Molecular mechanisms of statin-associated myotoxicity

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Molecular mechanisms of statin-associated myotoxicity

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To my parents

Acknowledgement

At the end of this journey, I would like to acknowledge all those who have supported me during my PhD study.

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A journey is easier when you travel together.
To each of you, I am grateful.
Grazie.

Contents

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

Important abbreviations

BSA	Bovine serum albumin
DMSO	Dimethyl sulphoxide
4E-BP1	Eukaryotic translation initiation factor 4 E binding protein
eIF4E	Eukaryotic initiation factor 4 E
FoxO	Forkhead box O
GGOH	Geranylgeranyol
HMG-CoA	Hydroxy-methylglutaryl-coenzyme A
IGF-1	Insulin-like growth factor- 1
MaFbx	Muscle atrophy F-box
mTOR	Mammalian target of rapamycin
MuRF-1	Muscle RING-finger protein-1
OCR	Oxygen consumption rate
PI3K	Phosphoinositide 3-kinase
ψm	Membrane potential

Summary

Statins, hydroxyl-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are cholesterol-lowering drugs that are majorly used to treat hypercholesterolaemia and dyslipidaemia implicated in the pathogenesis of coronary heart disease and atherosclerosis [1]. They are generally considered safe drugs, but there are a number of reports of skeletal muscle damage associated with their use [2]. The myotoxicity ranges from a mild clinical syndrome consisting of benign myalgia to rare but life-threating rhabdomyolysis [3]. These side-effects can impact on quality of life and compliance, and in extreme cases lead to death [4].

Because millions of people in the world are currently taking statins every day, it is an urgent task to uncover the mechanism by which statins lead to side effects [5].

This thesis includes two published papers and one still in preparation.

Our first paper presents a comparison between three different statins on the market: simvastatin, atorvastatin and rosuvastatin. Since there are differences among statins in terms of their efficacy and toxicity, we aimed to analyze the different molecular mechanisms that may contribute to the diverse grade of toxicity between simvastatin, atorvastatin and rosuvastatin.

Simvastatin and atorvastatin appear to have a higher than average risk of myotoxicity contributing to the highest number of cases of rhabdomyolysis among statins [6] [7]. On the contrary rosuvastatin, the most hydrophilic statin, appears to have a reduced myotoxicity [8] [7].

C2C12 myotubes were exposed to 10 μM or 50 μM simvastatin, rosuvastatin or atorvastatin for 24 hours. We demonstrated that myotubes were more susceptible to simvastatin and atorvastatin than to rosuvastatin treatment. Therefore, difference between rosuvastatin and atorvastatin or simvastatin could point to possible mechanisms of toxicity.

The cytotoxicity of simvastatin and atorvastatin was associated with a drastic and dose-dependent impairment of AKT signaling cascade that led to inhibition

of the protein synthesis, increase of the protein degradation and promotion of apoptosis. Conversely, rosuvastatin blocked AKT signaling only at high concentration and to a lesser extent compared with the other two statins. The reduced effect on cytotoxicity and AKT signaling inhibition in C2C12 myotubes treated with rosuvastatin was accompanied with normal protein synthesis and absence of protein degradation and apoptosis. These results provide evidence that an impairment of AKT signaling pathways might be a causative factor in statin-induced myotoxicity.

Our second paper expands on these previous results by showing that the myotoxicity, and with it, the impairment of AKT signaling, can be prevented by the addition of IGF-1.

IGF-1 is well known for exerting an anabolic effect on skeletal muscle [9] by activating IGF-1/AKT pathway [10]. Therefore we investigated whether IGF-1 could antagonize the myotoxicity induced by statins.

Myotubes were exposed to 10 μM simvastatin and/or 20 ng/ml IGF-1 for 18 hours. Simvastatin-induced myotoxicity was completely antagonized by IGF-1. Moreover, the protective effect of IGF-1 was mediated by the activation of IGF-1/AKT pathway that led to a suppression of atrophic markers and apoptosis, and simultaneously triggered pro-synthetic pathways. These studies provide new insight into the prevention of statin toxicity and may herald new discoveries for the treatment of statin-induced myalgia.

The final paper takes the work of the previous two papers and places it into a novel system: the cardiac muscle. Statins are primarily prescribed to cure and prevent cardiovascular disease. Thus, cardiac side-effects may be masked by falsely attributing them to the underlying disease.

In this paper, we investigated on the effect of simvastatin in cardiomyocyte *in vitro* and *in vivo*. We treated H9c2 rat cardiomyocytes with 10 μM and 100 μM simvastatin for 24 hours. H9c2 cells showed a reduction in the mitochondrial membrane potential and energetic impairment linked to mitochondrial dysfunction. Consequently, the cellular ATP level was decreased. This decrease led to the activation of AMPK, nuclear translocation of FoxO3, upregulation of atrogin-1 and initiation of apoptosis. We confirmed these results *in vivo*. We demonstrated that the treatment of mice with simvastatin 5 mg/kg/day for 21

days impaired the activity of several enzyme complexes of the electron transport chain in cardiomyocytes and increased mRNA expression of atrogin-1 and markers of apoptosis. This is the first study that shows energetic impairment linked to atrophy and apoptosis induced by statins in the heart, and warrants further investigation to assess statin safety in susceptible patients.

1 Statins

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are a class of drugs used to reduce blood cholesterol level. They inhibit the synthesis of cholesterol binding to the enzyme HMG-CoA reductase at nanomolar concentrations and leading to competitive displacement of the natural substrate, HMG-CoA, which binds only at micromolar concentrations (Fig. 1).

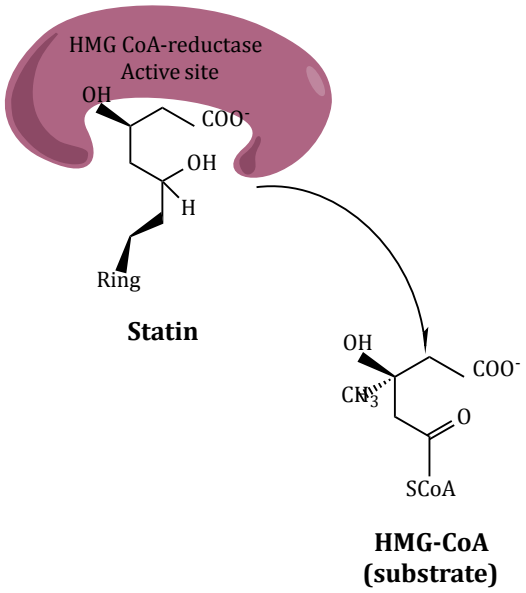


Fig. 1. Mechanism for statin inhibition of HMG-CoA reductase. Statins competitively inhibit HMG-CoA reductase leading to competitive displacement of the natural substrate, HMG-CoA

HMG-CoA reductase is the first and the rate-limiting enzyme of the cholesterol biosynthesis. It catalyzes the conversion of HMG-CoA to mevalonic acid [11] [12](Fig. 2).

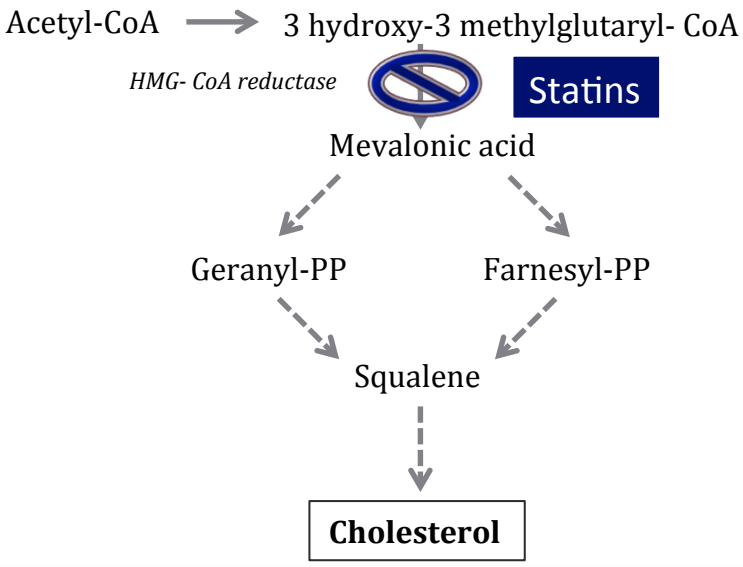


Fig. 2. Schematic representation of cholesterol biosynthetic pathway. Cholesterol is a 27 carbons compound synthesized from acetyl-CoA which is the precursor to all sterol molecules. First, Acetyl-CoA is converted to 3-hydroxy-3 methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. Then, HMG-CoA is converted to mevalonate by HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis. Through a series of other intermediates, mevalonate is converted to cholesterol. Cholesterol is either synthesized de novo in hepatocytes or absorbed from the diet. Inhibition of HMG-CoA reductase by statins curtails not only hepatic cholesterol but also all isoprenoid intermediates. Insufficient levels of isoprenoid species (i.e. geranyl pyrophosphate and farnesyl pyrophosphate) cause numerous cellular disfunctions, including altered cell signaling by inhibition of prenylation, a crucial post-translational modification for many proteins, loss of membrane integrity due to deficient cholesterol synthesis, etc.

In addition, the decreased cholesterol production in the liver is accompanied by an increased synthesis of hepatic LDL receptors, which promotes the clearance of low-density lipoprotein (LDL) and very low density lipoprotein (VLDL) from the bloodstream [13] (Fig. 3).

While all statins share the pharmacophore (a dihydroxyheptanoic acid segment unit and a ring system with different substituents) responsible for the binding to HMG-CoA reductase, important differences exist among statins that distinguish their synthesis, lipophilicity, pharmacokinetic properties, drug-food interactions and, LDL-lowering potency [14].

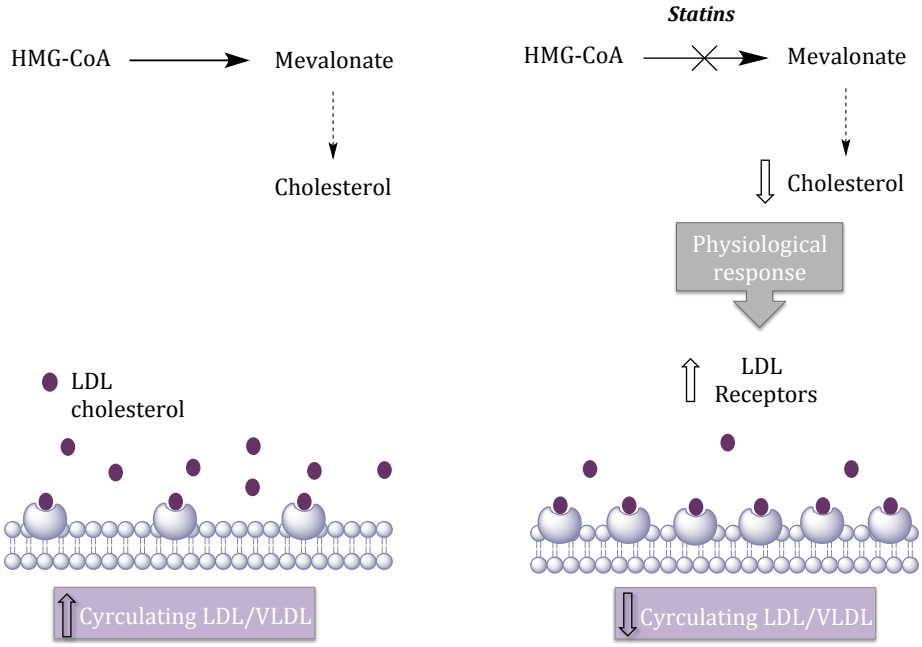
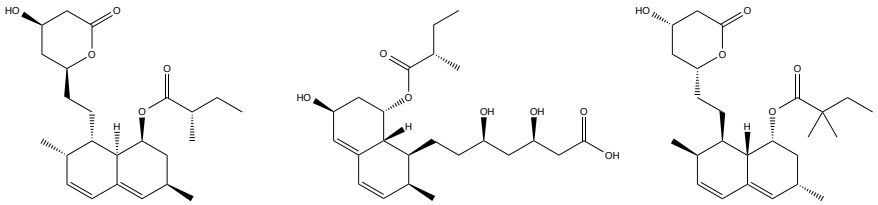


Fig. 3. Cholesterol-lowering action of statins. Statins inhibit HMG-CoA reductase leading to a final decrease in the intracellular content of cholesterol. To compensate this diminution, cells respond increasing the number of LDL receptors on the cell membrane, Consequently, the LDL is taken up more rapidly and its level in the plasma falls.

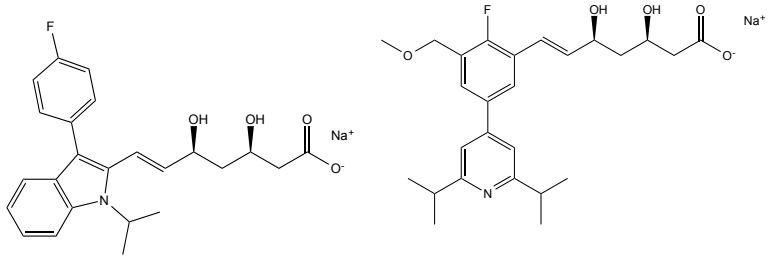
Statins are commonly classified into two types: type 1, natural or fungal-derived statins, and type 2, synthetic statins. The functional difference between natural and synthetic statins relies on their ability to interact and inhibit the HMG-CoA reductase and, on their lipophilicity [15]. Type 1 statins (i.e. lovastatin, pravastatin and, simvastatin) (Fig. 3) exhibit binding via a decalin-ring structure that resemble the first statin ever discovered, mevastatin [16].

Differences in statin structure and binding characteristics may partially contribute to differences in the inhibition of HMG-CoA reductase efficacy and other pharmacologic properties. For example, rosuvastatin is one of the most potent statin, it is relatively hydrophilic, it has a greater number of bonding interactions with the catalytic site of HMG-CoA reductase compared with most of the other statins and, it is the most well tolerated statin in the market [14]. Type 2 statins (i.e. fluvastatin, cerivastatin, atorvastatin, and rosuvastatin) (Fig. 5) exhibit additional binding via their fluorophenyl group [17].

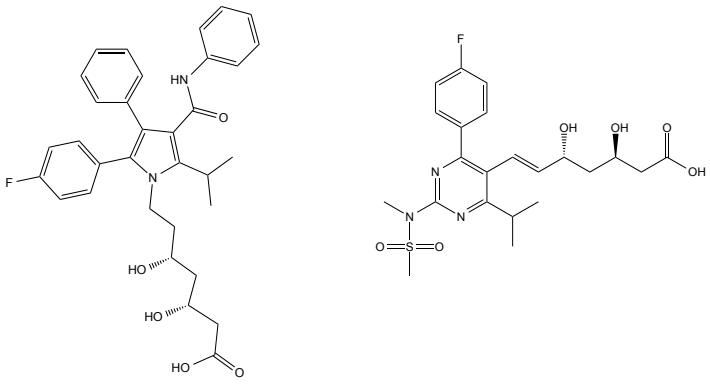


Lovastatin Pravastatin Simvastatin

Fig. 4. Type 1 statins. Lovastatin, pravastatin and simvastatin exhibit binding via a decalin ring structure.



Fluvastatin Cerivastatin



Atorvastatin Rosuvastatin

Fig. 5. Type 2 statins. Fluvastatin, cerivastatin, atorvastatin and, rosuvastatin have larger groups linked to the HMG-like moiety than the type 1 statins. One of the main differences between the type 1 and type 2 statins is the replacement of the butyryl group of type 1 statins by the fluorophenyl group of type 2 statins.

1.1 Why study statins?

Statins are widely used to prevent cardiovascular disease (CVD) and hyperlipidemia. Since CVD is the worldwide leading cause of death and results in a huge burden of mortality and morbidity, statins are among the most prescribed drugs in Western countries [18].

Although they are generally well tolerated, clinical observations suggest that statin exposure may exhibit dose-dependent skeletal muscle damage.

The clinical spectrum of myotoxicity ranges from a mild clinical syndrome consisting of nonspecific myositis and myalgias (occurrence of 1 - 5% in patients) to life-threatening rhabdomyolysis [2]. Rhabdomyolysis is a clinical condition characterized by destruction of skeletal muscle tissue. The most severe consequence of rhabdomyolysis is the kidney failure and occasionally death due to the release of breakdown products into the bloodstream, leading to accumulation of them in kidney tubules. Rhabdomyolysis is the most feared adverse event associated with statin therapy but fortunately rare for statin monotherapy at standard doses [19]. The withdrawal of cerivastatin in 2001 from the market worldwide due to reports of fatal rhabdomyolysis (52 deaths of drug-related rhabdomyolysis within 4 years), generated substantial alerts concerning the safety profile of the available statins [20]. Graham et al. showed a low incidence of rhabdomyolysis (0.44 in 10.000 patient years) for monotherapy with atorvastatin, pravastatin or simvastatin. However, adverse events have a dramatic clinical relevance due to their impact on quality of life and reduce compliance of millions of people taking statins every day [19].

For all these reasons, it is a matter of great urgency to elucidate the mechanisms by which statins lead to side effects.

1.2 Role of pharmacokinetics and predisposing factors in statin-induced myopathy

Myopathies are associated with all statins even though some statins have a higher risk [21]. Side effects are usually dose-dependent. Therefore, pharmacokinetic properties and any factor (i.e. polymorphisms or drug- drug interactions) that increases the serum concentration of the drug may predispose

to myopathy [22].

In vitro and *in vivo* experiments suggest that lipophilic statins (such as simvastatin, lovastatin, atorvastatin, pitavastatin and cerivastatin) are more likely to affect the skeletal muscle than the hydrophilic statins (such as pravastatin, rosuvastatin, and fluvastatin) [7] [23]. Lipophilic compounds, indeed, tend to achieve higher levels of exposure in non-hepatic tissues (e.g. muscle), since they penetrate into peripheral tissues by passive diffusion enhancing their potential for myotoxic effects [24]. Moreover, they undergo hepatic metabolism via the cytochrome P450 (CYP450) system which makes them more subjected to drug-drug interaction [7].

In contrast, hydrophilic statins tend to be more hepatoselective since they depends on an active transport process to enter the hepatocytes and exert their effects. Moreover, they are minimally metabolized by the cytochrome P450 enzyme system before elimination, therefore, they are less involved in any clinically relevant drug-drug interactions with agents that induce or inhibit CYP450 isoenzymes [6].

Possible pharmacokinetic interactions of statins with other drugs deserve particular attention. Because statins are prescribed on a long-term basis, many patients might receive pharmacological treatments for concomitant conditions during the course of statin use [25]. Moreover, all statins, except pravastatin, are metabolized by CYP450 isoenzymes in the liver [26]. Three commonly prescribed statins (simvastatin, lovastatin and atorvastatin) are metabolized by CYP3A4 [27]. CYP3A4 is the most important CYP isoenzymes for drug metabolism, it metabolizes more than 50% of prescribed drugs, leading to a huge risk of drug-drug interactions [28].

Both CYP450 inhibitors and inducers play an important role in disposition of statin, in terms of their plasma levels and the risk of statin-induced adverse effects [26].

Neuvonen et al. [29] reported that simvastatin-associated muscle disorders were 6-fold higher when patients were taking CYP3A4 inhibitors at the same time but, there were no change in patients taking CYP3A4 inhibitors in combination with pravastatin (which is not metabolized by CYP3A4).

Cytochrome P450 inhibitors are agents that inhibit the hepatic enzymes, leading

to increased plasma levels of statins and greater risk of adverse effects. While, cytochrome P450 inducers are agents that induce the hepatic enzymes, leading to decreased plasma levels of statins and a subsequent decreased bioavailability of statin [30]. In table 1 are reported the most common inhibitors and inducers which influence statin metabolism.

Statin-fibrate combination therapy deserves particular precautionary warnings because myopathy can occur with either drug alone, and the effects may be additive [20] [4].

Shek et al. showed that a co-administration of statin and fibrate increases the risk of myopathy associated with CK elevations with an incidence of approximately 0.12%. Although all fibrates have been associated with cases of CK elevations and myopathy in combination with statins, the risk of developing myopathy and rhabdomyolysis with gemfibrozil was 10- to 15-fold higher compared to other fibrates. The leading cause of this higher toxicity is that gemfibrozil exhibits greater inhibitory potency towards specific CYP450 and UDP-glucuronosyltransferase (UGT) isoenzymes that leads to a reduced statin clearance [31] [32] [33].

