Fluvastatin and atorvastatin affect calcium homeostasis of rat skeletal muscle fibers in vivo and in vitro by impairing the sarcoplasmic reticulum/mitochondria Ca\textsuperscript{2+}-release system

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Abbreviations: HMGCoA reductase, 3-hydroxy-methyl-glutaryl Coenzyme A reductase; EDL, extensor digitorum longus; [Ca$^{2+}$]$_{i}$, intracellular calcium concentration; MT, mechanical threshold; PKC, Ca-dependent protein kinase C; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; PTP, permeability transition pore; RR, ruthenium red; CsA, cyclosporine A; SOCE, store-operated calcium entry

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Abstract
The mechanism by which the 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) induce skeletal muscle injury is still under debate. By using fura-2 cytofluorimetry on intact extensor digitorum longus (EDL) muscle fibers, here we provided the first evidence that two months in vivo chronic treatment of rats with fluvastatin (5 mg kg⁻¹ and 20 mg kg⁻¹) and atorvastatin (5 mg kg⁻¹ and 10 mg kg⁻¹) caused an alteration of calcium homeostasis. All treated animals showed a significant increase of resting cytosolic calcium [Ca²⁺]ᵢ, up to 60% with the higher fluvastatin dose and up to 20% with the other treatments. The [Ca²⁺]ᵢ rise induced by statins administration was not due to an increase of sarcolemmal permeability to calcium. Additionally, the treatments reduced caffeine responsiveness. In vitro application of fluvastatin caused changes of [Ca²⁺]ᵢ resembling the effect obtained after the in vivo administration. Indeed fluvastatin produced a shift of mechanical threshold for contraction toward negative potentials and an increase of resting [Ca²⁺]ᵢ. By using ruthenium red and cyclosporine A, we determined the sequence of the statins-induced Ca²⁺ release mechanism. Mitochondria appeared as the cellular structure responsible for the earlier event leading to a subsequent large sarcoplasmic reticulum Ca²⁺ release. In conclusion we suggest that calcium homeostasis alteration may be a crucial event for myotoxicity induced by this widely used class of hypolipidemic drugs.
Introduction

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are the most useful agents for treatment of hypercholesterolemia. By blocking the rate-limiting step catalyzed by this enzyme in the mevalonate pathway, de novo synthesis of cholesterol is prevented and low-density-lipoprotein cholesterol uptake into cells is promoted (Goldstein and Brown, 1990). The different statins currently available (simvastatin, atorvastatin, lovastatin, pravastatin, fluvastatin, rosuvastatin) are generally well tolerated in patients. However, side effects may arise in skeletal muscle, ranging from transient increase in creatine kinase (CK), muscle pain and cramps to myositis and potentially life-threatening rhabdomyolysis (Bellosta et al., 2004; Rosenson, 2004). Despite the numerous studies describing myopathy in animals and humans, the molecular mechanism of statin-induced myotoxicity has not been completely elucidated. A variety of hypotheses have been formulated to explain such toxicity, including impairment of glycoprotein synthesis in the muscle membrane as well as reduction of ubiquinone concentration in mitochondria causing severe deficits in energy metabolism (Evans and Rees, 2002).

Interestingly, it was also proposed that an alteration of structures involved in Ca\(^{2+}\) homeostasis could play a pivotal role in producing myocyte injury. After 2-3 months chronic treatment of rats with simvastatin, the voltage threshold for contraction (mechanical threshold, MT), a calcium-sensitive index of excitation-contraction coupling, was shifted toward more negative potentials in fast-twitch muscle fibers (Pierno et al., 1999), an effect which is compatible with an increase of resting cytosolic calcium concentration ([Ca\(^{2+}\)]). Moreover, \textit{in vitro} studies showed a statin-induced elevation of [Ca\(^{2+}\)], and suggested a possible interference of the drug with intracellular Ca\(^{2+}\) stores (Nakahara et al., 1994; Inoue et al., 2003; Sirvent et al., 2005) which in turn might be responsible of cell damages via activation of Ca\(^{2+}\)-dependent proteolitic enzymes (Sacher et al., 2005). Importantly, all statins-induced effects on skeletal muscle are strictly dependent on their lipophilicity, showing highly hydrophilic pravastatin no muscle toxicity even at high doses (Nakahara et al., 1994; Pierno et al., 1995; Pierno et al., 1999). Recently, a multidisciplinary study
aimed to the identification of the cellular and tissue targets of a chronic treatment with newer powerful and lipophilic statins, such as fluvastatin and atorvastatin, on rat skeletal muscle was conducted (Pierno et al., 2006). Both statins resulted more potent than simvastatin in producing skeletal muscle injury. Indeed, although no histological changes were observed on muscle fibers, both statin treatments produced a significant negative shift of the potentials of MT at which fibers contract. In addition, statins altered muscle excitability by reducing resting chloride conductance (gCl), the electrical parameter sustained by the voltage-gated chloride channel CLC-1 critical for the maintenance of membrane stability. Since CLC-1 is negatively regulated by the Ca^{2+}-dependent protein kinase C (PKC) (De Luca et al., 1998; Rosenbohm et al., 1999), the observed reduction of gCl supports the hypothesis that statins administration could effectively interfere with calcium handling mechanism.

