The AKT/mTOR signaling pathway plays a key role in statin-induced myotoxicity

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ABSTRACT

Statins are drugs that lower blood cholesterol levels and reduce cardiovascular morbidity and mortality. They are generally well-tolerated, but myopathy is a potentially severe adverse reaction of these compounds. The mechanisms by which statins induce myotoxicity are not completely understood, but may be related to inhibition of the AKT signaling pathway. The current studies were performed to explore the down-stream effects of the statin-associated inhibition of AKT within the AKT signaling pathway and on myocyte biology and morphology in C2C12 myotubes and in mice in vivo. We exposed C2C12 myotubes to 10 μM or 50 μM simvastatin, atorvastatin or rosuvastatin for 24 h. Simvastatin and atorvastatin inhibited AKT phosphorylation and were cytotoxic starting at 10 μM, whereas similar effects were observed for rosuvastatin at 50 μM. Inhibition of AKT phosphorylation was associated with impaired phosphorylation of S6 kinase, ribosomal protein S6, 4E-binding protein 1 and FoxO3a, resulting in reduced protein synthesis, accelerated myofibrillar degradation and atrophy of C2C12 myotubes. Furthermore, impaired AKT phosphorylation was associated with activation of caspases and PARP, reflecting induction of apoptosis. Similar findings were detected in skeletal muscle of mice treated orally with 5 mg/kg/day simvastatin for 3 weeks. In conclusion, this study highlights the importance of the AKT/mTOR signaling pathway in statin-induced myotoxicity and reveals potential drug targets for treatment of patients with statin-associated myopathies.

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1. Introduction

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are currently the most often prescribed and the most effective cholesterol-lowering drugs. They are generally well-tolerated but can rarely be associated with potentially severe adverse reactions, particularly on skeletal muscle [1,2]. Statin-associated muscle injuries vary from mild myopathy to potentially lethal rhabdomyolysis, a condition characterized on skeletal muscle [1,2]. In a large study in the USA, the incidence of rhabdomyolysis leading to hospitalization was zero for pravastatin, approximately 0.5 per 10,000 person-years for cerivastatin [6]. In combination with a fibrate, the incidence rose by a factor of 10 or more.

While a high exposure to statins is a clearly established risk factor for statin-associated myopathy [1,2,7], the molecular mechanisms leading to muscle damage, in particular rhabdomyolysis, in patients treated with statins are less clear [3]. Different factors could play a role; for example impaired mitochondrial function [8,9], induction of skeletal muscle breakdown due to increased expression of atrogin-1/MaFbx [10], reduction of skeletal muscle protein synthesis [11], inhibition of small GTPases due to impaired prenylation [12] and/or impaired creatine synthesis [13].

Previous work from our group suggested that simvastatin-induced myotoxicity might be related to inhibition of the phosphorylation and thereby activation of AKT [14]. As shown in Fig. 1, the AKT signaling pathway is essential for muscle growth during development and regeneration. AKT functions as a key regulator of both protein synthesis and degradation by activating the protein kinase mammalian target of rapamycin (mTOR) [15] and by inhibiting forkhead box O (FoxO) transcription factors [16]. Moreover, AKT is directly involved in the regulation of cell survival through the suppression of apoptosis by blocking the activation of caspases [17].

Activation of mTOR by AKT leads to phosphorylation of the ribosomal protein S6 kinase (S6K) at Thr 389, which phosphorylates and thereby activates the ribosomal protein S6 (rpS6) [18]. Furthermore, activation of 10,000 person-years for cerivastatin [6]. In combination with a fibrate, the incidence rose by a factor of 10 or more.
mTOR leads to phosphorylation of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), thereby disrupting its interaction with eIF4E and making eIF4E available for mRNA translation [19]. Impaired activation of mTOR is therefore expected to reduce protein synthesis.

Forkhead members of the O class (FoxO) form a family of transcription factors involved in protein breakdown and apoptosis. Several insults such as apoptosis, oxidative stress and/or cytokine release activate FoxO3a by dephosphorylation, which is followed by nuclear translocation of the dephosphorylated protein [20]. Activation of FoxO3a is associated with muscle atrophy, since nuclear translocation of FoxO3a triggers the expression of mediators of proteolysis such as muscle RING-finger protein-1 (MuRF-1) and muscle atrophy F-box (atrogin-1/MaFbx) [21,22].