Not only the concomitant administration of lipid-lowering agents, but also genetic factors and drug transporter expression are variables that could affect the concentration-effect relationship.

It has been demonstrated that the organic anion transporting polypeptide (OATP) is important for the hepatic uptake of hydrophilic statins [22]. The hepatic transporter OATP1B1 is the main transporter of statins into the liver. A single nucleotide polymorphism in the SLCO1B1 gene that encodes the OATP1B1 leads to decreased hepatic uptake, increased plasma statin levels which, in turn, causes myopathy [34] [35].

	Statins	Inhibitors	Inducers
CYP3A4	Simvastatin, lovastatin, atorvastatin	Ketoconazole, itraconazole, fluconazole, erythromycin, clarithromycin, tricyclic anti-depressants, nefazodone, venlafaxine, fluvoxamine, fluoxetine, sertraline, cyclosporine A, tacrolimus, mibefradil, diltiazem, verapamil, protease inhibitors, midazolam, corticosteroids, grapefruit juice, tamoxifen, and amiodarone	Barbiturates, phenytoin, phenobarbital, barbiturates, rifampin, dexamethasone, cyclophosphamide, carbamazepine, omeprazole, troglitazone
CYP2C9	Fluvastatin, rosuvastatin	Ketoconazole, fluconazole, sulfaphenazole	Rifampin, phenobarbital, phenytoin, troglitazone

Table 1: list of the most common inhibitors and inducers or CYP involved in statin metabolism

2 Potential mechanisms of statin-induced myopathy

In the recent years enormous effort has been made to uncover the molecular mechanisms of statin-induced myotoxicity and several causes are discussed in literature [21] [36] [11].

2.1 Inhibition of the mevalonate pathway

The most likely reason for adverse drug reactions associated with statins is linked to their mechanism of action [37] [26]. The inhibition of the HMG-CoA reductase leads not only to a blockage of cholesterol synthesis but also to a reduced production of all intermediates such as mevalonate and various isoprenoid derivatives (Fig. 1) enabling statins to affect many cellular processes [38].

Masters et al. showed that the replacement of mevalonate abolished the changes associated with statin treatment [24]. Whereas the downstream inhibition of the enzymes squalene synthase or squalene epoxidase does not cause myotoxycity [39] [40]. Taken together, these findings suggest that statin-induced myotoxicity is most likely due to the reduction in the synthesis of crucial intermediary isoprenoid derivatives such as geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) and not to the reduction in cholesterol synthesis. Indeed, GPP and FPP are responsible for the prenylation of various proteins essential in a variety of cellular signaling pathways, transportation, cell growth and transformation processes that enhance cell-membrane integrity and support intracellular metabolic pathways [41-43].

Evidence for the involvement of dysprenylation in statin-induced myotoxicity is provided by studies that revealed that the addition of geranylgeraniol (GGOH) rescues statin-treated cells from apoptosis [41].

Moreover, statin-related inhibition of dolichol synthesis impairs N-glycosylation of cell surface proteins such as the insulin like growth factor -1 receptor (IGF-1R). Correct α –subunit glycosylation is essential for the cleavage of the pro-receptor and, therefore, for the formation of mature functional IGF-1R [44, 45] [46].

Coenzyme Q10 (CoQ10), also known as ubiquinone, is synthesized via

mevalonate pathway. CoQ10 is a lipophilic component of the respiratory chain that transfers electrons from complexes I and II to complex III. CoQ10 has a key role in the oxidative phosphorylation in mitochondria and consequently in ATP production. Furthermore, it has antioxidant properties and serves as a membrane stabilizer. Due to the central role of ubiquinone in the respiratory chain, it was hypothesized that its deficit mediates statin-induced myopathy. However, no direct association between decreased CoQ10 levels in myocytes and myopathy has ever been demonstrated in any human or animal study [3, 47]. Moreover, oral CoQ10 supplementation did not prevent the risk of statin-related myopathy [48].

2.2 Direct effects on the mitochondrial electron transport chain

The mitochondria electron transport chain (ETC) is composed of four multiprotein complexes and it is an essential for the cellular energy production (Fig. 6).

Via a series of redox reactions, electrons are transferred from an electron donor (NADH or QH2) to a terminal electron acceptor (O_2). These redox reactions release energy which is used to actively pump protons from the mitochondrial matrix into the intermembrane space through three proton pumps (Complex I, III and IV). This generates a proton gradient that drives ATP synthesis via oxidative phosphorylation at the ATP synthase [49].

Independent studies observed vacuolization of mitochondria and disrupted cristae pointing to a primary involvement of mitochondria in the pathogenesis of statin-induced muscle atrophy [50, 51].

Schick et al. detected a decrease in mitochondrial DNA in patients treated with high doses of simvastatin again indicating the involvement of a mitochondrial damage [52]. In a clinical setting, patients under statin therapy emerged a significantly increased serum lactate/pyruvate ratio, which points to a mitochondrial dysfunction accompanied by a compensatory increase in glycolysis [53].

Several studies revealed that statins directly inhibit complexes of the electron transport chain (ETC) [54] [55] [56]. The inhibition occurs immediately and on

isolated mitochondria, suggesting that statins have a direct effect on mitochondria [57] [58].

Since mitochondria are involved not only with bioenergetics but also with oxidative damage and apoptosis, a direct inhibition of the ETC would have dramatic effects: energy levels would drop down, mitochondrial integrity would be compromised, and apoptosis triggered [58].

However, it is worth to highlight that many studies investigating the toxic effect on mitochondrial ETC use very high concentrations of statins [59]. Whether localized concentrations of statins could reach levels high enough in the mitochondria of patients is still a question of debate [60].

Nevertheless, the effect of statins on mitochondria in patients with genetic variations in the complexes or transporters may increase the susceptibility to muscle symptoms [60].

Further evidence of a direct effect of statins on the mitochondrial ETC is provided by Sirvent et al. [61]. They showed that statin treatment affects cellular Ca^{2+} homeostasis by inducing an efflux of Ca^{2+}. They suggested that this is caused maily by lipophilic statins which are able to diffuse into muscle fibers and directly inhibit one or more complexes of the mitochondrial ETC.

Altered Ca^{2+} homeostasis in the muscles triggers apoptosis via distinct targets, leading to muscle dysfunction and dysregulation. For instance, activation of Ca^{2+}-dependent protein phosphatase such as calcineurin leads to altered gene transcription, activation of Ca^{2+}-dependent endonucleases causes apoptotic DNA degradation or activation of Ca^{2+}-dependent proteases such as calpain induces apoptosis [62].

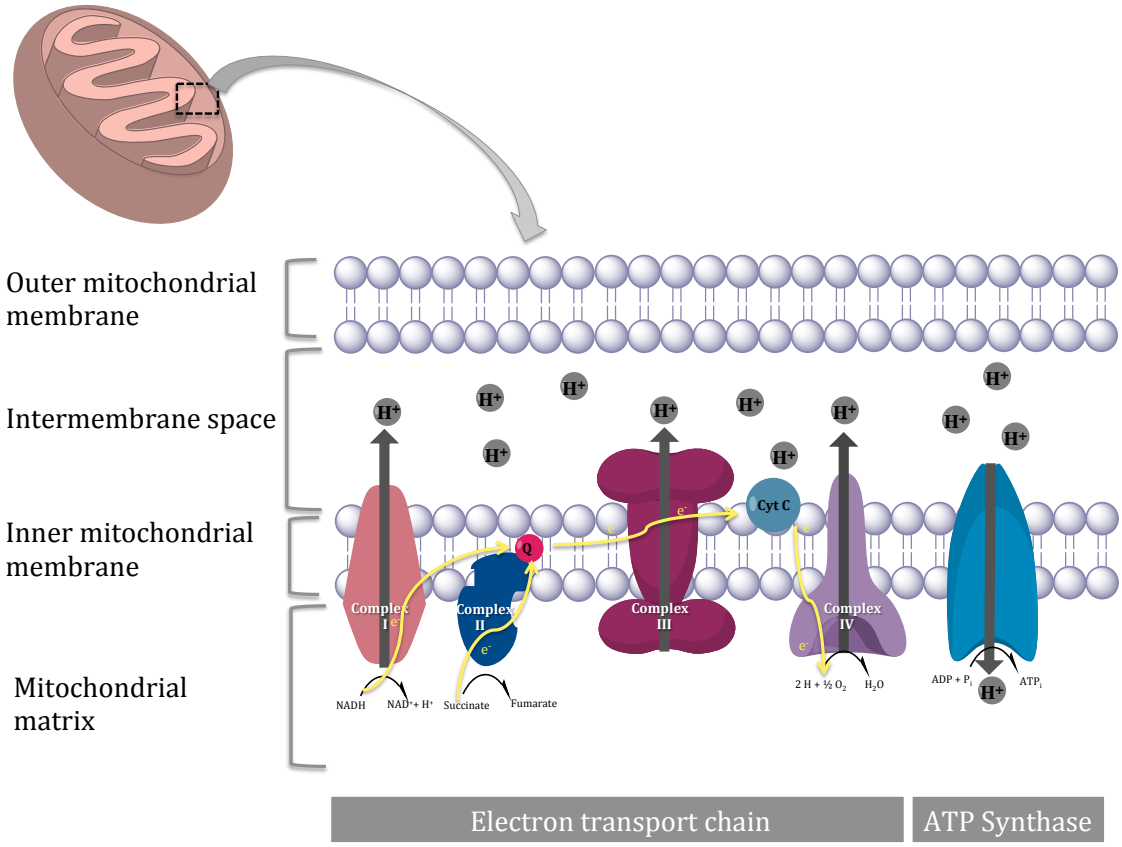


Figure 6. The mitochondrial respiratory chain. Electrons enter the respiratory chain via the complex I and are transferred to ubiquinone (Q). Additional electrons are delivered from the oxidation of complex II via $FADH_2$ and are also transferred into the ubiquinone pool. The Q-cycle contributes electrons to the cytochrome c via complex III. Complex IV catalyzes the reduction of oxygen to water. The electron transport chain and the ATP production are coupled by a proton gradient across the inner mitochondrial membrane. This electrochemical gradient is used by the ATP synthase to generate ATP via oxidative phosphorylation.

3 The insulin-like growth factor 1 pathway

The insulin-like growth factor 1 (IGF-1) is a 70 amino acid peptide with a high degree of sequence homology to insulin [63].

IGF-1 pathway provides a potent proliferative signaling system that stimulates growth and differentiation in many different cell types and it is considered one of the most important signaling pathway for inducing skeletal muscle hypertrophy and cell survival [9] (Fig. 7).

IGF-1 signals primarily through the IGF-1 receptor (IGF-1R), a tyrosine kinase receptor that consists of $\alpha_2\beta_2$ heterotetramers held together by disulfide bridges [64].

When IGF-1 binds to the extracellular α subunits of the receptor, the intracellular β subunits undergo a conformational change leading to the autophosphorylation of intracellular tyrosine. This event causes an activation of phosphorylation cascade of several substrates, including the insulin receptor substrate family of proteins (such as Insulin receptor substrate 1 and others) [65]. Once phosphorylated, these docking proteins activate downstream intracellular signaling through the cytoplasmic phosphatidylinositol 3-kinase (PI3K) pathway leading to a subsequent phosphorylation and activation of AKT.

AKT is a serine-threonine protein kinase that plays a key role in controlling vital cellular functions such as cell survival/apoptosis, cell cycle progression and proliferation [66].

AKT inhibits protein degradation by phosphorylating and, thus inhibiting, the nuclear translocation of the transcription factors Forkhead box O (FoxO) family [67] [68]. FoxO factors are required for the transcriptional regulation of the ubiquitin ligases atrogin-1, also called muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF-1), leading to the ubiquitylation of muscle proteins and their subsequent degradation via the proteasome [69] [70].

Furthermore, AKT stimulates protein synthesis via phosphorylation of the mammalian target of rapamycin (mTOR). In turn, mTOR mediates phosphorylation and activation of S6 kinase (S6K), and in parallel, phosphorylation and inactivation of 4E-BP1, a repressor of translation initiation.

S6K directly phosphorylates the 40S ribosomal protein S6, increasing the translation of mRNA while, phosphorylated 4E-BP1 activates eukaryotic translation initiation factor 4E (eIF4E) which binds an mRNA cap and triggers the translation [71] [72] [73].

In addition, AKT promotes cell survival regulating several proteins involved in apoptosis.

The primary target is Bad, a pro-apoptotic protein that interacts with the anti-apoptotic Bcl-2 at the mitochondrial membrane. Upon phosphorylation on Ser 136 by AKT, BAD dissociates from Bcl-2 and its pro-apoptotic function is blocked [74] [75] [76].

Moreover, AKT promotes survival also through inactivating caspases, thus preventing the initiation of the pro-apoptotic machinery [77] [78] [79].

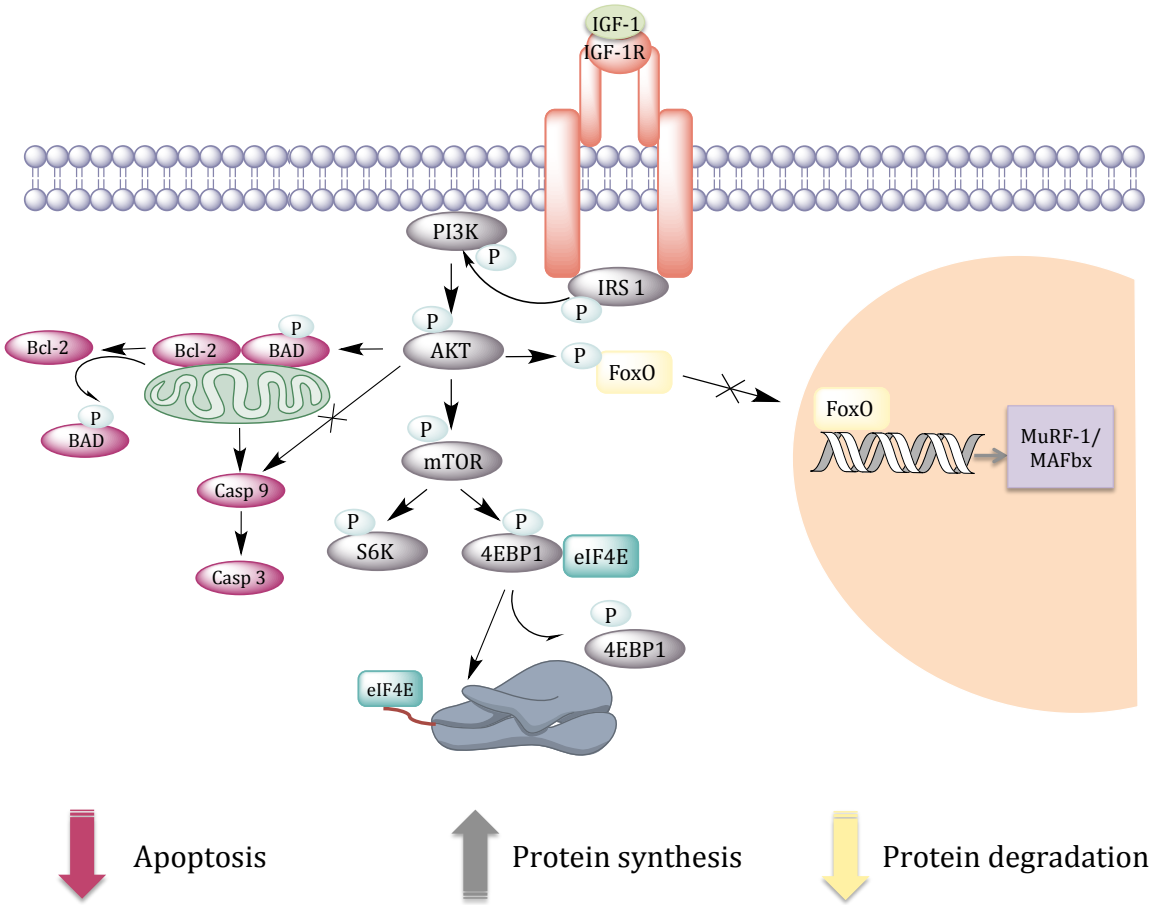


Fig. 7. A simplified scheme of the IGF-1 pathway.

3.1 What is the link between the IGF-1 signaling pathway and statin-induced toxicity?

Recently Mullen et al. revealed a decrease in AKT phosphorylation hence leading to the induction of E3 ubiquitin ligase atrogin-1 during statin-treatment *in vitro*. As a consequence, skeletal muscle fibers undergo to cell death [56].

Reduced AKT phosphorylation leads to an increased nuclear translocation of FoxO transcription factors and subsequent upregulation of atrophy markers MuRF-1 and MAFbx [80]. In the case of FoxO3, activation was demonstrated to be sufficient to induce atrophy [80], a finding, which was subsequently supported by the atrophic phenotype induced by the transgenic expression of FoxO1 [81].

In addition, *in vitro* studies demonstrated that the ubiquitin-proteasome proteolytic genes, MAFbx and MuRF-1, are responsible for promoting protein degradation in dexamethasone-induced atrophy in differentiated myotubes [82]. Similarly, Hanai et al. highlighted the importance of MAFbx in statin-induced myopathy. They showed that atrogin-1 knockout mice and knockdown zebrafish are resistant to statin-induced myopathy [83].

Conversely, genetic activation of AKT was shown to be able to block the atrophy-associated upregulation in MAFbx and MuRF-1 transcription [9] by inactivating FoxO transcription factors [80] [9].

IGF-1 is a hypertrophy-inducer agent [84]. It increases muscle mass by stimulating the AKT pathway [10], resulting in the downstream activation of targets which are required for protein synthesis [79] and inactivation of targets which are involved in protein degradation [9].

IGF-1 treatment has an integral role in antagonizing [70] [9] [65] or attenuating settings of skeletal muscle atrophy [85] acting through the AKT signaling pathway [9, 63].

Taken together, these findings lead to the conclusion that dysregulation of the IGF-1/AKT pathway may be a key step in statin-induced muscle damage.

However, the precise mechanism, of how statins interfere with IGF-1/AKT signaling hence leading to the induction of MAFbx in muscle cells is still not solved.

As mentioned previously, statins reduce dolichol production, which is required for the N- glycosylation of the IGF-1R [86]. A decrease in mature IGF-1R could lead to reduced AKT phosphylation, increased nuclear translocation of FoxO1 and FoxO3a, and an upregulation in MAFbx.

In addition, the dysprenylation of small GTPases, that are crucial in many signaling pathways [86] and could also be involved in IGF-1/AKT signaling [87].

Aims

This thesis has three main aims. Firstly, we aimed to uncover the effect of three common prescribed statins, simvastatin, atorvastatin and rosuvastatin, on skeletal muscle. As simvastatin is known to be toxic on C2C12 myotubes and to negatively regulate the AKT signaling pathway [56], it was our main goal to understand whether the AKT signaling cascade plays a role in the statin-induced myotoxicity. For this reason, we conducted our experiments on myotubes derived from the C2C12 skeletal muscle cell line, and we induced them with 10 μM or 50 μM of simvastatin, atorvastatin or rosuvastatin. We initially analyzed differential patterns of protein involved in the AKT signaling pathway, and looked for differences in these components. Then, we correlated the protein expression pattern with the atrophic effects. This was the first time such analysis would have been performed, and would point to possible mechanisms for statin-induced skeletal muscle toxicity.

The second aim of this thesis was to expand our previous findings by investigating whether IGF-1 could reduce or block the simvastatin-induced toxicity. Indeed, IGF-1 has a major anabolic effect in various cell lines [70, 88]. Moreover, IGF-1/AKT pathway is a central player regulating muscle mass as it activates protein synthesis and inhibits protein degradation [10]. We aimed to uncover whether the activation of IGF-1/AKT pathway is sufficient to have a prevention of statin-induced myotoxicity in C2C12 myotubes. This study has a potential impact on the discovery of new therapeutic avenue to treat statin-induced toxicity.

The final aim of this thesis was to look for toxicity of statins in cardiac muscle. This topic has a great relevance since the prevention of cardiovascular disease is the primary reason for prescribing statins. Statins have been reported to inhibit mitochondrial function in various cell lines [37] and induce skeletal muscle atrophy [21, 56]. We shed light on whether simvastatin affects cardiac mitochondria leading to cardiac atrophy. We used *in vivo* and *in vitro* models to present, for the first time, evidence of simvastatin-induced toxicity in

cardiomyocytes linked to a bioenergetic failure. This data opens new questions concerning statin safety and, in the same time, might be an explanation for the observed idiosyncratic toxicity, which affects only a small minority of patients taking statins.