On the basis of the above findings, we performed a two months in vivo administration with fluvastatin and atorvastatin to rats and evaluated ex vivo the effect of drug treatment on resting [Ca^{2+}], by fura-2 cytofluorimetry on tendon to tendon intact fibers of extensor digitorum longus (EDL) muscle. In parallel, to further elucidate the statin-induced Ca^{2+} release mechanism, we characterized the statin cellular effects after acute in vitro application.

Our data showed that both statins caused a sustained increase in cytosolic Ca^{2+} levels by interfering with intracellular stores such as mitochondria and sarcoplasmic reticulum. Considering the crucial role of resting calcium ions in skeletal muscle function and maintenance, our findings contribute to a better understanding of the mechanism responsible for cramps, myalgia and other muscular side effects induced by this widely used class of hypolipidemic drugs.
Methods

Animals care and in vivo drug treatment

Animal care and in vivo drug treatment was approved by the Italian Health Department and the Institutional Animal Care and Use Committee according to Italian law DL 116/92 and the European Community Directive (86/609/EEC). Male Wistar rats (Charles River Laboratories, Italy), initially weighing 300-350 g were used. The animals were housed individually in rat appropriate cages and fed with approximately 30 g day$^{-1}$ of a commercial rodent chow (Charles River, 4RF21) and tap water ad libitum. Rooms were maintained at constant temperature (22-24°C) and exposed to a light cycle of 12 h day$^{-1}$ (8.00 a.m – 8.00 p.m.). The animals were subdivided in six experimental groups as follows: the first group (9 rats) was chronically administered with 5 mg kg$^{-1}$ day$^{-1}$ of fluvastatin, the second (6 rats) with 20 mg kg$^{-1}$ day$^{-1}$ of fluvastatin, the third (6 rats) with 5 mg kg$^{-1}$ day$^{-1}$ of atorvastatin, the fourth (10 rats) with 10 mg kg$^{-1}$ day$^{-1}$ of atorvastatin, the fifth (6 rats) only with the vehicle (aqueous methylcellulose, CMC) used to dissolve the drugs and the sixth (7 rats) was an untreated control group. During the treatment period all animals showed normal body weight gain and appeared in good health, with the exception of rats chronically treated with high doses of fluvastatin (20 mg kg$^{-1}$) (for details see Pierno et al., 2006). The doses of drugs were chosen on the basis of data present in the literature regarding human and rodents, as previously reported (Pierno et al., 2006). Fluvastatin and atorvastatin dissolved in CMC (0.5%) suspension were administered orally via an esophageal cannula, once a day, for 2 months. For each rat, the weight related dose was formulated so that the maximal volume of drug-containing suspension was 1 ml. Since the results obtained from the two control groups (untreated and CMC treated rats) were similar, we have combined and showed data as unique control.

In vivo study: determination of the fore-limb muscle strength.
The forelimb muscle force of control and treated rats was evaluated before and every week until the end of the treatment by means of a grip strength meter (Columbus Instruments, USA). Five rats per group (5 rats treated with fluvastatin 5 mg/kg, 5 rats treated with fluvastatin 20 mg/kg, 5 rats treated with atorvastatin 5 mg/kg, 5 rats treated with atorvastatin 10 mg/kg and 5 control rats) were analysed. For this measure, the rats were allowed to grasp a triangular ring connected to a force transducer and then gently pulled away until the grip was broken. The transducer saved the force value at this point, as a measure of the maximal resistance the animal can develop with its forelimbs (De Luca et al., 2003). Five measures (1-5) were taken from each animal within 3 min and each value was normalized with respect to the weight of the animal. We calculated the difference between the 5th measure and the 1st measure in each animal as a muscle fatigability index. The mean values (± S.E.M.) of this difference of control and of treated rats were then analysed for significance (by ANOVA test).

