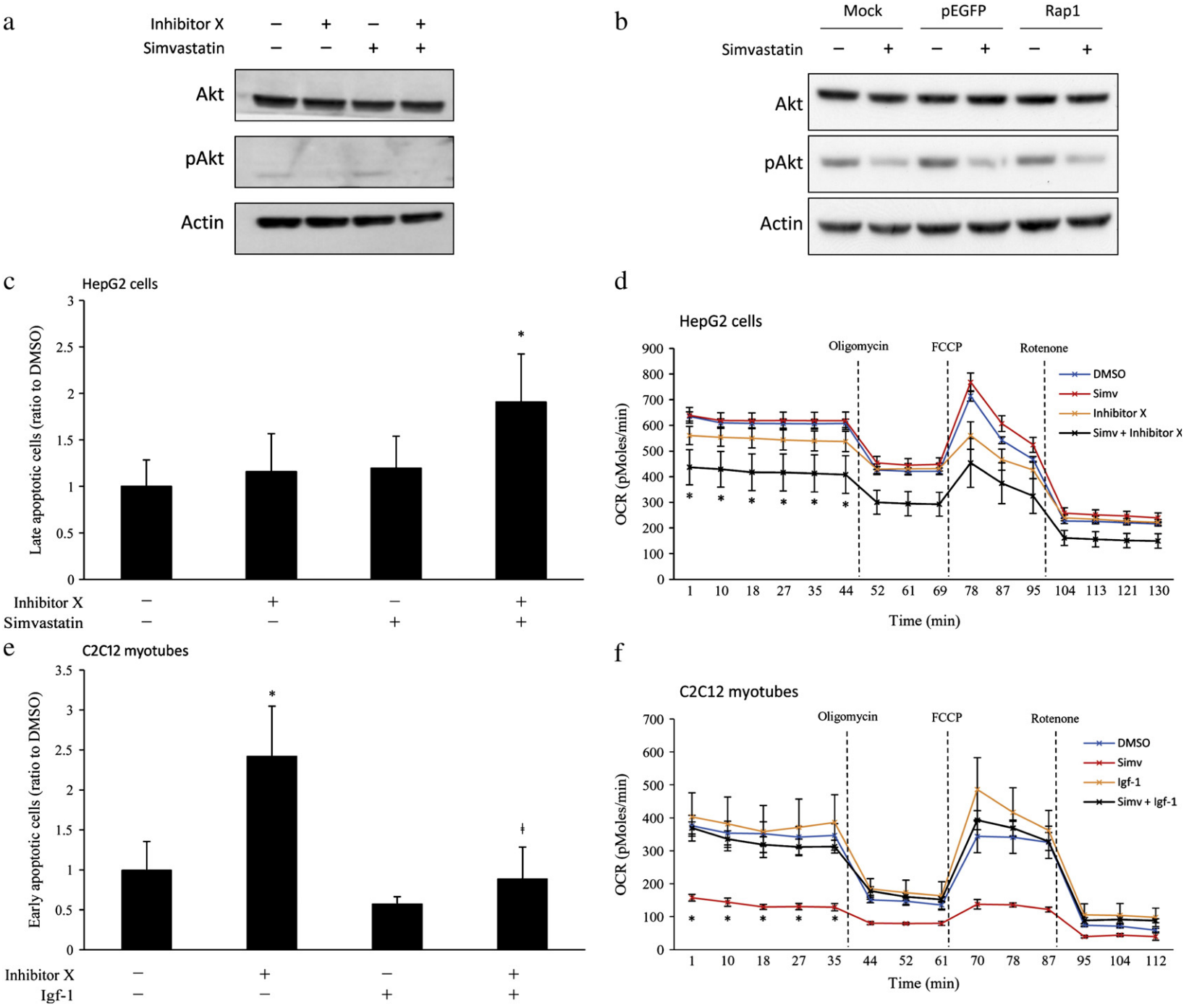
To test our theory, we inhibited Akt activity in HepG2 cells, and activated Akt in C2C12 myotubes. Coincubation of simvastatin with Akt inhibitor X in HepG2 cells led to a lowering of mitochondrial respiration and cell viability. This suggests that the ability of HepG2 cells to increase Akt phosphorylation 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 signaling in statin-induced myotoxicity, and adds to this knowledge by showing that Igf-1/Akt signaling can rescue mitochondrial function in simvastatin-treated cells [14,28]. 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 as Igf-1 is not known to bind to complexes of the ETC (although no studies have investigated whether Igf-1 could inhibit transport of simvastatin in mitochondria — which would not rule out direct inhibition).