Paper one

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

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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.

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 by massive destruction of muscle fibers and release of their contents into the bloodstream [3,4]. While muscle pain affects up to 20% of statin users [5], more severe adverse muscular reactions are much rarer [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 simvastatin and atorvastatin and 5.3 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

Abbreviations: MaFbx, muscle atrophy atrophy F-box; mTOR, mammalian target of rapamycin (component of mTORC1 and mTORC2); FoxO, forkhead box O; MuRF-1, muscle RING-finger protein-1; S6K, ribosomal protein S6 kinase; rpS6, ribosomal protein S6; 4E-BP1, initiation factor 4E binding protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PFA, paraformaldehyde; ABTS, 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]; PDK1, phosphoinositide-dependent kinase-1; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2

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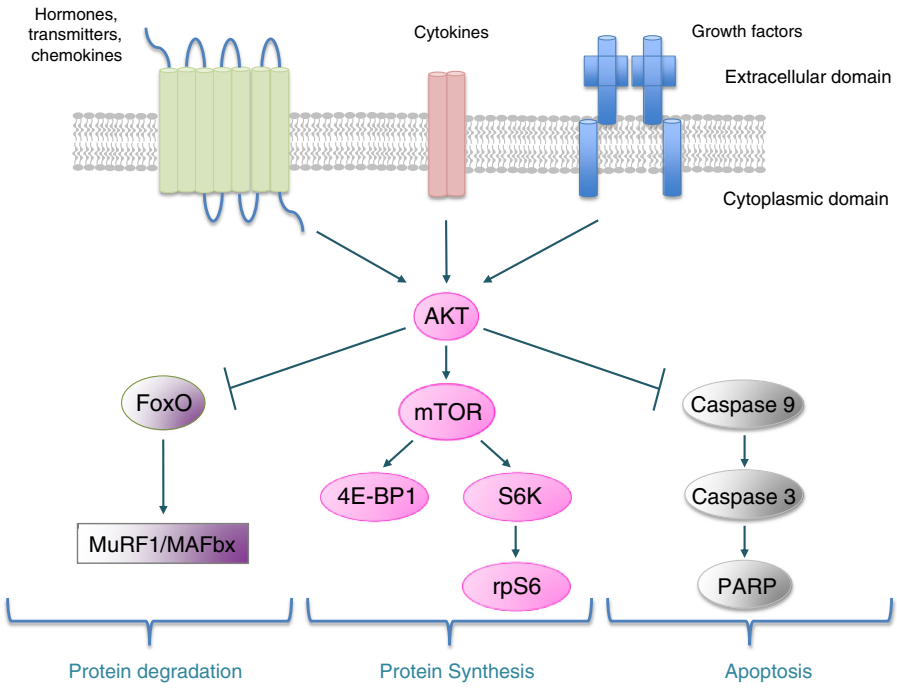

Fig. 1. Schematic representation of the AKT/mTOR signaling pathway. Upon activation by hormones, cytokines or growth factors, AKT translocates to the plasma membrane and is phosphorylated on Ser 473 and on Thr 308. Once activated, AKT phosphorylates downstream cytosolic and nuclear effectors inducing protein synthesis and blocking apoptosis and protein degradation.

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% CO_2 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 (Sutzfeld, 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 (p70S6K pT389 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, rosuvastatin or atorvastatin for 24 h. Afterwards, mRNA was extracted and purified using the Qiagen RNeasy 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′AGTGAGGACCGGCTACTGTG3′ and reverse 5′GATCAAACGTTGCGAATCT3′. MuRF-1: forward 5′CCTG CAGAGTGACCAAGGA3′ and reverse 5′GGCGTAGAGGGTGTCAAACT3′. 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′-CATGGCCTTCCGTGTTCC TA-3′ and reverse 5′CCTGCTTCACCACCTTCTTGA-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. Nonspecific 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 CellInsight 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 RNeasy 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 T308), 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

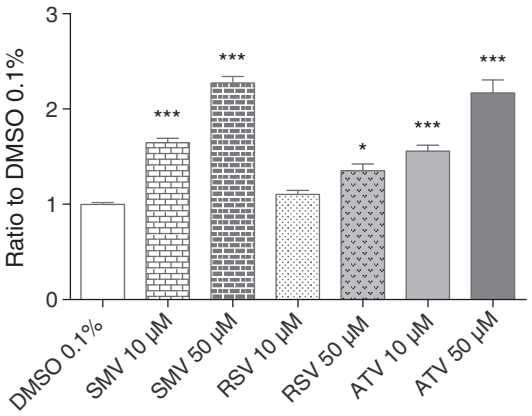

Fig. 2. Cytotoxicity of statins in C2C12 myotubes. C2C12 myotubes were exposed to 10 μM or 50 μM simvastatin (SMV), rosuvastatin (RSV) or atorvastatin (ATV) for 24 h, and cytotoxicity was determined by adenylate kinase release using the Toxilight assay. Results were normalized to the control incubations (DMSO 0.1%). Data represent the mean ± SEM of three independent measurements carried out in triplicates. *$P < 0.05$ and ***$P < 0.001$ *vs.* DMSO control.

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

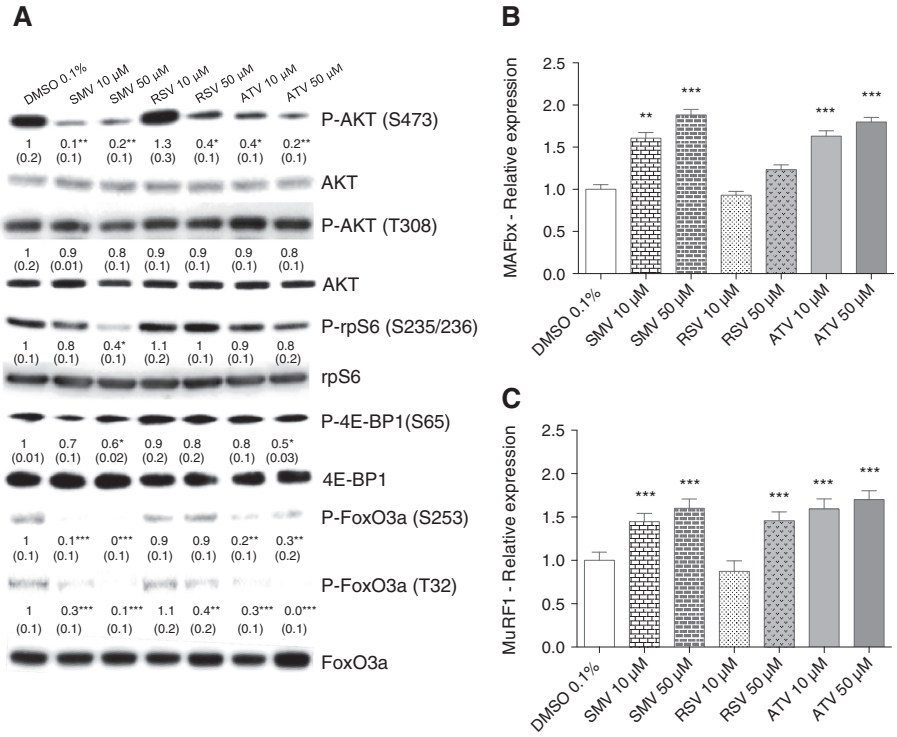

Fig. 3. Effect of statins on components of the AKT/mTOR pathway in C2C12 myotubes. C2C12 myotubes were cultured in serum-free medium for 24 h and then treated with 10 or 50 μM simvastatin (SMV), rosuvastatin (RSV) or atorvastatin (ATV) for 24 h. 0.1% DMSO was used as a negative control. A: Western blot analysis of the expression of phosphorylated AKT (P-AKT), phosphorylated ribosomal protein S6 (P-rpS6), phosphorylated 4E-binding protein 1 (P-4E-BP1), phosphorylated Forkhead box protein O3a (P-FoxO3a) and the respective total proteins. B. and C. mRNA expression of MAFbx and MuRF-1 after statin treatment was determined by real-time PCR. GAPDH was used as housekeeping gene. Data represent the mean ± SEM of three to four independent experiments carried out in triplicates. **$P < 0.01$ and ***$P < 0.001$ *vs.* DMSO control.

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

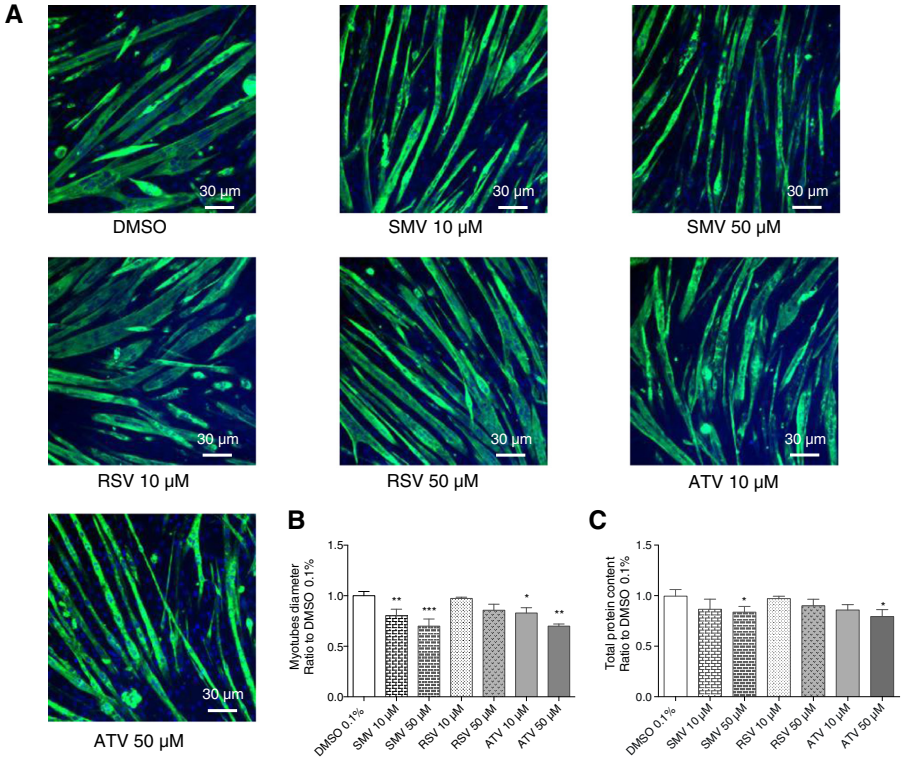

Fig. 4. Statin-induced C2C12 myotube atrophy. C2C12 myotubes were treated with 10 or 50 μM simvastatin (SMV), rosuvastatin (RSV) or atorvastatin (ATV) for 24 h. 0.1% DMSO was used as a negative control. A. Photomicrographs of myotubes fixed and stained for myosin heavy chain (green) and with DAPI (blue). 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.

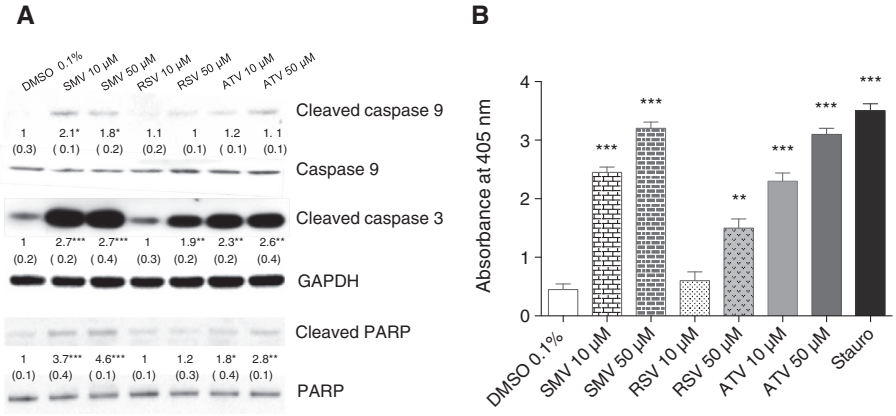

Fig. 5. Activation of apoptosis by statins in C2C12 myotubes. Myotubes were treated with 10 or 50 μM simvastatin (SMV), rosuvastatin (RSV) or atorvastatin (ATV) for 24 h. 0.1% DMSO was used as a negative control. A. Myotubes were lysed and centrifuged to remove cell debris. Aliquots of the supernatants 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.

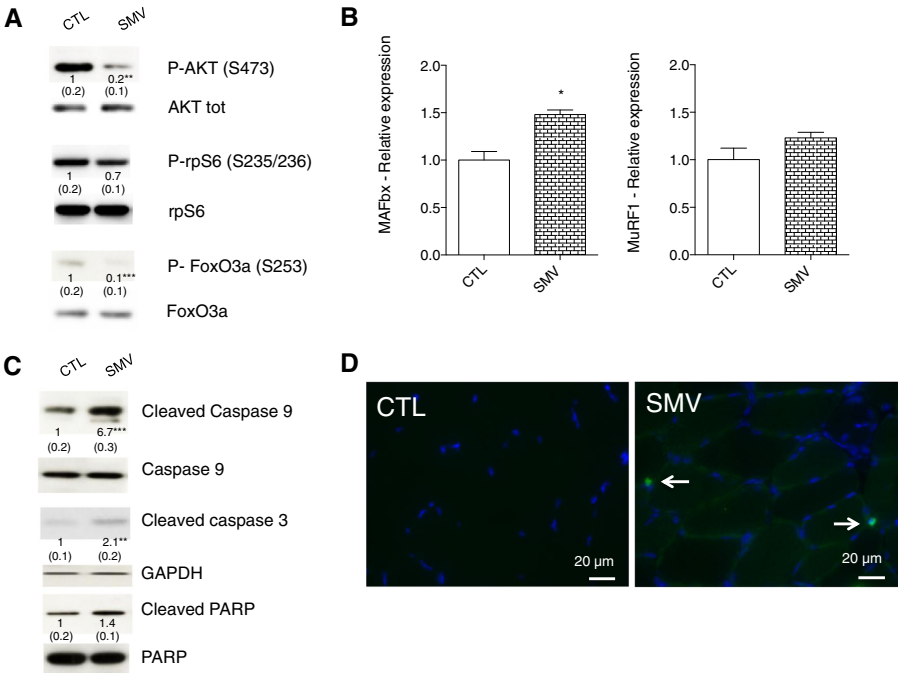

Fig. 6. Effect of statins on the components of the AKT/mTOR pathway and apoptosis *in vivo*. C57BL/6 J mice were treated with 5 mg/kg simvastatin (SMV) per day for 21 days. Analyses were performed using gastrocnemius muscle. A. Western blot analysis of the expression of phosphorylated AKT (P-AKT), phosphorylated 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).

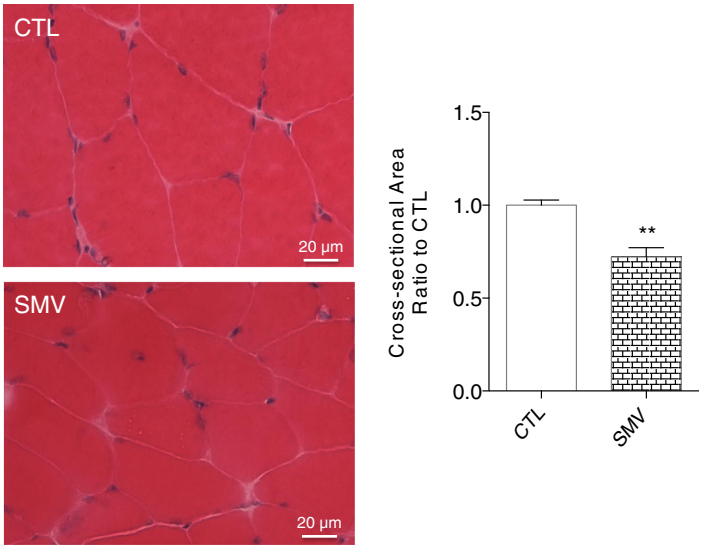

Fig. 7. Statin-induced muscle fiber atrophy *in vivo*. Mice were treated with 5 mg/kg/day for 21 days simvastatin (SMV) or with saline (CTL). Gastrocnemius muscles were removed and frozen sections were stained with hematoxylin/eosin. The area of the individual myofibers was decreased in mice treated 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 degradation *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 cells types [8,9,35]. In our previous study in L6 cells (a rat skeletal muscle cell line) [8], we postulated a mitochondrial mechanisms 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 [1,2,6]. 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.

Sources of funding

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Conflicts of interest

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

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Paper two

IGF-1 prevents simvastatin-induced myotoxicity

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1 Abstract

Statins are drugs used to lower cholesterol levels. They are generally well-tolerated, but their use is frequently associated with dose-dependent skeletal muscle damage. The mechanisms by which statins induce myotoxicity are still not completely understood. Statins have been shown to negatively regulate the AKT signaling cascade with an unclear mechanism, whereas insulin-like growth factor (IGF-1) is known to activate AKT signaling pathay and promote muscle growth.

The objective of the present study was to measure the effect of IGF-1 on simvastatin-induced toxicity in C2C12 myotubes.

Myotubes were exposed to 10 μM simvastatin and/ or 20 ng/ml IGF-1 for 18 hours. Simvastatin inhibited IGF-1 pathway blocking protein synthesis, enhancing breakdown of myofibrillar proteins, while also triggering apoptosis. Conversely, simvastatin-induced myotoxicity was completely antagonized by IGF-1. Moreover, the protective effect of IGF-1 was mediated by the activation of IGF-1/AKT signaling cascade that led to a suppression of atrophic markers and apoptosis, and simultaneously triggered pro-synthetic pathways. These results were confirmed by the maintenance of myotubes morphology.

This study provides new insight into the prevention of statin toxicity and may herald new discoveries for the therapeutic intervention of the statin-induced myalgia.

Keywords: Simvastatin; IGF-1; Protein synthesis; Protein degradation; Apoptosis

2. Introduction

Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are cholesterol lowering medications. They reduce morbity and mortality from coronary heart disease and mitigate the risk of stroke [1].

Statins are generally well-tolerated, however, they can also produce potentially severe adverse reactions, particularly on the skeletal muscle [2]. Skeletal muscle injuries vary from mild myopathy to serious and clinically dangerous rhabdomyolysis, a clinical condition characterized by the destruction of the skeletal muscle fibers and release of their contents into the bloodstream [3, 4].

Previous work from our group showed that simvastatin-induced myotoxicity might be related to AKT inhibition [5]. AKT, also called protein kinase B (PKB), is a major effector of IGF-1 pathway (Fig.1). IGF-1/ AKT pathway plays a major role in the regulation of skeletal muscle growth. IGF-1 is considered a promising anti-atrophic agent because of its ability to promote hypertrophy [6]. It has been demonstrated that administration of IGF-1 ameliorates atrophic settings [7] [8] and that IGF-1 is sufficient to induce significant skeletal muscle hypertrophy via the AKT signaling cascade in animal models [9, 10] and muscle cell culture systems [11, 12]. By contrast, skeletal muscle atrophy has been associated with a decreased ability to activate the IGF-1/AKT pathway [13].

The hypertrophic effects of IGF-1 are mediated by the IGF-1 receptor (IGF1R), a tyrosine kinase receptor which signals through the AKT pathway. AKT controls both protein synthesis, by promoting the activation of the mammalian target of rapamycin (mTOR) and, protein degradation, by phosphorylating and thereby suppressing the activation of forkhead members of the O class (FoxO) transcription factors [14-16].

mTOR controls protein translation, through phosphorylation of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), and protein transcription, through phosphorylation of the ribosomal protein S6 kinase (S6K) [17-21].

AKT suppresses the nuclear translocation of the FoxOs transcription factors [16]. FoxO factors are required for the transcriptional regulation of two ubiquitin ligases in muscle: muscle atrophy F-box (MAFbx) also called atrogin-1 and,

muscle ring finger 1 (MuRF-1). These E3 ubiquitin ligases conjugate ubiquitin to muscle proteins (i.e. myosin), targeting them for degradation by the ubiquitin–proteasome system. Growth factors or stress agents regulate FoxOs activation through a shuttling mechanism [22]. Growth factors stimulate the phosphorylation of FoxO proteins, which in turn provokes the nuclear exclusion and facilitates their degradation [19]. Whereas, stress agents cause FoxOs dephosphorylation, thereby accelerating their nuclear translocation and subsequent activation [23-25] [26].