**Dissection of native muscle fibres**

For *ex vivo* and *in vitro* experiments, extensor digitorum longus (EDL) muscle were removed from the animal under deep urethane anaesthesia (1.2 g/Kg body weight). Soon after the surgery, the rats still anaesthetized, were euthanized by anaesthetic overdose. EDL muscle of the different groups of treated rats as well as of control rats were pinned in a dissecting dish containing 95% O2/ 5% CO2-gassed normal physiological solution at room temperature (22 °C) for further dissection. Small bundles of 10-15 fibers arranged in a single layer were dissected lengthwise, tendon to tendon, with the use of microscissors, as described elsewhere (Fraysse et al., 2003).

**Fura-2 fluorescence measurements in intact muscle fibres**

Calcium measurements were performed using the membrane-permeant Ca\(^{2+}\) indicator fura-2 acetoxymethyl ester (fura-2 AM, Molecular Probes-Invitrogen, Italy). Loading of muscle fibers was performed for 2 hours at 25°C in normal physiological solution containing 5 µM fura2-AM mixed
to 0.05 % (v/v) Pluronic F-127 (Molecular Probes). After loading, muscle fibers were washed with normal physiological solution and mounted in a modified RC-27NE experimental chamber (Warner Instrument Inc., Hamden, USA) on the stage of an inverted Eclipse TE300 microscope (Nikon, Japan) with a x40 Plan-Fluor objective (Nikon, Japan). The mean sarcomere length was set to 2.5-2.7 µm. Fluorescence measurements were made using a QuantiCell 900 integrated imaging system (Visitech International Ltd, Sunderland, UK) as previously described (Fraysse et al., 2003; Fraysse et al., 2004).

During experiments, pairs of background subtracted images of fura-2 fluorescence (510 nm) after excitation at 340 nm and 380 nm were acquired and ratiometric images (340/380 nm) were calculated for each muscle fiber of the preparation using QC2000 software. Subsequently fluorescence ratio values were converted to the resting cytosolic calcium [Ca$^{2+}$] (nM), after a calibration procedure, using the equation: [Ca$^{2+}$]$_i$ = (R-R$_{\text{min}}$)/(R$_{\text{max}}$-R)*K_D*$\beta$ where R is the ratio of fluorescence excited at 340 nm to that excited at 380 nm; K_D is affinity constant of fura-2 for calcium which was taken as 145 nM (Molecular Probes); $\beta$ is a parameter according to Grynkiewicz et al. (1985) and was determined experimentally in situ in ionomycin-permeabilized muscle fibers as previously described (Fraysse et al., 2003). R$_{\text{min}}$ and R$_{\text{max}}$ were determined in muscle fibers incubated in Ca$^{2+}$-free normal physiological solution containing 10 mM EGTA and in normal physiological solution, respectively.

**Determination of sarcolemmal permeability to divalent cations**

The manganese quench technique was used to estimate the sarcolemmal permeability to divalent cations. Mn$^{2+}$ enters via the same routes as Ca$^{2+}$ but accumulates inside the cell. As Mn$^{2+}$ quenches the fluorescence of fura-2, the reduction of the intensity of fura-2 fluorescence can be used as an indicator of the time integral of Mn$^{2+}$ influx (Parekh and Penner, 1997). Muscle preparations were perfused for 2 min with normal physiological solution containing 0.5 mM Mn$^{2+}$ as a surrogate of CaCl$_2$ (quenching solution). To measure the Mn$^{2+}$ influx through the store-operated Ca$^{2+}$ channels
(SOC) a proper protocol for SOCE activation was used (Kurebayashi and Ogawa, 2001; Zhao et al., 2005; Zhao et al., 2006). Particularly, the fibers were perfused with 10 μM thapsigargin and 40 mM caffeine for 15 min in zero Ca²⁺ extracellular solution to induce SR Ca²⁺ depletion; successively 0.5 mM Mn²⁺ was added to extracellular solution. During the quenching protocol the fluorescence of fura-2 excited at 360 nm was acquired at 1 Hz.