It is so far unknown how statins can reduce Igf-1/Akt signaling and lead to toxicity. Previous work, including studies from our own lab, shows 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 [5,44,45]. GGPP is important for the geranylgeranylation of proteins, and many geranylgeranylated proteins are required for cell signaling. Our previous work shows that Rap1, a small GTPase, has reduced geranylgeranylation after simvastatin treatment in C2C12 myotubes [6]. Other groups show that Rap1 potentiates Igf-1 signaling, and Baviera et al. have postulated that Rap1 links cAMP signaling to Igf-1/Akt signaling [27]. 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 [46]. Others also show that Rap1 can be found in mitochondria [47,48]. cAMP is also located in mitochondria, and thought to regulate mitochondrial function [49,50]. 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

Fig. 4. The influence of Igf-1/Akt signaling on simvastatin-induced mitochondrial toxicity in HepG2 cells and C2C12 myotubes. Immunoblots for total and phosphorylated Akt in (a) HepG2 cells treated for 24 h with 10 μM simvastatin and/or 5 μM Akt inhibitor X, and (b) transfected C2C12 myotubes treated for 6 h with 10 μM simvastatin. 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 10 μM simvastatin and/or 5 μM 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) A comparison of OCRs in HepG2 cells treated with 10 μM simvastatin and/or 5 μM 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 10 μM simvastatin and/or 10 ng/ml 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) A comparison of OCRs in C2C12 myotubes treated with 10 μM simvastatin and/or 10 ng/ml 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.


a
Inhibitor X
Simvastatin
Akt
pAkt
Actin
b
Mock
pEGFP
Rap1
Simvastatin
Akt
pAkt
Actin
c
HepG2 cells
Late apoptotic cells (ratio to DMSO)
Inhibitor X
Simvastatin
d
HepG2 cells
Oligomycin
FCCP
Rotenone
DMSO
Simv
Inhibitor X
Simv + Inhibitor X
OCR (pMoles/min)
Time (min)
e
C2C12 myotubes
Early apoptotic cells (ratio to DMSO)
Inhibitor X
Igf-1
f
C2C12 myotubes
Oligomycin
FCCP
Rotenone
DMSO
Simv
Igf-1
Simv + Igf-1
OCR (pMoles/min)
Time (min)

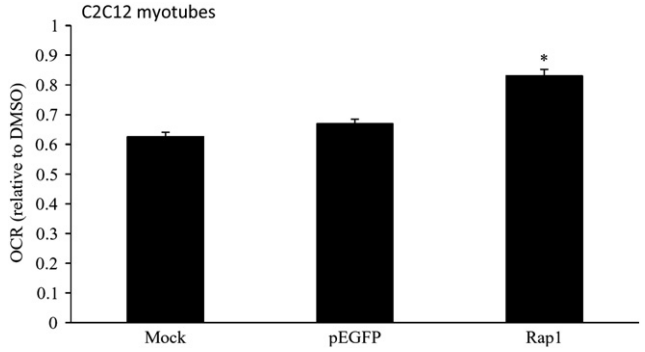


Fig. 5. The effect of expressing constitutively active Rap1 on mitochondrial toxicity in C2C12 myotubes. C2C12-mock, C2C12-pEGFP and C2C12-Rap1 myotubes were treated with DMSO or 10 μM simvastatin for 6 h. OCR was measured on a XF24 analyzer. Results are indicative of three independent experiments carried out in quintuplicate. *$p<0.05$ versus C2C12-mock.