Taking into account our previous observations regarding the effect of simvastatin on AKT activation [14] and the central role of AKT for skeletal muscle protein metabolism and integrity [15–17], the principle aims of the current study were 1. to investigate whether inhibition of the phosphorylation of AKT is specific for simvastatin or can also observed for other statins, 2. to investigate the downstream effects of the inhibition of AKT on target proteins involved in apoptosis, protein degradation and protein synthesis, and 3. to demonstrate that these effects cannot only be shown in vitro in cultured myotubes but also in vivo in mice treated with simvastatin.

2. Materials and methods

2.1. Chemicals

Simvastatin lactone (Sigma-Aldrich, St. Louis, MO, USA) was converted into the active acid following the protocol of Bogman et al. [23]. We prepared stock solutions (10 mM and 50 mM) in dimethylsulfoxide (DMSO) for simvastatin and in water for rosuvastatin and atorvastatin. We stored them at −20 °C. All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), except where indicated.

2.2. Cell lines and cell culture

C2C12 myoblasts were originally obtained from the American Type Culture Collection (ATCC) and kindly provided by Novartis (Basel, CH). We grew cells at 37 °C and 5% CO2 in a humidified cell culture incubator and we passaged them using trypsin. We initially seeded 150,000 myoblasts per well in a 6-well plate, and grew them for 2 days in growth medium consisting of high glucose (4.5 g/L) Dulbecco’s Modified Eagle Medium (DMEM) containing GlutaMAX (Invitrogen, Basel, Switzerland) and 10% heat-inactivated fetal bovine serum (Gibco, Paisley, UK). Afterwards, we induced cell differentiation using high glucose DMEM supplemented with 2% horse serum (Gibco, Paisley, UK) for 3 days. A morphological analysis of the cell cultures showed that 83 ± 3% of the nuclei were located in tubes. Then, we incubated the cell cultures in serum-free DMEM (Invitrogen, Basel, Switzerland) for 24 h before the addition of the test compounds. Since simvastatin had been dissolved in DMSO, we used control incubations containing 0.1% DMSO. This DMSO concentration has been shown not to be cytotoxic [24]. After 24 h treatment, we collected the cells to examine the expression of genes and activation of proteins of interest.

2.3. Animals

The animal study was approved by the cantonal veterinary authority (License 2659) and was performed in accordance with the guidelines...
from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Seven weeks old male C57BL/6 mice were obtained from Charles River Laboratories (Suttfeld, Germany) and were housed under controlled conditions at a 12 h/12 h light/dark cycle with free access to water and a standard pellet chow. After 7 days of acclimatization, the mice were randomly divided into two groups of eight animals. We treated the mice either with simvastatin (5 mg/kg/day) dissolved in water (SMV group) or water (CTL group) by oral gavage for 3 weeks. The body weight of the animals and food intake were recorded every 2 days.

2.4. Sample collection

After 21 days of treatment, mice were anesthetized with an intraperitoneal application of ketamine (100 mg/kg) and xylazine (10 mg/kg). Gastrocnemius muscle samples were frozen in liquid nitrogen immediately after excision. Since the tissue samples were obtained from living animals (mice were anesthetized), the time between sampling and freezing was only few seconds. Samples were kept at −80 °C until analysis.

2.5. Cytotoxicity assay

We used the ToxiLight BioAssay Kit (Lonza, Basel, Switzerland) to investigate the cytotoxicity of the compounds on C2C12 myotubes. The release of adenylate kinase was measured according to the manufacturer’s manual using luminescence with a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland).