Moreover, AKT is directly involved in regulating cell survival through the suppression of apoptosis by phosphorylating and, therefore blocking, the pro-apoptotic protein BAD and, consequently activating the caspase cascade [27].

In this work, we aimed to investigate and to better understand the role of IGF-1 in the prevention of simvastatin-induced myotoxicity.

Since we have previously shown that simvastatin triggers apoptosis and atrophy of C2C12 myotubes by inactivating AKT pathway, we investigated whether IGF-1 could protect from simvastatin-induced atrophy. For this purpose, we first studied the effects of simvastatin and IGF-1 on C2C12 myotubes cytotoxicity. Further, we characterized the effects of the combination of the two agents on atrophic and apoptotic markers.

This study increases our understanding of the importance that IGF-1 has on the statin-associated muscle atrophy and opens the door to a future development of specific modulators of IGF-1/ AKT signaling cascade for the therapeutic prevention of statin-induced myotoxicity.

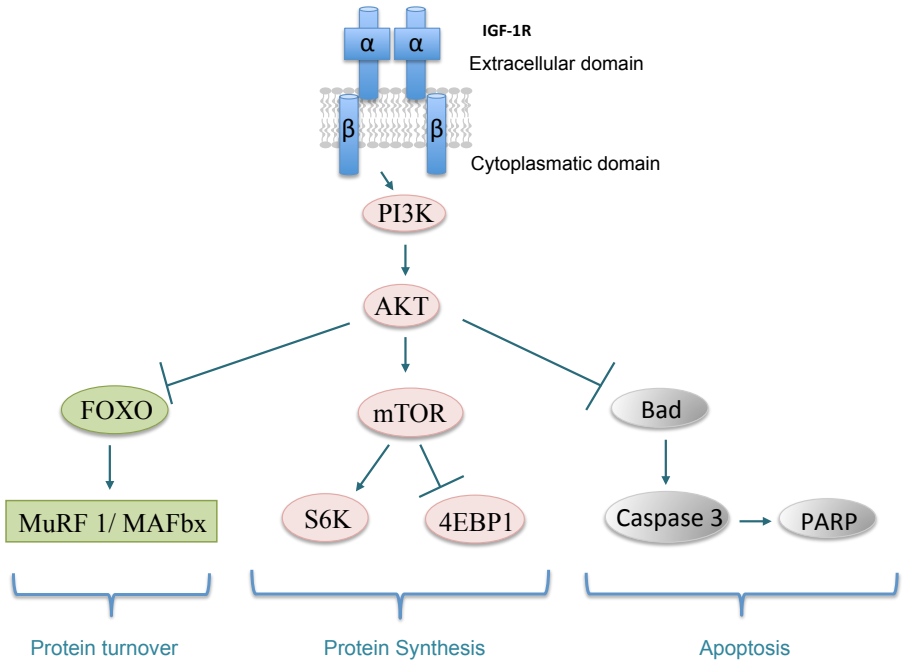

Fig. 1. A simplified scheme of the IGF-1/AKT pathway and its main components. Binding of IGF-1 to its receptor leads to its autophosphorylation and autoactivation. Subsequently, AKT translocates to the plasma membrane and is phosphorylated on Ser 473. Once activated, AKT inhibits protein degradation by phosphorylating and thus repressing FoxOs transcription factors, and stimulates protein synthesis via the mammalian target of rapamycin (mTOR). Moreover, AKT also blocks apoptosis inactivating the pro-apoptotic protein Bad.

3. Materials and Methods

3.1 Chemicals

We converted simvastatin into the active acid by using the protocol of Bogman et al. [28]. Stock solutions were prepared in DMSO and stored at -20°C. All other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where indicated.

3.2 Cell lines and cell culture

C2C12 myoblasts were from the American Type Culture Collection kindly provided by Novartis (Basel, Switzerland). We grew cells at 37°C in a humidified 5% CO_2 cell culture incubator and we passaged them using trypsin. Cell number

was determined using a Neubauer hemacytometer and viability using the trypan blue exclusion method.

We initially seeded 150,000 myoblasts per well in a 6-well plate, and grew them for 2 days in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX, high glucose (4.5 g/l) (Invitrogen, Basel, Switzerland) containing 10% heat-inactivated fetal bovine serum (Gibco, Paisley, UK).

Afterwards, we induced the differentiation using high glucose DMEM (Invitrogen, Basel, Switzerland) supplemented with 2% horse serum (Gibco, Paisley, UK), for 3 days. Then, we incubated the cultures in serum-free DMEM (Invitrogen, Basel, Switzerland) for 24 h before the addition of the compounds to test. Cell culture supplements were all purchased from GIBCO (Paisley, UK). DMSO was used as a control and its concentration was always 0,1%. After 18 h treatment, we collected cells and we proceed to the analysis of interest.

3.3 Cytotoxicity

We used the ToxiLight BioAssay Kit (Lonza, Basel, Switzerland) to investigate the cytotoxicity of the compounds on C2C12 myotubes. This assay measures the release of adenylate kinase, a marker for loss of cell membrane integrity. After drug incubation, we added 100 µl assay buffer to 20 µl supernatant from drug-treated cell culture medium, and we measured luminescence after incubation in the dark for 5 min, using a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland).

3.4 Cell lysis and immunoblotting

After drug incubation, we homogenized C2C12 myotubes in phosphosafe buffer (EMD Millipore, USA). We centrifuged the samples at 1600 *g* at 4°C and collected the supernatant. We determined the whole protein content for each sample using BCA Protein Assay Kit (Pierce, Thermo Scientific, Rockford, U.S.A). Lysates were separated on 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Basel, Switzerland) under reducing conditions and transferred to polyvinylidendifluoride membranes (EMD Millipore, Massachusetts, USA). We blocked the membranes with 5% nonfat dry milk in DPBS (Gibco, Paisley, UK)

containing 0.1% Tween-20 (Sigma-Aldrich, MO, USA) for 1 h at room temperature. We incubated 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 1:2000 of secondary antibody (Santa Cruz Biotechnology, USA) in 5% nonfat milk in PBS-T, washed them and developed the immunoreactive bands with 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 loading differences, the scanning units obtained from test proteins were divided by scanning units obtained from the housekeeping protein GAPDH.

3.5 Subcellular fractionation

We used the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Thermo Scientific, Rockford, U.S.A) to separate cytoplasmic and nuclear protein fractions. After drug incubation, we harvested C2C12 myotubes with trypsin-EDTA and pelleted them by centrifuging at 500 × *g* for 5 minutes. We resuspended the pellet with ice-cold CER I and incubated on ice for 10 minutes. We added ice-cold CER II, mixed and incubated on ice for 1 minute. Then, we centrifuged the tube for 5 minutes at 16,000 *g* and we transferred the supernatant (cytoplasmic extract) to a clean pre-chilled tube. In order to isolate the nuclear extract, we resuspended the pellet in ice-cold NER, vortexing for 15 seconds every 10 minutes, for a total of 40 minutes. At the end, we centrifuged the tube at 16,000 *g* for 10 minutes and we transferred the supernatant (nuclear extract) fraction to a clean pre-chilled tube.

3.6 FoxO3a translocation assay

After fixing the cells with 4% PFA, we permeabilized the myotubes with 0,2% Triton in PBS, we blocked the nonspecific binding with 10% BSA in PBS and we incubated with FoxO3a antibody (Cell Signaling Technology, USA) diluted 1:500 in PBS overnight. The day after, we washed the cells and we incubated with

Alexa Fluor 594 F (AB') (Invitrogen, Basel, Switzerland) diluted 1:1000 in PBS. We then stained the nuclei with DAPI (Invitrogen, Basel, Switzerland).

The distribution of FoxO3a between the two sub-cellular compartments nucleus and cytoplasm was analyze and measured on a single cell level in C2C12-cells by use of the Cytoplasm to Nucleus Translocation BioApplication software designed for the ArrayScan HCS Reader (Cellomics Inc) as described earlier [29]. The average intensity of 1000 cells/well was quantified using a 20X objective. Cellular focusing and definition of the nuclear region was performed in channel 1 (nuclear channel) according to the Hoechst-33342 nuclear dye after a fixed exposure time of 80 ms, FoxO3a dependent fluorescence was visualized within channel 2 at 488 nm (300 ms). Within the target channel 2 an algorithm was utilized to define two masks either corresponding to each nucleus, identified within channel 1, or the cytoplasm beyond the nuclear region; the average pixel intensity within the separate masks of the target cannel were measured and results were reported as mean Nucleus-Cytoplasm Intensity-Differences.

3.7 Real-Time Polymerase Chain Reaction (RT-PCR)

We treated C2C12 myotubes with the drug of interest for 18 h. Afterwards, RNAs were extracted and purified using the Qiagen RNeasy mini extraction kit (Qiagen, Hombrechtikon, Switzerland). The purity and quantity of RNA were evaluated with the NanoDrop 2000 (Thermo Scientific, Wohlen, Switzerland) and cDNA was synthesized from 10 µg RNA using the Qiagen omniscript system.

The real-time PCR was performed in triplicate using SYBR green (Roche Diagnostics, Rotkreuz, Basel). We used primers specific for the atrophic genes MAFbx (forward: 5'AGTGAGGACCGGCTACTGTG3',reverse: 5'GATCAAACGTTGCGAATCT3'and MuRF-1 (forward: 5'CCTGCAGAGTGACCAAGGA3', reverse: 5'GGCGTAGAGGGTGTCAAACT3').

Real time PCR was executed by using the ViiA7 software.

We calculated relative quantities of specifically amplified cDNA with the comparitive-threshold cycle method. GAPDH was used as endogenous reference (forward: 5′-CATGGCCTTCCGTGTTCCTA-3′; reverse: 5′CCTGCTTCACCACCTTCTTGA-3′). No-template and no-reverse-transcription controls ensured that unspecific amplification could be excluded.

3.8 Immunostaining and diameter measurement

After fixing the cells with 4% PFA, we permeabilized the myotubes with 0,2% Triton in PBS, we blocked the nonspecific binding with 10% BSA in PBS and we incubated with myosin heavy chain (MHC) (EMD Millipore, Massachusetts, USA) diluted 1:1000 in PBS overnight. The day after, we washed the cells and we incubated with Alexa Fluor 488 F (AB') (Invitrogen, Basel, Switzerland) diluted in PBS. We then mounted cells with ProLong Gold antifade reagent with DAPI (Invitrogen, Basel, Switzerland). To measure diameters, we took four pictures per well and from each picture we measured the biggest 30 diameters using Cell R software (Olympus Imaging Australia Pty Ltd, Tokyo, Japan). Afterwards, we calculated the average for all the diameters measured in each well.

3.9 Apoptotic DNA fragmentation assay

We used Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals, Mannheim, Germany) to evaluate the Apoptotic DNA laddering under the conditions described by the manufacturer. To isolate DNA, cells were incubated for 10 min with 200 µL of lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, and 20% Triton X-100, pH 4.4). Following the incubation period, we added 100 µL of isopropanol to each tube, vortexed the samples, added them to filter tubes, and centrifuged for 1 min at 5000 g. Filter tubes were washed twice with 500 µL of wash buffer (20 mM NaCl and 2 mM Tris-HCl, pH 7.5). Then, 200 µL of elution buffer (10 mM Tris, pH 8.5) was added to each filter tube, and samples were centrifuged at 6000 g for 1 min. DNA content and integrity were determined by measuring samples absorbance at 260 nm using a NanoDrop 2000 (Thermo Scientific, Wohlen, Switzerland). We used 1% agarose gel in TBE buffer (10 mM Tris, 80 mM boric acid, 1 mM EDTA) containing ethidium bromide (Sigma) to separate and visualize DNA bands. DNA was added at 5 µg/lane. Gel images were captured using a Bio-Rad Gel Doc 2000 Imager with Quantity One software (Bio-Rad Laboratories).

3.10 Protein synthesis assay

We determined the protein synthesis using the Click-iT® Plus OPP Protein

Synthesis Assay Kits (Invitrogen, Basel, Switzerland) following manufacturers' instructions. In brief, C2C12 myotubes were treated for 18 h with the compounds of interest. Afterwards, the medium containing the treatment was removed and Click-iT® OPP was added into the well. We incubated cells for 1 hour. Then we fixed and permeabilized them with 0.5% Triton X 100. This step was followed by incubation for 30 minutes of medium containing Click-iT® OPP reaction cocktail. We remove the reaction cocktail and add the NuclearMask™ Blue Stain working solution incubating for 30 minutes at room temperature. We finally wash out the HCS NuclearMask™ Blue Stain solution and we proceeded to imaging and analysis.

3.11 Statistical Methods

All the data were analyzed using the mean +/- standard error of the mean (SEM) and 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).

4. Results

4.1 IGF-1 reduces the simvastatin-induced cytotoxic effects in a dose-dependent manner

Cytotoxicity was determined by measuring adenilate kinase (AK) release (Fig. 2). Incubation time and concentration were chosen based on results of previous experiments which showed that, the treatment of 10 μM simvastatin for 18 h was the shortest incubation and the lowest concentration in which cell death was significantly increased [5]. AK release in C2C12 myotubes treated with simvastatin for 18 h was around 1.7 fold higher than that of control cells, indicating that simvastatin directly affects the cell membrane integrity and has a cytotoxic effect. Interestingly, cells co-treated with simvastatin and increasing concentration of IGF-1 showed a dose dependent decrease in AK release

compared to simvastatin- treated cells. The total prevention by IGF-1 was observed at concentrations ranging from 10 ng/ml to 100 ng/ml, whereas, lower concentrations prevented simvastatin-induced toxicity only to a lesser extent.

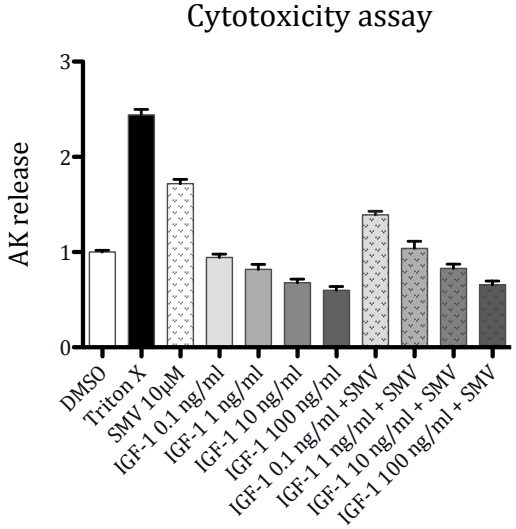

Fig. 2. IGF-1 counteracted the cytotoxic effects induced by simvastatin. C2C12 myotubes were incubated with simvastatin and/or increasing concentration of IGF-1 for 18 h. Cell cytotoxicity was quantified using Toxilight assay and expressed as ratios to the DMSO control. 0.1% DMSO and triton X were used as a negative and positive control respectively. Data represent the mean ± SEM of four independent experiments carried out in triplicates.

4.2 IGF-1R inhibitor blocks the IGF-1 preventive effect

To test whether the IGF-1-mediated prevention depends on the IGF-1 receptor, we performed cell cytotoxicity assay using NVP-AEW541, a specific inhibitor of IGF-1 receptor [30] [31]. We incubated rising concentrations of NVP-AEW541 for 18 h to define that 10 µM was the minimum concentration required for effective inhibition of the IGF-1 receptor (data not shown).

The co-incubation of C2C12 myotubes with NVP-AEW541, simvastatin, and IGF-1 for 18 h resulted in an increase in AK release of up to 2.1 fold above control levels and up to 1.1 fold above the simvastatin and IGF-1 co-treatment.

To further evaluate whether the increased AK release did not actually reflect the cytotoxicity of NVP-AEW541, we analyzed the cytotoxicity of the compound alone. The experiments revealed that NVP-AEW541-treated cells had a slightly increase of AK release of up to 1.4 above control levels and the co-incubation of NVP-AEW541 together with IGF-1 (20 ng/ml) did not abolish this effect. Hence,

IGF-1 receptor triggers the prevention of simvastatin-induced toxicity and IGF-1 pathway is crucial for the viability of the cells, indeed, if IGF-1 receptor is blocked, the cell viability decreases (Fig. 3a). Moreover, we analyzed the inactivation of the receptor by western blot just to confirm our hypothesis. As expected, the inhibitor reduced the phosphorylation of the receptor under basal conditions and the addition of IGF-1 could not restore the basal phosphorylation (Fig. 3b).

4.3 IGF-1 inhibits the protein degradation triggered by simvastatin

We previously showed that simvastatin blocks AKT activation [32]. AKT activation inhibits protein degradation by retaining forkhead transcription factors (FoxOs) in the cytoplasm and, therefore, inhibiting the transcription of several genes [26] [15]. Here, we tested how the localization of FoxO3a is regulated in the C2C12 myotubes cotreated with simvastatin and IGF-1. Subcellular fractionation and western blot analysis for FOXO3a were performed. Under normal condition FOXO3a was predominantly detected in the cytoplasm. By contrast, simvastatin promoted Foxo3a nuclear accumulation and this effect was abolished by the coincubation with IGF-1 (Fig. 4a). These results were also confirmed by Foxo's traslocation assay. We observed a nuclear accumulation of FoxO3a after simvastatin treatment and, a sequestered FoxO3a in the cytoplasm after the cotreatment IGF-1 and simvastatin (Fig. 4b). Collectively, these data indicate that the shuttling and, therefore, the subsequent activation of FoxO3a caused by simvastatin are prevented by IGF-1.

Furthermore, to understand the role of FoxO3a in simvastatin-treated myotubes, we investigated on the mRNA expression of the ubiquitin ligase gene MuRF-1 and MAFbx. These ubiquitin ligases are markers for muscle atrophy and moreover, FoxO3a plays an important role in the transcriptional activation of MuRF-1 and MAFbx [33, 34]. While simvastatin induced a robust expression of mRNAs specific for MuRF-1 and MAFbx, the co-treatment with IGF-1 prevented completely this effect (Fig. 5).

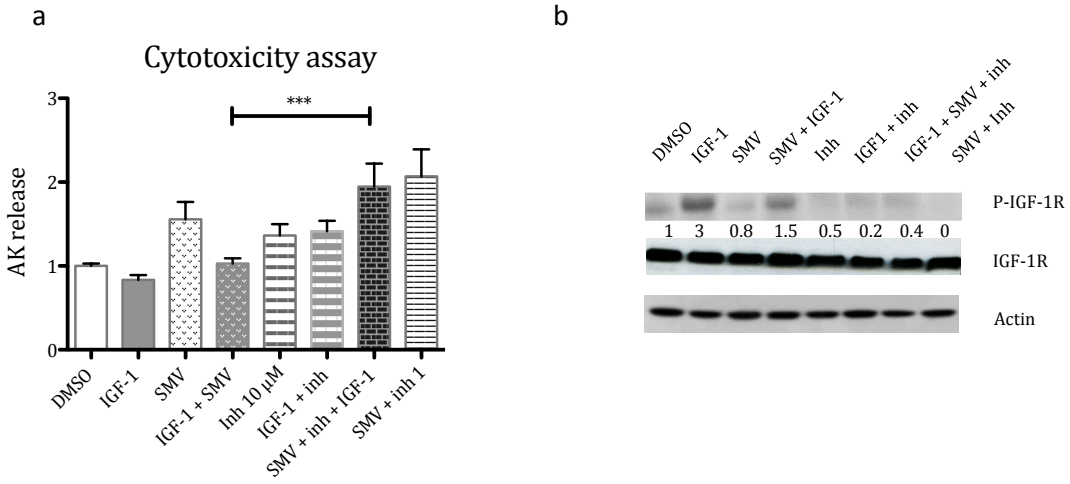

Fig. 3. IGF-1 receptor inhibitor (NVP-AEW541) blocked the IGF-1-mediated prevention accompanying cytotoxicity. (A) Inhibition of IGF-IR signaling-mediated by NVP-AEW541 was determined in C2C12 myotubes. Cells were pretreated with 10 µM NVP-AEW541 for 2 h and then stimulated with simvastatin and/or IGF-1 for 18 h. (A) Cell cytotoxicity was quantified using Toxilight assay and expressed as ratios to the DMSO control. (B) Representative blots of the expression of phospho- IGF-1 receptor. The expression of the housekeeping gene GAPDH was analyzed for standardization. Data represent the mean ± SEM of four independent experiments carried out in triplicates. ***P < 0.001 *vs*. DMSO control.

