

Simvastatin impairs myoblast proliferation and myotube formation of C2C12 myoblasts and in mouse skeletal muscle

Gerda M Sanvee

University Basel

Jamal Bouitbir

University of Basel

Stephan Kraehenbuehl (✉ stephan.kraehenbuehl@usb.ch)

Universitatsspital Basel <https://orcid.org/0000-0001-8347-4145>

Research Article

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Abstract

Statins reduce cardiovascular complications in patients with high LDL-cholesterol but are associated with myopathy. We investigated the possibility that statins impair skeletal muscle regeneration by assessing simvastatin toxicity on C2C12 myoblasts and myotubes and mouse skeletal muscle. Simvastatin increased plasma membrane permeability and decreased the cellular ATP content in both myoblasts and myotubes, but with a stronger effect on myoblasts. While insulin prevented cytotoxicity up to 8 hours after addition of simvastatin to myotubes, prevention in myoblasts required simultaneous addition. Mevalonate and geranylgeraniol also prevented simvastatin-associated cytotoxicity on myoblasts and myotubes. Simvastatin impaired the phosphorylation of the insulin receptor (IR β), Akt ser473 and S6rp, and increased phosphorylation of AMPK thr172 in both myotubes and myoblasts, which was prevented by insulin and mevalonate. Simvastatin impaired oxygen consumption and increased superoxide production by myoblasts and myotubes and induced apoptosis via cytochrome c release. In addition, simvastatin impaired proliferation and fusion of myoblasts to myotubes by inhibiting the expression of the nuclear transcription factor MyoD and of the metalloprotease ADAM-12. Decreased expression of the proliferation factor Ki-67 and of ADAM-12 were also observed in gastrocnemius of mice treated with simvastatin. In conclusion, myoblasts were more susceptible to the toxic effects of simvastatin and simvastatin impaired myoblast proliferation and myotube formation. Impaired muscle regeneration represents a new mechanism of statin myotoxicity and may be important in statin-associated myopathy.

Introduction

Statins are drugs used to reduce serum LDL-cholesterol concentrations. Their pharmacological effect is confined to the liver, where they inhibit HMG-CoA reductase, a key enzyme in the mevalonate pathway. Inhibition of HMG-CoA reductase impairs the biosynthesis of cholesterol and of different non-sterol intermediates such as dolichol, farnesyl and ubiquinone and thereby increases the expression of LDL receptors on the surface of hepatocytes.(Bouitbir et al. 2020) In patients with increased LDL-cholesterol plasma concentrations, statins reduce mortality and morbidity caused by cardiovascular diseases. (Fulcher et al. 2015) Statins are generally well tolerated with only few adverse reactions. A frequently observed adverse reaction is myopathy, ranging from myalgia with or without elevation of serum creatine kinase activity to rhabdomyolysis.(Armitage 2007; Bouitbir et al. 2020) Depending on the definitions used, myopathy affects up to 30% of patients in a dose-dependent fashion.(Alfirevic et al. 2014; Norata et al. 2014)

Skeletal muscle accounts for approximately 40% of body weight and is important for a variety of processes such as generation of force and heat, locomotion and energy metabolism.(Frontera, Ochala 2015) Myogenesis is a tightly regulated process, eventually leading to the formation of new muscle fibers. In this process, stem cells (so called satellite cells) are activated to mononucleated precursor cells or myoblasts, which proliferate in the presence of myogenic factors involved in gene regulation and eventually fuse to form multinucleated myotubes that can further differentiate into functional myofibers. (Zammit 2017) Myogenic regulatory factors (MRFs) are for instance MyoD, MRF4, Myf5 and myogenin;

they regulate myogenesis(Hernandez-Hernandez et al. 2017; Megeney, Rudnicki 1995; Zammit 2017) by controlling the synthesis of important muscle proteins such as actin, troponin and myosin heavy chains. (Burattini et al. 2004) In the postnatal skeletal muscle tissue, satellite cells are located in the periphery of muscle fibers, between the sarcolemma and the basement membrane, and can be activated, proliferate and fuse to replace myotubes in order to maintain muscle mass after muscle injury. Defects in these processes can lead to several musculoskeletal disorders.(Chal, Pourquie 2017)

C2C12 cells are considered as a good model to study skeletal muscle development.(Burattini et al. 2004; Maglara et al. 2003) They derive from the C3H murine cell line and represent immortalized myoblasts, similar to quiescent satellite cells in myofibers.(Burattini et al. 2004; Yaffe, Saxel 1977) Upon serum withdrawal, C2C12 myoblasts can differentiate to plurinucleated myotubes that are precursors of mature myofibers.(Berendse et al. 2003) During the differentiation from myoblasts to myotubes, expression of myogenin and myosin heavy chain (MHC) increase. Additionally, C2C12 myotubes contain sarcomeres and are able to contract and generate force.(McMahon et al. 1994)

The processes leading to statin-associated muscle symptoms are currently not fully elucidated. Statins have been reported to impair mitochondrial function, increase reactive oxygen species (ROS) production and deplete mitochondrial ATP synthesis in several *in vitro* models as well as *in vivo* in animals and in humans.(Bouitbir et al. 2020) Defects in prenylation due to inhibition of the mevalonate pathway by statins have been linked to impaired function of several small G proteins and increased apoptosis of myocytes.(Bouitbir et al. 2020) In order to understand the mechanisms leading to statin-induced muscle damage, our group previously worked with C2C12 myotubes and identified inhibition of the insulin receptor/Akt/mTORC signaling pathway as a key mechanism leading to simvastatin-induced myotoxicity. (Bonifacio et al. 2015; Bonifacio et al. 2017; Sanvee et al. 2019b) Since mTORC1 is important for the activation of satellite cells,(Rodgers et al. 2014) we hypothesized that statins could impair satellite cell activation and thereby muscle regeneration. In order to test this hypothesis, we studied the toxicity of simvastatin on C2C12 myoblasts and myotubes and characterized the effect of simvastatin on different stages of post-natal myogenesis in the C2C12 cell model and in mice treated with simvastatin. We could show that myoblasts are more susceptible than myotubes to the toxic effects of simvastatin and that simvastatin impairs their proliferation, resulting in the inhibition of myotube formation. Statins are toxic for myoblasts and impair myotube formation, which may delay muscle regeneration after skeletal muscle injury.

Methods

Chemicals

Simvastatin lactone (Sigma-Aldrich, St-Louis, USA) was converted to the active acid form by hydrolysis. (Bogman et al. 2001) 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. Mevalonolactone (Sigma-Aldrich, USA) was diluted in water and stocks were

stored at -20°C. Dolichol C₁₀₀ was purchased from ARC (American Radiolabeled Chemicals, USA) and stock solutions were prepared in chloroform:methanol (2:1). Geranylgeraniol was purchased from Sigma-Aldrich and stock solutions were prepared in ethanol.

Animals

Male C57BL/6J mice (n = 20, age 16–18 weeks) were acclimatized one week prior to the start of the study and housed in a standard facility with 12h light-dark cycles and controlled temperature (21–22°C). The mice were fed a standard pellet chow and water *ad libitum*. Experiments were accepted by the cantonal veterinary authority of Basel, Switzerland (License 2847) and were performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Study design and simvastatin administration to mice

Mice were randomly divided into two groups (n = 10 per group) after one week of acclimatization. Group (1) was treated with water for 21 days and group (2) with simvastatin 5 mg/kg/day for 21 days (n = 10). This dose of simvastatin was chosen according to our previous study.(Bonifacio et al. 2015) Simvastatin was dissolved in water and the animals were treated once daily by oral gavage. Food and water consumption and changes in body weight were recorded every day.

Sample collection

After treatment for 21 days, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Tissues were collected immediately after reaching anesthesia, weighed, and muscle samples were quickly frozen in isopentane cooled by liquid nitrogen and stored at -80°C for later analysis.