2.6. Cell lysis and immunoblotting

After incubation with different compounds, we homogenized C2C12 myotubes in Phosphosafe buffer (EMD Millipore, USA). Then, we centrifuged the samples at 1600 ×g for 10 min at 4 °C. We then collected the supernatant and determined the protein content in each supernatant using the BCA Protein Assay Kit (Pierce, Thermo Scientific, Rockford, USA). Supernatants were applied on 4–12% Bis–Tris polyacrylamide gels (Invitrogen, Basel, Switzerland) for protein separation and run under reducing conditions. After separation, proteins were transferred to polyvinylidendifluoride membranes (EMD Millipore, Massachusetts, USA). We blocked the membranes with 5% nonfat dry milk in phosphate buffered saline (PBS) (Gibco, Paisley, UK) containing 0.1% Tween-20 (Sigma-Aldrich, MO, USA) (PBS-T) for 1 h at room temperature before incubation overnight with the primary antibody (Cell Signaling Technology, USA) diluted 1:1000 in blocking buffer. The day after, we incubated the blots for 1 h with the secondary antibody (Santa Cruz Biotechnology, USA) diluted 1:2000 in 5% nonfat milk in PBS-T. Then, we washed the membranes and developed the immunoreactive bands using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, United Kingdom). Chemiluminescent images were scanned using an HP ScanJet 8300 (Hewlett-Packard Co., Palo Alto, CA) and band intensities of the scanned images were analyzed using the National Institutes of Health Image J program (version 1.41). To correct for loading differences, the scanning units obtained for the test proteins were divided by the scanning units obtained for either the respective total protein or for housekeeping protein GAPDH.

The ELISA for the quantification of S6K phosphorylation at T389 was obtained from Abcam, Cambridge, UK (pT389 S6K PhosphoTracer ELISA Kit). The ELISA was performed exactly using the protocol of the provider described in the protocol book.

2.7. Real-time polymerase chain reaction (RT-PCR)

We treated C2C12 myotubes with 10 μM and 50 μM of simvastatin, rosvastatin or atorvastatin for 24 h. Afterwards, mRNA was extracted and purified using the Qiagen RNAeasy mini extraction kit (Qiagen, Hombrechtikon, Switzerland). RNA concentration and integrity were evaluated with the NanoDrop 2000 (Thermo Scientific, Wohlen, Switzerland) and cDNA was synthesized from 10 μg RNA using the Qiagen omniscript system. We performed the real-time PCR analysis using SYBR green (Roche Diagnostics, Rotkreuz, Basel). We assessed mRNA expression for genes associated with muscle atrophy using the following primers. MAFbx: forward 5′-AGTAGGGACCGCCTACTGT3′ and reverse 5′-GATCAAAACTCCGAACT3′. MuRF-1: forward 5′-CTG CAGAATGACAAAGA3′ and reverse 5′-GGCGTAGAGGGTTGCTAAACT3′. Real time PCR was performed using the Viia7 software (Life technologies, Switzerland). We calculated relative quantities of specifically amplified cDNA with the comparative–threshold cycle method using GAPDH as the housekeeping gene (forward 5′-CATGGCTTCTCCTTGTCC TA-3′ and reverse 5′- CCTGCTTCACACCTTTGTA-3′). Controls for non-specific amplification were run without reverse transcription.

2.8. Immunostaining and diameter measurement

To analyze changes in myotube diameter, myotubes were stained following the protocol of Minetti et al. [25]. Briefly, myotubes were fixed with 4% PFA (Paraformaldehyde) and permeabilized with 0.2% Triton. Non-specific binding was blocked with goat serum (Gibco, Paisley, UK) followed by incubation with anti-myosin heavy chain (anti-MHC) (EMD Millipore, Massachusetts, USA) diluted 1:1000 in PBS and subsequently with Alexa fluor 488 (Invitrogen, Basel, Switzerland) diluted 1:2000 in PBS. To measure diameters, we used CellSens Insight Technology (Thermo scientific, Wohlen, Switzerland). The protein content of the cells was determined as described in Section 2.6.

2.9. Apoptotic DNA fragmentation ELISA

Apoptosis-associated DNA fragmentation was quantified using the cell death detection ELISA kit from Roche (Roche Applied Science, Indianapolis, IN) by assessing the cytosolic histone-associated mono- and oligo-nucleosomes. Briefly, the extracted nuclei-free cytosolic fraction was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti- DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically after incubation with 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) for 10 min at 20 °C. The change in color was measured at 405 nm using a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland). Measurements were performed in triplicate with all samples analyzed on the same microtiter plate in the same setting. The OD reading was normalized to the protein content of the incubations.

