

mTORC2 is the Primary Target for Simvastatin-Associated Toxicity in C2C12 Cells and in Mouse Skeletal Muscle

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Research Article

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Abstract

Statins decrease the serum LDL-cholesterol concentration and reduce the risk for cardiovascular diseases but can cause myopathy, which may be related to mTORC inhibition. In the current study, we investigated which mTORC is inhibited by simvastatin and by which mechanisms. In C2C12 myoblasts and myotubes and mouse gastrocnemius, simvastatin was cytotoxic and inhibited S6rp and Akt Ser473 phosphorylation, indicating inhibition of mTORC1 and mTORC2, respectively. In contrast to simvastatin, the mTORC1 inhibitor rapamycin did not inhibit mTORC2 and was not cytotoxic. Like simvastatin, knock-down of Rictor, an essential component of mTORC2, impaired Akt Ser473 and S6rp phosphorylation and was cytotoxic for C2C12 myoblasts, suggesting that mTORC2 inhibition is an important myotoxic mechanism. The investigation of the mechanism of mTORC2 inhibition showed that simvastatin impaired Ras farnesylation, which was prevented by farnesol but without restoring mTORC2 activity. In comparison, Rap1 knock-down reduced mTORC2 activity and was cytotoxic for C2C12 myoblasts. Simvastatin impaired Rap1 prenylation and function, which was prevented by geranylgeraniol. In addition, simvastatin and the complex III inhibitor antimycin A caused mitochondrial superoxide accumulation and decreased the activity of mTORC2, which could partially be prevented by the antioxidant MitoTEMPO. In conclusion, mTORC2 inhibition is an important mechanism of simvastatin-induced myotoxicity. Simvastatin inhibits mTORC2 by impairing geranylgeranylation of Rap1 and by inducing mitochondrial dysfunction.

Introduction

Statins are lipid-lowering drugs that inhibit a key enzyme in the mevalonate pathway, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. They exert their effects in the liver, where they inhibit the *de novo* formation of cholesterol [1], which is associated with activation of SREBP-2 and increased expression of LDL-cholesterol receptors on the surface of hepatocytes [2]. Statins are used for the treatment of hypercholesterolemia and are associated with decreased mortality and morbidity due to cardiovascular diseases [3]. However, inhibition of the HMG-CoA reductase not only impairs the biosynthesis of cholesterol, but also the production of intermediates of cholesterol synthesis such as isoprenoids, which are important for protein prenylation and proper cell function.

Statins are effective drugs and are in general well tolerated. However, they can cause adverse reactions mainly affecting skeletal muscle with clinical manifestations ranging from myalgia to potentially fatal rhabdomyolysis [4–6]. Statin-induced myopathy affects up to 30% of the patients treated with these drugs, depending on the definition of myopathy [3; 7; 8]. Recently, new-onset diabetes has been reported as an adverse reaction of statins and the prevalence of patients affected is estimated up to 30% [9; 10].

Several theories are proposed to explain statin-associated myopathy, comprising defects of protein prenylation, mitochondrial dysfunction, induction of muscle atrophy and/or apoptosis [11]. Work from us and from other research groups revealed the insulin receptor/Akt/mTOR signaling pathway inhibition as an important contributor to statin-induced myopathy [12–16]. For instance, we recently reported that

simvastatin induces insulin resistance and decreases glucose uptake into skeletal muscle. We showed that this is a consequence of the inhibition of Akt activation in myocytes, leading to reduced GLUT4 translocation to the cell membrane and impaired glucose absorption [15].

Thus, Akt inhibition appears to be a key event in the induction of muscle damage by statins. Akt is a serine/threonine kinase that regulates growth, apoptosis and atrophy in skeletal muscle [17; 18]. For full activation, Akt needs to be phosphorylated on two residues; on threonine 308, which is performed by the kinase PDK1 downstream of the insulin receptor, and on serine 473, which is realized by the protein complex mTORC2 [19]. We demonstrated that Akt serine 473 phosphorylation was reduced in C2C12 myotubes and mice treated with simvastatin, indicating impaired activity of mTORC2 [12–15].

mTOR is a serine/threonine kinase involved in cell growth regulation. It is a component of two distinct multiprotein complexes: mTORC1, which is rapamycin-sensitive and mTORC2, which was identified as rapamycin-insensitive [20–24]. mTORC1 is activated by nutrients and growth factors and promotes protein synthesis through phosphorylation of the kinase S6rp and the elongation factor 4-EBP1 [25; 26]. Akt activates mTORC1 indirectly by phosphorylating and thereby inhibiting the tuberous sclerosis 1/2 (TSC1/2) complex, which leads to stimulation of the small GTPase Rheb and activation of mTORC1 [27–30]. mTORC2 is activated by growth factors [31–34] and Akt, which can phosphorylate the mTORC2 components Rictor and mSin1 [35]. mTORC2 regulates cytoskeleton remodeling, cellular proliferation as well as glucose uptake and metabolism [36].

Simvastatin inhibits both mTORC1 and mTORC2 in myocytes, as illustrated by decreased protein synthesis due to impaired phosphorylation of S6rp and 4-EBP1 and impaired phosphorylation of Akt Ser473 [12–15]. We have also indications that simvastatin inhibits the signaling between the insulin receptor and Akt, which consequently affects Akt activity and downstream pathways [15]. Moreover, we showed that mTOR Ser2448 phosphorylation was inhibited by simvastatin [15]. However, since mTORC2 is needed for full mTORC1 activation, the contribution of the inhibition of mTORC1 and mTORC2 to the myotoxicity of simvastatin could so far not be differentiated.

Considering the strong effect of simvastatin on the phosphorylation of Akt Ser473 [12–15], we hypothesized that mTORC2 inhibition is the primary event of simvastatin-induced myotoxicity. To challenge this hypothesis, we conducted the current study aiming to assess the individual contribution of mTORC1 and mTORC2 to simvastatin-induced myotoxicity. For that, we compared the effects of the specific mTORC1 inhibitor rapamycin and of mTORC2 knockdown with those of simvastatin on C2C12 cells. Using this approach, we could demonstrate that mTORC1 inhibition did not explain simvastatin-induced toxicity, whereas abrogation of mTORC2 led to similar effects on C2C12 cells than treatment with simvastatin. Finally, we could show that impaired prenylation of the small GTPase Rap1 and oxidative stress are the main mechanisms by which simvastatin inhibits mTORC2 in C2C12 cells.

Materials And Methods

Chemicals

Simvastatin lactone (Sigma-Aldrich, St-Louis, USA) was converted to the active acid form by hydrolysis [37] for cell experiments. Stock solutions were obtained after dilution in DMSO (Sigma-Aldrich, USA) and stored at -20°C. Human insulin was purchased from Sigma-Aldrich, stored at 4°C and stock solutions were prepared in medium. Rapamycin was purchased from Sigma-Aldrich and diluted in DMSO. Mevalonolactone (MEVA) (Sigma-Aldrich, USA) was diluted in water and stocks were stored at -20°C. Farnesol (FOH) was purchased from Sigma-Aldrich (Sigma-Aldrich, USA) and stock solutions were prepared in DMSO. Geranylgeraniol (GGOH) was purchased from Sigma-Aldrich and stock solutions were prepared in ethanol. MitoTEMPO (MITOT.) was obtained from Sigma-Aldrich and stocks were obtained after dilution in water. Antimycin A (Ant.A) (Sigma-Aldrich, St-Louis, USA) stock solutions were obtained after preparation in ethanol.

Animal experiments

The animal experiments were accepted by the cantonal veterinary authority of Basel, Switzerland (License 3035) and were conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Experiments were done on male C57BL/6J mice (n = 30) with an age of 18–23 weeks and a bodyweight of 29–35 g. They were acclimatized one week before the start of the study and housed in a standard facility with 12h light-dark cycles and controlled temperature (21–22°C). The mice had free access to a standard pellet chow diet and water ad libitum.

Mice were randomly divided into four groups (n = 6 per group for control mice [Ctrl], and mice treated with 5 and 10 mg/kg simvastatin per day, and n = 12 per for mice treated with 25 mg/kg per day). Control mice were treated with vehicle (saline containing 8.3% EtOH:Tween80 [1:1, v/v]) for 21 days. Groups 2 to 4 were treated with 5, 10, or 25 mg/kg simvastatin per day (Sigma-Aldrich, St-Louis, USA) dissolved in saline containing 8.3% EtOH:Tween80 (1:1, v/v) [38]. Mice were treated once a day by oral gavage. Food and water consumption, and body weight changes were monitored daily during the study.

After 21 days of treatment, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg, Graeb AG, Switzerland) and xylazine (20 mg/kg, Graeb AG, Switzerland). Gastrocnemius muscle was collected immediately after anesthesia, weighed, and quickly frozen in isopentane cooled by liquid nitrogen and stored at -80°C for later analysis. We separated the superficial part of the gastrocnemius and quadriceps muscle (mostly glycolytic, with a low mitochondrial content) from their deep parts (oxidative-type muscle with a high mitochondrial content) macroscopically [39; 40].