Cell line and maintenance

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: 10 mg/mL). Three 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

For membrane integrity assessment, 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.(Felser et al. 2013) For rescue experiments, cells were first exposed to 10 μM simvastatin for 3, 6, 8, 12 hours prior addition of 10 or 100 ng/mL insulin. To determine metabolic activity of cells, we quantified 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 (Männerdorf, Switzerland).

Western blots

Western blots were prepared as previously described.(Sanvee et al. 2019b) Briefly, cells were grown and differentiated on 6-well culture plates and treated with the test compounds for 24 hours. After treatment, they were 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. The cell supernatants were then collected and protein contents were determined using the BCA Protein Assay kit (Pierce, Thermo Scientific, USA). 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-insulin receptor β (Tyr1361) (ab60946; Abcam, UK), insulin receptor β (3025; Cell Signaling Technology, USA), phospho-Akt (Ser473), Akt (4060 and 2920; Cell Signaling Technology, USA), phospho-AMPK alpha (Thr172), AMPK alpha (2535 and 2532; Cell Signaling Technology, USA), phospho-S6 ribosomal protein (Ser235/236), S6 ribosomal protein (4858 and 2217; Cell Signaling Technology, USA), SOD2 (ab74231; Abcam, UK), Myosin Heavy Chain (MHC) (05-716; Millipore, USA), cleaved and pro-caspase-3 (9662; Cell Signaling Technology, USA), cytochrome c (ab133504; Abcam, UK). GAPDH (ab8245; Abcam, UK) was diluted 1:6000. 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.

Cellular oxygen consumption

For cellular respiration measurement, C2C12 were seeded in growth medium in Seahorse XF 24-well culture plates coated with Poly-D-Lysine (Sigma-Aldrich, USA) at 25'000 cells per well. They were allowed to attach overnight or to differentiate to myotubes and were treated for 24 hours with the drugs. Respiration was determined in live cells using a Seahorse XF24 analyzer (Seahorse Biosciences, North Billerica, MA, United States) as described before.(Felser et al. 2013) Cells were incubated in unbuffered DMEM medium (4 mM L-glutamate, 1 mM pyruvate, 1 g/L glucose, 63.3 mM sodium chloride, pH 7.4)

and kept in a CO₂-free incubator at 37°C for at least 40 minutes for equilibrating. Then the plate was transferred to the analyzer. Basal oxygen consumption rate (OCR) was determined in the presence of glutamate/pyruvate (4 and 1 mM, respectively). Then 1 µM oligomycin was injected to determine the oxidative leak (inhibition of ATP-linked respiration). Afterwards, 1 µM FCCP was used to stimulate maximally the mitochondrial respiration and then 1 µM rotenone was used to determine the non-mitochondrial respiration (complex I inhibitor). The non-mitochondrial respiration was subtracted from the basal, oxidative leak and maximum respiration. After analysis, protein content in each well was determined after removing the supernatant using sulforhodamine B and absorbance was measured at 490 nm using a Tecan M200 Pro Infinity plate reader (Männerdorf, Switzerland).

Mitochondrial superoxide production

Generation of mitochondrial reactive oxygen species was determined using MitoSOX Red (Invitrogen, Basel, Switzerland). C2C12 cells were seeded in black costar 96-well plates at 5'000 cells per well. They were treated for 24 hours with simvastatin, insulin and/or mevalonate. 100 µM Antimycin A was used for 1 hour as positive control. After treatment, medium was removed from the cells and replaced by a 2.5 µM MitoSOX solution in Dulbecco's phosphate-buffered saline (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 (Männerdorf, Switzerland). Signal was calculated related to protein content.

MTT assay (cell proliferation)

Cell proliferation was assessed using the ability of the cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to water-insoluble purple formazan. MTT (Sigma-Aldrich, USA) was dissolved in DPBS to 5 mg/mL, pH 7.4. The solution was filter-sterilized and kept at 4°C protected from light. Cells were seeded at 5'000 cells per well in 96-well culture plates and were incubated with medium containing test compounds 6 hours after seeding, once attached. Two sets of proliferating myoblasts were obtained one day and 2 days after seeding. Fused myoblasts were obtained 3 days after seeding. Cells were treated for 24 hours with the test compounds. Afterwards, 10 µL MTT solution was added per well in growth medium (to a final concentration of 0.45 mg/mL) for 4 hours at 37°C. Formazan precipitates formed were dissolved using 100 µL Solubilization solution (16% sodium dodecyl sulfate dissolved in 40% dimethylformamide in 2% glacial acetic acid, pH 4.7) and absorbance was recorded at 570 nm using a Tecan M200 Pro Infinity plate reader (Männerdorf, Switzerland).

Real-time PCR

RNA extraction from myoblasts and mice skeletal muscle 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'; MyoD forward 5'- TTGAGAGATCGACTGCAGCA – 3' and reverse 5'-ACTTCTGCTCTTCCCTTCCC – 3'; ADAM12 forward 5'- AATTAACAGCCCTCCCGTCA – 3' and reverse 5'-TAGACGAAGCCCTGCAGAAA – 3'; GAPDH forward 5'-CATGGCCTTCCGTGTTTCTA-3' and reverse 5'-CCTGCTTCACCACCTTCTTGA-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 (GAPDH).

Cytosolic cytochrome c quantification

Cytochrome c present in the cytosol was quantified using the Mitochondria/Cytosol Fractionation Kit (ab65320, Abcam, UK). Cells were seeded in T-75 culture flask and treated with test compounds for 24 hours prior fractionation. Mitochondrial and cytosolic fractions were collected according to manufacturer's instructions and cytochrome c content in the cytosol was determined by western blotting (cytochrome c [ab133504; Abcam, UK]). GAPDH quantification was used for the cytosolic compartment specificity.

Statistical analysis

Results are presented as mean \pm SEM. All data were analyzed by unpaired Student's t test (comparison of two groups) or one-way ANOVA with Newman-Keuls's post-hoc test (comparison of multiple groups) with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, US). Significance was determined as $p < 0.05$.

Results

Simvastatin-induced cytotoxicity was more accentuated in C2C12 myoblasts compared to myotubes and geranylgeraniol prevented simvastatin-induced cytotoxicity in both cell models.

To assess membrane toxicity of simvastatin in myoblasts and myotubes, we determined the release of adenylate kinase (AK) in the medium in both cell types exposed for 24 hours. Simvastatin was toxic for both myoblasts and myotubes, but the effect on myoblasts was stronger (Fig. 1A). The insulin receptor pathway is essential for muscle cell growth, survival and metabolism.(Bonifacio et al. 2015; Carnagarin et al. 2015) We previously showed that insulin could prevent the decrease in cell viability of C2C12 myotubes treated with simvastatin.(Sanvee et al. 2019a) Here, we studied the effect of insulin on myotubes and myoblasts exposed to insulin. Insulin alone did not induce membrane toxicity in either cell type. At a physiological concentration (10 ng/mL), insulin attenuated simvastatin-associated toxicity on myotubes, but not on myoblasts. At a supraphysiological concentration (100 ng/mL), insulin attenuated the simvastatin-associated toxicity on myoblasts and completely prevented the toxicity on myotubes. Similar effects were observed when the decrease in the cellular ATP pool, a marker of metabolic activity, was analyzed in both cell models after treatment with simvastatin and/or insulin. As shown in Fig. 1B, simvastatin significantly depleted ATP in myoblasts and myotubes. At a physiological concentration,

insulin prevented this depletion in myotubes, while only the supraphysiological insulin concentration prevented this effect also in myoblasts (Fig. 1B).

To further investigate the difference in susceptibility to simvastatin of myoblasts and myotubes, we added insulin certain periods of time after exposure to simvastatin. In myoblasts (shown in supplementary Fig. S1A-C), insulin did not prevent cytotoxicity if added after exposure to simvastatin. In contrast, in myotubes, insulin prevented cytotoxicity up to 8 h after exposure to simvastatin (Fig. S1D-F).