2.10. Histological analysis of muscle tissue

Muscle samples were frozen in isopentane. Frozen sections were stained with hematoxylin–eosin. H&E staining photographs were captured on an Olympus BX61 microscope (Olympus, Hamburg, Germany). After staining, we selected random muscle fibers with a distinct cell membrane and we excluded elongated fibers indicating an oblique section. We employed Image J (version 1.41) software to measure muscle fibers within 4 muscle cross-sections from 4 different mice belonging to each group. We then calculated the mean and the respective SEM.

2.11. mRNA extraction of muscle tissue

mRNA was extracted and purified using the Quiagen RNAeasy mini extraction kit (Hombrechtikon, Switzerland) with a DNA digestion step to ensure RNA quality. RNA quality was evaluated with the NanoDrop 2000 (Thermo Scientific, Wohlen, Switzerland). We then
synthesized cDNA using the Quiagen omniscript system and used 10 ng of cDNA for quantitative RT-PCR performed as described above.

### 2.12. Immunoblotting of muscle tissue

Expression of components of the AKT signaling pathway and of apoptosis pathways was checked by Western Blotting. We homogenized frozen muscle samples with a Micro-Dismembrator S (Sartorius, Göttingen, Germany) during 1 min at 2000 rpm. We then resuspended the tissue powder in protein extraction reagent (T-PER, thermo Scientific, Wohlen, Switzerland) containing a protease inhibitor cocktail (Roche AG, Basel, Switzerland), centrifuged at 9000 g at 4 °C for 5 min, collected the supernatant and determined protein levels.

Protein separation, blotting and quantification of the separated proteins were performed as the in vitro samples described above.

### 2.13. TUNEL assay

TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) staining of myonuclei positive for DNA strand breaks was performed using a commercially available fluorescence detection kit (Life Technologies, Zug, Switzerland). Cross sections (10 µm) of muscles cut with a cryostat microtome were fixed with 4% paraformaldehyde for 15 min and the fixed sections were permeabilized with 2 mg/mL proteinase K. The TUNEL reaction mixture containing terminal deoxynucleotidyltransferase (TdT) and fluorescein-labeled dUTP was added to the sections in portions of 50 µL and then incubated for 60 min at 37 °C in a humidified chamber in the dark. After incubation, the sections were rinsed three times in PBS for 1 min each. Following embedding with Prolong diamond antifade mountant with DAPI (Life Technologies, Zug, Switzerland), the sections were investigated with a fluorescence microscope (Olympus BX61, Germany; 40× objective).

### 2.14. Statistical methods

Data are presented as mean ± SEM. Statistical significance (*P < 0.05; **P < 0.01, ***P < 0.001) was determined using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. We performed all the statistical analyses using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, US).

### 3. Results

#### 3.1. Statins are toxic for C2C12 myotubes

The cytotoxicity of simvastatin, rosuvastatin, and atorvastatin for C2C12 myotubes was determined after exposure for 24 h by measuring the release of adenylate kinase (AK) (Fig. 2). Treatment with 10 µM or 50 µM simvastatin or atorvastatin was associated with cell death at both concentrations. In contrast, rosuvastatin was cytotoxic only at 50 µM.