Cell cultures

C2C12 myoblasts (American Type Culture Collection, USA) were kindly provided by Novartis (Basel, Switzerland). Cells were cultured in Dulbecco's Modified Eagle Medium – GlutaMAX supplemented with 10% fetal bovine serum (FBS) and 1% HEPES (Gibco, UK). Cells were maintained at 37°C in a humidified

5% CO₂ cell culture incubator. Cells were passaged using trypsin upon reaching approximately 60% confluence and seeded in appropriate well plates prior differentiation into myotubes.

To obtain myotubes, three days after seeding, medium was replaced by differentiation medium (DM) containing DMEM-Glutamax and 1% HEPES supplemented with 2% horse serum (Gibco, UK) and 0.03 % insulin (stock: 10mg/mL). Three additional days later, medium was changed for differentiation medium without insulin. Myotubes were then treated for 24 hours with compounds of interest in serum-free differentiation medium.

Membrane toxicity and cellular ATP content

As described previously [15], to assess membrane integrity, cells (5'000 cells/well) were seeded in a 96-well plate and treated for 24 hours with compounds of interest. Membrane toxicity was determined using the ToxiLight™ (Lonza, Basel, Switzerland) assay [41]. The metabolic activity of cells was determined with the quantification of intracellular ATP using the CellTiterGlo Luminescent cell viability assay (Promega, Switzerland), in accordance with the manufacturer's instructions. 0.1 % DMSO was used as negative control, and 1% Triton-X 100 was used as positive control for toxicity assays. Luminescence was measured with a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland).

Western blots

Western blotting was carried out as described in [15]. Briefly, cells were grown and differentiated on 6-well culture plates and treated with the test compounds for 24 hours. After treatment, they were washed twice with cold PBS (Gibco, UK) and lysed in Phosphosafe buffer (EMD Millipore, USA) for 5 minutes on ice. Cell lysates were centrifuged at 1'600 g for 10 minutes at 4°C. Supernatants were then collected and protein contents were determined using the BCA Protein Assay kit (Pierce, Thermo Scientific, USA). For mouse samples, 20 to 40 mg of glycolytic gastrocnemius was homogenized using a microdismembrator for 1 minute at 2000 rpm (Satorius Stedim Biotech, France). Homogenates were lysed on ice for 15 minutes in Phosphosafe (EMD Millipore, USA). Then, the mixtures were centrifuged for 10 minutes at 4°C at maximal speed and supernatants were collected and protein content determined. After dilution with lithium dodecyl sulfate sample buffer (Invitrogen, Switzerland) and heating at 93°C for 5 minutes, proteins were resolved on NuPAGE™ 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Switzerland) at 140 volts. Gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, USA). Membranes were incubated for 1 h in 5% nonfat dry milk in PBS containing 0.1% Tween-20 (Sigma-Aldrich, USA) blocking solution. Then, membranes were incubated overnight with the following primary antibodies diluted 1:1000 in the blocking solution: phospho-mTOR (Ser2448) and mTOR (5536 and 2983; Cell Signaling Technology, USA), phospho-Akt (Ser473), Akt (4060 and 2920; Cell Signaling Technology, USA), phospho-S6 Ribosomal protein (Ser235/236), S6 ribosomal protein (4858 and 2217; Cell Signaling Technology, USA), Rheb (13879; Cell Signaling Technology, USA), Rictor (2114; Cell Signaling Technology, USA), Ras (610001; BD Biosciences), unprenylated Rap1 and total Rap1 (sc-398755 and sc-373968 respectively; Santa Cruz Biotechnologies, USA). Anti-SOD2 (ab16956; Abcam, UK) and anti-GAPDH (ab8245; Abcam, UK) were diluted 1:5000 and 1:6000, respectively. Secondary antibodies (Santa Cruz

Biotechnologies, USA) were used for 1 h diluted 1:2000 in the blocking solution. Membranes were then washed, and protein bands were developed using the Clarity™ Western ECL Substrate (Bio-Rad Laboratories, USA). Protein expression was quantified using the Fusion Pulse TS device from Vilber Lourmat (Oberschwaben, Germany). Equal loading of the samples was checked using the quantity of housekeeping gene GAPDH.

Transfection for Rictor and Rap1 knock-down

For mTORC2 inhibition, we used RNA interference for the mTORC2 protein Rictor and Rap1 siRNA to inhibit Rap1 expression in C2C12 myoblasts. Rap1 siRNA (mouse), Rictor siRNA (mouse) and Control siRNA-A were purchased from Santa Cruz Biotechnology (sc-61479 and sc-37007, respectively; USA). The transfection was done using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. Briefly, C2C12 myoblasts were seeded in 6-well plates or in 96-well plates and the transfection was performed 24 hours after seeding. The Lipofectamine and siRNAs were diluted separately in Opti-MEM (Gibco, UK). For both Rap1 and Rictor, we used 60 pmol and 10 pmol siRNA per well for 6-well and 96-well plates, respectively. For mock siRNA, we used 30 pmol and 5 pmol siRNA for 6-well and 96-well plates, respectively.

Diluted siRNAs and diluted Lipofectamine were then mixed and added dropwise to the cells. The transfection efficiency was first assessed 1 to 3 days after. For treatments, myoblasts were transfected and treated 24 hours later with compounds of interest.

Real-time PCR

As done previously [15], RNA extraction from C2C12 cells was performed using the Qiagen RNeasy mini extraction kit (Qiagen, Switzerland) according to the manufacturer's instructions. The RNA concentration and purity of each mRNA sample was determined with the NanoDrop 2000 (Thermo Scientific, Switzerland). cDNA was then obtained from 1 µg RNA using the Qiagen Omniscript system (Qiagen, Switzerland). The amplification reactions were performed using SYBR green (Roche Diagnostics, Switzerland) and specific forward and reverse primers.

The following primers were used: Ki-67 forward 5' – GCCATAACCCGAAAGAGCAG – 3' and reverse 5'-CCAGTTTACGCTTTGCAGGT – 3'; Atrogin-1 forward 5' – AGTGAGGACCGGCTACTGTG – 3' and reverse 5'-GATCAAACGCTTGCGAATCT – 3'; Rheb forward 5'- ATA TGG TGG GGA AAG TGC AG -3' and reverse 5'-AAT TGT CAC ATC ACC GAG CA -3'; Rictor forward 5'- ACTTGTCCTCTGTGCTTCA – 3' and reverse 5'-AGCCTCACTTCATGCTTCCT – 3'; 18 S forward 5'- AGT CCC TGC CCT TTG TAC ACA – 3' and reverse 5'-CGA TCC GAG GGC CTC ACT A -3'. Real time PCR was performed using the ViiA7 software (Life Technologies, Switzerland) on an ABI PRISM 7700 sequence detector (PE Biosystems, Switzerland). The $\Delta\Delta C_t$ method was used to determine relative gene expression levels and the values were normalized to the housekeeping gene (18S).

Mitochondrial superoxide accumulation

Generation of mitochondrial superoxide was determined using MitoSOX Red (Invitrogen, Basel, Switzerland). C2C12 were seeded and differentiated or transfected with siRNA in black costar 96-well plates. They were treated for 24 hours with simvastatin and/or MitoTEMPO and/or geranylgeraniol and/or farnesol. 100 μ M Antimycin A was used for 1 hour as positive control. After treatment, medium was removed from the cells and replaced by a 2.5 μ M MitoSOX solution in DPBS for 10 minutes in the dark. Fluorescence was measured afterwards at 510 nm emission and 580 nm excitation using a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland). Signal was calculated related to protein content.

Statistical analysis

Results are presented as mean \pm SEM. All data were analyzed using one-way ANOVA with Newman-Keuls's post-hoc test (comparison of multiple groups) with GraphPad Prism 8 (GraphPad Software, La Jolla, CA, US). Significance was determined as $p < 0.05$.

Results

Simvastatin inhibits phosphorylation of Akt Ser473 and S6rp in C2C12 cells and mouse skeletal muscle.

As shown in Fig. 1, treatment with simvastatin inhibited the phosphorylation of Akt Ser473 and S6rp in C2C12 myoblasts (Fig. 1A), C2C12 myotubes (Fig. 1B) and in mouse skeletal muscle (Fig. 1C). These findings show that simvastatin inhibits the activity of mTORC1 (inhibition of S6rp phosphorylation) and of mTORC2 (inhibition of Akt Ser473 phosphorylation) *in vitro* and *in vivo*. In the following, C2C12 myoblasts and myotubes were used to find out the mechanisms of simvastatin-associated mTORC inhibition and myotoxicity.

Rapamycin inhibits mTORC1 in C2C12 myotubes but is not cytotoxic and does not affect Akt Ser473 phosphorylation.