HMG-CoA reductase inhibitors not only impair the biosynthesis of cholesterol, but also the biosynthesis of mevalonate and of other isoprenoids, which are important for several cellular functions and signaling. (Alizadeh et al. 2017) We treated myotubes and myoblasts with simvastatin alone and/or mevalonate, dolichol and geranylgeraniol to investigate if replenishment of mevalonate pathway metabolites could alleviate simvastatin-induced toxicity. In addition to adenylate kinase release in the medium, the cellular ATP pool was also assessed. Overall, toxic effects of simvastatin were higher in C2C12 myoblasts. Myoblasts exposed to 10 μ M simvastatin showed a 4.5-fold increase in membrane toxicity and a significant decrease in the cellular ATP content (Fig. 1C). Mevalonate alone did not affect membrane integrity in myoblasts and significantly reduced the toxicity of simvastatin in a concentration-dependent manner (Fig. 1C). Dolichol failed to prevent simvastatin-induced AK release and ATP depletion in the co-treatment setting. In contrast, geranylgeraniol (10 and 50 μ M) completely prevented simvastatin-associated membrane toxicity when applied together with simvastatin (Fig. 1C) and also maintained ATP levels in myoblasts (Fig. 1C). In myotubes, the same toxicity pattern was observed (Fig. 1D). However, compared to myoblasts, the toxicity associated with simvastatin was less strong and mevalonate in the two concentrations used could not prevent the adenylate kinase release upon simvastatin treatment (Fig. 1D).

Simvastatin inhibited cellular respiration in C2C12 myoblasts whereas myotubes were less sensitive to simvastatin.

As described in Fig. 1B to D, simvastatin decreased the ATP content in C2C12 myoblasts and myotubes, which was compatible with impaired function of the mitochondrial respiratory chain. We therefore analyzed mitochondrial oxidative metabolism in C2C12 myoblasts and myotubes using the SeahorseXF24 analyzer. In C2C12 myoblasts (shown in Fig. 2A), simvastatin impaired basal, leak and maximal oxygen consumption. Insulin slightly increased basal, but did not affect leak or maximal oxygen consumption. The addition of insulin to simvastatin did not prevent the toxic effect of simvastatin, whereas the addition of mevalonate was partially preventive. In C2C12 myotubes (shown in Fig. 2B), simvastatin decreased basal, leak and maximal respiration, but less than in myoblasts. Insulin alone slightly stimulated basal oxygen consumption and prevented the toxicity of simvastatin completely. Mevalonate also prevented the inhibition of oxygen consumption by simvastatin completely.

When normalized to the protein content, we observed a significant decrease in the basal and maximal oxygen consumption rate of myoblasts treated with 10 μ M simvastatin, which could be prevented by co-treatment with insulin or mevalonate (supplementary Fig. S2A). In myotubes, only the maximal oxygen

consumption was significantly decreased by simvastatin (supplementary Fig. S2B). Insulin and mevalonate exhibited no significant effect on the respiration of myotubes and prevented the toxic effect of simvastatin. Interestingly, myotubes displayed a higher maximal respiration compared to the precursor cells (suggesting a higher mitochondrial content), while basal respiration was higher in myoblasts (Fig. 2A and B).

Simvastatin increased mitochondrial superoxide production in C2C12 myoblasts and stimulated SOD2 expression in myoblasts and myotubes

Impairment of the mitochondrial respiration chain can increase the production of reactive oxygen species (ROS). We quantified the production of superoxide anion in C2C12 muscle cells treated with simvastatin, insulin and/or mevalonate for 24 hours using MitoSOX red, a cationic agent transported into the mitochondrial matrix that can be oxidized by superoxide. Incubation with 100 μ M antimycin A for 1 h was used as a positive control. In myoblasts, simvastatin strongly stimulated mitochondrial superoxide production and insulin could not prevent this effect (Fig. 3A). In contrast, mevalonate could at least partially prevent mitochondrial simvastatin-induced mitochondrial superoxide accumulation (Fig. 3A). In myotubes, on the other hand, simvastatin led to an only slight (non-significant) increase of superoxide accumulation (Fig. 3A). Insulin and mevalonate did not affect superoxide production in the absence or presence of simvastatin (Fig. 3A).

We evaluated the antioxidant capacities in each type of cells with the quantification of superoxide dismutase 2 (SOD2) expression, as SOD2 degrades superoxide anions in the mitochondrial matrix. We not only found that myotubes have a higher basal expression of SOD2 compared to myoblasts (Fig. 3B and C), but also that treatment with simvastatin stimulated the SOD2 expression in both cell types (Fig. 3B and C).

The IR/Akt signaling was similarly impaired in myoblasts and myotubes treated with simvastatin and insulin was more effective in preventing this toxicity than mevalonate.

We have previously demonstrated in C2C12 myotubes and in mice that inhibition of the IR/Akt/mTOR pathway by simvastatin is an important mechanism of statin-induced myotoxicity.(Bonifacio et al. 2015; Bonifacio et al. 2017; Bouitbir et al. 2016; Mullen et al. 2011) However, it is unknown whether this inhibition occurs also in C2C12 myoblasts. To investigate IR/Akt signaling, we treated myoblasts and myotubes for 24 hours with 10 μ M simvastatin with or without addition of insulin (10 or 100 ng/mL) or mevalonate (10 or 50 μ M) and performed western blots for phosphorylated and total forms of key factors of this pathway. We observed that the phosphorylation of the insulin receptor β , Akt Ser473 and ribosomal protein S6 (S6rp) dropped when myoblasts or myotubes were treated with simvastatin (Fig. 4A and B). Insulin alone stimulated the phosphorylation of these proteins in both myoblasts and myotubes. In combination with simvastatin, insulin prevented the drop associated with simvastatin for Akt Ser473 and S6rp and partially also for the IR β in both myotubes and myoblasts (Fig. 4A and B). In the presence of mevalonate, phosphorylation was maintained for the IR β , Akt Ser473 and S6rp in myoblasts and myotubes. In the presence of simvastatin, mevalonate was not able to prevent impaired phosphorylation

of the IR β in myotubes and myoblasts and of S6rp in myotubes, but could partially prevent impaired phosphorylation of Akt Ser473 in myotubes and myoblasts and of S6rp in myoblasts (Fig. 4A and B). Remarkably, simvastatin and mevalonate increased the synthesis of the total IR β in both cell types (Fig. 4A and B). (Sanvee et al. 2019a) In contrast to the reduced phosphorylation observed for the previously mentioned proteins, phosphorylation of AMPK alpha was stimulated by simvastatin in both myotubes and myoblasts (Fig. 4A and B). Insulin did not significantly affect the phosphorylation of AMPK alpha in myotubes and myoblasts irrespective of the presence of simvastatin. Mevalonate had no effect on the phosphorylation of AMPK alpha in myoblasts but impaired AMPK phosphorylation in myotubes.

Mevalonate prevented the impairment of myoblast proliferation by simvastatin.

In skeletal muscle, progenitor cells need to proliferate and fuse in order to form mature myofibers. Satellite cells are a subpopulation of muscle cells needed for muscle regeneration in case of injury. (Burattini et al. 2004; Chal, Pourquie 2017; McCroskery et al. 2003) Clinical observations in patients treated with statins are compatible with the hypothesis that statins could inhibit skeletal muscle regeneration. We therefore wondered whether simvastatin could impair proliferation of myoblasts. We used the MTT assay as a readout of cellular proliferation in myoblasts differentiating to myotubes. Simvastatin significantly decreased the proliferation of myoblasts at day one and two post-seeding, while this effect was lost as soon the cells were confluent at day three post-seeding (Fig. 5A). Insulin and mevalonate did not impair the proliferation of myoblasts and mevalonate was able to prevent the impairment of proliferation by simvastatin at day one and day two post-seeding (Fig. 5A).