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.

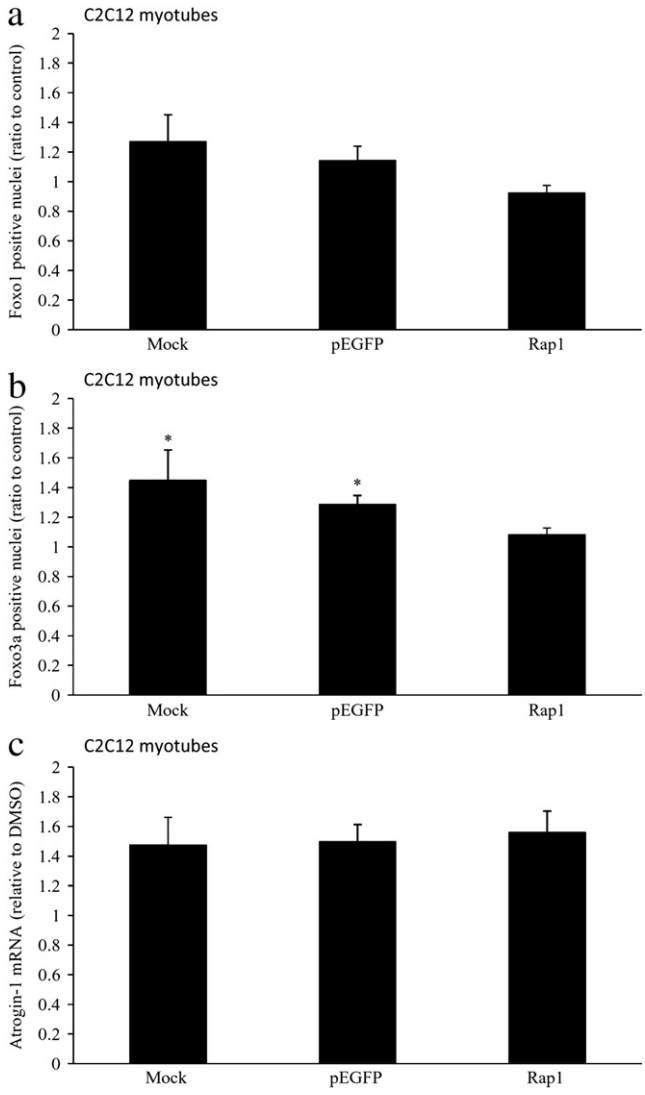


Fig. 6. The effect of expressing constitutively active Rap1 on atrogin-1 induction in C2C12 myotubes. (a) Co-localization of Foxo1 with nuclei. Results are the mean of 3 independent experiments. (b) Co-localization of Foxo3a with nuclei. (c) Atrogin-1 mRNA levels were also measured in transfected C2C12 myotubes. The results are means of three independent experiments. *$p<0.05$ versus DMSO control.

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 signaling. We aim to further investigate the role of Rap1 in the Igf-1/Akt signaling pathway by increasing this efficiency.

Our data has, for the first time, provided a potential explanation for the difference in susceptibility to toxicity between liver HepG2 cells and skeletal muscle C2C12 myotubes. The effect of statins on Akt phosphorylation is 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 signaling, and a potential partial explanation for simvastatin-induced toxicity.