We first compared simvastatin-induced toxicity and the toxic effects of specific mTORC1 inhibition on C2C12 cells. We treated C2C12 myotubes with 100 nM rapamycin, a specific inhibitor of mTORC1, to investigate membrane toxicity and ATP content (Fig. 2A and 2B). Simvastatin led to a significant 2-fold increase in AK release (reflecting disturbed membrane integrity) and to a concordant depletion of intracellular ATP (Fig. 2A and 2B). Insulin prevented the toxic effects of simvastatin in co-treatment (Fig. 2A and 2B). Interestingly, and in contrast to simvastatin, rapamycin decreased AK release alone or in-co-treatment with simvastatin and/or insulin (Fig. 2A and 2B). To confirm that mTORC1 was inhibited, we quantified the phosphorylation of the S6rp, a marker of mTORC1 activity. The phosphorylation of S6rp (Ser235/236) was almost completely abolished in all incubations containing rapamycin (Fig. 2C). As established previously [14], also simvastatin significantly decreased the phosphorylation of the S6rp (Fig. 2C), but to a lesser extent than rapamycin (Fig. 2C). To determine if inhibition of mTORC1 influences the activity of mTORC2, we analyzed the phosphorylation of Akt Ser473, a substrate of mTORC2. Whereas simvastatin decreased Akt Ser473 phosphorylation, rapamycin alone did not affect the phosphorylation of Akt Ser473 (Fig. 2D). Co-treatment with simvastatin and rapamycin decreased the

phosphorylation of Akt Ser473, whereas in the presence of the combination insulin and rapamycin, Akt Ser473 phosphorylation was maintained (Fig. 2D).

mTOR is a protein kinase that can be phosphorylated and is a component of both mTORC1 and mTORC2. As shown in supplementary Fig. 1 (Fig. S1), rapamycin impaired Ser2448 phosphorylation of mTOR to a larger extent than simvastatin, whereas insulin did not affect mTOR phosphorylation. Interestingly, simvastatin mitigated the effect of rapamycin on Ser2448 mTOR phosphorylation, suggesting an interaction between the two compounds.

Rheb is a small GTPase, which is membrane-anchored and therefore prenylated, and which is important for the activation of mTORC1 [27–30]. As shown in supplementary Fig. 2A (Fig. S2A), simvastatin, but not rapamycin or insulin, impaired the prenylation of Rheb. In contrast, mRNA expression of Rheb was not significantly affected by simvastatin or rapamycin (Fig. S2A).

These data demonstrate that, in contrast to simvastatin, specific mTORC1 inhibition with rapamycin did neither induce myotoxicity, nor reduce mTORC2 activity or impair Rheb prenylation. Simvastatin inhibited mTORC1 by impairing the phosphorylation of Akt Ser473 and Rheb prenylation. However, the cytotoxicity of simvastatin could not be explained by inhibition of mTORC1.

mTORC2 inactivation in C2C12 myoblasts leads to a similar toxicity and impairment of Akt signaling as treatment with simvastatin.

We showed in several studies that simvastatin significantly and strongly inhibits the phosphorylation of Akt at the serine 473 site [12–15]. Since Akt Ser473 phosphorylation is performed by mTORC2, we hypothesized that mTORC2 inhibition might be the primary event in simvastatin-induced muscle damage and myocyte cell death. To test this hypothesis, we investigated whether mTORC2 inactivation could induce similar toxic effects than those observed with simvastatin. Using RNA interference, we knocked down Rictor to abrogate mTORC2 activity. siRNA targeted against Rictor decreased Rictor gene and protein expression in C2C12 myoblasts, while control siRNA did not affect Rictor expression (Fig. 3C; supplementary Fig. 3A and 3B). The transfection was efficient and stable for at least 72 hours on the protein level.

We then evaluated the effects of mTORC2 inhibition on membrane integrity (AK release into the medium) and on the cellular ATP content. Cells with Rictor knock-down treated with DMSO displayed similar toxicities (AK release and ATP content) as simvastatin-treated control cells (Fig. 3A and 3B). Control siRNA-transfected cells showed a significantly higher AK release with simvastatin (10-fold increase), simvastatin and insulin (7-fold-increase), and, in a less pronounced manner, simvastatin and 100 μ M mevalonate (5-fold increase) compared to DMSO-treated cells (Fig. 3A). Similar effects were observed on the ATP content of the C2C12 myoblasts (Fig. 3B). Insulin, mevalonate, geranylgeraniol and farnesol applied alone did not induce toxic effects in control-transfected cells (Fig. 3A and 3B). Geranylgeraniol prevented the effects of simvastatin completely (SMV + GGOH 50 μ M), whereas farnesol and mevalonate were less effective in preventing the toxicity of simvastatin (Fig. 3A and 3B). In contrast, the addition of

mevalonate, geranylgeraniol or farnesol did not prevent the toxicity associated with Rictor knock-down (Fig. 3A and 3B). Simvastatin increased the toxicity of Rictor knockdown, but in a less than additive manner (Fig. 3A and 3B).

To find out whether mTORC2 knock-down leads to similar defects on Akt signal transduction than simvastatin, we investigated the activation of Akt (phosphorylation on serine 473) and of the downstream effector S6rp. As shown in Fig. 3C-E, phosphorylation of Akt Ser473 was significantly decreased in Rictor siRNA-transfected compared to control cells, showing that mTORC2 is responsible for Akt Ser473 phosphorylation. Phosphorylation levels of Akt Ser473 were similar between simvastatin-treated control cells and C2C12 cells with Rictor knock-down, and simvastatin increased the inhibition of the phosphorylation of Akt Ser 473 when Rictor was absent. Mevalonate and geranylgeraniol maintained Akt Ser 473 phosphorylation in presence of simvastatin, while they were ineffective in cells with Rictor knock-down. Interestingly, insulin was able to maintain Akt Ser473 phosphorylation in Rictor knock-down cells, possibly due to remaining mTORC2 activity. Similar effects were observed for the phosphorylated form of the S6rp, a downstream effector of Akt and mTORC1.

Since mTORC2 stimulates cellular proliferation [36], we also assessed the effect of Rictor siRNA transfection and simvastatin on Ki-67 mRNA expression, which is a marker of cell proliferation [42]. As shown in supplementary Figure S4, both simvastatin and Rictor knockdown caused a dramatic decrease in Ki-67 mRNA expression, indicating impaired C2C12 myoblast proliferation. Insulin could not prevent this decrease, whereas mevalonate partially prevented the decrease in Ki-67 mRNA associated with simvastatin, but not with Rictor knockdown.

These results clearly show that most toxic effects and most effects on Akt signal transduction of simvastatin in C2C12 cells can be explained by inhibition of mTORC2. The data also indicate that the mechanisms of mTORC2 inhibition involves prenylation defects since replenishment with isoprenoids could prevent the cytotoxicity associated with simvastatin, but not the cytotoxicity associated with Rictor knock-down.

mTORC2 inactivation in simvastatin-treated myotubes is independent of Ras prenylation.

Since the addition of isoprenoids prevented cytotoxicity and impaired Akt activation by simvastatin, we investigated the role of proteins needing prenylation as a reason for impaired mTORC2 activity caused by simvastatin. Prenylation is performed with two products of the mevalonate pathway, geranylgeranyl pyrophosphate and farnesyl pyrophosphate, and up to 2% of cellular proteins are modified with this lipid post-translational modification [43]. Prenylation is necessary for certain proteins that need attachment to cellular membranes to be active. The investigation of simvastatin's effects on cell viability and Akt phosphorylation shown Fig. 3 revealed that mainly geranylgeraniol could prevent cytotoxicity and impaired activation of mTORC2, indicating that diminished prenylation is a probable mechanism contributing to toxic effects of simvastatin in skeletal muscle cells.

To assess this possibility, we first examined the prenylation of Ras. Ras is a prenylated small GTPase that activates the PI3K pathway and promotes proper mTORC2 activation [33; 44]. Cells treated with simvastatin displayed impaired Ras prenylation (Fig. 4A), as demonstrated by an increased unprenylated fraction of this GTPase. Neither mevalonate and nor geranylgeraniol restored Ras prenylation in the presence of simvastatin (Fig. 4A). However, geranylgeraniol completely prevented the decrease in Akt Ser473 phosphorylation (reflecting mTORC2 activity) in C2C12 myotubes treated with simvastatin (Fig. 4B), suggesting that inhibition of Ras prenylation by simvastatin is independent of impaired Akt Ser473 phosphorylation. As the addition of mevalonate and geranylgeraniol did not restore Ras prenylation in the presence of simvastatin, we assessed the effect of farnesol on Ras. After 24 hours of incubation, farnesol did not prevent impaired prenylation of Ras by simvastatin (Fig. 4C), nor did it improve impaired P-Akt Ser473 phosphorylation (Fig. 4D). However, after 48 hours of incubation, farnesol increased prenylation of Ras (Fig. 4C), but still failed to improve the phosphorylation of Akt Ser473 (Fig. 4D).

These results indicate that geranylgeraniol, but not farnesol, can prevent the effect of simvastatin on Akt phosphorylation but in a manner that is independent of Ras prenylation.

Geranylgeraniol enables prenylation of Rap1 in the presence of simvastatin and prevents cytotoxicity associated with simvastatin.

Rap1 is a small GTPase that has been shown to regulate the activity of mTORC2 in the amoeba *Dictyostelium discoideum* (Khanna, 2014). Considering its role in mTORC2 activation in amoeba, we investigated a possible function of Rap1 in the activation of mTORC2 and in the toxicity of simvastatin on C2C12 myoblasts and myotubes. As shown in Fig. 5A and 5B, simvastatin inhibited the prenylation of Rap1 efficiently. This effect could not be prevented by the addition of mevalonate, but by 50 μ M geranylgeraniol. A lower geranylgeraniol concentration (10 μ M) decreased the non-prenylated Rap1 fraction numerically, without reaching statistical significance. Mevalonate was not able to prevent the cytotoxicity of simvastatin completely, whereas geranylgeraniol was protective already at 10 μ M (Fig. 5C).