#### 3.2. Statins affect the AKT signaling pathway in C2C12 myotubes

We have shown previously that simvastatin impairs AKT activation in C2C12 myotubes [14]. In order to answer the question whether this effect is specific for simvastatin or can be observed also for other statins, we investigated the effects of three different statins on components of AKT signaling pathway. Because the activity of the proteins involved in the AKT signaling pathway is mainly regulated by phosphorylation, we examined the changes in the phosphorylation status of the key proteins and related them to the expression level of the respective total protein (see Fig. 1). As shown in Fig. 3A, simvastatin and atorvastatin significantly inhibited the phosphorylation of AKT at S473 (but not at T389), of 4E-BP1 at S65 and of FoxO3a at S253 and T32 in a concentration-dependent way. Simvastatin significantly inhibited the phosphorylation of rpS6 at S235/236, whereas atorvastatin showed only a numerical, but not a statistically significant inhibition. The high concentration of rosuvastatin showed a significant 60% reduction of AKT phosphorylation at S473 as well as a significant reduction of FoxO3a phosphorylation at T32. Phosphorylation of S6K at T389 was significantly (*P < 0.05) inhibited by simvastatin (53 and 87% at 10 and 50 µM, respectively), by simvastatin (8 and 15% at 10 and 50 µM, respectively) and by 50 µM rosuvastatin (5%) (determined by ELISA and therefore not shown in Fig. 3).

Since the AKT-associated phosphorylation of FoxO3a prevents the induction of muscle-specific atrophy genes such as the E3 ubiquitin ligases atrogin-1/MAFbx and MuRF-1 [22], we investigated the mRNA expression of atrogin-1/MAFbx and MuRF-1. As shown in Fig. 3B and C, atrogin-1/MAFbx and MuRF-1 mRNA expression were increased concentration-dependently in response to simvastatin and atorvastatin and, to lesser extent, also in response to rosuvastatin. These results suggest that statins, in particular simvastatin and atorvastatin, may promote skeletal muscle atrophy.

#### 3.3. Statins have atrophic effects on C2C12 myotubes

It is well established that activation of the AKT/mTOR signaling pathway is implicated in skeletal muscle hypertrophy in vitro and in vivo [21, 26,27]. To determine whether statins could induce an atrophic phenotype due to the inhibition of AKT, we examined responses of myotubes to 10 µM and 50 µM simvastatin, atorvastatin or rosuvastatin. C2C12 myotubes were treated with the different statins for 24 h and assayed for changes in myotube diameter. As shown in Fig. 4A and B, treatment with simvastatin and atorvastatin resulted in a distinct atrophic phenotype, with a concentration-dependent decrease in myotube diameter. In contrast, rosuvastatin did not significantly reduce the diameter of myotubes. In agreement with myotube atrophy, simvastatin and atorvastatin were both associated with a significant decrease in the total protein content of the myotubes, whereas rosuvastatin decreased the protein content only numerically without reaching statistical significance (Fig. 4C).

#### 3.4. Statins induce apoptosis of myotubes

It is well established that inhibition of the AKT signaling pathway is associated with apoptosis [17,28]. We therefore analyzed whether the
inhibition of the AKT signaling pathway by statins is linked with apoptosis of C2C12 myotubes. Indeed, treatment of C2C12 myotubes with 10 μM or 50 μM simvastatin or atorvastatin for 24 h was associated with increased expression of several markers of apoptosis, such as the cleaved forms of caspase 9, caspase 3 and PARP (Fig. 5A). In contrast, 10 μM rosuvastatin was not associated with cleavage of the caspases or of PARP, whereas 50 μM rosuvastatin caused a smaller increase in markers of apoptosis than simvastatin or atorvastatin. In order to verify that this increase in markers of apoptosis is associated with apoptosis, we evaluated DNA fragmentation which is a feature of apoptotic cell death. As expected, simvastatin and atorvastatin induced DNA fragmentation in a concentration-dependent fashion starting at 10 μM, while rosuvastatin increased DNA fragmentation only at 50 μM (Fig. 5B).

3.5. Simvastatin impairs the AKT signaling pathway and induces apoptosis in mice in vivo

To further confirm the role of the AKT pathway in statin-induced myotoxicity, we treated the mice orally with simvastatin 5 mg/kg body weight/day for 21 days. We then analyzed skeletal muscle proteins involved in the AKT pathway. Similar to the in vitro findings, simvastatin significantly inhibited the phosphorylation of AKT at S473 and FoxO3a at S253, resulting in up-regulation of mRNA of atrogin-1/MAFbx, a gene involved in muscle atrophy (Fig. 6A and B). We then analyzed whether the inhibition of the AKT signaling pathway by simvastatin was linked to apoptosis in murine skeletal muscle. Indeed, treatment with simvastatin for 21 days induced the cleavage of caspases 9 and 3, which are markers of apoptosis (Fig. 6C). In addition, TUNEL positive myonuclei, representing DNA strand breaks and indicating apoptosis, could only be detected in the gastrocnemius of mice treated with simvastatin and not in control mice (Fig. 6D). These findings demonstrated that the results obtained in vitro are reproducible in mice in vivo.