In order to confirm impaired proliferation of myoblasts in the presence of simvastatin, we quantified mRNA expression of Ki-67, a marker of cellular proliferation, in myoblasts treated with simvastatin for 24 hours and in gastrocnemius of mice treated for 21 days with simvastatin. In accordance with the results of the proliferation assay, we observed a strong repression of Ki-67 mRNA expression with simvastatin in myoblasts (Fig. 5B) and mouse skeletal muscle (Fig. 5C). In C2C12 myoblasts, we observed no change of Ki-67 mRNA expression in the presence of insulin or mevalonate alone (Fig. 5B). While insulin did not prevent the impairment of Ki-67 mRNA expression by simvastatin, co-treatment with mevalonate prevented this decrease completely (Fig. 5B). These results suggested that simvastatin impairs the proliferation of C2C12 myoblasts and of satellite cells in mouse skeletal muscle and that intermediates of the cholesterol synthesis pathway are important for C2C12 myoblast proliferation.

Simvastatin repressed regeneration factors expression in C2C12 myoblasts and mouse skeletal muscle.

Next, we investigated whether markers of cell differentiation and fusion to myotubes were affected in C2C12 myoblasts or mice were treated with simvastatin. For that, we measured the mRNA expression of MyoD and ADAM12, which are markers of differentiation and fusion, respectively. (Berkes, Tapscott 2005; Cao et al. 2003; Galliano et al. 2000; Yagami-Hiromasa et al. 1995) Simvastatin (10 μ M) significantly reduced MyoD and more severely ADAM-12 gene expression in cells (Fig. 6A and B). Co-treatment with insulin failed to prevent this effect in C2C12 myoblasts (Fig. 6A and B), whereas addition of mevalonate

maintained the mRNA expression of MyoD and ADAM-12 in the presence of simvastatin (Fig. 6A and B). Similarly, simvastatin significantly repressed the expression of ADAM-12 in mice gastrocnemius (Fig. 6D), whereas no such effect was observed for MyoD mRNA expression (Fig. 6C).

Insulin and mevalonate prevented apoptotic cascade activation in C2C12 myotubes, but not in myoblasts.

We showed that simvastatin decreased the viability of myotubes and myoblasts (Fig. 1) and caused mitochondrial dysfunction in myoblasts (Figs. 2 and 3). To investigate the mechanism of cell death caused by simvastatin, we studied markers of apoptosis in the two cell models. After 24 hours of treatment with simvastatin, there was a significant release of cytochrome c from the mitochondria into the cytosol in myoblasts and in myotubes (Fig. 7A and B). In myoblasts, this increase was attenuated, but not completely prevented, by co-treatment with insulin or mevalonate in myoblasts (Fig. 7A). In myotubes, the simvastatin-induced increase in cytosolic cytochrome c was completely prevented by co-treatment with mevalonate and partially by insulin (Fig. 7B).

Next, we evaluated the activation of the apoptotic cascade by assessing the formation of the cleaved form of pro caspase-3. In myoblasts, simvastatin, whether applied alone or together with insulin or mevalonate, significantly stimulated the cleavage of caspase-3 (Fig. 7C). In myotubes, caspase-3 activation was also significantly increased by simvastatin (Fig. 7D). In this cell model, insulin and mevalonate were able to prevent almost completely caspase-3 activation by simvastatin (Fig. 7D).

Discussion

The current study shows that simvastatin is more toxic on myoblasts than myotubes and that simvastatin inhibits the proliferation of myoblasts and their fusion to myotubes.

As described already in previous publications, simvastatin was cytotoxic for myotubes and this toxicity could be prevented by insulin up to 8 hours after the addition of simvastatin (Fig.S1).(Bonifacio et al. 2015; Bonifacio et al. 2017; Sanvee et al. 2019a) Regarding AK release and cellular ATP content, the two markers of cytotoxicity investigated, cytotoxicity of simvastatin was more accentuated for myoblasts than myotubes. A striking difference between the susceptibility of myoblasts and myotubes was for instance the finding that insulin was not able to prevent simvastatin-associated cytotoxicity in myoblasts if administered after the addition of simvastatin.

As suggested by the observed decrease in the cellular ATP content and the reduced oxygen consumption in the presence of glutamate and pyruvate, simvastatin impaired the function of the mitochondrial electron transport chain. Mitochondrial dysfunction associated with statins has been reported in previously and is an important reason for statin-associated myotoxicity.(Bonifacio et al. 2016; Bouitbir et al. 2011; Kaufmann et al. 2006; Schirris et al. 2015; Sirvent et al. 2008) Mitochondrial dysfunction was less apparent when cellular oxygen consumption was expressed per protein than per cell count. For the determination of the protein content, the cell supernatant is removed and the protein content is

determined in the adhering cells. Cells that do not adhere to the surface are damaged, but they do not necessarily have to be non-viable; the relation to the protein content may therefore underestimate mitochondrial dysfunction. Independently of the method used to express cellular oxygen consumption, mitochondria in myoblasts appeared to be damaged by simvastatin to a higher degree than in myotubes, explaining the more accentuated cytotoxicity for myoblasts. This is also reflected by the production of mitochondrial ROS, which was higher in myoblasts than in myotubes exposed to simvastatin. Since inhibition of mainly complex I and III of the electron transport chain is associated with increased mitochondrial ROS production,(Lenaz 2001; Markevich, Hoek 2015) this supports the observation that mitochondrial dysfunction was more accentuated in myoblasts than in myotubes.

Simvastatin caused spilling of cytochrome c into the cytosol and cleavage of caspase-3, which was more accentuated in myoblasts than in myotubes. This finding indicates initiation of apoptosis triggered by mitochondrial dysfunction.(Orrenius et al. 2007) The observation that both spilling of cytochrome c into the cytoplasm and cleavage of caspase-3 were more pronounced in myoblasts than myotubes again supports the notion that mitochondrial damage was more accentuated in myoblasts than in myotubes.

In contrast to cytotoxicity and mitochondrial damage, the effects on the insulin signaling pathway were not much different between myoblasts and myotubes, suggesting that cytotoxicity and impaired insulin signaling have different mechanisms. Simvastatin decreased the phosphorylation of the IR β , Akt Ser473 and S6rp by 60–90% in both myoblasts and myotubes. The results in myotubes correspond well with our previous investigations.(Bonifacio et al. 2015; Bonifacio et al. 2017; Sanvee et al. 2019b) Insulin was able to prevent the decrease in phosphorylation by simvastatin at least partially in both cell systems investigated. Since the phosphorylation of Akt Ser473 is considered to be specific for mTORC2, (Sarbasov et al. 2005; Yang et al. 2015) our results indicate that mTORC2 can be activated by increased insulin signaling in both cell types. The prevention of phosphorylation by mevalonate was less accentuated than for insulin, suggesting that, at least in the cell models used, protein prenylation is less important than insulin signaling for the function of the protein kinases in the insulin signaling pathway.