To study the role of Rap1 in simvastatin-associated cytotoxicity in more detail, we performed a knock-down of Rap1 in C2C12 myoblasts using a siRNA approach (Fig. 5D). Rap1 knock-down was associated with a numerical increase in AK release and ATP depletion, which was augmented by simvastatin (Fig. 5E and 5F). The addition of mevalonate prevented the effect of simvastatin on AK release completely and of the combination simvastatin/Rap1 knock-down partially. In comparison, the addition of geranylgeraniol prevented the effect of simvastatin and the combination simvastatin/Rap1 knock-down on AK release and ATP depletion completely.

These results support a role of Rap1 in the toxicity of simvastatin on C2C12 cells and indicate that prenylation plays a role in the function of Rap1.

Rap1 knock-down causes similar effects on the Akt pathway, atrogin-1 mRNA and Ki-67 mRNA expression as simvastatin.

Next, we assessed the role of Rap1 in the function of mTORC1 and mTORC2 and in the downstream effects. Knock-down of Rap1 was associated with a clear drop in the Akt Ser473 phosphorylation, proving the importance of Rap1 for mTORC2 activation (Fig. 6A). Simvastatin showed the expected decrease in Akt Ser473 phosphorylation and increased the effect of Rap1 knock-down. The addition of mevalonate partially and of geranylgeraniol completely prevented the effect of simvastatin on Akt Ser473 phosphorylation.

In addition, treatment with simvastatin and Rap1 knock-down were associated with an increase in atrogin-1 mRNA expression and a decrease in the mRNA expression of Ki-67 (supplementary Fig. S5). Atrogin-1 belongs to the F-box protein family and forms one of four subunits of the ubiquitin protein ligase complex SCFs [45]. Atrogin-1 expression can be regarded as a marker of muscle atrophy and has been shown to be increased in C2C12 cells and mice exposed to simvastatin [46–48]. Mevalonate and geranylgeraniol prevented the increase in atrogin-1 mRNA in simvastatin-treated C2C12 myoblasts, but not in cells with Rap1 knock-down (supplementary Fig. S5A). In addition, mevalonate prevented the mRNA decrease in Ki-67 partially and geranylgeraniol completely in C2C12 myoblasts treated with simvastatin, but not in cells with Rap1 knock-down (supplementary Fig. S5B).

The experiments show that Rap1 activates mTORC2 and support the role of Rap1 in the toxicity of simvastatin on C2C12 cells.

Simvastatin increases mitochondrial ROS production which can be mitigated by antioxidants and which contributes to impaired function of mTORC2 and cytotoxicity.

The lower cytotoxicity of Rap1 knockdown compared to simvastatin (Fig. 5D and 5E) despite similar effects on the mTORC2/Akt pathway (Fig. 6B) suggested that the cytotoxicity of simvastatin was not only due to inhibition of mTORC2 but could have additional components. We and others have shown repeatedly that statins impair the function of the mitochondrial electron transport chain [46; 49; 50], which is associated with mitochondrial ROS production [40]. As shown in Fig. 7A, mitochondrial accumulation of ROS was increased in C2C12 myoblasts treated with simvastatin, whereas Rap1 knock-down was not associated with mitochondrial ROS accumulation. Geranylgeraniol prevented ROS accumulation completely, indicating that geranylgeraniol can act as a ROS scavenger. As expected, also the complex III inhibitor antimycin A was associated with mitochondrial ROS accumulation, which could be prevented by geranylgeraniol or by the antioxidant MitoTEMPO. Since mitochondrial ROS accumulation is often associated with increased SOD2 expression [51; 52], we determined SOD2 protein expression by western blotting. As shown in Fig. 7B, simvastatin and Rap1 knockdown increased SOD2 protein expression numerically compared to control siRNA treated cells, whereas the combination simvastatin and Rap1 knockdown increased SOD2 expression significantly. This was partially prevented by mevalonate and completely by geranylgeraniol. Next, we aimed to find out whether mitochondrial dysfunction contributes to simvastatin-associated cytotoxicity and inhibition of mTORC2 in C2C12 myotubes. As shown in

Fig. 7C, simvastatin and antimycin A increased mitochondrial ROS accumulation also in C2C12 myotubes, which could be prevented by MitoTEMPO. As expected, simvastatin and antimycin A caused AK release from C2C12 myotubes, which could be prevented by the addition of MitoTEMPO (Fig. 7D). Similarly, simvastatin and antimycin A caused a drop in the ATP content of C2C12 myotubes (Fig. 7E). This drop could be prevented by MitoTEMPO for simvastatin, but not for antimycin A. Simvastatin and antimycin A impaired Akt Ser473 phosphorylation, which could be partially (simvastatin) or completely (antimycin A) be prevented by MitoTEMPO (Fig. 7F).

These results show that mTORC2 can also be inhibited by mitochondrial ROS accumulation and that mitochondrial dysfunction contributes to the cytotoxicity of simvastatin.

Discussion

Statins are associated with different types of skeletal muscle toxicity, including muscle atrophy, impairment of muscle regeneration and myocyte apoptosis and necrosis [13; 47; 48; 53]. Previous studies have shown that inhibition of mTORC1 and mTORC2 as well as mitochondrial toxicity may be involved in statin-associated myotoxicity [11]. In the current studies, we investigated the contribution of these mechanisms to simvastatin-induced myotoxicity in C2C12 cells and in mice.

We first investigated the role of mTORC1 using rapamycin for mTORC1 inhibition. Rapamycin is an antifungal macrolide forming a complex with the 12 kDa FK506-binding protein (FKBP12), which inhibits mTORC1 in an allosteric manner [54]. Our data showed that rapamycin inhibited mTORC1 but was not cytotoxic for C2C12 myotubes. This was in clear contrast to simvastatin, which also inhibited mTORC1, but was cytotoxic. A second important difference between rapamycin and simvastatin was the finding that only simvastatin, but not rapamycin, inhibited Akt Ser473 phosphorylation, which is phosphorylated by mTORC2 (Fig. 2D) [21]. These findings showed that inhibition of mTORC1 alone did not explain the toxicity of simvastatin on C2C12 cells and, importantly, that mTORC1 inhibition did not affect mTORC2 activity in this cell model. In other reports, an impairment in the activity of mTORC1 was associated with a decrease in the function of mTORC2 [55–57]. The difference to the findings in the current study may be explained by the short incubation time in our experiments (24 hours) and by the different cell types used.

The inhibition of mTORC1 by simvastatin, which is demonstrated in the current study by decreased phosphorylation of S6rp, can be explained by impaired phosphorylation of Akt Ser473 (Fig. 2D). Akt activates mTORC1 indirectly via inhibition of the tuberous sclerosis 1/2 (TSC1/2) complex and stimulation of the small GTPase Rheb [27–30]. Simvastatin reduced the prenylation of Rheb (supplementary Fig. 2A), which impairs Rheb function [29]. Prenylation is needed for membrane anchoring of Rheb, which is essential for mTORC1 recruitment and activation [28; 58]. In agreement with our findings, Basso *et al.* showed that inhibition of Rheb farnesylation impairs the phosphorylation of S6rp, which is a function of mTORC1 [59]. Taken together, these results indicated that impaired phosphorylation of Akt Ser473 and reduced prenylation of Rheb were responsible for the impairment of

the mTORC1 activity by simvastatin. Impaired activity of mTORC1 could explain muscle atrophy associated with statins [12], but not cytotoxicity caused by simvastatin in the current study.

Our previous studies suggested that mTORC2 inhibition is the primary insult of simvastatin in mice and C2C12 cells [12–15]. Since there are no specific mTORC2 inhibitors available, we used siRNA knock-down to produce C2C12 cells with reduced mTORC2 activity. Knock-down of Rictor was associated with increased AK release, ATP depletion and inhibition of Akt Ser473 phosphorylation in C2C12 myoblasts, which was enhanced by the addition of simvastatin (Fig. 3). As these findings were similar to simvastatin-induced toxicity, we believe that mTORC2 inhibition is indeed an important mechanism of simvastatin-associated myotoxicity. Possibilities to explain the increase in cytotoxicity of Rictor knock-down by simvastatin include inhibition of the remaining mTORC2 activity and additional toxicity of simvastatin unrelated to mTORC2.

Multiple studies have demonstrated the protective effects of geranylgeraniol and mevalonate in presence of statins, and also their ability to maintain different processes impaired by statins such as for instance glucose transport, insulin receptor signaling and cell viability [47; 53; 60–62]. The main mechanism postulated to explain these findings is related to the fact that, as discussed above, small GTPases need prenylation to exert their role in cellular metabolism and function. Based on these considerations, we investigated whether impaired Ras prenylation would be a mechanism leading to mTORC2 inhibition, since there is a crosstalk between the Ras pathway and PI3K/Akt signaling. Activation of the Ras pathway stimulates PI3K activity and the production of phosphatidylinositol triphosphate (PIP3) [33], which can activate mTORC2 [33; 34]. It appeared to be possible that impaired PI3K activity due to lacking Ras stimulation could lower PIP3 levels and thereby decrease mTORC2 activation [33; 34]. In support of this notion, Mullen *et al.* described that Ras farnesylation was impaired with simvastatin in C2C12 myotubes [63]. In our experiments, we confirmed that simvastatin impaired Ras farnesylation (Fig. 4). Surprisingly, however, the addition of farnesol for 24 h to the incubations containing simvastatin did not increase the prenylated fraction of Ras (Fig. 4C). After an incubation for 48 h with farnesol and simvastatin, the prenylated fraction of Ras increased (Fig. 4C), but the activity of mTORC2 in the presence of simvastatin remained low (Fig. 4D). This indicated that Ras prenylation is a slow process and that reduced prenylation of Ras could not explain inhibition of mTORC2 by simvastatin. Therefore, other proteins requiring prenylation had to limit the activity of mTORC2 in the presence of simvastatin.