3.6. Simvastatin reduces the skeletal muscle fiber area in mice

To investigate whether inhibition of AKT by simvastatin could induce muscle atrophy also in vivo in mice, we examined the area of myofibers in mice treated with simvastatin for 21 days. Indeed, treatment with simvastatin resulted in a significant reduction of the myofiber area (Fig. 7). The results show that simvastatin can induce muscle fiber atrophy not only in vitro, but also in mice in vivo.

4. Discussion

The current investigations were based on a previous study from our laboratory in which we showed that simvastatin interferes with AKT phosphorylation in C2C12 myotubes, but not in HepG2 cells [14]. The results of the current study confirm and expand these findings in several ways. First, we could demonstrate that the inhibition of AKT phosphorylation in C2C12 myotubes is not specific for simvastatin, but can be observed also for the other statins investigated, particularly for the lipophilic atorvastatin. Second, we could demonstrate the consequences.
Fig. 4. Statin-induced C2C12 myotube atrophy. C2C12 myotubes were treated with 10 or 50 µM simvastatin (SMV), rosuvastatin (RSV) or atorvastatin (ATV) for 24 h. 0.1% DMSO was used as a negative control. A. Photomicrographs of myotubes fixed and stained for myosin heavy chain (green) and with DAPI (blue). B. Quantification of the myotube diameters. C. Total protein content of the myotubes, determined using the BCA Protein Assay Kit after lysis of the myotubes. The protein content of control cells (DMSO 0.1%) was 3.06 mg/10^6 cells. Data were normalized to the DMSO control and are expressed as the mean ± SEM of four independent experiments. *P < 0.05 vs. DMSO control.

Fig. 5. Activation of apoptosis by statins in C2C12 myotubes. Myotubes were treated with 10 or 50 µM simvastatin (SMV), rosuvastatin (RSV) or atorvastatin (ATV) for 24 h. 0.1% DMSO was used as a negative control. A. Myotubes were lysed and centrifuged to remove cell debris. Aliquots of the supernatants were used for the Western blot analysis for the expression of cleaved caspase 9, cleaved caspase 3, cleaved PARP and the respective total proteins. GAPDH was used as a loading control. Quantification of the relative protein expression is indicated in numbers below the blots. B. Apoptotic DNA fragmentation induced by statins was determined by measuring the cytosolic mono- and oligo-nucleosomes with ELISA. Data are presented as mean ± SEM of three independent experiments. DMSO 0.1% and 100 nM staurosporin were used as a negative and positive control, respectively. **P < 0.01 and ***P < 0.001 vs. DMSO control.
Fig. 6. Effect of statins on the components of the AKT/mTOR pathway and apoptosis in vivo. C57BL/6J mice were treated with 5 mg/kg simvastatin (SMV) per day for 21 days. Analyses were performed using gastrocnemius muscle. A. Western blot analysis of the expression of phosphorylated AKT (P-AKT), phosphorylated ribosomal protein S6 (P-rpS6), phosphorylated forkhead box protein O3a (P-FoxO3a) and the respective total proteins. B. mRNA expression of MuRF-1 and MAFbx after statin treatment was determined by real-time PCR. GAPDH was used as a housekeeping gene. C. Western blot analysis of the expression of cleaved caspase 9, cleaved caspase 3, cleaved PARP and the respective total proteins. GAPDH was used as loading control. Quantification of relative protein expression is indicated in numbers below the blots. D. TUNEL staining for the detection of myonuclei undergoing apoptosis. Data are presented as mean ± SEM of n = 8 animals per group. **P < 0.01 and ***P < 0.001 vs. control (CTL).