### Mechanical threshold for contraction (MT)

Soon after the removal from the rat, EDL muscles, tied at the end of each tendon, were place on a glass rod located in a 25 ml muscle bath chamber maintained at 30°C and continuously perfused with 95% O₂/5%CO₂-gassed normal physiological solution. The mechanical threshold (MT) for contraction was determined using a two-microelectrode point voltage clamp method in the presence of 3 μM tetrodotoxin, as described previously (Pierno et al., 2006). Depolarizing current pulses of increasing duration (5-500 ms) were given repetitively at a rate of 0.3 Hz, while the impaled fibers were viewed continuously with a stereomicroscope (100x magnification). The command voltage was increased until contraction was just visible and the threshold membrane potential at this point was read from a digital sample-and-hold voltmeter. The mean threshold membrane potential $V$ (mV) ± SEM (n fibres) was plotted as a function of the pulse duration $t$ (ms) and the relationship was fit using the equation

$$V(t) = \frac{[H - R \cdot \exp(-t/\tau_R)]}{[1 - \exp(-t/\tau_R)]},$$

where $H$ is the holding potential (-90 mV), $R$ (mV) is the rheobase voltage and $\tau_R$ (ms) is the time constant to reach $R$. The MT values were expressed as the fitted $R$ parameter along with the standard error that was determined from the variance-covariance matrix in the non linear least squares fitting algorithm.

### Solution and chemical compounds

The normal physiological solution contained (in mM): 148 NaCl, 4.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 0.44 NaH₂PO₄, 12 NaHCO₃, 5.5 glucose. The pH of all solutions was adjusted to 7.3-7.4 by bubbling them with 95% O₂/5% CO₂. The calcium free-solution had the same composition of the normal
physiological solution excepted that CaCl$_2$ was omitted and 10 mM of ethylene glycol bis(β-aminoethyl ether)- $N,N',N''$-tetracetic acid (EGTA) was added. The quenching solution had the same composition except that 0.5 mM MnCl$_2$ was substituted for CaCl$_2$.

All chemicals cited above and ionomycin, caffeine, ruthenium red, cyclosporine A, thapsigargin, were purchased from Sigma (St. Louis, MO, USA).

Commercially available fluvastatin (Lescol®, fluvastatin sodium; Novartis Pharmaceuticals Corporation. A.I.C. n. 029163021) and atorvastatin (Torvast®, atorvastatin calcium; Pfizer Ireland Pharmaceuticals. A.I.C. n. 033007042) in CMC (0.5%) suspension were used for in vivo chronic treatment to rats. For in vitro experiments fluvastatin sodium salt purchased from Calbiochem was used.

**Statistical analysis**

The analysis of variance (ANOVA) followed by Bonferroni’s t-test was used to evaluate statistical differences between treated and control groups. For the in vitro studies, significance levels were calculated using Student’s unpaired t-test.
Results

In vivo study

Effects of in vivo chronic treatment with fluvastatin and atorvastatin on the activity-dependent change of fore-limb muscle strength

We have measured the forelimb muscle strength in control and in all treated groups of rats. In each animal we have normalized the forelimb strength measured at different time points to body weight so to minimize the influence of the body weight gain. At the first analysis no differences were found in the normalized force between control and all the rats treated with the different drugs. In particular, the forelimb force measured at the 8th week of treatment in 5 mg/kg fluvastatin and 20 mg/kg fluvastatin was 2.20 ± 0.07 g/g (n = 5), 2.49 ± 0.09 g/g (n = 5) with respect to the control value 2.24 ± 0.07 g/g (n = 5). However, when we considered the difference between the 1st and the 5th measure, read after 3 min from the first one, we found that this value was strongly decreased in 20 mg/kg fluvastatin-treated rats. Moreover the decrease was more greater how much greater was the duration of the treatment (Fig. 1). No difference was found in the rats treated with atorvastatin at both dosages (data not shown).

Thus, we found that fluvastatin at high doses was able to significantly increase muscle fatigability in terms of reduced force production due to prolonged activity.

Ex vivo studies

Effects of in vivo chronic treatment with fluvastatin and atorvastatin on skeletal muscle calcium homeostasis

Visible light microscopic changes and voltage-dependent calcium release
As useful indexes of the health state of the muscle fibres (Fraysse et al., 2004; Dulhunty, 1992), we first investigated the effect of the \textit{in vivo} statins chronic treatment on the gross morphologic fibers aspect and on the excitation-calcium release coupling. To this purpose, we examined skeletal muscle fibers samples by visible light microscopy and exposed them to a depolarizing high potassium solution.