Considering that geranylgeraniol was able to prevent the decrease in mTORC2 activity in the presence of simvastatin completely (Fig. 4B), Rap1 was regarded to be a possible candidate. Rap1 must be geranylgeranylated and coordinates several signaling pathways, including PI3K/Akt signaling, which regulate for instance cytoskeleton rearrangement, vesicle translocation, motility, and apoptosis [64]. In support of a role of Rap1, it had been shown that the prenylated Rap1 fraction decreases in C2C12 cells treated with statins [53; 63], which was associated with cell death. Furthermore, geranylgeranyl transferase inhibitors induced similar effects on C2C12 cells as statins [63]. Although Rap1 appears to have a role in mTORC1 activation [65], much less is currently known about a possible link between Rap1 and mTORC2 activity. It has been demonstrated that Rap1 regulates Ras-mediated activation of TORC2

in the amoeba *Dictyostelium discoideum* [44]. The current study demonstrates that Rap1 is essential for mTORC2 activity in C2C12 myoblasts and that geranylgeranylation is essential for Rap1 function. Inhibition of geranylgeranylation of Rap1 by simvastatin impaired the activation of mTORC2, which inhibited full activation of Akt and mTORC1 with the described downstream effects.

ATP depletion and oxidative stress have been reported to impair autoregulation and assembly of mTORC2, restricting the activity of this protein complex [66; 67]. These reports are supported by the results with antimycin A obtained in the current study (Fig. 7). Antimycin A caused mitochondrial ROS accumulation, suppressed cellular ATP levels, and decreased Akt Ser473 phosphorylation in C2C12 myotubes. Simvastatin showed similar effects on mitochondrial ROS and cellular ATP levels, but to a minor extent. The addition of MitoTEMPO, a specific mitochondrial antioxidant, not only reduced mitochondrial ROS accumulation, but also prevented the inhibition of Akt Ser473 phosphorylation by simvastatin and antimycin A. This suggests that mitochondrial dysfunction induced by statins is an additional mechanism to decrease the activity of mTORC2. Mitochondrial dysfunction may therefore contribute to statin-associated myotoxicity by contributing to mTORC2 inhibition and by directly affecting skeletal muscle energy metabolism.

In conclusion, the current study demonstrates that mTORC2 is an important target of simvastatin-associated myotoxicity. Impaired mTORC2 activity causes a decrease in Akt Ser473 phosphorylation, which reduces mTORC1 activation and restricts downstream effects of Akt and mTORC1. Statins impair the activity of mTORC2 by inhibiting the geranylgeranylation of the small GTPase Rap1 and by causing mitochondrial dysfunction with ROS accumulation. Mitochondrial dysfunction may also contribute independently of impaired mTORC2 activation to simvastatin-associated myotoxicity.

Declarations

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Conflicts of interest: The authors have declared that no conflict of interest exists.

Availability of data: All data are available on request.

Author contribution: Gerda M. Sanvee: Conceptualization, Methodology, Investigation, Data curation, Illustrations, Writing. Leonie Hitzfeld: Methodology, Investigation. Jamal Bouitbir: Supervision. Stephan Krähenbühl: Conceptualization, Supervision, Writing and Editing.

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Figures

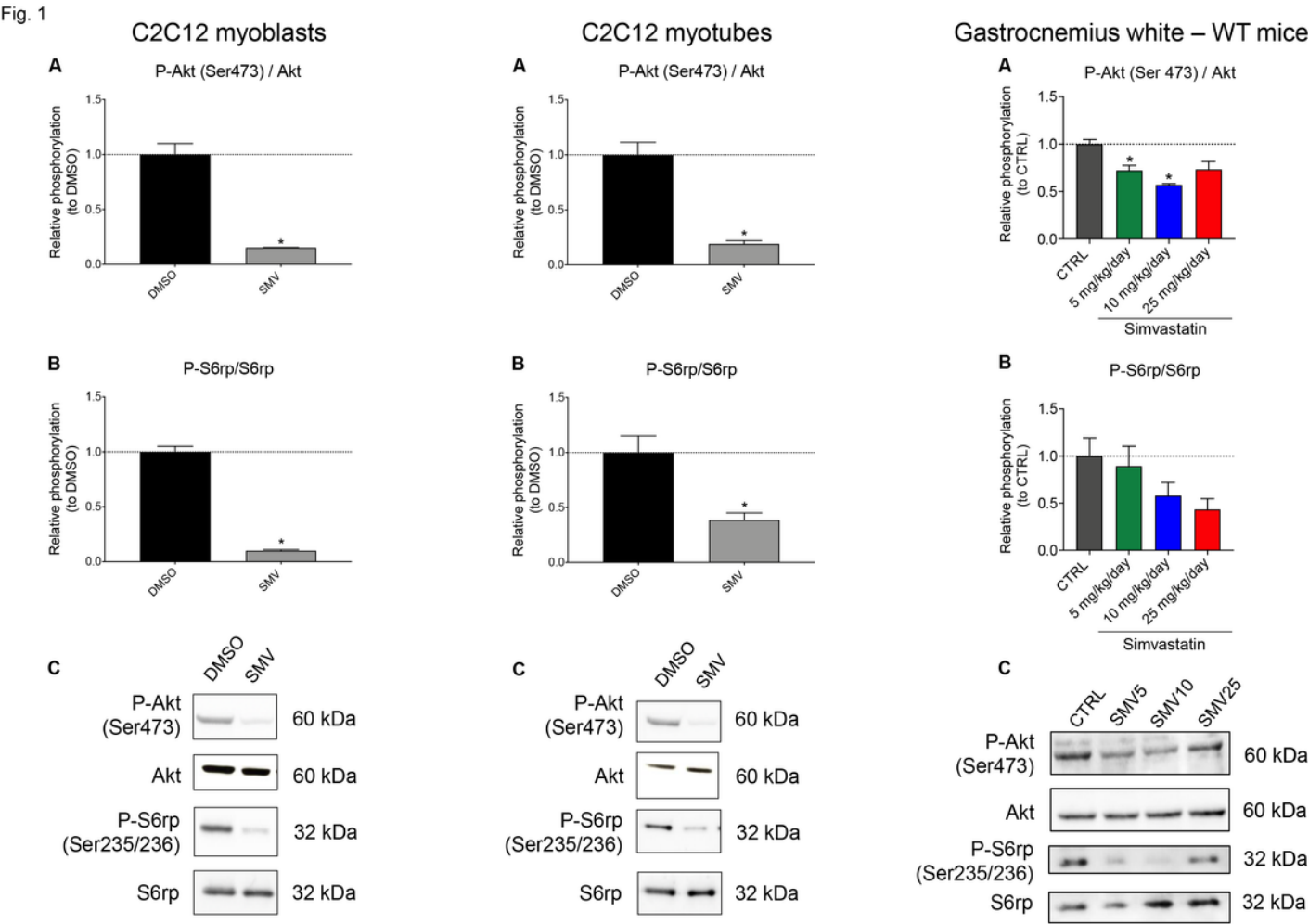


Figure 1

Simvastatin inhibits phosphorylation of Akt Ser473 and S6rp in C2C12 cells and mouse skeletal muscle. C2C12 myoblasts and myotubes were treated with simvastatin for 24 hours. Mice were treated with simvastatin at the doses indicated for 3 weeks by oral gavage. The results in mice were obtained in the white part of the gastrocnemius muscle. Data represent the mean \pm SEM of at least three independent experiments. * $P < 0.05$ versus 0.1 % DMSO or control (CTRL) mice. SMV: simvastatin, S6rp: S6 ribosomal protein.