Fig. 7. Statin-induced muscle fiber atrophy in vivo. Mice were treated with 5 mg/kg/day for 21 days simvastatin (SMV) or with saline (CTL). Gastrocnemius muscles were removed and frozen sections were stained with hematoxylin/eosin. The area of the individual myofibers was decreased in mice treated with simvastatin. Data are plotted as the mean ± SEM of n = 8 animals per group. *P < 0.05 vs. control (CTL).
of impaired AKT phosphorylation both within the AKT signaling pathway and for important cellular processes related to AKT signaling such as skeletal muscle protein synthesis and breakdown as well as apoptosis. Third, we could show that these effects of statins are not only an in vitro phenomenon, but can also be detected in mice treated with a simvastatin dose which is comparable to the doses used in humans.

The fact that all statins investigated were cytotoxic and inhibited the AKT signaling pathway (the more hydrophilic rosuvastatin to a lesser extent than the lipophilic simvastatin and atorvastatin) is compatible with the assumption that the mechanism of AKT inhibition is associated with the mode of action of the statins, namely inhibition of cholesterol synthesis at the level of hydroxymethylglutaryl-CoA (HMG)-CoA reductase. It has been postulated that intermediates between HMG and cholesterol, in particular farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, which are important for protein prenylation [12], can be diminished in skeletal muscle of patients treated with statins to such an extent that myopathy can occur [3]. This hypothesis is supported by the observation that the addition of mevalonate, but not of cholesterol, could at least partially prevent myotoxicity associated with lovastatin in rabbits [29]. It is difficult, however, to explain by this mechanism why statins impair the AKT signaling pathway. A possible explanation may be related to the observations that prenylation is important for the function of small GTPases [12] and that statins can inhibit their prenylation [30]. Rab1, a prenylated small GTPase, has been shown to stimulate AKT phosphorylation [31] and to possibly be involved in statin-associated muscle damage [12,14]. Since overexpression of Rab1 could not normalize AKT phosphorylation in C2C12 myotubes exposed to simvastatin [14], the precise role of statin-induced loss of Rab1 prenylation for impaired AKT activation is currently not clear and needs further investigation.

A second possibility to explain the effect of statins on the AKT signaling pathway is by the well-established mitochondrial toxicity of these compounds [8,9,32,33]. By impairing the function of the mitochondrial respiratory chain, statins decrease the skeletal muscle ATP and increase the ADP content [34], leading to the activation of AMPK. AMPK activation is associated with impaired phosphorylation of AKT, as shown in a recent study describing the toxicity of simvastatin cultured H9c2 cardiomyocytes and in mice in vivo [35].

Interestingly, all statins investigated impaired AKT phosphorylation at S473, but not at T308 (Fig. 3A). AKT phosphorylation at S473 is performed by mTORC2, whereas AKT phosphorylation at T308 is performed by phosphoinositide-dependent kinase-1 (PDK1), a component of the Igf-1 receptor/AKT pathway [36]. Taking into account that statins not only impaired the activity of mTORC2, but also of mTORC1 (impaired phosphorylation of S6K, rpS6 and 4E-BP1), a third possible mechanism is impairment of mTOR, a component of both TORC1 and TORC2 [37] by a so far not identified mechanism.

Independently of the mechanism leading to impaired AKT phosphorylation, the inhibition of the AKT signaling pathway had several important consequences on C2C12 cells and also on skeletal muscle of mice treated with simvastatin. Statins that inhibited the phosphorylation of S6K, which impaired phosphorylation of the ribosomal protein S6 (Fig. 3A) have an important role in mRNA translation and protein synthesis [18]. Although we did not directly determine protein synthesis by C2C12 myotubes in the current study, the observed reduction in the protein content of C2C12 myotubes is compatible with impaired protein synthesis, which is likely a consequence of impaired rpS6 phosphorylation.