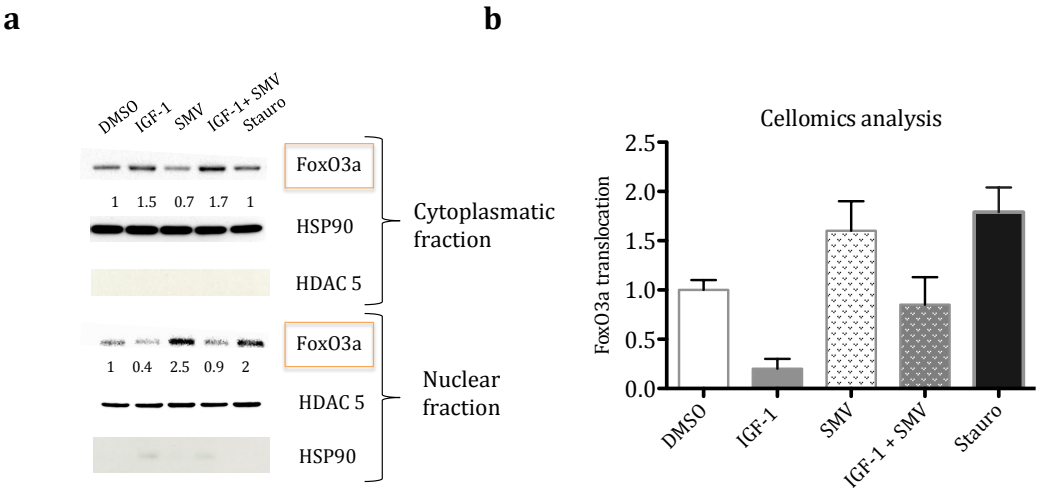

Fig. 4. IGF-1 blocked the FoxO3a activation induced by simvastatin. Cells were induced with IGF-1 and/or simvastatin for 18 h. 0.1% DMSO and staurosporine were used as negative and positive control respectively. **(a)** Cells were lysed and subcellular fractionation was performed. FoxO3a was detected in the different cellular subfractions. HSP90 and HDAC were used as loading control for the cytoplasmatic fraction and for the nuclear fraction respectively. Data represent the mean ± SEM of four independent experiments carried out in triplicates. **(b)** C2C12 myotubes were stained using FoxO3a antibody. Nuclei were stained using DAPI. FoxO translocation after the treatment with the indicated compounds was measured to evaluate Foxo's activity. Data represent the mean ± SEM of four independent experiments carried out in triplicates.

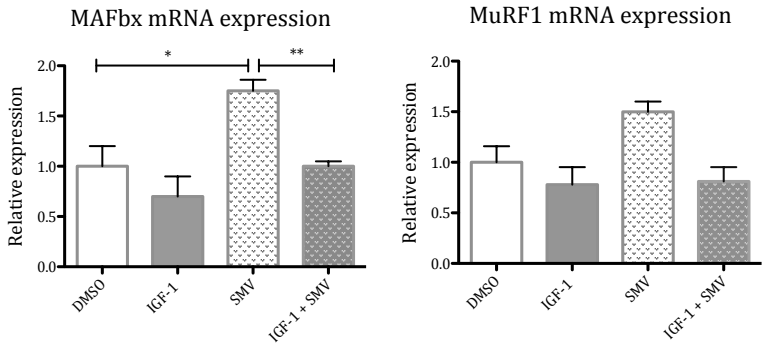

Fig. 5. IGF-1 completely blocked simvastatin-upregulated MAFbx/MuRF-1 expression in C2C12 myotubes. Expression of the indicated ubiquitin ligase genes was determined by real-time PCR. Data represent the mean ± SEM of three independent experiments carried out in triplicates. **P* < 0.05 and ***P* < 0.01 *vs.* DMSO control.

4.4 IGF-1 blocks the myotubes atrophy induced by simvastatin

C2C12 myotubes were treated, fixed and stained with an antibody against the myofibrillar protein myosin heavy chain (MHC). Immunofluorescence staining revealed that exposure to 10 μM simvastatin reduced significantly the diameter of myotubes, while the exposure to simvastatin and IGF-1 did not change the size of the myotubes. This result underscores that IGF-1 blocks the development of the muscle atrophy induced by simvastatin (Fig. 6).

4.5 IGF-1 prevents apoptosis in simvastatin-treated C2C12 myotubes

To investigate the effect of IGF-1 on simvastatin-induced apoptosis, we evaluated the activity of key mediators in the apoptotic signaling cascade such as the pro-apoptotic protein BAD, caspase 3 and, PARP. As expected, simvastatin induced a dramatic activation of the apoptotic markers whereas, the co-incubation of IGF-1 and simvastatin inactivated pro-apoptotic proteins (Fig. 7a). Moreover, we analyzed the apoptotic DNA fragmentation, one of the hallmarks of apoptotic cell death. As shown in figure 7b, simvastatin treatment induced apoptotic DNA laddering which was totally prevented by the co-incubation with IGF-1. These data suggest that IGF-1 is able to abrogate the apoptosis induced by simvastatin.

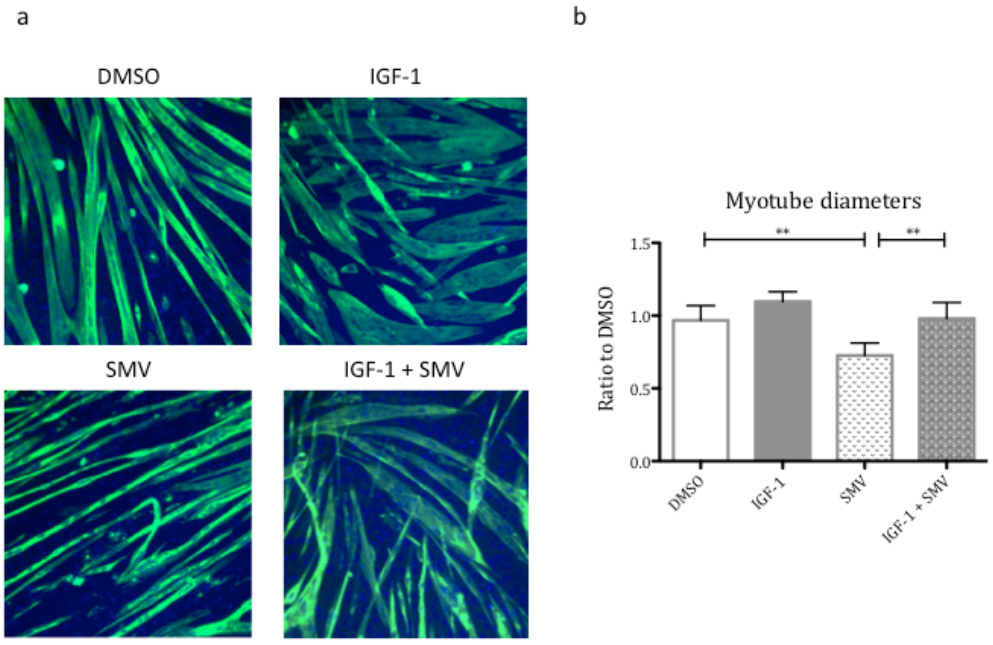

Fig. 6. IGF-1 blocked myotubes atrophy induced by simvastatin. C2C12 myotubes were treated with 0,1% DMSO, 20 ng/ml IGF-1, and/or 10 μM simvastatin for 18 h. **(a)** Representative immunostaining using MHC antibody. **(b)** Myotube diameter after the treatment with the indicated compounds was measured to evaluate myotube atrophy. Data represent the mean ± SEM of four independent experiments carried out in triplicates. **P < 0.01 vs. DMSO control.

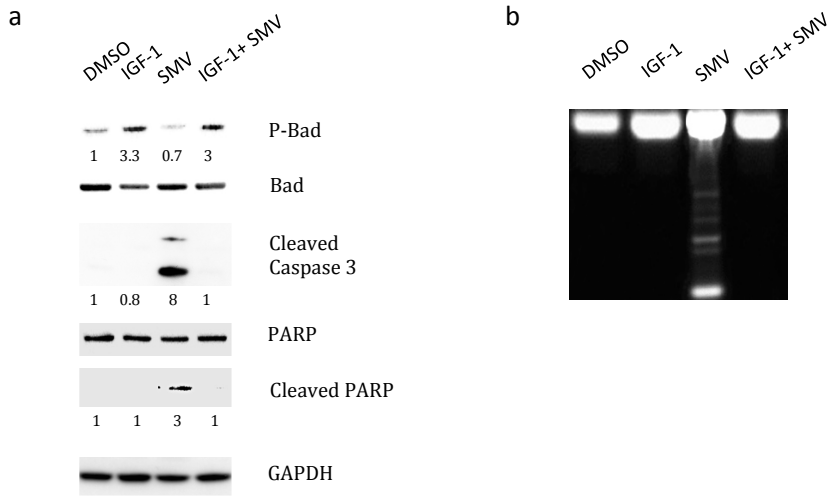

Fig. 7. IGF-1 abolished apoptosis induced by simvastatin treatment. C2C12 myotubes were treated with 0.1% DMSO, 20 ng/ml IGF-1, and/or 10 μM simvastatin for 18 h. Whole cell lysate were subjected to either Western blot analysis or DNA laddering assay. **(a)** Shown are representative blots of apoptotic markers. GAPDH levels are used as a loading control. Fold stimulation of the apoptotic proteins was quantitated with Image J (numbers below bands). **(b)** Ladder-like pattern of DNA fragmentation into oligonucleosome-length fragments was observed after simvastatin treatment suggesting an apoptotic event. Results are representative of three independent experiments.

4.6 IGF-1 restores the protein synthesis blocked by simvastatin

To further understand the molecular mechanisms contributing to the protective effect of IGF-1 on simvastatin-treated C2C12 myotubes, we examined the expression of AKT downstream effectors involved in protein synthesis.

As shown in figure 8, IGF-1 remarkably abolished the specific inhibition of AKT pathway induced by simvastatin restoring the levels of expression of phospho-AKT, phospho-S6K and phospho-4EBP1.

Next, we examined whether the myoprotective effect of IGF-1 observed in our C2C12 model could be attributed, at least in part, to a restoration of the protein synthesis. Using a fluorescent labeled methionine, we were able to visualize the overall protein synthesis after the treatment with the drug of interest. Simvastatin-treated myotubes appeared to have a decrease in methionine content compared to control cells. By contrary, the cotreatment IGF-1 and simvastatin showed a complete restoration of the protein synthesis (Fig. 9a). In addition, to confirm these results, we measured the total protein content isolated from C2C12 myotubes after treatments. As expected, simvastatin-treated cells showed a significant decrease in the overall protein content which is not seen in the co-treated cells (Fig. 9b). Taken together, these findings indicate that IGF-1 is able to reactivate the protein synthesis blocked by simvastatin through the activation of the AKT pathway, and the protein synthesis plays a role in the prevention of statin– induced toxicity.

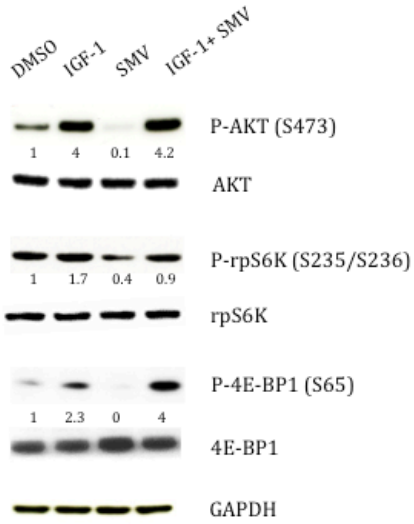

Fig. 8. The protective effects of IGF-1 are correlated with an efficient restoration of the AKT pathway. C2C12 myotubes were treated with the indicated compounds for 18 h. Whole cell lysates were subjected to western blot analysis. Representative blots of basal expression levels of phospho- AKT Ser 473, phospho-rpS6 Ser 235/236 and phospho-4E-BP1 Ser 65. The relative levels of indicated proteins were quantitated with Image J and normalized to the total protein (numbers below bands). GAPDH was used as a loading control. Data are representative of four independent experiments.

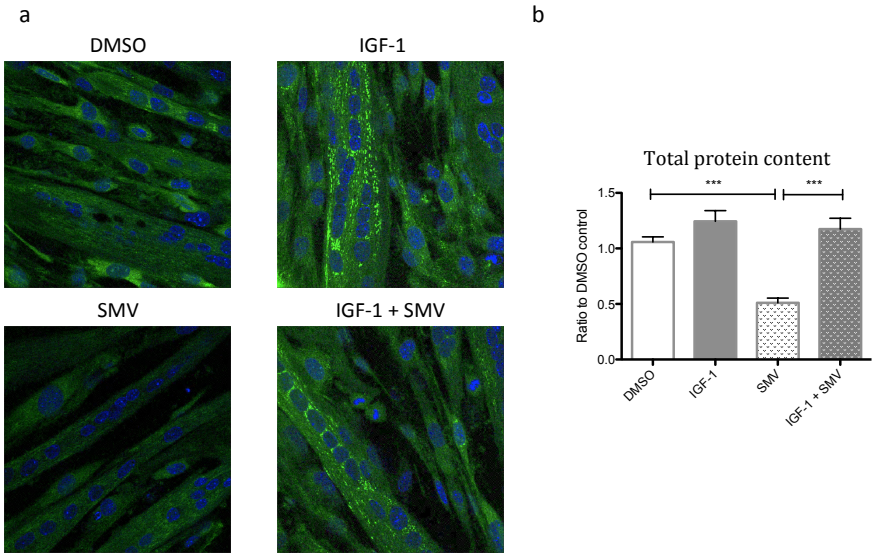

Fig. 9. IGF-1 restored the protein synthesis blocked by simvastatin. C2C12 myotubes were treated with the indicated compounds for 18 h. (a) Representative immunohistochemical staining of methionine in C2C12 myotubes. (b) Total protein content isolated from C2C12 myotubes after treatment with the indicated compounds. Data represent the mean ± SEM of four independent experiments carried out in triplicates. ***P < 0.001 vs. DMSO control.

5. Discussion

The present study demonstrates for the first time that IGF-1 acts as a master regulator in simvastatin-induce myotoxicity by coordinating simultaneously the activation of protein synthesis and, the repression of protein degradation and apoptosis.

Previous study reported that statins interfere with AKT phosphorylation, which might be related to the toxicity of statin in skeletal muscle [32, 35]. In addition, statins have been demonstrated to induce apoptosis in various type of cells [36, 37], whereas IGF-1 pathway is an essential anti-apoptotic molecular pathway that confers the survival to cells [38] [39] and stimulates muscle growth by inhibiting the protein degradation [6]. Recent studies showed that IGF-1/AKT pathway could dominantly reverse the atrophy-inducing effects of dexamethasone [40] [26]; whereas no data exist concerning the direct impact of IGF-1 on statin treated muscle cells.

We therefore explored the possibility whether IGF-1 could also reverse the myotoxic effects induced by simvastatin in C2C12 myotubes. We incubated myotubes with 10 μM simvastatin and/or increasing concentrations of IGF-1 for 18 h. The co-incubation with simvastatin and IGF-1 revealed a dominantly dose-dependent reduction in statin-induced cytotoxicity. It is worth to notice that simvastatin concentration exceeds the peak reached in the plasma of patients which is about 1 μM [41]. However, localized concentration in skeletal muscle could be higher, especially if statins are combined with cytochrome P450 inhibitors or with transporters' dysfunction [42].

Further, to confirm the key role of the IGF-1/AKT pathway in blocking statin-induced cytotoxicity, we exploited a pharmacological approach. We used a specific inhibitor of IGF-1 R [30], NVP-AEW541, and we analyze the cytotoxic profile. The co-incubation of C2C12 myotubes with NVP-AEW541, simvastatin, and IGF-1 for 18 hours resulted in an increase in AK release, blocking completely the survival signals triggered by IGF-1. Hence, IGF-1 induces the prevention of simvastatin-induced toxicity through the activation of its receptor.

Moreover, since IGF-1 has been shown to repress protein degradation retaining FoxO3a in the cytoplasm [43], we analysed the FoxO3a translocation into the

nucleus and the subsequent induction of atrophic markers MuRF-1 and MAFbx after the treatment with IGF-1 and/or simvastatin. In this study we showed that IGF-1 through activation of AKT retains FoxO3a in the cytoplasm and prevents the upregulation of MuRF-1 and MAFbx induced by simvastatin. The inactivation of this proteolytic pathway by IGF-1 indicates that the prevention of simvastatin-associated muscle atrophy results not only from a suppression in cell death, but also from a maintenance of myofibrillar proteins. Indeed, using C2C12 myotubes, we showed that simvastatin directly stimulates muscle catabolism affecting the myotube morphology, and interestingly, this catabolic effect is completely blocked by IGF-1.

In addition, since several works implicate IGF-1 in blocking apoptosis in various cell types [44] [45] [46] and, IGF-1 is known to exert anti-apoptotic effects blocking the activation of caspases [27], we explored the possibility that the protective effect of IGF-1 on simvastatin-induced cytotoxicity, might be also related to an inhibition of apoptosis. Our data confirmed our hypothesis. IGF-1 negatively regulated apoptotic markers such as BAD, caspase 3 and cleaved PARP leading to a complete inhibition of apoptosis. Indeed, the apoptotic DNA laddering assay confirmed that IGF-1 completely prevents the apoptosis induced by simvastatin. These findings are consistent with previous work that reported that IGF-1/AKT signaling interferes with drug-induced apoptosis [47] and pointed out that the anti-apoptotic and anti-atrophy signals of IGF-1 interfere with the toxicity of simvastatin.

IGF-1 is a potent anabolic factor and animals treated with IGF-1 reported a generalized increase in protein synthesis [48]. Activation of IGF-1 receptor by its ligand initiates intracellular signaling cascade that results in the stimulation of AKT signaling pathway [40, 49]. In skeletal muscle, IGF-1 directly stimulates AKT inducing a phosphorylation cascade that leads to an inhibition of protein breakdown but also to stimulation of the protein synthesis [50]. Therefore, we extended our investigation focussing on two downstream effectors of mTOR involved in protein synthesis: S6K and 4E-BP1. These proteins, once phosphorylated by mTOR, upregulate the protein translation maschinery and promote the protein translation [51] [52]. Our data demonstrate that the AKT/mTOR/S6K pathway impacted by simvastatin can be completely rescued by

the addition of IGF-1.

We finally then shed light on the role of the AKT/mTOR/S6K pathway in the IGF-1-induced prevention of muscle wasting associated with simvastatin, by exploring the protein synthesis and the overall protein content. Immunostaining pictures showed that the expression of methionine was markedly reduced in simvastatin-treated myotubes and completely restored in myotubes treated with IGF-1 and simvastatin. These results were further confirmed by the measurement of total protein in C2C12 myotubes after treatment with IGF-1 and/or simvastatin. Simvastatin induced a significant decrease of the whole protein content which is completely prevented by the addition of IGF-1.

While previous studies have shown that IGF-1 elicits muscle hypertrophy [48] [53] [54] and inhibits [55] or ameliorates muscle atrophy [56], we demonstrate for the first time that IGF-1 can counteract the atrophy stimuli induced by simvastatin by suppressing catabolic pathways associated with MAFbx and MuRF-1 and triggering anabolic signaling associated with S6K and 4EBP1.

In addition, IGF-1 and insulin downstream signalling are largely overlapping. Our data may also partially explain reported risk of statins in inducing type 2 diabetes [57]. Indeed, statin treatment may result in a truncated insulin response caused by the impairment of AKT phosphorylation. Diabetic setting due to an impaired activation of AKT in response to insulin has been then described in several independent studies [58] [59] [60].

In summary, our study provides first evidence that the IGF-1 blocks simvastatin-induced myotoxicity by potently restoring the IGF-1/AKT signaling pathway suppressed by simvastatin.

Hence, inducing IGF-1R may be a promising approach to overcome the skeletal muscle toxicity caused by statins. Therefore, our *in vitro* data merit a confirmation *in vivo* to test the clinical effects of a compound IGF-1-like for the treatment of myalgia associated with statins.

Conflict of Interest

The authors declare no conflict of interest.