Simvastatin had a strong negative effect on the proliferation and differentiation of myoblasts and inhibited the fusion to myotubes. C2C12 myoblasts can be regarded as murine satellite cells that can be activated and differentiated to myotubes, which resemble muscle fibers.(Burattini et al. 2004) After having activated the C2C12 myoblasts by substrate deprivation, we studied markers of myoblast proliferation, differentiation and fusion to myotubes for assessing the effect of simvastatin on muscle regeneration. Our investigations show clearly that simvastatin impairs C2C12 cell proliferation as assessed by the MTT test and by the determination of mRNA expression of Ki-67, a marker of cell proliferation.(Dumont et al. 2015) C2C12 differentiation, investigated by MyoD mRNA expression, (Hernandez-Hernandez et al. 2017) and fusion to myotubes, assessed visually and by mRNA expression of the metalloprotease ADAM-12 needed for myoblast fusion,(Yagami-Hiromasa et al. 1995) were also impaired by simvastatin. Analysis of Ki-67 and ADAM-12 mRNA expression in mice skeletal muscle revealed a similar picture, indicating that the same processes can be observed also *in vivo*. The protein complex mTORC1 has been described to have an important role in the transition of quiescent satellite

cells to alert cells, thus in the activation of stem cells.(Rodgers et al. 2014) Since simvastatin inhibits activation of mTORC1 (indirectly by inhibition of mTORC2, which causes incomplete activation of Akt and, consequently, impaired activation of mTORC1(Bonifacio et al. 2015; Mullen et al. 2011)), impaired proliferation of myoblasts by simvastatin can be explained by mTORC1 inhibition. The consequence of impaired myoblast activation and proliferation is restrained differentiation and fusion since less myoblasts enter the terminal parts of the regeneration process.

This negative effect of simvastatin on myoblast proliferation and myotubes formation may be clinically relevant, since it could impair muscle regeneration after muscle injury in patients treated with statins. In support of this notion, it is well established that heavy exercise can induce or worsen myopathy in patients treated with statins. While the prevalence of myopathy can reach 30% in patients not performing heavy exercise,(Alfirevic et al. 2014; Norata et al. 2014) it may be as high as 75% in elite athletes treated with a statin.(Sinzinger, O'Grady 2004) It is conceivable that muscle injury associated with heavy exercise in statin users can cause prolonged muscular symptoms because statins impair muscle regeneration.

In conclusion, myoblasts were more susceptible to the toxic effects of simvastatin, in particular regarding the impairment in mitochondrial function. The number of satellite cells may therefore be reduced in statin users. In addition, simvastatin impaired the proliferation of C2C12 myoblasts and myotube formation, possibly by inhibition of mTORC1. Impaired muscle regeneration may play an important role in statin-associated myopathy.

Declarations

- This work was supported by a grant of the Swiss National Science Foundation to SK (31003A_156270)
- None of the authors reports a conflict of interest regarding this study
- Animal experiments were accepted by the cantonal veterinary authority of Basel, Switzerland (License 2847) and were performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.
- All authors have seen and accepted the last version of the manuscript
- Author contribution:
 - Gerda Sanvee performed the experiments, interpreted the data and wrote the draft of the manuscript
 - Jamal Bouitbir designed the study, supervised the lab work and wrote the draft of the manuscript
 - Stephan Krähenbühl designed and financed the study, discussed the data and wrote the final version of the manuscript

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Figures

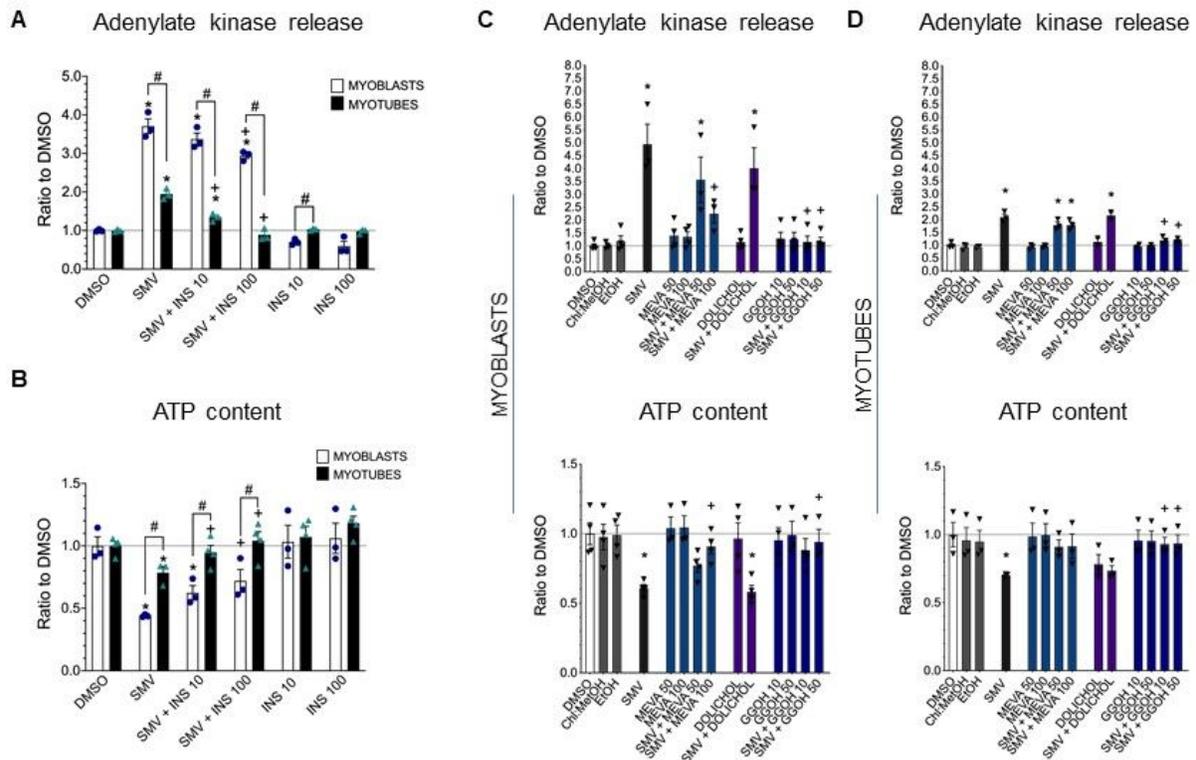


Figure 1

Effects of simvastatin, insulin and cholesterol biosynthesis intermediates on membrane integrity and intracellular ATP of C2C12 myoblasts and myotubes. Membrane toxicity was assessed by release of adenylate kinase (AK) into the medium and metabolic activity was assessed by the depletion of intracellular ATP. Treatment with DMSO 0.1% was used as control for simvastatin, 0.15% chloroform:methanol (2:1) as a control for dolichol and 0.1% ethanol as a control for geranylgeraniol. A. Membrane toxicity in C2C12 myoblasts and myotubes after treatment with 10 μ M simvastatin and/or 10 or 100 ng/mL insulin for 24 hours. B. ATP content in C2C12 myoblasts and myotubes after treatment with 10 μ M simvastatin and/or 10 or 100 ng/mL insulin for 24 hours. Membrane integrity and ATP content C. in myoblasts and D. in myotubes treated for 24 hours with 10 μ M simvastatin and/or 50 and 100 μ M mevalonolactone and/or 10 μ M dolichol and/or 10 and 50 μ M geranylgeraniol. Data represent the mean \pm SEM of at least three independent experiments. Data were analyzed by one-way ANOVA with Newman-Keuls's post-hoc test. * P <0.05 versus respective control incubations; + P <0.05 versus 10 μ M simvastatin; # P <0.05 myoblasts versus myotubes. SMV: simvastatin, INS: insulin, MEVA: mevalonolactone, GGOH: geranylgeraniol, EtOH: ethanol, Chl:metOH: chloroform:methanol.