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References

[1] Scandinavian Simvastatin Survival Study Group, Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S), Lancet 344 (1994) 1383–1389.
[2] M. Endres, U. Laufs, Z. Huang, T. Nakamura, P. Huang, M.A. Moskowitz, J.K. Liao, Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8880–8885.
[3] M.S. Brown, J.R. Faust, J.L. Goldstein, I. Kaneko, A. Endo, Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase, J. Biol. Chem. 253 (1978) 1121–1128.
[4] S. Matzno, S. Yasuda, S. Juman, Y. Yamamoto, N. Nagareya-Ishida, K. Tazuya-Murayama, T. Nakabayashi, K. Matsuyama, Statin-induced apoptosis linked with membrane farnesylated Ras small G protein depletion, rather than geranylated Rho protein, J. Pharm. Pharmacol. 57 (2005) 1475–1484.
[5] A. Dricu, M. Wang, M. Hjertman, M. Malec, H. Blegen, J. Wejde, M. Carlberg, O. Larsson, Mevalonate-regulated mechanisms in cell growth control: role of dolichyl phosphate in expression of the insulin-like growth factor-1 receptor (Igf-1R) in comparison to Ras prenylation and expression of c-myc, Glycobiology 7 (1997) 625–633.
[6] P.J. Mullen, B. Lüscher, H. Scharnagl, S. Krähenbühl, K. Brecht, Effect of simvastatin on cholesterol metabolism in C2C12 myotubes and HepG2 cells, and consequences for statin-induced myopathy, Biochem. Pharmacol. 79 (2010) 1200–1209.
[7] J. Davignon, Beneficial cardiovascular pleiotropic effects of statins, Circulation 109 (2004) 39–43.
[8] J. Li, Y.M. Sun, L.F. Wang, Z.Q. Li, W. Pan, H.Y. Cao, Comparison of effects of simvastatin versus atorvastatin on oxidative stress in patients with coronary heart disease, Clin. Cardiol. 33 (2010) 222–227.
[9] J.A. Staffa, J. Chang, L. Green, Cerivastatin and reports of fatal rhabdomyolysis, N. Engl. J. Med. 346 (2002) 539–540.
[10] C.M. Ballantyne, A. Corsini, M.H. Davidson, H. Holdaas, T.A. Jacobson, E. Leiterdorf, W. März, J.P. Reckless, E.A. Stein, Risk for myopathy with statin therapy in high-risk patients, Arch. Intern. Med. 163 (2003) 553.
[11] N. Chalasani, H. Aljadhey, J. Kesterson, M.D. Murray, S.D. Hall, Patients with elevated liver enzymes are not at higher risk for statin hepatotoxicity, Gastroenterology 125 (2004) 1287–1292.
[12] R.M. Guthrie, D.R. Martin, The safety of Rosuvastatin: effects on renal and hepatic function, Expert. Opin. Drug. Saf. 6 (2007) 573–581.
[13] P. Kaufmann, M. Török, A. Zahno, K.M. Waldhauser, K. Brecht, S. Krähenbühl, Toxicity of statins on rat skeletal muscle mitochondria, Cell. Mol. Life Sci. 63 (2006) 2415–2425.
[14] P. Cao, J. Hanai, P. Tanksale, S. Imamura, V.P. Sukhatme, S.H. Lecker, Statin-induced muscle damage and atrogin-1 induction is the result of a geranylgeranylation defect, FASEB J. 23 (2009) 2844–2854.
[15] K. Folkers, P. Langsjoen, R. Willis, P. Richardson, L.J. Xia, C.Q. Te, Lovastatin decreases coenzyme Q levels in humans, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 8931–8934.
[16] S. Nadanaciva, J.A. Dykens, A. Bernal, R.A. Capaldi, Y. Will, Mitochondrial impairment by PPAR agonists and statins identified *via* immunocaptured OXPHOS complex activities and respiration, Toxicol. Appl. Pharmacol. 223 (2007) 277–287.