Fig. 2

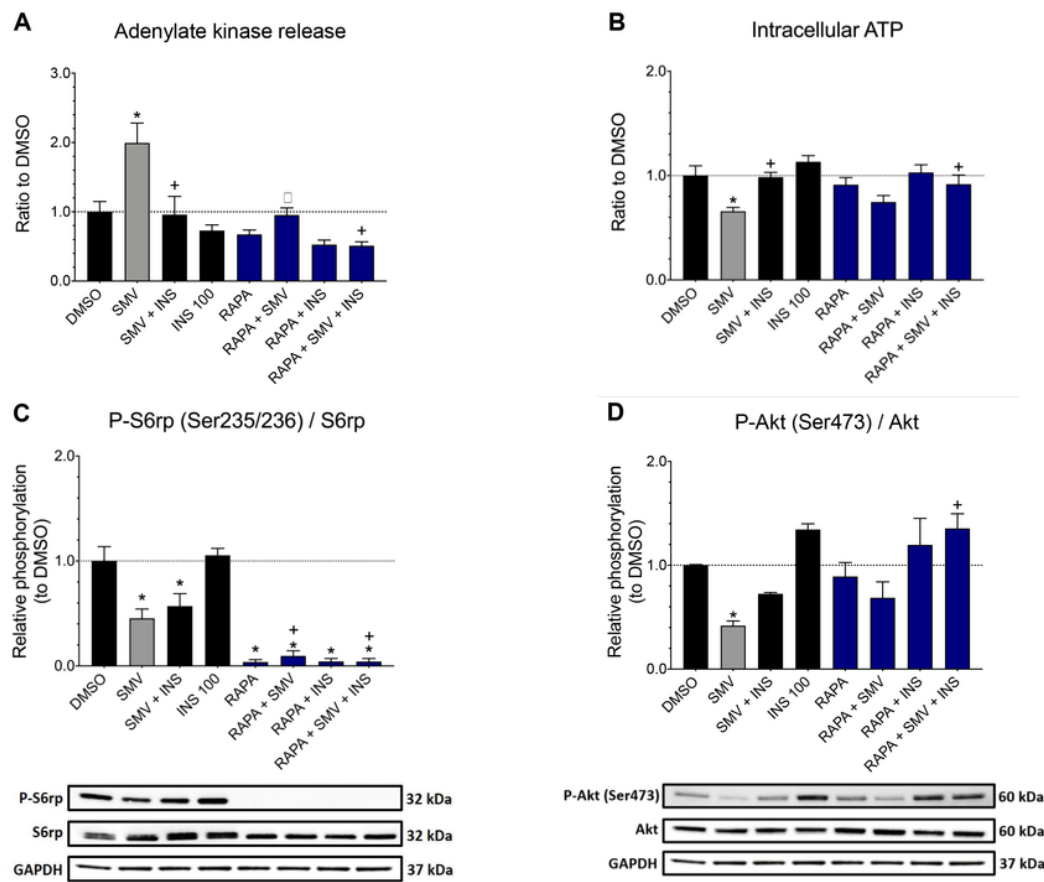


Figure 2

Rapamycin inhibits mTORC1 in C2C12 myotubes but is not cytotoxic and does not affect Akt Ser473 phosphorylation. C2C12 myotubes were treated for 24 hours with 10 μ M simvastatin and/or 100 ng/mL insulin and/or 100 nM rapamycin. 0.1% DMSO and 1% Triton-X 100 treatments were used as negative and positive controls, respectively. A. Membrane toxicity (AK release) in C2C12 myotubes. B. ATP content in myotubes. C. Expression and representative immunoblots of phosphorylated S6rp (Ser235/236) and its total form. D. Expression of phosphorylated Akt (Ser473) and Akt total. Representative immunoblots are shown. Data represent the mean \pm SEM of three independent experiments. * $P < 0.05$ versus 0.1 % DMSO; + $P < 0.05$ versus 10 μ M simvastatin, # $P < 0.05$ versus 100 nM rapamycin. SMV: simvastatin, INS: insulin, RAPA: rapamycin, S6rp: S6 ribosomal protein.

Fig. 3

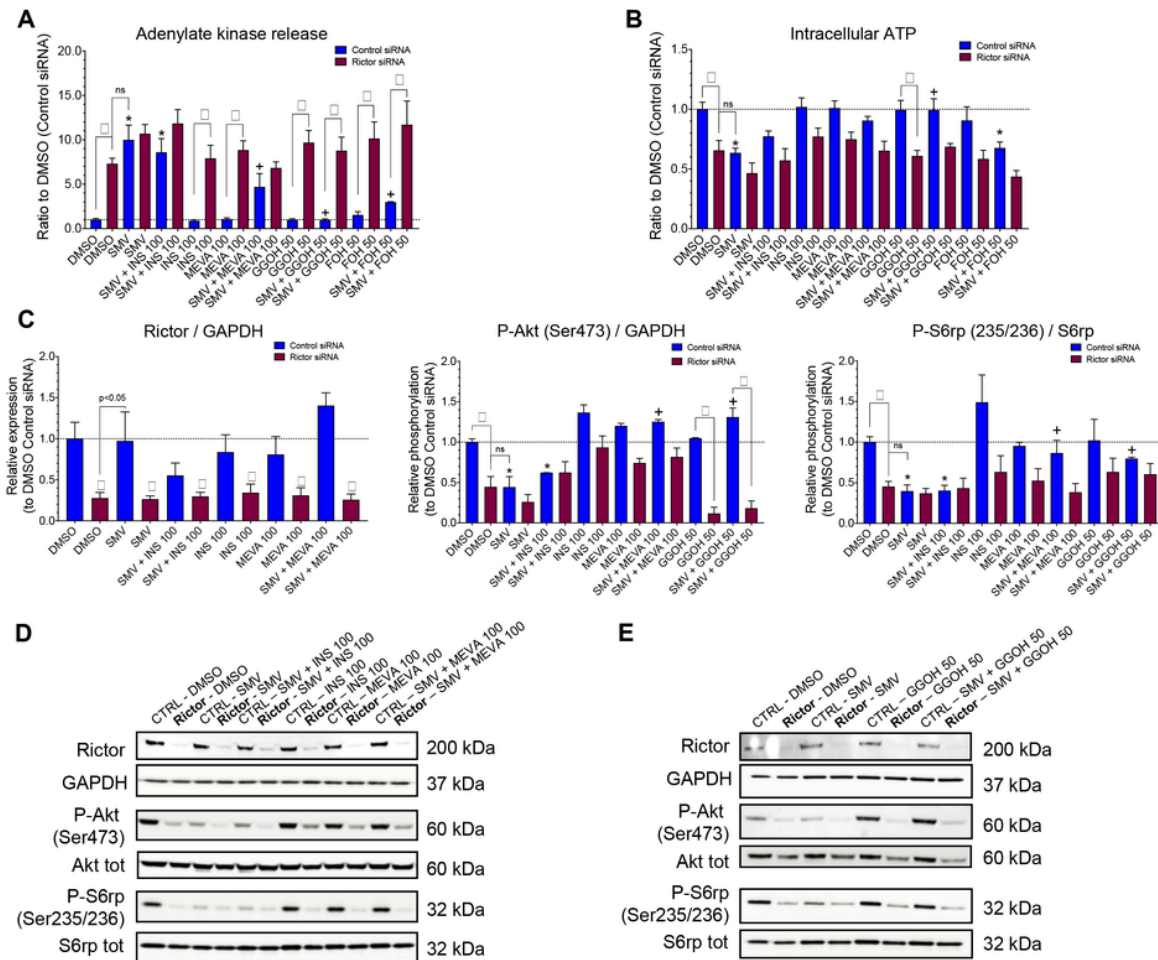


Figure 3

mTORC2 inactivation in C2C12 myoblasts leads to a similar toxicity as treatment with simvastatin. C2C12 myoblasts were transfected for 24 hours with control siRNA or 60 pmol Rictor siRNA and treated with 10 μ M simvastatin and/or 100 ng/mL insulin and/or 100 μ M mevalonate and/or 50 μ M geranylgeraniol and/or 50 μ M farnesol. 0.1% DMSO was used as negative control and 1% Triton-X 100 as positive control (not shown). A. Membrane toxicity (AK release) in transfected cells treated for 24 hours. B. Intracellular ATP in transfected cells treated for 24 hours. C. Quantification for Rictor, P-Akt Ser473 and P-S6rp 235/236 based on the immunoblots 3D and 3E. D and E. Representative immunoblots of Rictor, P-Akt and P-S6rp. Data represent the mean \pm SEM of at least three independent experiments. * $P < 0.05$ versus 0.1 % respective DMSO control sample; + $P < 0.05$ versus respective 10 μ M simvastatin sample. # $P < 0.05$ Rictor siRNA samples versus respective control siRNA samples. SMV: simvastatin, INS: insulin, MEVA: mevalonate, GGOH: geranylgeraniol, FOH: farnesol.