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Paper three

Simvastatin induces mitochondrial dysfunction and increases atrogin-1 expression in H9c2 cardiomyocytes and mice *in vivo*

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ORGAN TOXICITY AND MECHANISMS

Simvastatin induces mitochondrial dysfunction and increased atrogin-1 expression in H9c2 cardiomyocytes and mice in vivo

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Abstract Simvastatin is effective and well tolerated, with adverse reactions mainly affecting skeletal muscle. Important mechanisms for skeletal muscle toxicity include mitochondrial impairment and increased expression of atrogin-1. The aim was to study the mechanisms of toxicity of simvastatin on H9c2 cells (a rodent cardiomyocyte cell line) and on the heart of male C57BL/6 mice. After, exposure to 10 μmol/L simvastatin for 24 h, H9c2 cells showed impaired oxygen consumption, a reduction in the mitochondrial membrane potential and a decreased activity of several enzyme complexes of the mitochondrial electron transport chain (ETC). The cellular ATP level was also decreased, which was associated with phosphorylation of AMPK, dephosphorylation and nuclear translocation of FoxO3a as well as increased mRNA expression of atrogin-1. Markers of apoptosis were increased in simvastatin-treated H9c2 cells. Treatment of mice with 5 mg/kg/day simvastatin for 21 days was associated with a 5 % drop in heart weight as well as impaired activity of several enzyme complexes of the ETC and increased mRNA expression of atrogin-1 and of markers of apoptosis in cardiac tissue. Cardiomyocytes exposed to simvastatin in vitro or in vivo sustain mitochondrial damage, which causes AMPK activation, dephosphorylation and nuclear transformation of FoxO3a as well as increased expression of atrogin-1. Mitochondrial damage and increased atrogin-1 expression are associated with apoptosis and increased protein breakdown, which may cause myocardial atrophy.

Keywords Simvastatin · Atrogin-1 · Mitochondrial respiration · FoxO transcription factors · Apoptosis

Annalisa Bonifacio and Peter J. Mullen have contributed equally to this work.

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Introduction

Statins are among the most often prescribed drugs in Western countries. They are used frequently in patients with cardiovascular diseases because they reduce morbidity and mortality from coronary heart disease (Group 1994; LaRosa et al. 2005; Ridker et al. 2008), reduce the risk of stroke (Amarenco et al. 2006) and improve the walking distance in patients with peripheral arterial disease (Mohler et al. 2003). Their major site of action is the liver, where they inhibit HMG-CoA (hydroxyl-methyl-glutaryl-coenzyme A) reductase, the rate-limiting step in cholesterol biosynthesis. Inhibition of this pathway also impairs other processes, such as ubiquinone production and the isoprenylation and *N*-linked glycosylation of proteins (Matzno et al. 2005).

Altering these processes can reduce inflammation, oxidative stress and platelet adhesion (Davignon 2004), actions known as pleiotropic effects of statins.

Statins are generally well tolerated, but there are exposure-dependent adverse reactions, particularly on skeletal muscle. Myopathy is observed in 1.5–5 % of patients treated with statins (Joy and Hegele 2009), whereas rhabdomyolysis is much rarer (Graham et al. 2004). The most important risk factor for myotoxicity of statins is an increased exposure, for instance, due to impaired transport of statins into hepatocytes by OATP1B1 (Link et al. 2008) or due to impaired hepatocellular metabolism of statins caused by interactions with other drugs (Ratz Bravo et al. 2005; Roten et al. 2004). The molecular mechanisms how statins damage skeletal muscle are not completely clear. Frequently mentioned possibilities include impaired prenylation of critical proteins (Cao et al. 2009; Sakamoto et al. 2011), impaired mitochondrial function (Bouitbir et al. 2012; Kaufmann et al. 2006; Kwak et al. 2012; Larsen et al. 2013; Mullen et al. 2011; Sirvent et al. 2005) or impaired mitochondrial proliferation (Schick et al. 2007), accelerated skeletal muscle breakdown due to increased expression of atrogin-1 (Cao et al. 2009; Hanai et al. 2007) and impaired skeletal muscle protein synthesis (Tuckow et al. 2011).

Adverse reactions of statins on cardiac muscle have been much less intensively studied, but could also be clinically relevant. In two publications, cardiac muscle was described to be less sensitive to mitochondrial adverse reactions of statins than skeletal muscle (Bouitbir et al. 2012; Sirvent et al. 2005), findings which are compatible with the clinical experience that the heart is usually not affected in patients with statin-induced rhabdomyolysis (Joy and Hegele 2009; Roten et al. 2004). On the other hand, in vitro studies have clearly demonstrated that lipophilic statins such as simvastatin and lovastatin can be toxic on cardiac myocytes and can induce apoptosis (Demyanets et al. 2006; Kong and Rabkin 2004; Rabkin and Kong 2003; Rabkin et al. 2007). In addition, Bouitbir et al. demonstrated that cardiac mitochondria exposed to simvastatin in vivo react to a possible insult by increasing antioxidative mechanism and by proliferation (Bouitbir et al. 2012).

As mentioned above, mitochondrial damage and increased expression of atrogin-1 appear to be key findings for skeletal muscle injury associated with statins. Previous studies have shown that atrogin-1 is expressed in cardiac muscle and that it is upregulated during experimental heart failure (Adams et al. 2007; Mearini et al. 2010). Since there are so far no studies available about the effect of statins on mitochondrial function and the expression of atrogin-1 in cardiomyocytes, we decided to study the effect of simvastatin on these parameters on rodent cardiomyocytes in vitro and in mice in vivo. Furthermore, these studies allowed us to investigate possible links between mitochondrial damage and increased expression of atrogin-1.

Materials and methods

Chemicals

Simvastatin lactone (Sigma-Aldrich, St. Louis, MO, USA) was converted into the active acid following the protocol of Bogman et al. (2001). We prepared stock solutions of 10 and 100 mmol/L simvastatin in dimethylsulfoxide (DMSO) and stored them at −20 °C. All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), except where indicated.

Cell culture

H9c2 cardiomyocytes were provided by Dr Pfister (University Hospital Basel, Switzerland). They were grown in Dulbecco modified Eagle's medium (DMEM) containing 4.5 g/l glucose with Glutamax from Invitrogen (Basel, Switzerland) and supplemented with 10 % FBS, 5 mmol/L HEPES, 1 mmol/L sodium pyruvate and 500 μg/ml penicillin–streptomycin. Cells were kept at 37 °C in a humidified 5 % CO_2 cell culture incubator and were passaged using trypsin. The cell number was determined using a Neubauer hemacytometer and viability using the trypan blue exclusion method.

Myoblasts were seeded at 150,000 cells/well of a six-well plate (or equivalent) and grown in a humidified incubator with 5 % CO_2 at 37 °C for 2 days before drug treatment. We then treated them with simvastatin at 10 and 100 μmol/L. All incubations contained 0.1 % DMSO (including control incubations).

Animals

The experiments were performed in agreement with the Declaration of Helsinki and were approved by the cantonal veterinary authority (License 2659). Male C57BL/6 mice were purchased from Charles River Laboratories (Sutzfeld, Germany) and were adult age-matched (7 weeks old) housed in a standard facility with 12 h light–dark cycles and controlled temperature (21–22 °C). The mice were fed with a standard pellet chow and water ad libitum. After 7 days of acclimatization, the mice were divided into two groups: (1) eight mice (SMV group) treated with simvastatin by oral gavage dissolved in water at 5 mg/kg body weight/day for 21 days group and (2) eight mice (CTL group) treated with the same amount of water and served

as a control. Animals and food intakes were weighted every 2 days.

Sample collection

After the 21 days of treatment, the mice were anaesthetized with an intraperitoneal application of ketamine (100 mg/kg) and xylazine (10 mg/kg). The left ventricles were excised and conserved in ice-cold BIOPS buffer (10 mmol/L Ca-EGTA buffer, 0.1 µmol/L free calcium, 5.77 mmol/L ATP, 6.56 mmol/L $MgCl_2$, 20 mmol/L taurine, 15 mmol/L phosphocreatine, 0.5 mmol/L dithiothreitol and 50 mmol/L MES, pH 7.1) until analysis.

Heart samples were frozen in liquid nitrogen immediately after excision. Since the mice were anesthetized, tissues were obtained from living animals, and the time between sampling and freezing was only a few seconds. Samples were kept at −80 °C until analysis.

Cytotoxicity assay

In vitro cytotoxicity was assessed by the release of adenylate kinase (AK), which results from the loss of cell membrane integrity. AK was quantified using the ToxiLight assay kit supplied from Lonza (Basel, Switzerland). After incubation with simvastatin for 6 and 24 h, we used the protocol outlined in Mullen et al. (2010).

Intracellular ATP content

The cellular ATP content, a marker for metabolic cell activity and cell viability, was determined using a CellTiter Glo kit from Promega (Madison, USA) following the producer's instructions. In brief, 100-µL assay buffer was added to each 96-well containing 100-µL culture medium. After incubation in the dark for 30 min, luminescence was measured using a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland).

Mitochondrial membrane potential

To detect changes in mitochondrial membrane potential ($\Delta\psi_m$), we used tetramethylrhodamine ethyl ester (TMRE), which distributes across the inner mitochondrial membrane in accordance with the Nernst equation (Bursac et al. 1999). Cells were incubated with simvastatin for 24 h and then detached and incubated with 100 nmol/L TMRE for 30 min at 37 °C and 5 % CO_2. We used a Live/Dead® Near-IR Dead Cell stain kit from Invitrogen to exclude dead cells. Viable cells were analyzed with a DAKO Cyan cytometer. The mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was used as a control for depletion of the mitochondrial membrane potential.

Oxygen consumption

We measured intact cellular respiration with a Seahorse XF24 analyzer (Seahorse Biosciences, North Billerica, MA, USA). H9c2 myoblasts were seeded in Seahorse XF24-well cell culture microplates at 5,000 cells per well in 250-µL growth medium. We treated cells with simvastatin for 24 h and then replaced the medium with 750-µL medium containing Dulbecco modified Eagle's medium (DMEM) high-glucose (4.5 g/l) medium with Glutamax (Invitrogen, Basel, Switzerland) supplemented with 1 mmol/L sodium pyruvate, 25 mmol/L glucose and 15 mg phenol red. Cells were equilibrated to the medium for 45 min at 37 °C in a CO_2-free incubator and then transferred to the XF24 analyzer. We first measured the basal oxygen consumption rate (OCR). Then, we determined leak-state respiration, a measure for uncoupling of oxidative phosphorylation. For that, we added oligomycin (final concentration 2.5 µmol/L), an inhibitor of F_0F_1ATPase. An OCR higher than in control incubations is compatible with uncoupling by the toxicant investigated (Felser et al. 2013). This was followed by the addition of the chemical uncoupler FCCP (final concentration 1 µmol/L) to obtain maximum mitochondrial respiration. Finally, the complex I inhibitor rotenone (final concentration 1 µmol/L) was added for the determination of the residual respiration. Respiration rates were calculated as the mean of all determinations at a certain state (basal, leak and FCCP-stimulated respiration) after subtraction of residual respiration in the presence of rotenone.

Activity of enzyme complexes of the mitochondrial electron transport chain

Mitochondrial respiration was analyzed in permeabilized H9c2 myoblasts and in permeabilized myocardial fibers. Mitochondrial measurements were performed at 37 °C with an Oxygraph-2k system equipped with DatLab software (Oroboros Instruments, Austria). Multisubstrate and multiinhibitor protocols were used simulating the operation of the mitochondrial electron transport chain (ETC). H9c2 myoblasts were first permeabilized with digitonin (8.1 µg/million cells) before the activities of complexes I, II, III and IV were determined.

In a first incubation, we determined the activity of complex I and of complex III (see supplementary data for a more detailed description of the substrates and inhibitors used). First, we assessed the activity of complex I using glutamate and malate (final concentrations 10 and 5 mmol/L, respectively) as substrates in the presence of

ADP (final concentration 2.5 mmol/L). Then, we added oligomycin (final concentration 2.5 μmol/L) for the determination of the leak-state respiration. This was followed by the addition of FCCP (final concentration 1 μmol/L) to reach a full stimulation of the ETC and of cytochrome *c* (final concentration 10 μmol/L) to assess mitochondrial integrity. After that, we added rotenone (final concentration 1 μmol/L), an inhibitor of complex I. Now, we added duroquinol (0.5 mmol/L), an artificial substrate of complex III, and finally antimycin A (final concentration 2.5 μmol/L), an inhibitor of complex III.

In a second run, we assessed the activity of complex II and of complex IV (see supplementary data for a more detailed description of the substrates and inhibitors used). We first added succinate (final concentration 10 mmol/L), a substrate of complex II. Then, we added rotenone (final concentration 1 μmol/L) and ADP (final concentration 2.5 mmol/L). Then, we added oligomycin (final concentration 2.5 μmol/L) for the determination of the leak-state respiration. This was followed by the addition of FCCP (final concentration 1 μmol/L) to reach a full stimulation of the ETC and of cytochrome *c* (final concentration 10 μmol/L) to assess mitochondrial integrity. Then, we added antimycin A (final concentration 2.5 μmol/L) in order to block the electron transport between complex II and IV and finally *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) and ascorbate (final concentrations 0.5 and 2 mmol/L, respectively) as artificial substrates of complex IV. Respiration rates are expressed in picomoles O_2 per second per one million cells.

For the determination of the respiratory capacity of heart muscle fibers, the left ventricle of each heart was removed and transferred into 2 ml of ice-cold BIOPS buffer. After dissection, fiber bundles were permeabilized by gentle agitation for 30 min in ice-cold BIOPS solution supplemented with 50 μg/mL saponin. Permeabilized fibers were then washed in ice-cold BIOPS buffer for 10 min under shaking followed by washing two times in ice-cold mitochondrial respiration medium MiR05 (0.5 mmol/L EGTA, 1 g/l fatty acid free bovine serum albumin, 3 mmol/L $MgCl_2$, 20 mmol/L taurine, 10 mmol/L KH_2PO_4, 110 mmol/L sucrose, 60 mmol/L K-lactobionate and 20 mmol/L HEPES, pH 7.1). Prior to respirometry measurements, 2–3 mg of permeabilized muscle bundles were blotted, weighed and immediately used for respirometry measurements. In these preparations, activity of complex I, II and IV was analyzed (see supplementary data for a more detailed description of the substrates and inhibitors used). In order to determine the activity of complex I, we added glutamate and malate (final concentration 10 and 5 mmol/L, respectively) as substrates, followed by ADP (final concentration 2.5 mmol/L). Then, we added rotenone (final concentration 1 μmol/L) as an inhibitor of complex I. After that, we added succinate (final concentration 10 mmol/L) as a substrate of complex II, followed by antimycin A (final concentration 2.5 μmol/L) as an inhibitor of complex III. Finally, we added the artificial substrates ascorbate and TMPD (final concentration 2 and 0.5 mmol/L, respectively) for complex IV, which was followed by the addition of cytochrome *c* (final concentration 10 μmol/L) to assess mitochondrial integrity. Respiration rates are expressed in picomoles O_2 per second per gram wet weight.

Confocal microscopy

H9c2 cardiomyocytes were grown in Lab-Tek microscopy chambers (Nunc, NY, USA) and treated with simvastatin for 24 h. The cells were fixed with 4 % PFA, permeabilized with 0.2 % triton-X and then labeled with the appropriate antibody. We used DAPI to stain nuclei. We used a Zeiss LSM 710 confocal microscope to take the images, and Zen software to process them (Carl Zeiss, Switzerland).

Western blot analysis

After incubation with 0.1 % DMSO (control) or simvastatin (10 and 100 μmol/L) for 6 or 24 h, we lysed the H9c2 myoblasts with Phosphosafe extraction buffer (EMD Millipore, USA). We centrifuged the samples at 1,600*g* at 4 °C. Lysates were then resolved by electrophoresis on 4–12 % bis–tris polyacrylamide gels (Invitrogen, Carlsbad, USA) under reducing conditions and transferred to a polyvinylidendifluoride membrane (EMD Millipore, USA). We incubated the membranes with anti-phosphopeptide antibodies provided by Cell Signaling Technology (Danvers, USA). After washing, membranes were exposed to secondary antibodies (Santa Cruz Biotechnology, Dallas, USA).

Immunoblots were developed using enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK). Results obtained using phosphorylation-specific antibodies were corrected for total protein expression and are presented as the ratio to control incubations. Band intensities of the scanned images were quantified using the National Institutes of Health Image J program, version 1.41.

Annexin/PI staining

Apoptosis and necrosis were investigated using Annexin V and propidium iodide (PI) staining (Vybrant TM Apoptosis Assay Kit #2, from Invitrogen). After 24-h incubation with simvastatin, we stained detached cells and stained them with 5 μL Annexin V-AlexaFluor 488 and 1 μL propidium iodide (final concentration 1 μg/ml) in Annexin V binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$ in H_2O, pH 7.4). Cells were incubated for 15 min at room temperature and analyzed by flow cytometry using

a DAKO Cyan cytometer (Beckman Coulter, Marseille, France). Data were analyzed using FlowJo 9.3.2 software (Tree Star, Ashland, OR, USA).

Caspase 3/7 assay

Caspase 3/7 activity was determined using the luminescent Caspase-Glo 3/7 assay (Promega, Wallisellen, Switzerland). The assay was conducted according to the manufacturer's protocol.

Atrogin-1 mRNA expression

We treated H9c2 cardiomyocytes with simvastatin for 24 h. RNA was extracted and purified using the Qiagen RNeasy mini extraction kit, with a DNA digest step to ensure pure RNA.

Moreover, we extracted mRNA from the heart of C57BL/6 mice with Trizol reagent™ (Invitrogen, Basel, Switzerland) following the instructions of the producer. We synthesized cDNA using the Qiagen omniscript system and used 10 ng cDNA for quantitative RT-PCR. We used SYBR green with primers specific for atrogin-1 (forward primer: 5′-GAAGACCGCTACTGTGGAA-3′ and reverse primer: 5′-ATCAATCGCTTGCGGATCT-3′ for in H9c2 myoblasts and forward primer: 5′AGTGAGGACCGGCT ACTGTG3′; reverse primer: 5′GATCAAACGCTTGCG AATCT3′ for heart murine muscle fibers), Bcl-2 (forward primer: 5′GGTGGGCCCGGAACATCT3′ and reverse primer: 5′GGGCCCTACCGTCCTACTAAT3′) and Bax (forward primer: 5′-GGC TGG ACA CTG GAC TTC CT3′ and reverse primer 5′GGTGAGGACTCCAGCCACAA3′).

Relative quantities of specifically amplified cDNA were calculated with the comparative-threshold cycle method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acted as endogenous reference (forward primer: 5′-CATGGCCTTCCGTGTTCCTA-3′; reverse primer: 5′-CCTGCTTCACCACCTTCTTGA-3′), and no-template and no reverse transcription controls ensured nonspecific amplification could be excluded.

Statistical analysis

Statistical analysis was completed using the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA). All results are expressed as mean ± SD and evaluated with one-way ANOVA followed by the comparisons between incubations containing toxicants and the control group using Dunnett's posttest procedure. p values <0.05 (*) were considered significant.

Results

Simvastatin induces cytotoxicity in H9c2 cardiomyocytes

To firstly investigate whether simvastatin is directly toxic to cardiomyocytes, we performed AK release treating the H9c2 myoblasts for 6 and 24 h with 10 and 100 µmol/L simvastatin. We observed a significant AK release with 10 µmol/L simvastatin only after 24 h, whereas 100 µmol/L simvastatin was already toxic after 6 h (Fig. 1a). These results suggested that simvastatin could be associated with cardiac toxicity and led us to investigate in deep on the mechanisms of this toxicity using in vitro and in vivo models.