As illustrated in Fig. 2 (top), there was no light microscopic changes in EDL muscle fibers of rats treated with fluvastatin 5 mg Kg$^{-1}$ day$^{-1}$ and atorvastatin at both dosages compared to control rats. In contrast, fluvastatin-related muscle of rats treated at the dose of 20 mg Kg$^{-1}$ day$^{-1}$, showed a compromised muscle fibers integrity characterized by a remarkable collapse and disarray of sarcomeres. This structural change was accompanied by the alteration of the voltage-dependent Ca$^{2+}$ release. Indeed, the amplitude of the Ca$^{2+}$ transient induced by 100 mM K$^{+}$ depolarizing solution showed by this group of treated animals was reduced by about 55% with respect to the control rats (Fig. 2 bottom).

\textit{Measurements of resting $[Ca^{2+}]_i$}

In line with previous studies (Fraysse et al., 2003; Fraysse et al., 2006), EDL fast-twitch skeletal muscle fibers of adult control rats showed a value of resting $[Ca^{2+}]_i$ of 26.9 ± 0.8 nM. Both \textit{in vivo} fluvastatin and atorvastatin administration induced a significant increase of $[Ca^{2+}]_i$ in all treated groups, although the effect was more important for animals treated with fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$ (Fig. 3). Indeed, the resting $[Ca^{2+}]_i$ was of 31 ± 0.8 nM and 43 ± 2.3 nM in rats treated with fluvastatin 5 mg Kg$^{-1}$ day$^{-1}$ and fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$, respectively. In the case of atorvastatin, the maximal effect was already observed with the lower dose, reaching a 20% increase of $[Ca^{2+}]_i$.

\textit{Sarcolemmal permeability to divalent cations}
We used the Mn$^{2+}$ quenching technique to assess the possibility that resting calcium increase in muscle fibers of treated rats, in particular in fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$ treated animals, could be related to a greater calcium influx. As it can be seen in Fig. 4A, no modification of sarcolemmal permeability to divalent cations was observed after atorvastatin in vivo treatment. The mean quench rate of 3.06 ± 0.17 % min$^{-1}$ and of 3.20 ± 0.22 % min$^{-1}$ for atorvastatin 5 mg Kg$^{-1}$ day$^{-1}$ and 10 mg Kg$^{-1}$ day$^{-1}$ treated fibers completely overlapped the value of control rats (3.5 ± 0.15 % min$^{-1}$). Interestingly, although in vivo low dose of fluvastatin did not alter the Mn$^{2+}$ quench rate, it was drastically reduced to a value that was approximately 60% lower than that of control rats after the chronic treatment with fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$.

All together these data indicate that changes in muscle resting [Ca$^{2+}$], observed after the in vivo chronic treatment with fluvastatin and atorvastatin are not due to an increase of the sarcolemmal cationic permeability.

**Caffeine-induced calcium release**

Because the resting [Ca$^{2+}$], increase was not related to an increased resting permeability of sarcolemma, we investigated whether the statin-induced [Ca$^{2+}$], increase may be due to an internal calcium store depletion. To evaluate the sarcoplasmic reticulum (SR) functionality, we exposed muscle fibers to caffeine, which induces a calcium release from SR via activation of ryanodine receptors (RyR) (Rios and Pizarro, 1991). Since the maximal amplitude value of caffeine-induced calcium transient in rat EDL intact muscle fibers was obtained by application of 40 mM caffeine (Fraysse et al., 2003), we determined the caffeine responsiveness of the different animal groups at this dose.

Compared to control rats, a slight amplitude reduction of the calcium transient induced by 40 mM caffeine was observed after treatment with fluvastatin 5 mg Kg$^{-1}$ day$^{-1}$ and atorvastatin at both doses. The lowering of the responsiveness to caffeine was more marked in fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$ treated rats, showing a 80% reduction of the calcium transient amplitude (Fig. 4B).
Effect of *in vitro* application of fluvastatin on calcium homeostasis of skeletal muscle fibers

In order to gain insight into the cellular mechanism of statin-induced calcium handling alteration, we evaluated the drug effect after *in vitro* acute application on rat skeletal muscle fibers. On the basis of the results obtained with the *in vivo* statins administration, we focused our attention on fluvastatin that produced the most evident alteration of the calcium homeostasis in treated rats muscle fibers.

*Mechanical threshold and resting [Ca$^{2+}$]*

We previously demonstrated that muscle fibers from fluvastatin-treated rats needed less depolarization to contract showing a rheobase voltage (R) shifted of 3-4 mV toward more negative potentials as compared to control rats (Pierno et al., 2006).