[17] A. Feliciello, M.E. Gottesman, E.V. Awedimento, cAMP-PKA signaling to the mitochondria: protein scaffolds, mRNA and phosphatases, Cell. Signal. 17 (2005) 279–287.
[18] A. Rasola, M. Sciacovelli, F. Chiara, B. Pantic, W.S. Brusilow, P. Bernadi, Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 726–731.
[19] N. Hanawa, M. Shinohara, B. Saberi, W.A. Gaarde, D. Han, N. Kaplowitz, Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury, J. Biol. Chem. 283 (2008) 13565–13577.
[20] R. Fukuyama, T. Fujita, Y. Azuma, A. Hirano, H. Nakamuta, M. Koida, T. Komori, Statins inhibit osteoblast migration by inhibiting Rac-Akt signaling, Biochem. Biophys. Res. Commun. 315 (2004) 636–642.
[21] Y. Sekine, Y. Furuya, M. Nishii, H. Koike, H. Matsui, K. Suzuki, Simvastatin inhibits the proliferation of human prostate cancer PC-3 cells *via* down-regulation of the insulin-like growth factor 1 receptor, Biochem. Biophys. Res. Commun. 372 (2008) 356–361.
[22] L. Girnita, M. Wang, Y. Xie, G. Nilsson, A. Dricu, J. Wejde, O. Larsson, Inhibition of N-linked glycosylation down-regulates insulin-like growth factor-1 receptor at the cell surface and kills Ewing's sarcoma cells: therapeutic implications, Anticancer Drug Des. 15 (2000) 67–72.
[23] L. del Peso, M. González-García, C. Page, R. Herrera, G. Nuñez, Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt, Science 278 (1997) 687–689.
[24] A. Parcellier, L.A. Tintignac, E. Zhuravleva, B.A. Hemmings, PKB and the mitochondria: AKTing on apoptosis, Cell. Signal. 20 (2008) 21–30.
[25] S.J. Gardai, D.A. Hildeman, S.K. Frankel, B.B. Whitlock, S.C. Frasch, N. Borregaard, P. Marrack, D.L. Bratton, P.M. Henson, Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils, J. Biol. Chem. 279 (2004) 21085–21095.
[26] N. Majewski, V. Nogueira, R.B. Robey, N. Hay, Akt inhibits apoptosis downstream of BID cleavage *via* a glucose-dependent mechanism involving mitochondrial hexokinases, Mol. Cell. Biol. 24 (2004) 730–740.
[27] A.M. Baviera, N.M. Zanon, L.C. Navegantes, I.C. Kettelhut, Involvement of cAMP/Epac/PI3K-dependent pathway in the antiproteolytic effect of epinephrine on rat skeletal muscle, Mol. Cell. Endocrinol. 315 (2010) 104–112.
[28] J. Hanai, P. Cao, P. Tanksale, S. Imamura, E. Koshimizu, J. Zhao, S. Kishi, M. Yamashita, P.S. Phillips, V.P. Sukhatme, S.H. Lecker, The muscle-specific ubiquitin ligase atrogin-1/MAFbx mediates statin-induced muscle toxicity, J. Clin. Invest. 117 (2007) 3940–3951.
[29] K. Bogman, A.K. Peyer, M. Török, E. Kusters, J. Drewe, HMG-CoA reductase inhibitors and P-glycoprotein modulation, Br. J. Pharmacol. 132 (2001) 1183–1192.
[30] G. von Degenfeld, A. Banfi, M.L. Springer, H.M. Blau, Myoblast-mediated gene transfer for therapeutic angiogenesis and arteriogenesis, Br. J. Pharmacol. 140 (2003) 620–626.
[31] E. Gottlieb, S.M. Armour, M.H. Harris, B. Thompson, Mitochondrial membrane potential regulates matrix configuration and cytochrome *c* release during apoptosis, Cell Death Differ. 10 (2003) 709–717.
[32] H.T. Hornig-Do, G. Günther, M. Bust, P. Lehnartz, A. Bosio, R.J. Wiesner, Isolation of function pure mitochondria by supramagnetic microbeads, Anal. Biochem. 389 (2009) 1–5.
[33] P.D. Thompson, J. Clarkson, R.H. Karas, Statin-associated myopathy, J. Am. Med. Assoc. 289 (2003) 1681–1690.