Fig. 4

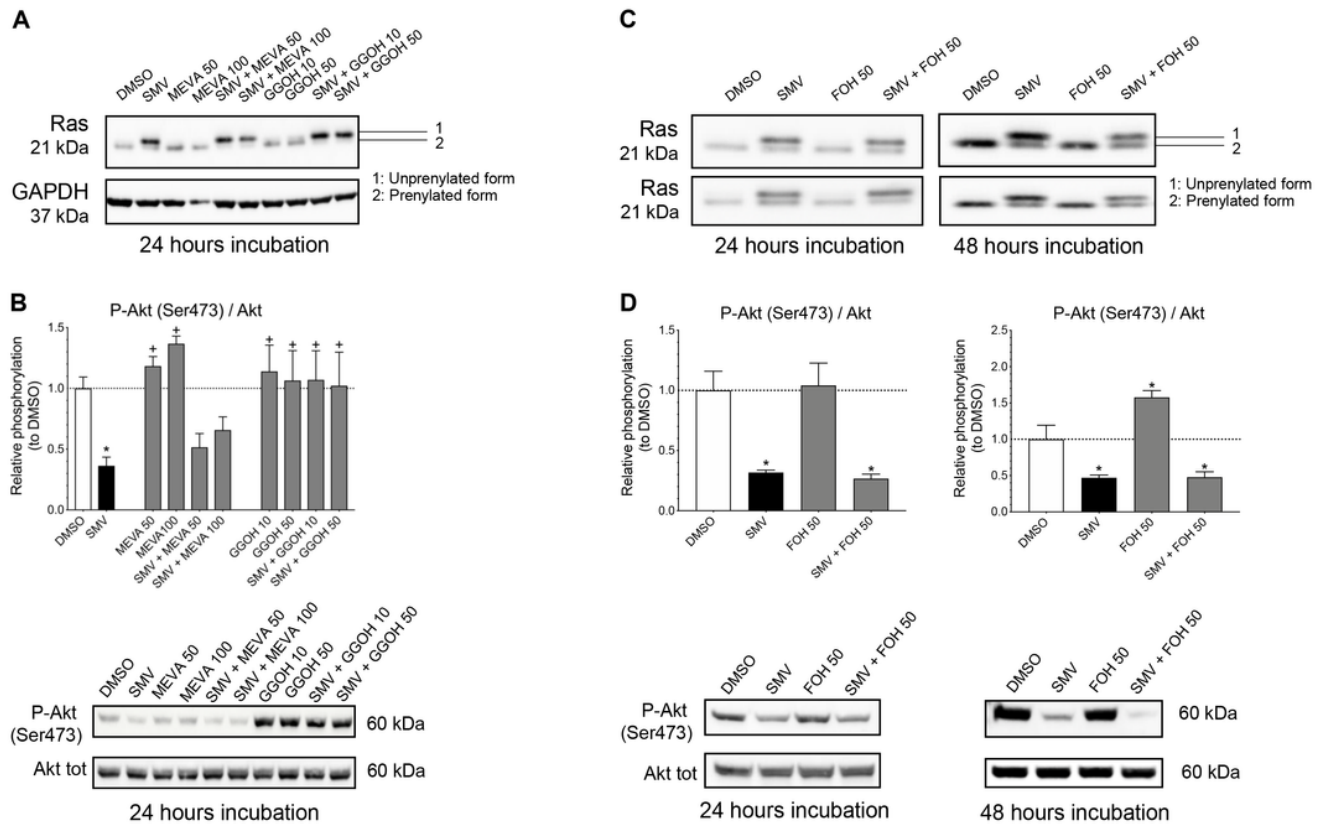


Figure 4

mTORC2 inactivation in simvastatin-treated myotubes is independent of Ras prenylation. Myotubes were treated for 24 hours or for 48 hours with 10 μ M simvastatin and/or 100 μ M mevalonate and/or 50 μ M geranylgeraniol and/or 50 μ M farnesol. 0.1% DMSO was used as negative control. A. Effect of mevalonate and geranylgeraniol on the prenylation of Ras in C2C12 myotubes. The upper band represents the non-prenylated Ras and the lower band is the prenylated fraction. B. Effect of mevalonate and geranylgeraniol on the phosphorylation of Akt Ser473 in C2C12 myotubes. C. Effect of farnesol on the prenylation of Ras in C2C12 myotubes. D. Effect of farnesol on the phosphorylation of Akt Ser473 in C2C12 myotubes. GAPDH expression was used for loading control for the immunoblots. Data represent the mean \pm SEM of three independent experiments. * $P < 0.05$ versus 0.1 % DMSO control; + $P < 0.05$ versus 10 μ M simvastatin. SMV: simvastatin, MEVA: mevalonate, GGOH: geranylgeraniol, FOH: farnesol.

Fig. 5

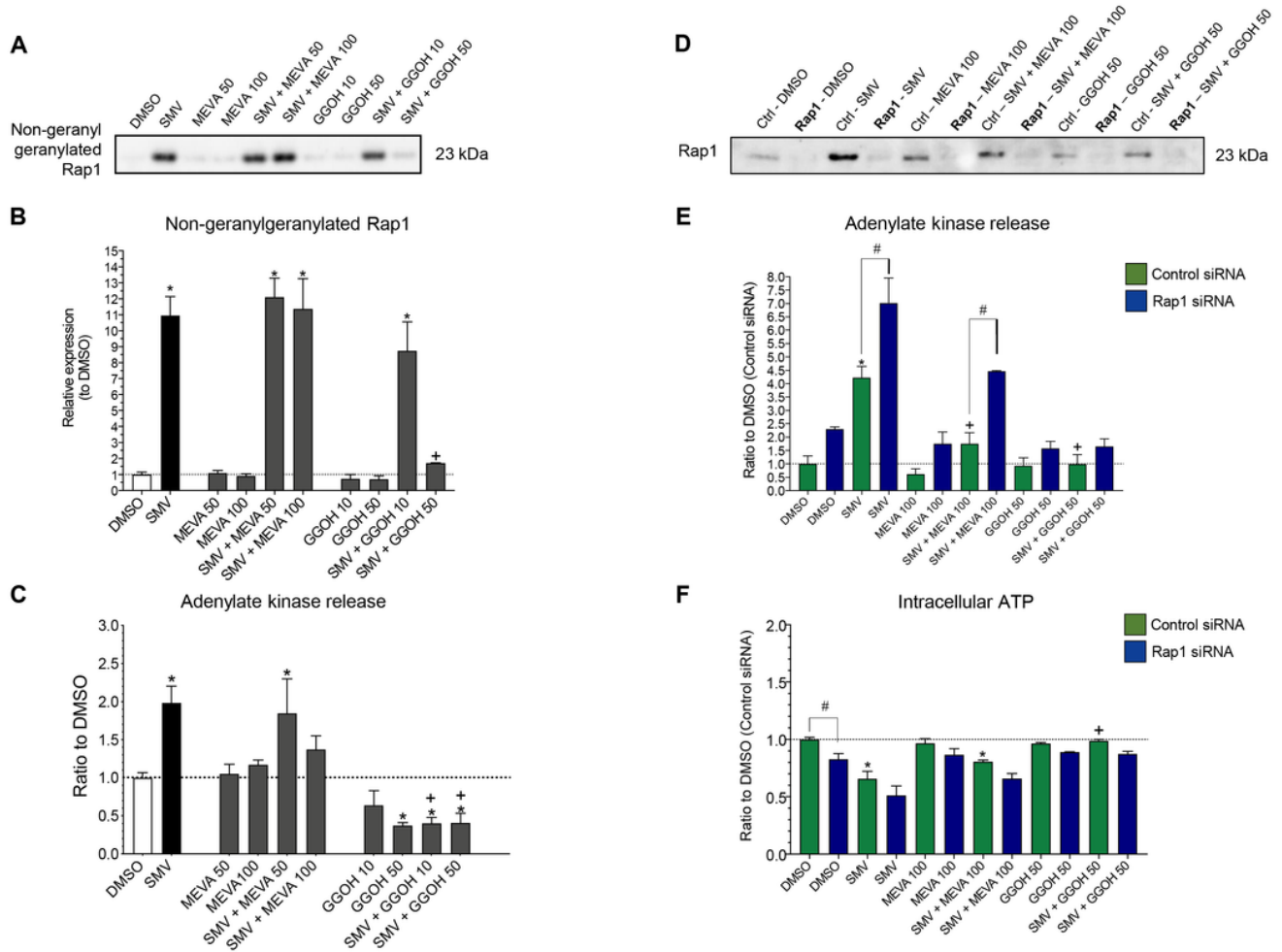


Figure 5

Geranylgeraniol enables prenylation of Rap1 in the presence of simvastatin and prevents cytotoxicity associated with simvastatin. A. C2C12 myotubes were treated for 24 hours with 10 μ M simvastatin, 50 or 100 μ M mevalonate and/or 10 or 50 μ M geranylgeraniol and unprenylated Rap1 expression was determined by immunoblotting. B. Quantification of non-prenylated Rap1. C. Membrane integrity (AK release) of C2C12 myotubes depending on prenylation of Rap1. D. C2C12 myoblasts were transfected for 24 hours with control siRNA or Rap1 siRNA (60 pmol siRNA) and treated for 24 hours with 10 μ M simvastatin, mevalonate and/or geranylgeraniol. Rap1 expression was determined by immunoblotting. E. Membrane integrity (AK release) of C2C12 myoblasts treated with control siRNA or Rap1 siRNA treated for 24 hours with 10 μ M simvastatin, mevalonate and/or geranylgeraniol. 0.1% DMSO was used as negative control. F. ATP content of C2C12 myoblasts treated with control siRNA or Rap1 siRNA treated for 24 hours with 10 μ M simvastatin, mevalonate and/or geranylgeraniol. 0.1% DMSO was used as negative control. Data represent the mean \pm SEM of at least three independent experiments. * P <0.05 versus 0.1 % respective DMSO control sample; + P <0.05 versus respective 10 μ M simvastatin sample. # P <0.05 Rap1 siRNA samples versus respective control siRNA samples. SMV: simvastatin, MEVA: mevalonate, GGOH: geranylgeraniol.