Accordingly, the *in vitro* application of fluvastatin on EDL muscle produced a negative shift of the voltage threshold for contraction. Particularly, the effect was observed already after application of 50 µM fluvastatin that shifted R of about 2 mV toward more negative potential with respect to the control value (data not shown) and became statistically significant at higher concentration. Indeed, as shown in Fig. 5, 200µM fluvastatin produced a marked shift of strength-duration curve at each pulse duration. The R value obtained by the fit of the experimental points were -66.0 ± 0.15 mV (n fibres) and -69.5 ± 0.25 mV (n fibres) in absence and in presence of 200µM fluvastatin, respectively.

In parallel, micromolar acute application of fluvastatin triggered a sustained rise of [Ca$^{2+}$]$_i$ in fura-2-loaded skeletal muscle fibers (Fig. 6 A). Interestingly, as shown in Fig. 6A reporting the effect of fluvastatin at the dosage of 200µM, the Ca$^{2+}$ transient exhibited a peculiar time course, characterized by a slow rising phase (lasting 15 min) followed by a more rapid and large increase of [Ca$^{2+}$]$_i$. Particularly, fluvastatin enhanced cytosolic calcium level from 28.0 ± 1.8 nM to a plateau
value of 124 ± 20 nM reached 20 min after the onset of drug application. When the drug was
removed, the fluvastatin-triggered Ca\(^{2+}\) increase slowly (over 15 min) returned close to the basal
resting [Ca\(^{2+}\)]\(_i\) (data not shown). As can be appreciated from the concentration-response relationship
curve (Fig. 6 B), such marked increase of [Ca\(^{2+}\)]\(_i\) was observed for dosage ≥ 200 \(\mu\)M. For dosage
below 200\(\mu\)M, fluvastatin effect was characterized by only a slow rising phase responsible for a
slight increase of [Ca\(^{2+}\)]\(_i\). Therefore in further investigations, we routinely applied fluvastatin at 200
\(\mu\)M.

\(\text{Mn}^{2+}\) quench rate

In agreement with the reduction of the cation membrane permeability observed in fluvastatin 20 mg
Kg\(^{-1}\) day\(^{-1}\) treated animals, \textit{in vitro} application of fluvastatin reduced fluorescence quench.
Particularly, the mean quench rate was 3.9 ± 0.15 \% min\(^{-1}\) and 2.89 ± 0.16 \% min\(^{-1}\) before and
after application of 200\(\mu\)M fluvastatin, respectively.

It has been proposed that store-operated calcium entry (SOCE) is essential for maintenance of Ca\(^{2+}\)
homeostasis by ensuring the refilling of intracellular calcium storage (Kurebayashi and Ogawa,
2001; Zhao et al., 2005; Zhao et al., 2006). Thus, a reduction of SOCE activity could account for
the reduced caffeine or K-induced calcium transient observed in muscle fibers after \textit{in vivo}
treatment with 20 mg Kg\(^{-1}\) day\(^{-1}\) fluvastatin.

In order to confirm this hypothesis, we evaluated the effect of fluvastatin on Mn\(^{2+}\) permeability after
SOCE activation. To this end, SR Ca\(^{2+}\) stores of muscle fibers were depleted by treatment with
thapsigargin and caffeine (Kurebayashi and Ogawa, 2001; Zhao et al., 2006; Liantonio et al., 2007).
As show in Fig. 7A, activation of SOCE induced by SR Ca\(^{2+}\) depletion led to a steep Mn\(^{2+}\)
quenching of fura-2 fluorescence. Interestingly, we found that the pre-incubation of muscle fibers
with 200\(\mu\)M fluvastatin significantly reduced the rate of Mn\(^{2+}\) entry following depletion of SR Ca\(^{2+}\)
store (Fig. 7A). Indeed, the mean quench rate resulted 9.1 ± 0.15 % min⁻¹ (n = 15) and 5.1 ± 0.12 % min⁻¹ (n = 13) in muscle fibers no-treated and fluvastatin treated respectively.

Investigation of the source of calcium release

Removal of external Ca²⁺ in the bath solution did not abolish the fluvastatin induced [Ca²⁺]ᵢ rise, producing an increase of 108 ± 22 nM, a value not significantly different from that obtained in presence of extracellular calcium (Fig. 7B).