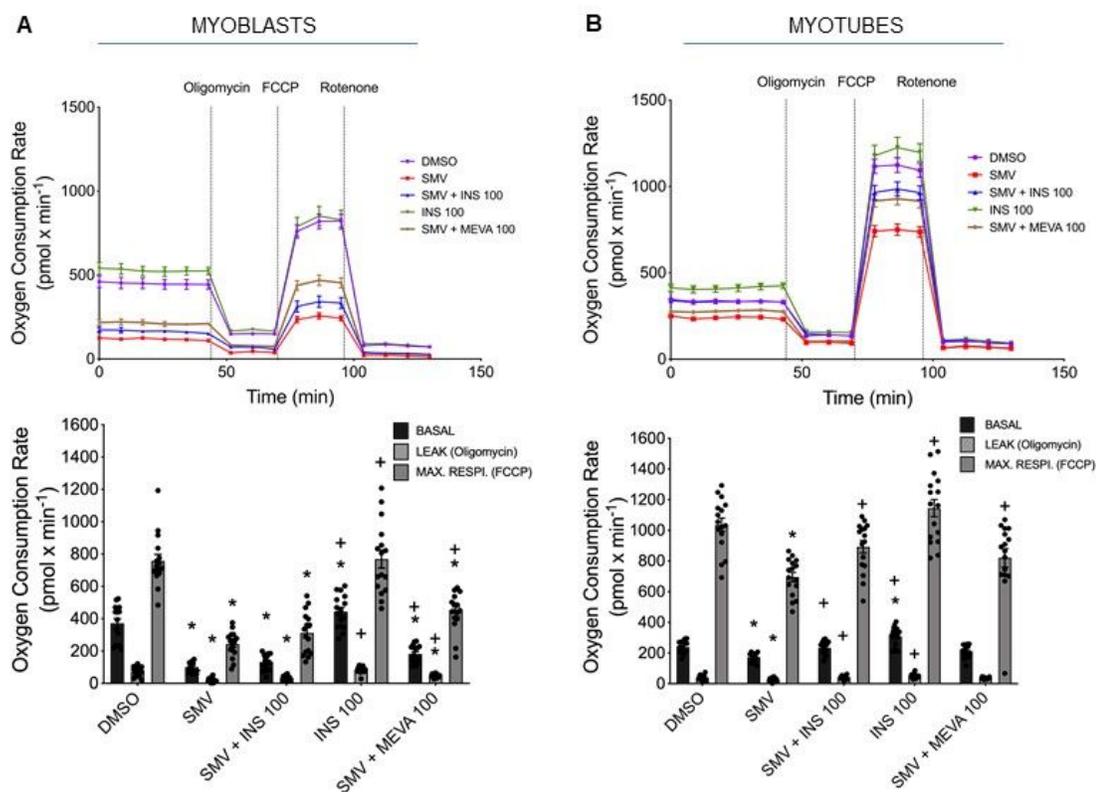


Figure 2

Effect of simvastatin on the function of the respiratory chain in C2C12 myoblasts and myotubes. A. Oxygen consumption rate (OCR) and corresponding quantifications in C2C12 myoblasts and in B. C2C12 myotubes following 24 hours exposure to 10 μ M simvastatin, 100 ng/mL insulin and/or 100 μ M mevalonolactone. Values are expressed per 25'000 cells. Data represent the mean \pm SEM of four independent experiments. Data were analyzed by one-way ANOVA with Newman-Keuls's post-hoc test. * $P < 0.05$ versus 0.1 % DMSO. SMV: simvastatin, INS: insulin, MEVA: mevalonolactone.

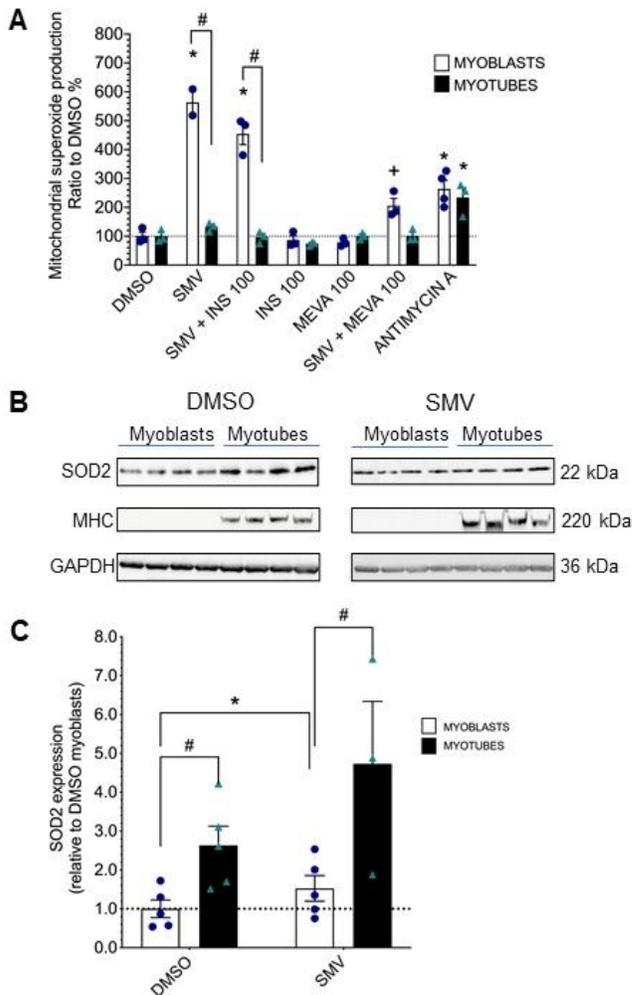


Figure 3

Mitochondrial superoxide accumulation and superoxide dismutase 2 (SOD2) expression in C2C12 cells treated with simvastatin. MitoSOX dye was used to quantify mitochondrial superoxide accumulation. DMSO 0.1% was used as a negative control and 100 μ M antimycin A was used as a positive control (1 hour exposure). A. Mitochondrial superoxide accumulation in C2C12 myoblasts and myotubes following 24 hours exposure to the drugs. B. Immunoblots and C. quantification of SOD2 and MHC in C2C12 cells treated with 0.1% DMSO and 10 μ M simvastatin for 24 hours. GAPDH was used as loading control. Data represent the mean \pm SEM of at least three independent experiments. Data were analyzed by one-way ANOVA with Newman-Keuls's post-hoc test. * $P < 0.05$ versus 0.1 % DMSO; + $P < 0.05$ versus 10 μ M simvastatin; # $P < 0.05$ myoblasts versus myotubes. SMV: simvastatin, INS: insulin, MEVA: mevalonolactone, SOD2: superoxide dismutase 2, MHC: myosin heavy chain.