Fig. 6

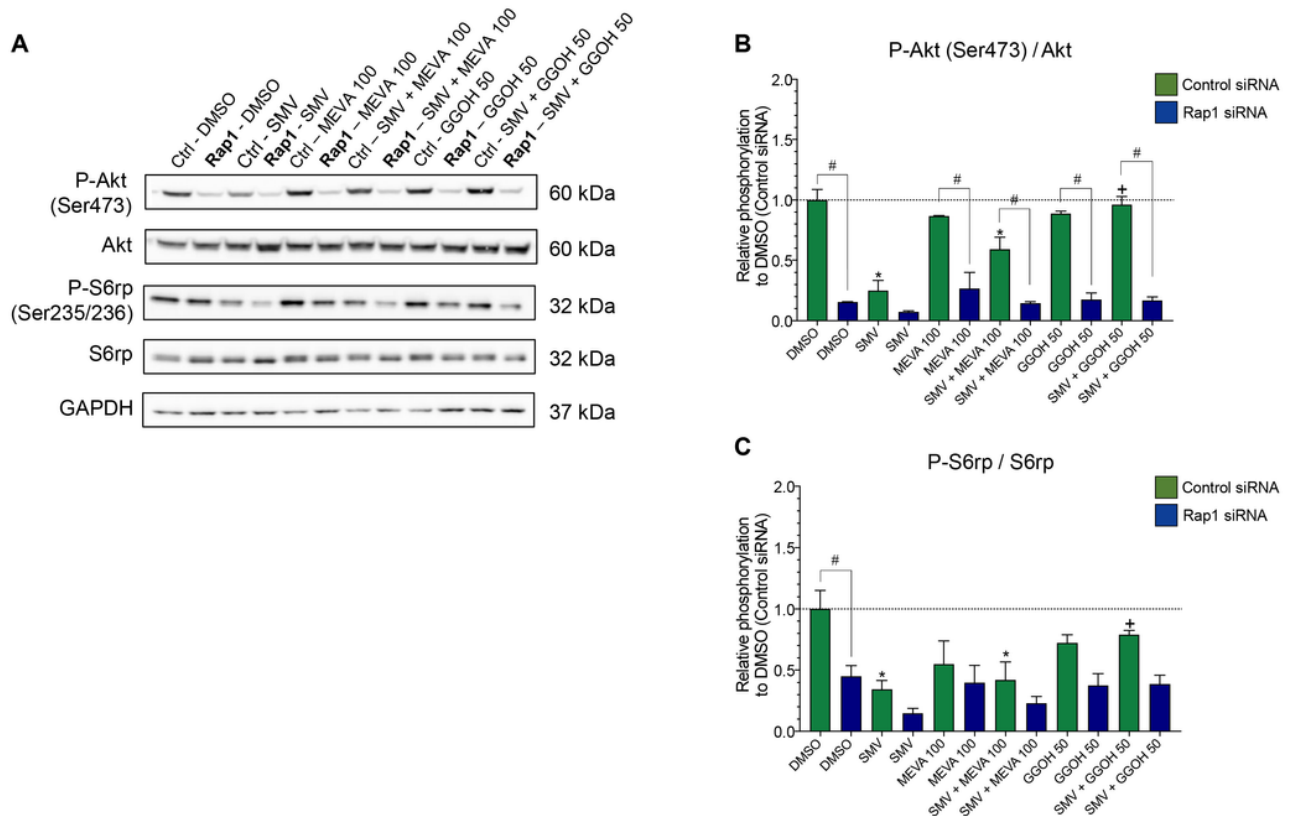


Figure 6

Rap1 knock-down causes similar effects on the Akt pathway as simvastatin. C2C12 myoblasts were transfected for 24 hours with control siRNA or Rap1 siRNA (60 pmol siRNA) and treated for 24 hours with 10 μ M simvastatin, mevalonate and/or geranylgeraniol. A. Representative immunoblot of phosphorylated (Ser 473) and total Akt, and phosphorylated (sER235/236) and total S6rp in control-siRNA and Rap1 siRNA transfected myoblasts after 24 hours of treatment. GAPDH expression was used for loading control. B. Quantification for the phosphorylation of Akt Ser473. C. Quantification for the phosphorylation of rpS6 Ser235/236. Data represent the mean \pm SEM of at least three independent experiments. * $P < 0.05$ versus 0.1 % respective DMSO control sample; + $P < 0.05$ versus respective 10 μ M simvastatin sample. # $P < 0.05$ Rap1 siRNA samples versus respective control siRNA samples. SMV: simvastatin, MEVA: mevalonate, GGOH: geranylgeraniol.

Fig. 7

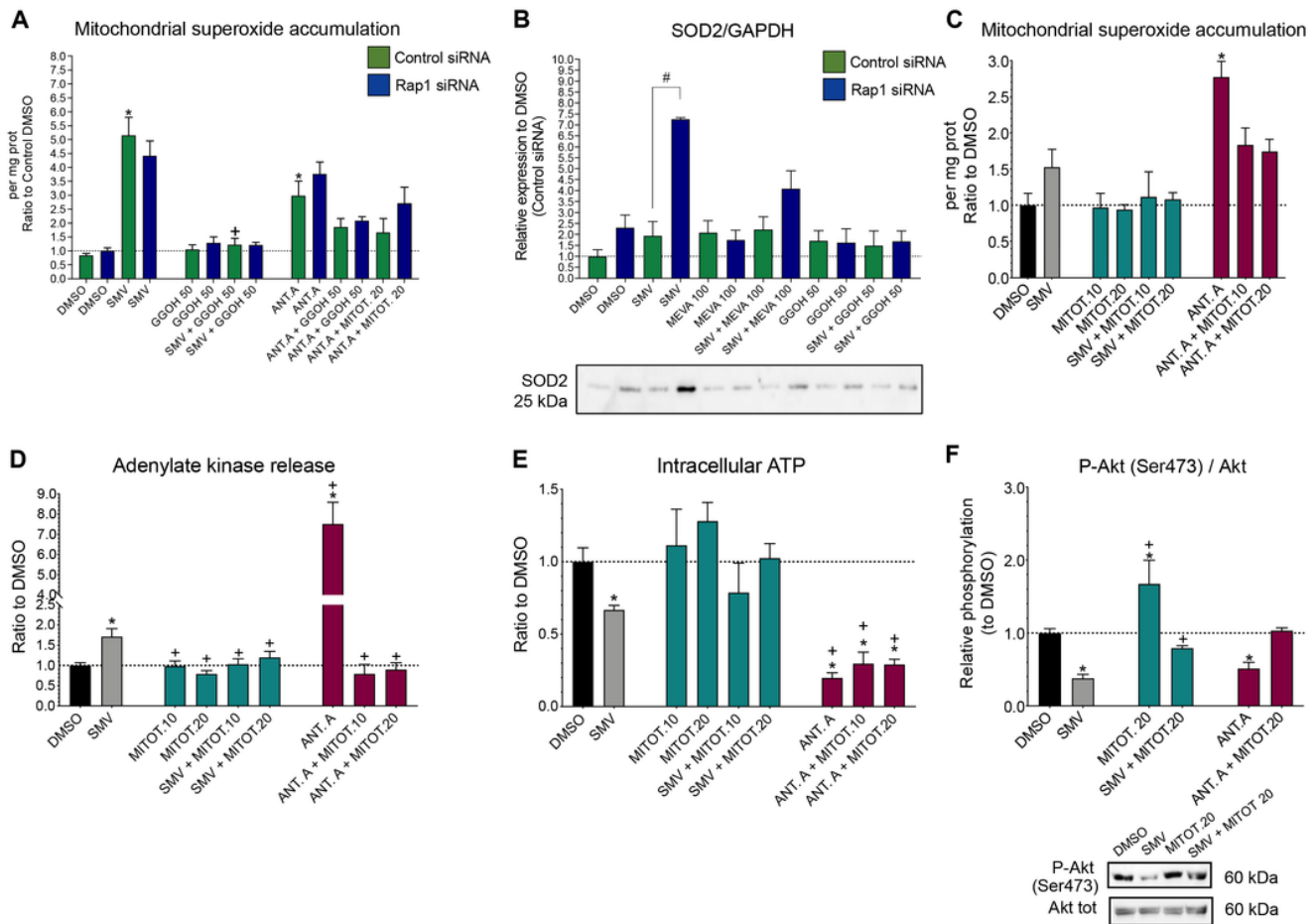


Figure 7

Simvastatin increases mitochondrial ROS production, which can be mitigated by antioxidants and which contributes to impaired function of mTORC2 and cytotoxicity. C2C12 myoblasts or myotubes were treated for 24 hours with 10 μ M simvastatin and/or 10 to 20 μ M MitoTEMPO and/or 10 to 50 μ M geranylgeraniol. MitoSOX Red dye was used to quantify mitochondrial superoxide production. 0.1% DMSO was used as negative control and 100 μ M antimycin A was used as positive control (1 hour exposure) A. Mitochondrial O₂⁻ accumulation in C2C12 myoblasts transfected with control siRNA or Rap1 siRNA. B. Protein expression of SOD2 in C2C12 myoblasts. C. Mitochondrial O₂⁻ accumulation in C2C12 myotubes treated with simvastatin or antimycin A. D. Membrane integrity (AK release) of C2C12 myotubes treated with simvastatin, MitoTEMPO, or antimycin A. E. Intracellular ATP of C2C12 myotubes treated with simvastatin, MitoTEMPO, or antimycin A. F. mTORC2 activity assessed by phosphorylation levels of Akt (Ser473) in C2C12 myotubes treated with simvastatin, MitoTEMPO, or antimycin A. Data represent the mean \pm SEM of three independent experiments. *P<0.05 versus 0.1 % DMSO; +P<0.05 versus 10 μ M simvastatin, #P<0.05 Rap1 siRNA samples versus respective control siRNA samples. SMV: simvastatin, MEVA: mevalonate, GGOH: geranylgeraniol, MITOT: MitoTEMPO, ANT.A: antimycin A.

Supplementary Files

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