Together with the lack of Mn²⁺ permeability enhancement after fluvastatin in vitro application (see above), this result strongly suggested that fluvastatin induced an increase [Ca²⁺]ᵢ likely by favoring a calcium release from intracellular stores. We thus attempted to identify the relevant intracellular source. We investigated the possible involvement of SR using ruthenium red, an inhibitor of the SR-Ca²⁺ release channels (RyR). Pretreatment with ruthenium red, at a concentration (5 µM) that completely prevented the 40 mM caffeine effect (Liantonio et al., 2007), significantly reduced the fluvastatin-induced [Ca²⁺]ᵢ increase, although a [Ca²⁺]ᵢ increase of 22.5 ± 1.2 nM was still observed (Fig. 6A; Fig. 7B). These data indicated that fluvastatin mobilized Ca²⁺ mainly from SR but that an other intracellular calcium store was involved. It is well known that mitochondria play a role in skeletal muscle [Ca²⁺]ᵢ (Saris and Carafoli, 2005). To assess a possible mitochondrial origin of the fluvastatin-induced [Ca²⁺]ᵢ increase, we evaluated the effect of such statin in the presence of cyclosporine A (CsA), an inhibitor of the mitochondrial permeability transition pore (PTP) (Crompton et al., 1999; Liantonio et al., 2007).

In agreement with previous study (Liantonio et al., 2007), the application of 2 µM CsA did not alter the baseline of [Ca²⁺]ᵢ (data not shown). Successively addition of 200µM fluvastatin failed to produce any [Ca²⁺]ᵢ increase (Fig. 7B). Thus, the inhibition of the mitochondrial PTP completely abolished the fluvastatin-induced [Ca²⁺]ᵢ increase.
Discussion

The aetiology of statin-induced skeletal muscle damage is still under debate. Considering the great number of statin-treated patients, the comprehension of mechanisms of statin-induced myotoxicity is determinant in order to prevent these adverse effects. Calcium ions exert a pivotal role in regulating muscle physiology (Berchtold et al., 2000) and an alteration of calcium homeostasis frequently results in a muscle damage. Accordingly, elevated resting $[Ca^{2+}]_{i}$ levels were found in pathophysiological conditions of skeletal muscle such as ageing (Navarro et al., 2001; Fraysse et al., 2006) or muscular dystrophy (Fraysse et al., 2004; De Luca et al., 2003). In the present study we investigated the potential relationship between statins myotoxicity and calcium homeostasis by assessing the effects of a chronic treatment with fluvastatin and atorvastatin on fura-2-loaded rat intact skeletal muscle fibers.

Either fluvastatin 5 mg Kg$^{-1}$ day$^{-1}$ and atorvastatin at both doses produced a significant increase in $[Ca^{2+}]_{i}$, with a concomitant decrease of the caffeine’s responsiveness and no changes in sarcolemmal calcium permeability. These findings clearly indicated a drug capability of altering calcium homeostasis by interfering with intracellular targets involved with calcium handling mechanisms. Importantly, fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$ treated animals, in addition to a marked increase of $[Ca^{2+}]_{i}$, showed a completely compromized sarcomere organization and a significant decrease of the depolarization-induced intracellular calcium peak. It might be possible that at this high drug dosage, together with the disruption of calcium homeostasis, a series of other cellular mechanisms may take place, all events accounting for the described detrimental effects. An interesting hypothesis could be that the fluvastatin administration would produce an alteration of the T-tubule membrane composition. Indeed, the T-tubule membrane system of striated muscle cells is highly enriched in cholesterol (Rosemblatt et al., 1981) and an alteration of the amount of this component caused a reduction of the depolarization-induced muscle tension (Launikonis et al., 2001). In support of this hypothesis, it was recently reported that skeletal muscle biopsies from
statins-treated patients are characterized by a breakdown of the T-tubular system and by subsarcolemmal ruptures (Draeger et al., 2006).

The degree of calcium homeostasis impairment induced by the statins administration well correlated with the \textit{in vivo} muscle function performance alteration. Indeed, rats treated with fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$ showed a reduced force production after prolonged activity thus indicating a fatigue-related decrease in muscle function of this animal group with respect to control rats.