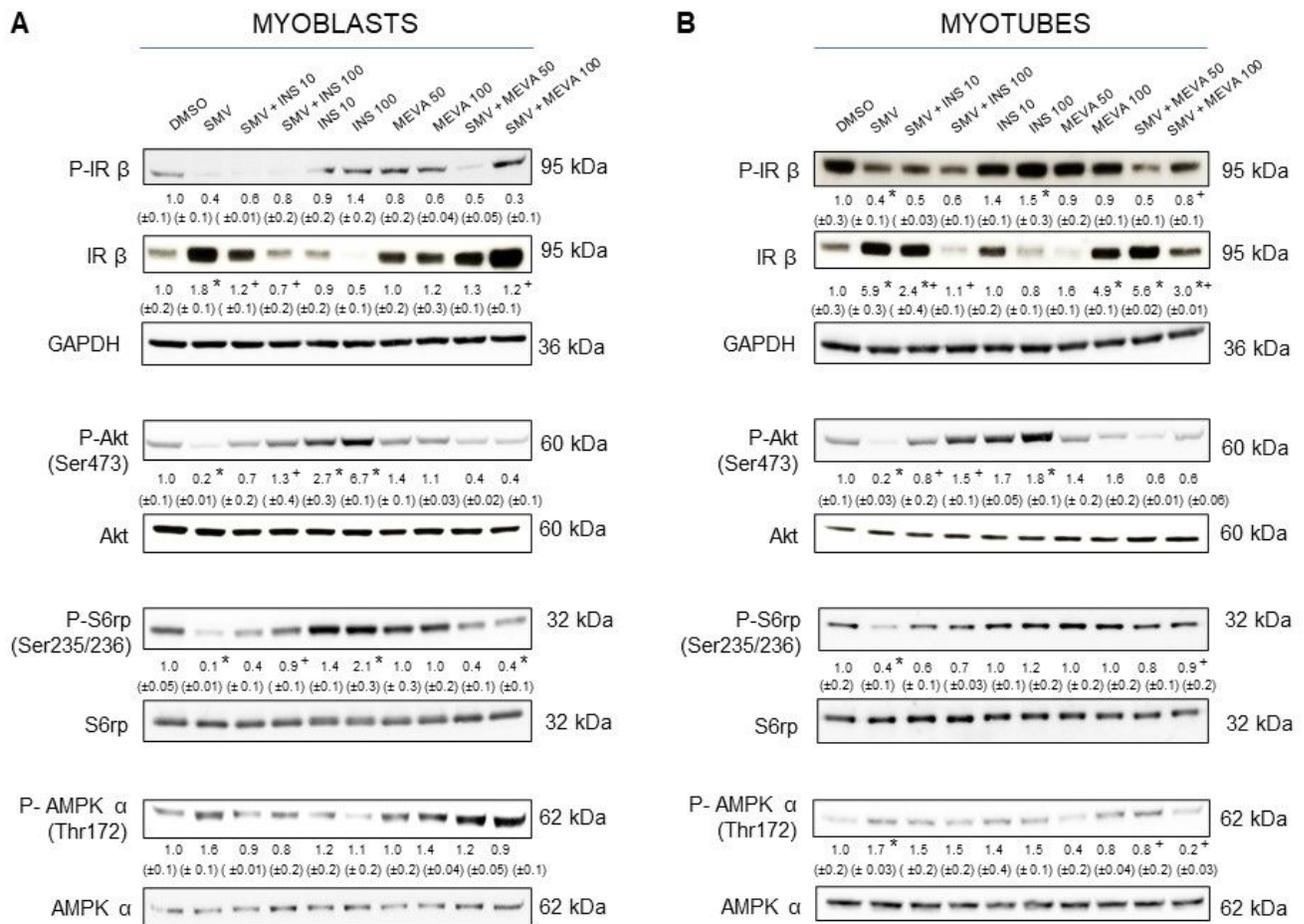


Figure 4

Effects of simvastatin, insulin and mevalonate on insulin receptor (IR)/Akt signaling in C2C12 myoblasts and myotubes. C2C12 myoblasts and myotubes were treated with 10 μ M simvastatin and/or 10 or 100 ng/mL insulin and/or 50 or 100 μ M mevalonate for 24 hours before protein lysate collection. GAPDH was used as loading control. A. Quantification and corresponding immunoblots of the expression of the phosphorylated and total forms of the insulin receptor β chain, Akt, AMPK and S6rp in C2C12 myoblasts. B. Quantification and immunoblots of the expression of the phosphorylated and total forms of the insulin receptor β chain, Akt, AMPK and S6rp in C2C12 myotubes. Data were analyzed by one-way ANOVA with Newman-Keuls's post-hoc test. Data represent the mean \pm SEM of at least three independent experiments. * $P < 0.05$ versus 0.1 % DMSO; + $P < 0.05$ versus 10 μ M simvastatin. SMV: simvastatin, INS: insulin, MEVA: mevalonolactone.

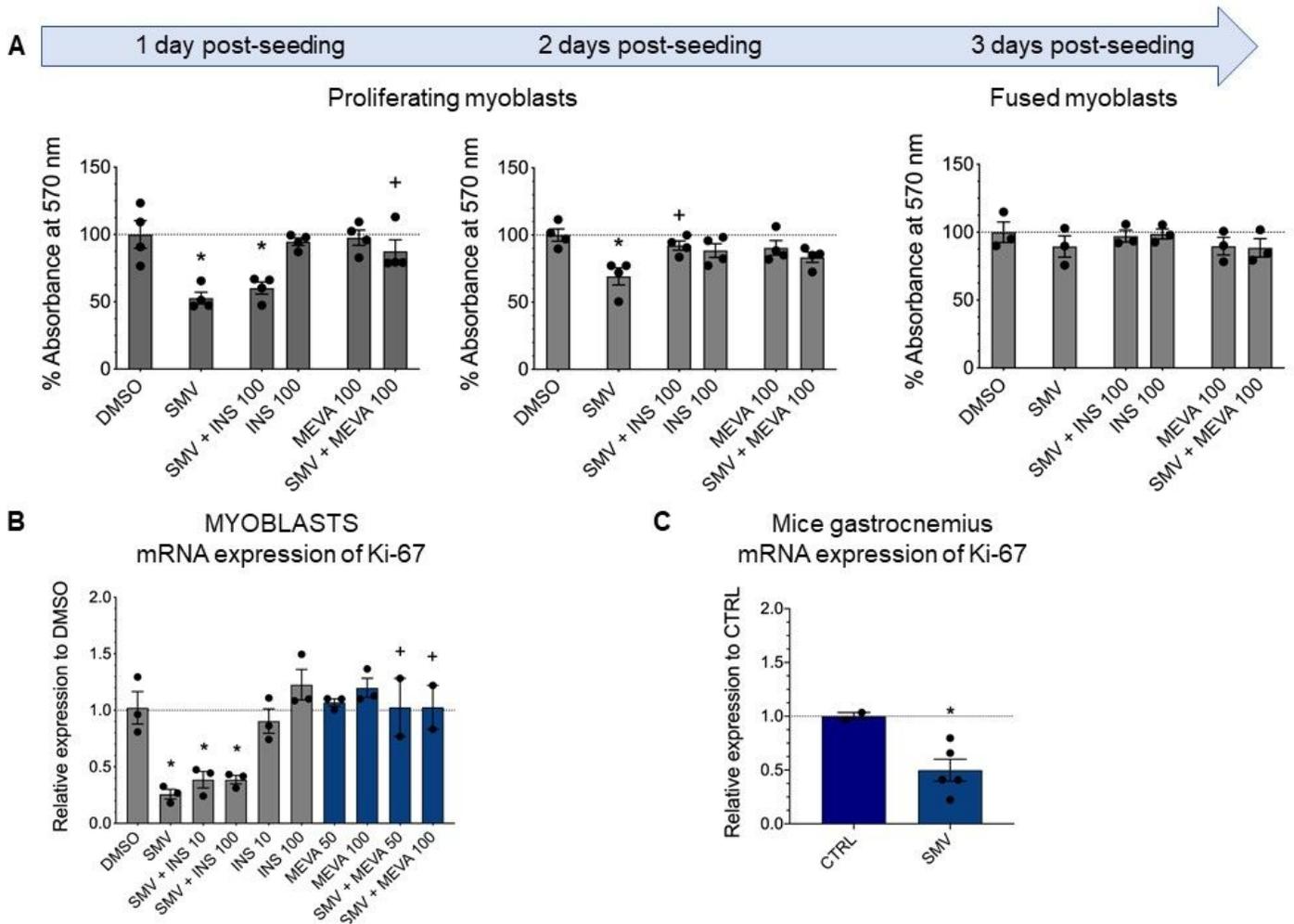


Figure 5

Influence of simvastatin on the proliferation of C2C12 myoblasts and in mouse skeletal muscle. C57BL/6 male mice were treated daily with simvastatin 5 mg/kg for 21 days. A. MTT assay was used to determine cellular proliferation of proliferating myoblasts (1 and 2 days post-seeding) and of fused myoblasts (3 days post-seeding) exposed to 10 μ M simvastatin, 100 ng/mL insulin and/or 100 μ M mevalonate for 24 hours. B. mRNA expression of the proliferation marker Ki-67 was determined by real-time PCR in proliferating myoblasts treated for 24 hours. C. mRNA expression of Ki-67 was determined by real-time PCR in gastrocnemius of mice treated for 21 days with water (CTRL) or simvastatin (SMV). Data in mice represent the mean \pm SEM; n = 10, *P<0.05. Data in C2C12 myoblasts represent the mean \pm SEM of at least three independent experiments (carried out in triplicates for the RT-PCR). Data were analyzed by one-way ANOVA with Newman-Keuls's post-hoc test (A, B) or by an unpaired t-test (C). *P<0.05 versus 0.1 % DMSO; +P<0.05 versus 10 μ M simvastatin. SMV: simvastatin, INS: insulin, MEVA: mevalonolactone, CTRL: control (water).