Simvastatin decreases the ATP content in H9c2 cardiomyocytes

Previous work showed an impairment in ATP after simvastatin treatment (Alfazari et al. 2013). To understand whether the key event of simvastatin-induced cardiotoxicity is an energetic impairment, we analyzed the cellular ATP content. The intracellular ATP content started to decrease already

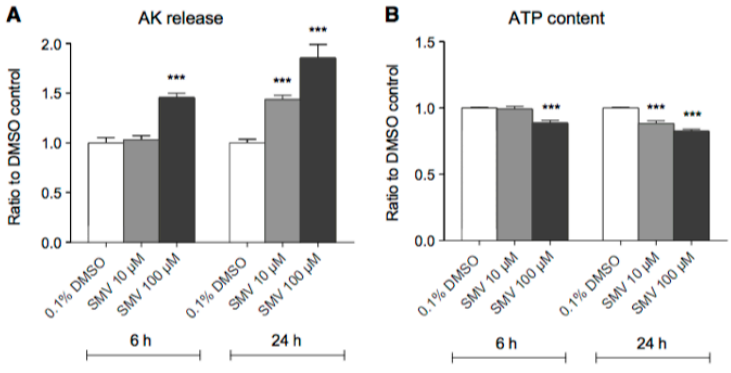

Fig. 1 Toxicity of simvastatin (SMV) on cardiomyocytes. H9c2 cardiomyocytes were incubated with DMSO, 10 or 100 µmol/L simvastatin for 6 and 24 h. We measured the release of adenylate kinase (AK) into the medium (**a**) and the cellular ATP content (**b**). DMSO-treated cells were used as a control. Results are expressed as ratios to the DMSO control. Each *bar* represents the mean of four independent experiments carried out in triplicate. ***$p < 0.001$ versus control

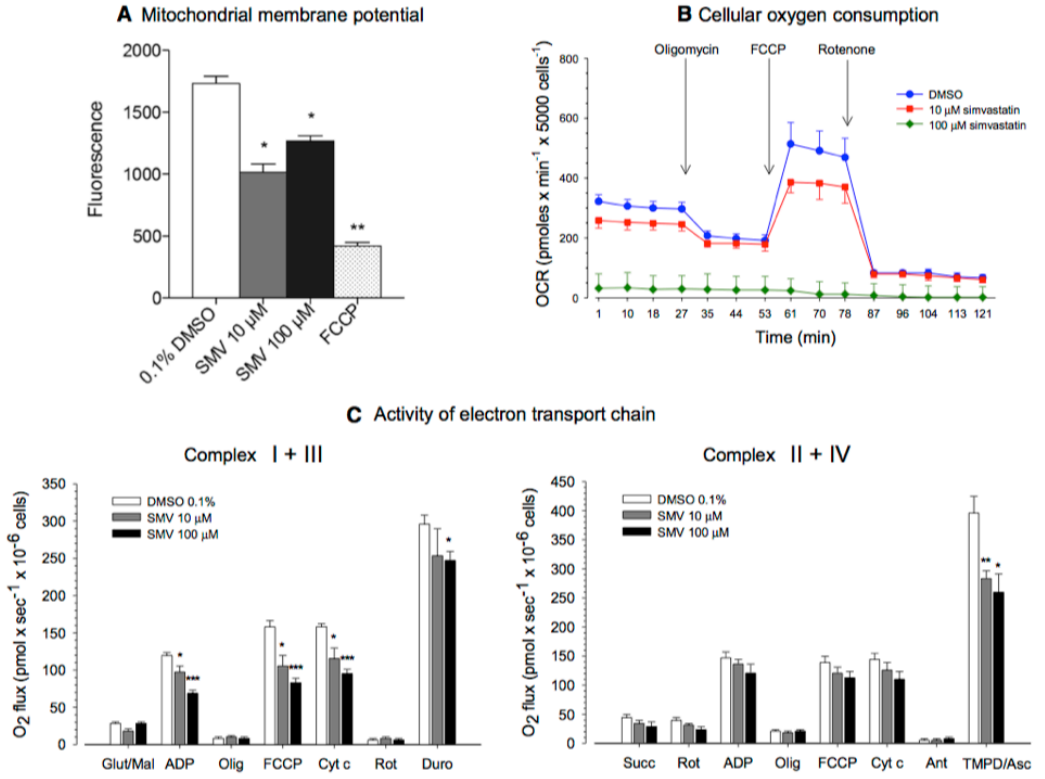

Fig. 2 Effect of simvastatin (SMV) on cellular oxygen consumption and mitochondrial function. **a** Membrane potential. Cells were incubated with DMSO, 10 or 100 µmol/L simvastatin for 24 h. TMRE was used to determine mitochondrial membrane potential, and near-IR dye from Invitrogen to exclude dead cells. The uncoupler FCCP was used as positive control. **b** Effect of simvastatin on cellular OCR. Cells were incubated with DMSO (*red lines*), 10 µM (*blue lines*) or 100 µM (*black lines*) simvastatin. OCRs are shown for 24-h incubation. 1 µM oligomycin, 15 µM FCCP and 1 µM rotenone (final concentrations) were added at the indicated points. **c** Effect of simvastatin on enzymes complexes of the ETC. The activity of complexes I to IV was determined as described in "Methods" section. Results are means of four independent experiments carried out in triplicate. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus control. *Glut* glutamate, *Mal* malate, *Olig* oligomycin, *FCCP* carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, *Cyt c* cytochrome *c*, *Rot* rotenone, *Duro* duroquinol, *Succ* succinate, *Ant* antimycin a, *TMPD* *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, *Asc* ascorbate (color figure online)

after 6 h for 100 µmol/L simvastatin, whereas 10 µmol/L simvastatin induced a decrease in ATP content only after 24 h (Fig. 1b). These results suggested that the energetic drop could be involved in the induction of cytotoxicity.

Simvastatin reduces mitochondrial membrane potential in H9c2 cardiomyocytes

It has been shown by our group and others that statins impair the mitochondrial function in other cell types (Bouitbir et al. 2012; Kaufmann et al. 2006; Sirvent et al. 2012). To investigate whether the reduction in the cellular ATP content is a consequence of mitochondrial impairment also in cardiomyocytes, we assayed for changes in the mitochondrial membrane potential ($\Delta\psi_m$) with the dye TMRE. A compromised mitochondrial $\Delta\psi_m$ is an indicator of impaired mitochondrial function (Felser et al. 2013). We pretreated the cells for 24 h with DMSO, 10 or 100 µmol/L simvastatin (Fig. 2a). H9c2 mitochondrial membrane potential was significantly reduced after treatment with both 10 and 100 µmol/L simvastatin. The positive control, FCCP, showed the expected marked reduction in mitochondrial membrane potential.

Simvastatin reduces oxygen consumption in intact H9c2 myocytes

To delve deeper into how simvastatin affects H9c2 mitochondria, we used a XF24 analyzer to determine changes

in OCR in simvastatin-treated intact H9c2 cardiomyocytes. Cellular consumption of oxygen mainly reflects mitochondrial metabolism (Felser et al. 2013). As shown in Fig. 2b, after 24-h treatment with 10 μmol/L simvastatin, baseline OCR values were significantly reduced (mean ± SD, $n = 4$; 179 ± 25 vs. 229 ± 21 pmol/min/5,000 cells in simvastatin-treated vs. control incubations, $p < 0.05$). After the addition of oligomycin, which blocks ATP production, the OCR was not different between simvastatin 10 μmol/L and control values (109 ± 18 vs. 122 ± 14 pmol/min/5,000 cells in simvastatin treated vs. control incubations), excluding uncoupling, suggesting that the baseline reduction is due impaired oxidative phosphorylation. After the addition of FCCP to determine maximum respiration, the inhibitory effect of simvastatin was confirmed (308 ± 46 vs. 414 ± 68 pmol/min/5,000 cells in simvastatin treated vs. control incubations, $p < 0.05$). Treatment with 100 μmol/L simvastatin resulted in a complete abolition of oxygen consumption, suggesting a complete inhibition of oxidative phosphorylation at this concentration.

Simvastatin impairs the function of the mitochondrial electron transport chain in vitro

In order to investigate on the decrease in oxygen consumption, we determined the function of the enzyme complexes of the ETC after treatment with vehicle (DMSO 0.1 %) or simvastatin (10 and 100 μmol/L) for 24 h with a high-resolution respirometry system (Oxygraph-2k). After exposure to simvastatin for 24 h, the respiratory capacities through complexes I and IV were significantly decreased for both concentrations in a dose-dependent way, whereas the function of complex III was significantly impaired only at 100 μmol/L simvastatin.

Moreover, the respiratory leak after the addition of oligomycin was not increased by the simvastatin-treated samples, indicating that simvastatin had no uncoupling effect in H9c2 myoblasts exposed for 24 h (Fig. 2c).

Simvastatin leads to AMPK activation, and subsequent FoxO3a nuclear translocation and upregulation of atrogin-1

The observed decrease in the cellular ATP content in H9c2 myoblasts exposed to simvastatin suggested that exposure to simvastatin could activate AMPK (5′ adenosine monophosphate-activated protein kinase; Towler and Hardie 2007a). AMPK is a metabolic master switch that regulates several metabolic processes including cellular uptake of glucose and metabolism of glucose and fatty acids (Mihaylova and Shaw 2011). Once activated, AMPK promotes ATP production by increasing catabolic activity while conserving ATP by switching off biosynthetic pathways (Hardie et al. 2012). As shown in Fig. 3a, both 10 and 100 μmol/L simvastatin remarkably triggered the activation of AMPK at both time points (6 and 24 h).

Activation of AMPK is associated with dephosphorylation of the nuclear transcription factor FoxO, which then can migrate into the nucleus (Chiacchiera and Simone 2010). As shown in Fig. 3b, we could observe a nuclear translocation of FoxO3a using confocal microscopy at 10 μmol/L. Once in the nucleus, FoxO3a acts as a transcription factor of different genes like, for instance atrogin-1, a key player in muscle protein degradation and therefore associated with muscle atrophy (Sandri et al. 2004). As shown in Fig. 3c, as expected, nuclear translocation of FoxO3a was associated with upregulation of atrogin-1 mRNA levels (Fig. 3d).

Simvastatin triggers apoptosis as the major form of cell death

We used Annexin V/PI staining to measure the type of cell death seen in H9c2 cardiomyocytes treated with simvastatin for 24 h. H9c2 cardiomyocytes treated with 10 μmol/L simvastatin showed a significant increase in early apoptotic cells when compared to control (6.3 vs. 3.0 %). This increase was concentration dependent; indeed, 100 μmol/L simvastatin showed a larger increase in early apoptotic cells (9.2 %; Fig. 4a). An increase in the late apoptotic fraction was only seen in cells treated with 100 μmol/L simvastatin (18.2 vs. 11.6 %; data not shown). In addition, we also analyzed the activation of caspase 3 by the caspase 3/7 glo kit and Western blots (Fig. 4c, d). In both assays, we could confirm an activation of caspase after simvastatin treatment. The stimulation was dependent on the concentration of simvastatin and on the incubation time.

Bax and Bcl-2 are two mitochondrial proteins involved in the regulation of apoptosis (Bagci et al. 2006), and an increased Bax/Bcl-2 ratio is associated with apoptosis (Perlman et al. 1999). Figure 4b shows a strong increase in the expression of Bax in cells incubated with simvastatin for 24 h and a reduced expression of Bcl-2 after exposure to simvastatin for 6 h and for 24 h. The Bax/Bcl-2 ratio was increased in a concentration- and time-dependent manner.

Simvastatin impairs mitochondrial functions in vivo

To confirm the role of simvastatin as a cardiac mitochondrial toxicant also in vivo, we treated mice orally with simvastatin 5 mg/kg body weight/day for 21 days. The heart weight was decreased by 5 % in simvastatin treated compared to control mice, but this decrease did not reach statistical significance (Fig. 5a). Similar to the in vitro findings, oxygen uptake by heart tissue was reduced in simvastatin-treated mice, indicating impaired mitochondrial function (Fig. 5b). Impaired oxygen uptake resulted from reduced

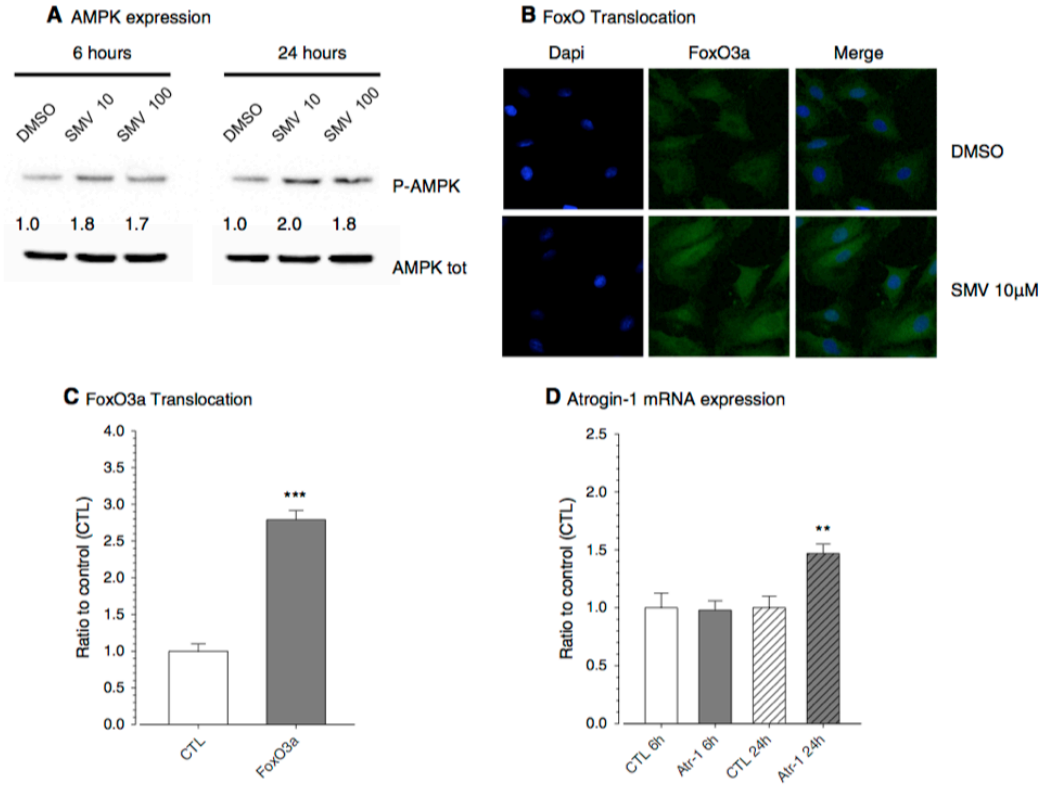

Fig. 3 Effect of simvastatin (SMV) on AMP-activated protein kinase (AMPK) activation, FoxO3a translocation and atrogin-1 expression in H9c2 cardiomyocytes. **a** Simvastatin induces AMPK phosphorylation in a concentration- and time-dependent fashion. **b** Treatment with simvastatin is associated with translocation of FoxO3a into the nucleus. **c** Numerical expression of the results (three independent experiments). **d** Atrogin-1 mRNA levels in H9c2 cardiomyocytes treated with 10 µM simvastatin for 6 or 24 h, relative to DMSO control (three independent experiments in triplicate). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control (CTL)

activities of complex I, II and IV of the ETC, agreeing well with the in vitro data. Identical to the in vitro results, there was an upregulation of atrogin-1 mRNA (Fig. 5c), and an increase in Bax mRNA as well as a decrease in Bcl-2 RNA, indicating increased apoptosis (Fig. 5d). These findings demonstrated that the results obtained in vitro could be reproduced in mice in vivo.

Discussion

This study presents several novel findings about the effects of the HMG-CoA reductase inhibitor simvastatin on cultured cardiomyocytes and the myocardium of mice. First, it demonstrates that exposure to simvastatin is cytotoxic for H9c2 cells in a dose- and time-dependent manner and that simvastatin impairs the function of enzyme complexes I and IV (and also of complex II after long-term exposure in vivo) of the ETC. Second, exposure of H9c2 cells to simvastatin is associated with the translocation of FoxO3a into the nucleus, which increases atrogin-1 mRNA expression. Third, treatment of mice with simvastatin at 5 mg/kg/day for 21 days inhibits the activity of the mitochondrial ETC and increases the expression of atrogin-1 mRNA in the heart.

In H9c2 cells, we started to observe cytotoxicity at a simvastatin concentration of 10 µmol/L after 24-h incubation. These data are in agreement with cytotoxicity experiments with cultured myotubes (Bouitbir et al. 2012; Kaufmann et al. 2006; Rabkin and Kong 2003). This simvastatin concentration is higher than the concentrations normally reached in vivo, which are below 5 µmol/L (Kwak

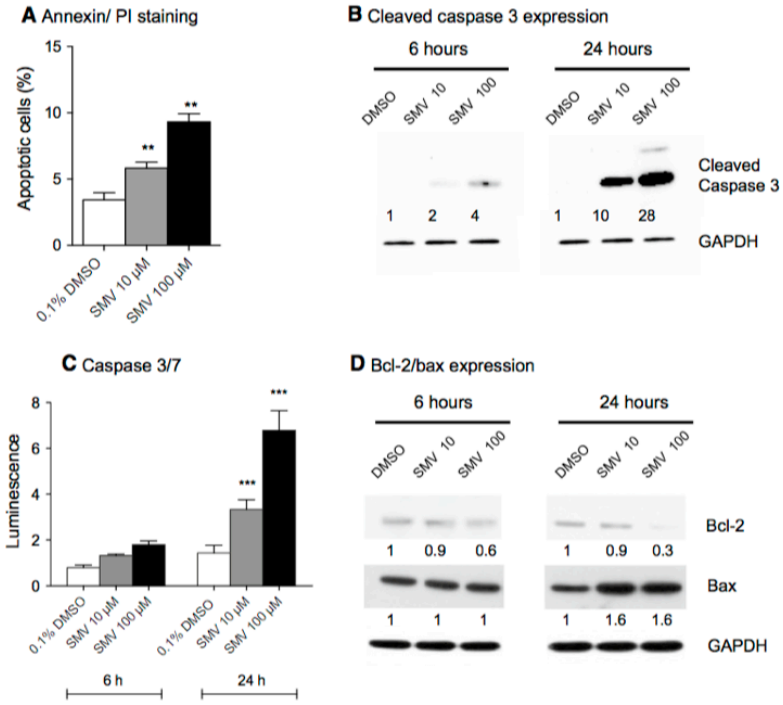

Fig. 4 Mechanisms of cell death after simvastatin (SMV) treatment in cardiomyocytes. **a** H9c2 myoblasts exposed for 24 h to DMSO, 10 or 100 µM simvastatin were labeled with Annexin V and PI and analyzed via flow cytometry. **b** Representative blot of cleaved caspase 3. **c** Caspase 3/7 activity after drug exposure for 6 and 24 h, expressed as ratio compared with DMSO control. **d** Representative blot of Bax and Bcl-2. Data in (**a**) and (**c**) represent the mean ± SEM of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control

et al. 2012). Nevertheless, the data are in agreement with the clinical findings in humans, showing that exposure is a strong risk factor for statin-associated myopathy (Link et al. 2008; Ratz Bravo et al. 2005; Roten et al. 2004). Based on the current and our previous data (Kaufmann et al. 2006), we assume that systemic concentrations in the low µmolar range are necessary for inducing myopathy in humans. Such concentrations can obviously be reached in patients with drug–drug interactions or impaired activity of OATP1B1.

As a vital organ rich in mitochondria and with a high oxygen need, the heart is susceptible for mitochondrial damage. In the presence of simvastatin, we initially observed a decrease in the electrical potential across the inner mitochondrial membrane ($\Delta\psi_m$), which represents an indicator of mitochondrial function (Felser et al. 2013). We believe that this change is not the result of cholesterol lowering by simvastatin, since the cholesterol precursor squalene does not reverse the effects of statins on cells (Thorpe et al. 2004), and the cellular cholesterol of cultured myotubes is maintained at cytotoxic simvastatin concentrations (Mullen et al. 2010). To confirm a toxic effect of simvastatin on mitochondria, we assayed for changes in oxygen consumption by H9c2 cells. Importantly, oxygen consumption by cardiomyocytes was reduced already after 6-h treatment with 10 µmol/L simvastatin, a time point when we did not observe cytotoxicity. This suggests that the changes in the mitochondrial function are a cause, rather than a consequence, of simvastatin-induced toxicity in H9c2 cells. This finding is new for cardiomyocytes and is in agreement with results reported from myotubes (Kaufmann et al. 2006; Liantonio et al. 2007).

In order to explore the effect of simvastatin on cardiac mitochondria in more detail, we measured the activity of the complexes of the ETC in permeabilized H9c2 cells. We could show that the exposure to simvastatin mainly inhibited the activity of the enzyme complexes I and IV of the ETC after 24 h of exposure. This is most probably a direct effect on the enzyme complexes and not an indirect effect due to a decrease in the mitochondrial ubiquinol pool.