[34] D.J. Graham, J.A. Staffa, D. Shatin, S.E. Andrade, S.D. Schech, L. La Grande, Incidence of hospitalised rhabdomyolysis in patients treated with lipid-lowering drugs, J. Am. Med. Assoc. 292 (2004) 2585–2590.
[35] F. Charatan, Bayer decides to withdraw cholesterol lowering drug, BMJ 323 (2001) 359.
[36] M. Werner, J. Sacher, M. Hohenegger, Mutual amplification of apoptosis by statin-induced mitochondrial stress and doxorubicin toxicity in human rhabdomyosarcoma cells, Br. J. Pharmacol. 143 (2004) 715–724.
[37] Y.L. Dai, T.H. Luk, C.W. Siu, K.H. Yiu, H.T. Chan, S.W. Lee, S.W. Li, S. Tam, B. Fong, C.P. Lau, H.F. Tse, Mitochondrial dysfunction induced by statin contributes to endothelial dysfunction in patients with coronary artery disease, Cardiovasc. Toxicol. 10 (2010) 130–138.
[38] P. Sirvent, J. Mercier, G. Vassort, A. Lacampagne, Simvastatin triggers mitochondria-induced Ca^{2+} signalling alteration in skeletal muscle, Biochem. Biophys. Res. Commun. 329 (2005) 1067–1075.
[39] W. Sun, T.S. Lee, M. Zhu, C. Gu, Y. Wang, Y. Zhu, J.Y. Shyy, Statins activate AMP-activated protein kinase *in vitro* and *in vivo*, Circulation 32 (2009) 675–679.
[40] O. Fromigué, Z. Hamidouche, P.J. Marie, Blockade of the RhoA-JNK-c-Jun-MMP2 cascade by atorvastatin reduces osteosarcoma cell invasion, J. Biol. Chem. 45 (2008) 30549–30556.
[41] T. Ogura, Y. Tanaka, T. Nakata, T. Namikawa, H. Kataoka, Y. Ohtsubo, Simvastatin reduces insulin-like growth factor-1 signaling in differentiating C2C12 myoblast cells in an HMG-CoA reductase inhibition-independent manner, J. Toxicol. Sci. 32 (2007) 57–67.
[42] E. Roudier, O. Mistafa, U. Stenius, Statins induce mammalian target of rapamycin (mTOR)-mediated inhibition of Akt signaling and sensitizes p53-deficient cells to cytostatic drugs, Mol. Cancer Ther. 5 (2006) 2706–2715.
[43] J.E. Mallinson, D. Constantin-Teodosiu, J. Sidaway, F.R. Westwood, P.L. Greenhaff, Blunted Akt/FOXO signalling and activation of genes controlling atrophy and fuel use in statin myopathy, J. Physiol. 587 (2009) 219–230.
[44] S.R. Lalani, G.D. Vladutiu, K. Plunkett, T.E. Lotze, A.M. Adesina, F. Scaglia, Isolated mitochondrial myopathy associated with muscle coenzyme Q10 deficiency, Arch. Neurol. 62 (2005) 317–320.
[45] S. Clarke, Protein isoprenylation and methylation at carboxy-terminal cysteine residues, Annu. Rev. Biochem. 61 (1992) 355–386.
[46] J. Qiao, F.C. Mei, V.L. Popov, L.A. Vergara, X. Cheng, Cell cycle-dependent subcellular localization of exchange factor activated by cAMP, J. Biol. Chem. 277 (2002) 26581–26586.
[47] S. Kim, A. Mizoguchi, A. Kikuchi, Y. Takai, Tissue and subcellular distributions of the smg-21/rap1/Krev-1 proteins which are partly distinct from those of c-ras p21s, Mol. Cell. Biol. 10 (1990) 2645–2652.
[48] M. Thomson, What are guanosine triphosphate-binding proteins doing in mitochondria, Biochem. Biophys. Acta 1403 (1998) 211–218.
[49] S. Sulimovici, B. Lunenfeld, Effect of gonadotrophins on adenylate cyclase of the outer and inner membrane subfractions of rat testis mitochondria, FEBS Lett. 41 (1974) 345–347.
[50] A.S. Fine, R.W. Egnor, E. Forrester, S.S. Stahl, Adenylate cyclase localization in unfixed specimens of rat oral mucosa and isolated mitochondria, J. Histochem. Cytochem. 30 (1982) 1171–1178.