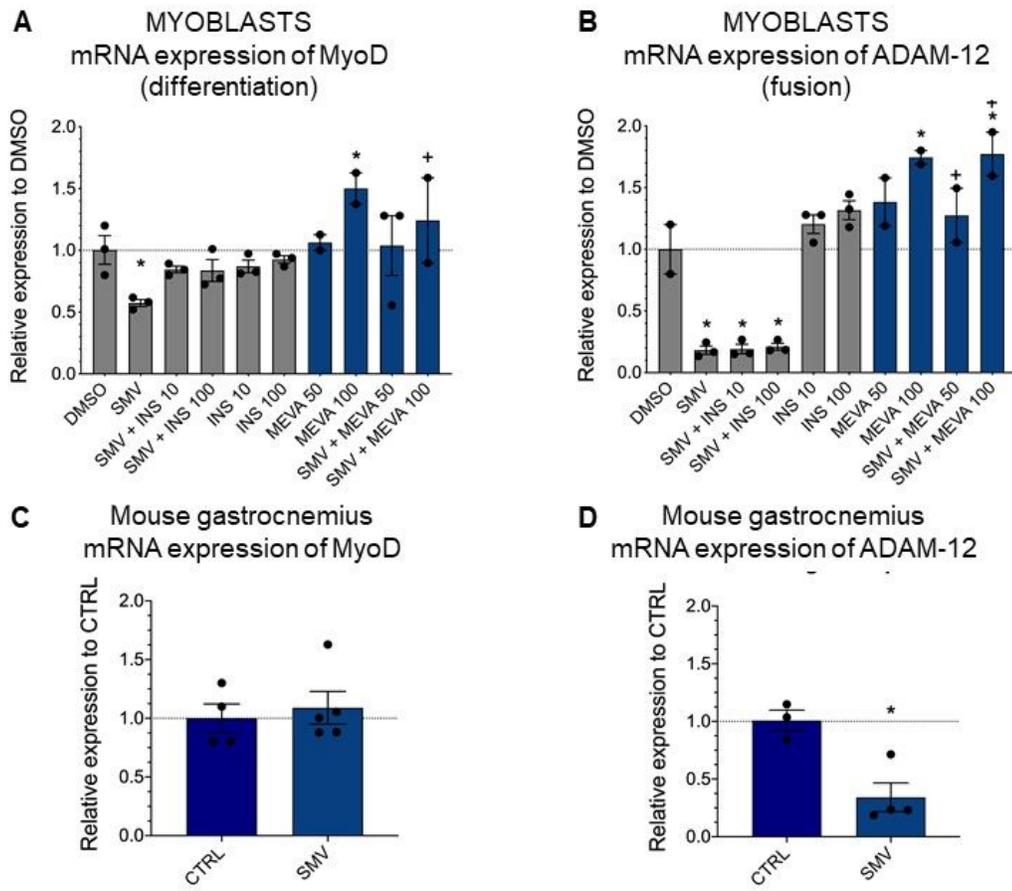


Figure 6

Expression of differentiation and fusion markers in C2C12 myoblasts and in mouse skeletal muscle. C57BL/6 male mice were treated daily with simvastatin 5 mg/kg for 21 days. A. mRNA expression of the differentiation marker MyoD was determined by real-time PCR in proliferating myoblasts exposed to simvastatin, insulin and/or mevalonate for 24 hours. B. mRNA expression of the fusion marker ADAM-12 was determined by real-time PCR in proliferating myoblasts exposed to simvastatin, insulin and/or mevalonate for 24 hours. C. mRNA expression of MyoD was determined by real-time PCR in gastrocnemius of mice treated with water (CTRL) or simvastatin (SMV). D. mRNA expression of the ADAM-12 was determined by real-time PCR in gastrocnemius of mice. Data in mice represent the mean \pm SEM; n = 10, *P<0.05. Data in C2C12 myoblasts represent the mean \pm SEM of at least three independent experiments (carried out in triplicates for the RT-PCR). Data were analyzed by one-way ANOVA with Newman-Keuls's post-hoc test (A, B) or by an unpaired t-test (C, D). *P<0.05 versus 0.1 % DMSO; +P<0.05 versus 10 μ M simvastatin. SMV: simvastatin, INS: insulin, MEVA: mevalonolactone, CTRL: control mice (water).

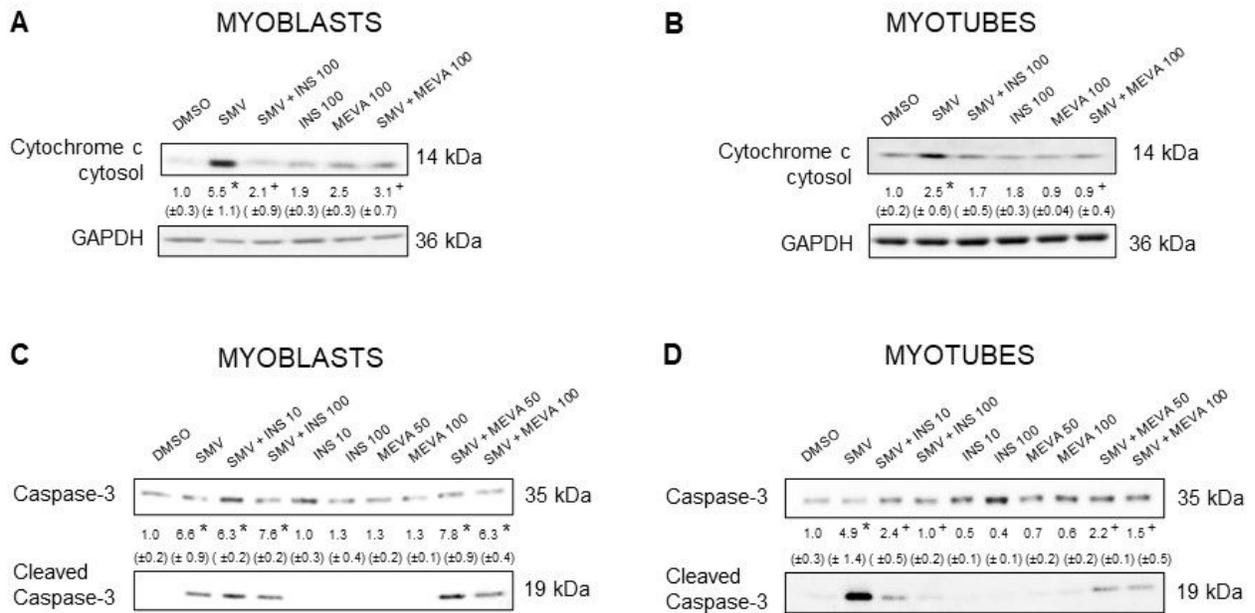


Figure 7

Apoptosis induction in C2C12 myoblasts and myotubes by simvastatin. Cytochrome c release from mitochondria to the cytosol and cleavage of the executioner caspase-3 was determined in cells treated for 24 hours with simvastatin, insulin and/or mevalonate. A. Immunoblots and quantification of cytochrome c in the cytosol in C2C12 myoblasts and B. in C2C12 myotubes. C. Representative immunoblots of procaspase-3 and cleaved caspase-3 in myoblasts and D. in C2C12 myotubes. GAPDH was used as loading control and compartment specificity. Data represent the mean \pm SEM of at least three independent experiments. Data were analyzed by one-way ANOVA with Newman-Keuls's post-hoc test. * $P < 0.05$ versus 0.1 % DMSO; + $P < 0.05$ versus 10 μ M simvastatin. SMV: simvastatin, INS: insulin, MEVA: mevalonolactone.

Supplementary Files

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