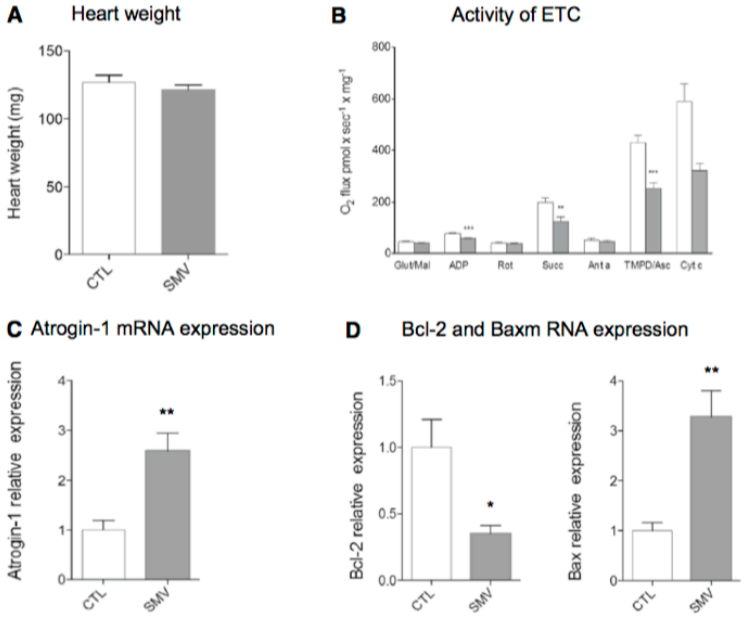

Fig. 5 Effect of simvastatin (SMV) on heart weight and cardiac atrogin-1 expression in C57BL/6 mice. Male C57BL/6 mice received water (CTL) or simvastatin (5 mg/kg/day) for 21 days. **a** Heart weight. Each data point represents the mean of eight hearts. **b** Activity of enzyme complexes of the ETC. **c** Atrogin-1 mRNA levels in seven simvastatin-treated mice relative to seven water control mice. **d** Bax/Bcl-2 mRNA levels in seven simvastatin-treated mice, relative to seven water control mice. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus control. *Glut* glutamate, *Mal* malate, *ADP* adenosine diphosphate, *Rot* rotenone, *Ant a* antimycin a, *TMPD* *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, *Asc* ascorbate, *Cyt c* cytochrome *c*

We have shown previously that exposure of myotubes to 10 μmol/L simvastatin for 24 h does not decrease the mitochondrial ubiquinone content (Mullen et al. 2010). More specific studies are needed to find out the precise mechanism how simvastatin impairs the function of the enzyme complexes of the respiratory chain.

Impairment of mitochondrial function can be associated with a decrease in the cellular ATP content, what we observed in cardiomyocytes exposed to 10 μmol/L simvastatin for 24 h (Fig. 1b). A drop in cellular ATP is associated with phosphorylation of AMPK, which activates and/or increases the expression of enzymes involved in catabolic processes for replenishing the cellular ATP stores (Mihaylova and Shaw 2011; Towler and Hardie 2007b). As explained in Fig. 6, activation of AMPK has several consequences. One of them is dephosphorylation of FoxO3, a nuclear transcription factor, which can travel into the nucleus and increase the expression of atrogin-1 (Sandri et al. 2004). As it has been shown previously for skeletal muscle (Cao et al. 2009; Hanai et al. 2007), we could demonstrate activation of AMPK followed by dephosphorylation and nuclear translocation of FoxO3 and finally increased expression of atrogin-1 also in cardiomyocytes.

In order to investigate whether these findings can be reproduced in vivo, we treated mice with simvastatin at 5 mg/kg/day for 21 days. This dose was calculated as described by Reagan-Shaw et al. (2008) and corresponds to approximately 0.4 mg/kg/day in humans, which is a normally used dose. Importantly, we could demonstrate mitochondrial dysfunction and increased atrogin-1 mRNA expression also in the myocardium of mice treated with simvastatin. Interestingly, the heart weight decreased numerically (by 5 %) in the simvastatin-treated mice, suggesting that the toxic effect of simvastatin on the heart could be associated with cardiac atrophy. On the one hand, atrophy-inducing effects of simvastatin could account for some of its cardiovascular benefits by counterbalancing cardiac hypertrophy and/or cardiac remodeling (Kang et al. 2009; Singh and Krishan 2010). On the other hand, statin-associated cardiac atrophy could have a negative effect on cardiac contractility, particularly in patients with heart failure.

Increased expression of atrogin-1 is associated with accelerated proteolysis and is therefore considered to be a major contributing factor to statin-induced myopathy (Hanai et al. 2007). Our previous (Kaufmann et al. 2006) and current data suggest that also increased apoptosis may play a role in muscle and/or cardiac toxicity associated with exposure to high systemic statin concentrations. Since in the current studies, mitochondrial damage was observed already after 6-h exposure to 10 μmol/L simvastatin, a time point when apoptosis was absent, apoptosis may have been triggered by a mitochondrial mechanism (Kaufmann et al. 2006). Importantly, the

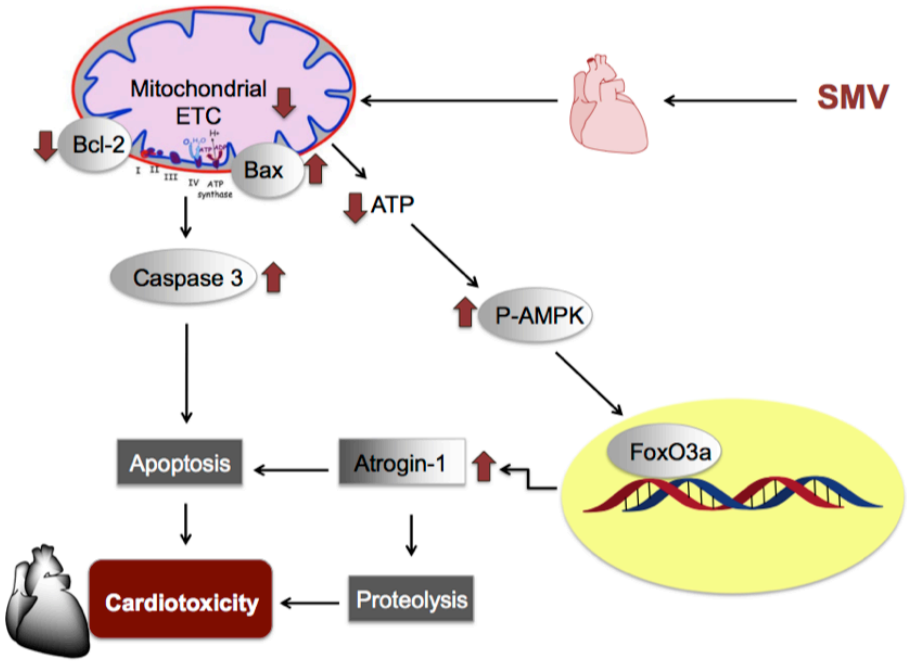

Fig. 6 Effect simvastatin on cardiomyocytes. Simvastatin (SMV) impairs the function of several enzyme complexes in mitochondria of cardiomyocytes. One consequence is a drop in cellular ATP, which is associated with activation of AMPK, nuclear translocation of FoxO3a and increased expression of atrogin-1. Atrogin-1 stimulates protein degradation. Another consequence is increased expression of Bax and decreased expression of Bcl-2, favoring apoptosis as shown by activation of caspases. Both apoptosis and increased protein degradation can be associated with cardiac atrophy

observed increase in the Bax/Bcl-2 ratio in the myocardium of mice treated with simvastatin suggests that statin-associated apoptosis also takes place in vivo. An increase in the Bax/Bcl-2 ratio indicates a high susceptibility to apoptotic stimuli (Vander Heiden and Thompson 1999).

Our study was not designed to answer the question why the heart is usually not affected in patients with rhabdomyolysis. Assuming that exposure is most probably not different between skeletal muscle and the myocardium, the most likely reason is a different susceptibility of myocytes and cardiomyocytes. In their study, Bouitbir et al. (2012) showed that ROS accumulation, which is associated with damage to the ETC (Felser et al. 2013), is much less in cardiomyocytes than in myocytes. This may be due to better defense mechanisms in cardiomyocytes such as, for instance, upregulation of SOD2, which metabolizes $O_2^{\cdot-}$.

In conclusion, our data show that cardiomyocytes exposed to simvastatin in vitro or in vivo sustain mitochondrial damage, causing AMPK activation, dephosphorylation and nuclear transformation of FoxO3 as well as increased expression of atrogin-1. Mitochondrial damage is associated with increased apoptosis. Increased protein degradation associated with atrogin-1 and increased apoptosis can lead to myocardial atrophy. Since AMPK is an important master switch for energy metabolism, further studies are necessary to study the effect of statins on signal transduction associated with metabolic processes.

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

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Conclusions and future perspectives

Since their introduction in the 1980s, statins have emerged as first-choice drugs for lowering serum cholesterol level [36]. Because of their pharmacological activity, they are the best selling prescription drugs in the Western Countries [1]. Although statins have a favorable safety profile, adverse drug reactions can occur [1]. The most relevant and well-documented side effect is myotoxicity, which ranges from mild myopathy to a potentially fatal rhabdomyolysis [37, 89]. Muscle toxicity associated with statins is dose-dependent, and the risk is amplified by drug interactions that increase statin's plasma levels and bioavailability [90, 91] [92].

Adverse events can occur with the use of any statin but the degree of risk within the therapeutic dose range varies among statins [2]. Despite the fact that statins are proven to be well-tolerated medications, currently there are millions of patients worldwide taking statins everyday thus reaching a significant number of people who present side effects [4]. Moreover they are chronic, generally life-long, lipid-lowering therapy meaning side effects, even though rare, adversely impact on the patient's quality of life and lead to noncompliance [11]. Due to all these reasons, there is an urgent need to discover precise mechanisms of statin-associated myotoxicity.

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

The primary objective of this study was to investigate on the molecular mechanisms of statin-induce toxicity in skeletal muscle. For this purpose, we used the well- characterized murine skeletal muscle model, the cell line C2C12, optimized for myotube formation. Previous study from our group showed that AKT is negatively regulated by simvastatin treatment in C2C12 myotubes [56]. Taking into consideration this previous finding, we examined the ability of three

structurally distinct statins to affect the AKT/mTOR signaling pathway with the aim of uncovering differences between them that might point out to their mechanism of toxicity in skeletal muscle. We chose three statins with different pharmacological and toxicological profiles: simvastatin, rosuvastatin and atorvastatin. Simvastatin and atorvastatin are both lipophilic drugs with a higher incidence for myopathies compared to maximum approved doses of other statins available on the market [22, 93]. Whereas, rosuvastatin is a relatively hydrophilic drug with a safer pharmacological profile than other statins [14, 94].
The comparison of the three statins revealed that myotubes were significantly more susceptible to simvastatin and atorvastatin than to rosuvastatin.
We supposed that lipophilic statins, such as simvastatin and atorvastatin, induce higher cytotoxic effects in skeletal muscle cells because of their physical-chemical properties [95]. Their lipophilic structure allows them to easily penetrate cell lipid bilayer membrane barriers [24] while hydrophilic statins, such as rosuvastatin, are membrane-impermeable and, therefore, they rely on an active transport process to enter cells and exert their effects [6].
When comparing the effect of these three different statins on AKT signaling pathway, we observed an inhibition of AKT and its downstream effectors in both simvastatin- and atorvastatin-treated C2C12 myotubes. In contrast, rosuvastatin exhibited a much weaker inhibition of AKT signaling cascade. These data are completely in line with the cytotoxicity data of these statins, and highlight the key role of AKT signaling pathway in cell survival [96] [97] [98].
Because of the importance of AKT in regulating several processes such as apoptosis, protein synthesis, protein breakdown, we investigated on the effect of the suppression of AKT pathway. We could confirm that the cytotoxicity is due to AKT inhibition that in turn, led to induction of atrophy and apoptosis. Indeed, simvastatin- and atorvastatin-treated C2C12 myotubes showed a reduction in whole protein content, a perturbation of myotubes morphology and induction of apopoptosis, whereas rosuvastatin-treated myotubes were affected only at high concentration and to a lesser extent than the other two statins.
In conclusion, this part of the thesis fully characterized the distinct responses to lipophilic and hydrophilic statins in C2C12 myotubes, identifing a key role of AKT/mTOR signaling pathway in statin-associated muscle toxicity.

Lipophilic statins cross the cell membrane of C2C12 myotubes by passive diffusion. Once inside the cells, they inhibit AKT/mTOR signaling pathway leading to activation of the atrophy pathways and promotion of the apoptotic cascade. All these effects stimulate muscle wasting. By contrary, hydrophilic statins have more difficulty to permeate inside muscle cells, and therefore to exert the inhibition of AKT/mTOR pathway associated with the consequent myotoxic effects.

Recently discovered pleiotropic effects [38] [12] and uses [99] [13] of statins attracted increasing interest in the scientific community. However, an effective treatment that counteracts muscle damage linked to statin use is not yet clinically available [3, 8]. Although further works are needed to understand how lipophilic statins efficiently block AKT/mTOR signaling pathway, this new understanding suggests several potentially therapeutic targets, such as inhibitors of FoxOs or activators of S6K, to prevent statin-associated myotoxicity.

Even though our findings provide critical insight into the molecular mechanisms of statin-induced myotoxicity, our *in vitro* data should be corroborated by *in vivo* studies in transgenic and knockout animal models involving components of the AKT/mTOR pathway to confirm our hypothesis and to identify a key factor for preventing statin-induced myotoxicity. Nevertheless, it is noteworthy that differences between *in vitro* and *in vivo* models and, clinical protocols might lead to different conclusions. For instance proteasome inhibitors, which have been successfully used to antagonize atrophy in different animal models [100] [101] in patients displayed cardiac complications [102].

Moreover, since AKT is at the intersection of different pathways, it plays major roles in several biological processes [78, 103] and, it has been also implicated in the molecular pathogenesis of several tumors [104] [105] [106]. Due to these reasons, it might be arduous to overcome these malign effects with pharmacologic manipulation.

Nonetheless, this study reveales a central role of AKT signaling cascade in muscle damage associated with statins and provides promising targets for developing novel therapeutic approaches.

IGF-1 prevents simvastatin-induced myotoxicity

In our previous work, we showed that AKT/mTOR signaling pathway has a crucial role in regulating muscle atrophy associated with statins. Moreover, simvastatin showed pronounced toxic effects on C2C12 myotubes. Therefore, in our second study, we investigated whether an AKT inducer could reduce myotoxicity associated with simvastatin.

IGF-1 is well known for its hypertrophic and anti-apoptotic effect [9] exerted by activating IGF-1/AKT pathway [10]. Moreover, recent studies showed that the IGF-1/AKT pathway could dominantly antagonize the atrophy induced by dexamethasone [9] [80]; whereas no data exist concerning the direct impact of IGF-1 on muscle atrophy associated with statins. Combining these previous findings, we assumed that IGF-1 might antagonize the myotoxicity induced by statins. This idea was further confirmed by our results showing that the simvastatin-induced cytotoxicity was significantly decreased by the addition of IGF-1. The prevention of cytotoxicity was strictly correlated by the activation of IGF-1R and its downstream signalling pathway. We demonstrated that activation of IGF-1 receptor by IGF-1 results in the reactivation of AKT signaling pathway inhibited by simvastatin. This reactivation impairs statin-induced muscle atrophy by stimulating both activation of protein synthesis and impairment of protein degradation and apoptosis.

Our data are in agreement with previous findings, showing that IGF-1/AKT pathway is essential for cell health, and decrease in AKT phosphorylation is correlated with increased muscular atrophic signals and displayed an increase in apoptosis [107, 108].

Moreover, it is noteworthy to mention that our data may partially explain reported risk of statins in inducing type 2 diabetes [111] [112]. IGF-1 and insulin share downstream signalling components, such as AKT [109]. Therefore, statin treatment may result in a truncated insulin response caused by the impairment of AKT phosphorylation [110].

In conclusion, IGF-1 reduces statin-induced cytotoxicity in a dose-dependent manner via reactivating IGF-1/ AKT signaling pathway and thus, exerting strong

anti-apoptotic, anti- atrophy and hypertrophic effects.

The data presented herein highlights that IGF-1 has a strong potential for the prevention or treatment of muscle atrophy associated with statins. Moreover, the IGF-1/AKT signaling cascade is unique since it controls both protein synthesis and protein degradation. Therefore, IGF-1 mimetics might be extremely useful for counteracting muscle atrophy and weakness.

Nevertheless, IGF-1 regulates many biological processes and its prolonged activation might be detrimental for muscle cells. Moreover, the diabetogenic effects [113] and the cancer risk associated with IGF-1 treatment [114] [115] might be difficult to overcome with pharmacological/ chemical manipulations. The development of a new generation of IGF-1 analogs that specifically target part of the AKT pathway in skeletal muscle is a goal for the field. For instance, ubiquitin ligases involved in degradation of sarcomeric proteins should be pursued. However, it should be considered that prolonged inhibition of protein degradation can have deterious conseguences on protein quality control and events such as accumulation of misfolded or aggregate-prone proteins could occur [116] [117].

In addition, also this study is mostly limited by *in vitro* analysis. Whether these results can be applied in human settings still needs further clarification. To confirm our hypothesis, our *in vitro* data should first be corroborated by *in vivo* studies.

Nonetheless, this study greatly enhances our knowledge on the protective effect of IGF-1 in statin-induced toxicity, and increases hope to develop efficient therapeutic approaches for counteracting muscle toxicity associated with statin use.

Simvastatin induces mitochondrial dysfunction and increases atrogin-1 expression in H9c2 cardiomyocytes and mice in vivo

While side-effects of statins in skeletal muscle are well-reported [2] [19] [3], very little is known of their effect on cardiac muscle. Therefore, the last aim of

this thesis was to investigate on the effects of simvastatin treatment in cardiomyocytes.

This work is particularly important as statins are first-line treatment of choice to prevent and cure cardiovascular disease. Thus, cardiac side-effects may be masked by falsely attributing them to the underlying disease.

Previous works showed that lovastatin reduces survival of cardiomyopathic hamsters treated with lovastatin [118] and cardiomyocyte viability [119].

In the present study, we showed that simvastatin elicited a concentration- and time-dependent decrease in cell viability in H9c2 rat cardiomyoblasts.

Accordingly, simvastatin exerted a reduction in ATP content linked to mitochondrial impairment. Consequently, the depression of the intracellular ATP levels activated AMPK, which in turn led to a nuclear translocation of Foxo3a , a subsequent up-regulation of atrogin-1 mRNA levels and induction of atrophy and apoptosis. Our *in vitro* observations were confirmed in C57BL/6 mice treated with 5 mg/kg body weight/day simvastatin for 21 days. These findings are in accordance with previous work that reported that, *in vitro,* lipophilic statins induce cytotoxicity of cardiac myocytes triggering apoptosis [120-123].

In conclusion, we showed that statin-induced cardiotoxicity is triggered by an mitochondrial impairment that lead to atrophy and apoptosis via AMPK/FoxO/Atrogin-1 signalling pathway. Moreover, apoptosis was also triggered by a separated mitochondrial signalling. Indeed, the ratio Bcl-2/Bax in simvastatin-treated mice was low.

Although these observations cannot yet be extrapolated in humans, our data show for the first time that simvastatin induces signs of atrophy in cardiomyocytes. It remains to be determined whether chronic therapy with statin in human could, in some circumstances, induce cardiac atrophy with consequent complications, analogous to the reduced survival observed in cardiomyopathic hamsters treated with lovastatin [118]. Neverthelss, in another point of view, if our findings would be confirmed in a clinical setting, statins could be used for the treatment of cardiomyocyte hypertrophy, which is strongly associated with obesity [124, 125]. It is worth to notice that the cardiotoxic effect could be just a consequence of high dose of simvastatin. The concentration we used *in vitro* exceeds the peak reached in the plasma of

patients which is about 1 μM [61]. However, localized concentration in a single organ could be higher, especially if statins are combined with cytochrome P450 inhibitors or with impaired activity of OATP1B1 [57] [29].

With the rising incidence and prevalence of cardiovascular diseases, the consequent increasing use of statins and, the inability of cardiomyocytes to regenerate, there is an urgent need to evaluate the statin safety in heart muscle. Therefore, further works are required to expand on this topic and fully determine any harmful effects of statins on heart in human.

Important points

In this study, we showed that C2C12 myotubes are more susceptible to lipophilic statins than to hydrophilic statins. The different susceptibility is correlated with the ability to inhibit AKT/mTOR signaling pathway. In addition, we showed the preventive effects of IGF-1 on statin-associated toxicity in skeletal muscle.

Moreover, we uncovered the toxicity of simvastatin on cardiac muscle. This toxicity is associated with the mitochondrial impairement, induction of atrophy and apoptosis.

Overall, this thesis unravels the causative mechanisms of statin-induced toxicity and has significant implications for development of new therapies to directly prevent statin-induced muscle damage.

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