In addition, AKT activation blocks protein degradative via the phosphorylation of FoxO3a, which impairs nuclear translocation of FoxO3a and the subsequent expression of the muscle atrophy markers MuRF-1 and atrogin-1/MAFbx [22,26]. As expected in the case of impaired AKT phosphorylation, we observed reduced phosphorylation of FoxO3a in the presence of statins, which was associated with increased mRNA expression of MuRF-1 and atrogin-1/MAFbx (in mice treated with simvastatin only the mRNA expression of atrogin-1/MAFbx was significantly increased). MuRF-1 and atrogin-1/MAFbx are associated with skeletal muscle protein degradation, offering an additional explanation for the observed decrease in the protein content of C2C12 myotubes and in skeletal muscle atrophy observed in mice treated with simvastatin.

Statins are known to induce apoptosis in various cell types [8,9,35]. In our previous study in L6 cells (a rat skeletal muscle cell line) [8], we postulated a mitochondrial mechanism for statin-associated apoptosis. Similar findings were reported by Kwak et al. [9], who investigated the effect of simvastatin in primary human myotubes. The current study shows that statins can induce apoptosis in C2C12 myotubes and in mice in vivo and indicates that statins can trigger apoptosis by inhibition of the AKT signaling pathway. This interpretation is compatible with our recent findings in cultured H9c2 cardiomyocytes [35] and with reports about statin-induced apoptosis in cancer cells [38].

Interestingly, in the current study, we consistently observed a lower toxicity of rosuvastatin compared to atorvastatin and simvastatin. In agreement with this finding, in publications reporting skeletal muscle adverse events for statins, it is generally agreed that lipophilic statins such as simvastatin are more toxic than the more hydrophilic compounds such as pravastatin [12,6]. Rosuvastatin, which is more hydrophilic than simvastatin or atorvastatin, has a good safety record regarding myotoxicity when used at the recommended dosage [39]. The results of the current study are in agreement with a previous study from our laboratory showing that pravastatin was less toxic than simvastatin and atorvastatin on cultured L6 cells and on isolated rat skeletal muscle mitochondria [8]. Two possible explanations for this finding are that hydrophilic statins have more difficulties to enter cells than lipophilic compounds or that rosuvastatin is per se less toxic for the AKT signaling pathway. The second possibility is less likely, since rosuvastatin is the most potent statin currently on the market and inhibition of HMG-CoA reductase is a likely toxicological mechanism. Regarding the first possibility, it is clearly established that different organic anion transport proteins (OATP), which are responsible for statin transport into the liver, are also expressed in skeletal muscle [40] and are important for the transport of statins into skeletal muscle [41]. At low concentrations, transport of both hydrophilic and lipophilic statins into myocytes appears to be achieved mainly by OATPs [40]. At the concentrations used in the current study, which may be reached in the systemic circulation when transport of statins into the liver and/or hepatic metabolism is blocked [41,42], diffusion may also play a role. When diffusion becomes important, lipophilic statins may reach higher intracellular concentrations than the hydrophilic compounds, possibly explaining that simvastatin and atorvastatin were more toxic in the current studies than rosuvastatin.

Statin-associated rhabdomyolysis is a rare event, usually occurring in patients treated with a therapeutic dose. Two well established risk factors include a high systemic exposure [1] and underlying metabolic muscle disease [43,44]. High systemic exposures in patients treated with a therapeutic dose can result from inherited malfunction of the transport mechanism into hepatocytes [7] or from drug–statin interactions [42,45,46]. Regarding drug–statin interactions, mainly (but not exclusively) the lipophilic statins are affected and the increase in the systemic exposure can be substantial, leading to serum concentrations in the low micromolar range [42]. The dependence of muscle toxicity with statin exposure is clearly demonstrated also by the current studies with C2C12 myotubes.

5. Conclusions

The current study demonstrates that statins are cytotoxic and impair AKT phosphorylation in C2C12 myotubes and in skeletal muscle of mice in vivo. The lipophilic simvastatin and atorvastatin showed a higher in vitro toxicity than the hydrophilic rosuvastatin. Impaired phosphorylation of AKT was associated with myofibrillar atrophy and apoptotic cell death of C2C12 myotubes. Similar findings were also obtained in skeletal muscle of mice treated with simvastatin. These findings may
have therapeutic implications, since activation of the AKT signaling pathway may improve myopathy in patients treated with statins.

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Conflicts of interest

None of the authors has any conflict of interest regarding this study.

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