In order to define the statin’s cellular mechanism of action leading to calcium homeostasis alteration, an \textit{in vitro} study was conducted. Importantly, micromolar acute application of fluvastatin caused changes of [Ca$^{2+}$]$_i$, resembling the effect obtained after the \textit{in vivo} administration. Indeed fluvastatin was capable of producing a shift of MT for contraction toward more negative potentials and an increase of resting [Ca$^{2+}$]. Furthermore, \textit{in vitro} fluvastatin application reduced the cation membrane permeability at rest, an effect that resulted more marked in condition of SOCE activation. These results allowed us to explain some of the statin-induced effects observed after \textit{in vivo} treatment. Indeed it was recently demonstrated that in skeletal muscle, SOCE is essential for the maintenance of Ca$^{2+}$ homeostasis by ensuring the refilling of intracellular calcium storage (Kurebayashi and Ogawa, 2001; Zhao et al., 2005; Zhao et al., 2006). The direct statin SOCE inhibition would lead to a chronic depletion of the intracellular stores, thus justifying the drastic reduction of the K- and caffeine-induced Ca$^{2+}$ transients characterizing the muscle fibers of fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$ treated animals. The \textit{in vivo} and \textit{in vitro} characterization of the type of the voltage-insensitive permeable calcium current effectively involvement into the SOCE related effect induced by statins, will be very useful to define the role and the relevance of such phenomenon in mediating the statin-induced myotoxicity.

Accordingly to quench measurements performed on both \textit{in vivo} and \textit{in vitro} statin-treated fibres, the amplitude of the fluvastatin-induced increase of [Ca$^{2+}$], did not vary after withdraw of extracellular calcium, thus strongly indicating that the drug effect on resting [Ca$^{2+}$], are not due to an increase of the sarcolemmal cationic permeability but rather to an internal Ca$^{2+}$ store depletion.
By using appropriate tools, we determined the sequence of the statins-induced Ca$^{2+}$ release mechanism. Mitochondria appeared as the cellular structure responsible for the earlier event leading to a sustained [Ca$^{2+}$], elevation. Indeed, the partially and the completely lack of effect of fluvastatin observed in presence of ruthenium red and CsA, respectively, led us to propose that a mitochondrial Ca$^{2+}$ efflux represent the early event which in turn caused the subsequent large SR Ca$^{2+}$ release. Importantly, our findings represent the first direct evidence of such mechanism on intact skeletal muscle fibers where the e-c coupling system functionality is ensured, and are in agreement with previous studies performed on cultured myoblasts as well as on skinned fibers (Nakahara et al., 1994; Inoue et al., 2003; Sirvent et al., 2005).

In support to a direct activity of fluvastatin on mitochondria, it was reported that various lipophilic statins produced alterations in mitochondrial function associated with dissipation of the electric potential across the inner mitochondrial membrane (Kaufmann et al., 2006; Velho et al., 2006), an event that could account for the opening of PTP observed in our investigations. Furthermore, tight structural association between the SR and mitochondria has been described in many cell types (Rizzuto et al., 1998) and recently a close functional connection between the two cellular structures has been reported in adult fast- and slow-twitch skeletal muscle (Shkryl et al., 2006). The statin-induced mitochondrial Ca$^{2+}$ efflux, although of small entity, might be highly localized near SR microdomains in close contact with mitochondria, resulting thus sufficient to activate a Ca$^{2+}$-induced Ca$^{2+}$ release from SR. Nevertheless, we can not exclude the hypothesis that the Ca$^{2+}$ movement triggered by the opening of PTP might activates a successive adequate cellular signal which in turn cause a direct stimulation of calcium efflux from SR.

Importantly, the in vitro reported mitochondrial calcium efflux could allow us also to explain the alteration of caffeine responsiveness observed after the in vivo administration. Indeed, since the RyR channels activity was depressed through a direct Ca$^{2+}$-dependent regulation (Fill and Copello, 2002), the reduced caffeine responsiveness particularly marked for fluvastatin 20 mg Kg$^{-1}$ day$^{-1}$ treated animals, could likely be due, other than to the SOCE inhibition, to an alteration of these SR
calcium release channels consequent to the persistent mitochondria-derived Ca\(^{2+}\) stimulation. Finally, we did not exclude that a reduced electrochemical driving force due to the increased cytosolic calcium amount could account for the limited caffeine effect.

All our data support the finding that statins induced an alteration in Ca\(^{2+}\) homeostasis in skeletal muscle fibers. The effects on Ca\(^{2+}\) signalling elicited by statins on vascular smooth muscle (de Sotomayor et al., 2005) and on endothelial cells (O’Meara et al., 2004; Heinke et al., 2004) explain some of the beneficial cardiovascular effects of HMG-CoA reductase inhibitors that are not related to their lipid-lowering properties. In contrast, a such effect on skeletal muscle could cause various cell damages strongly contributing to the pathogenesis of myopathy induced by statins. The most likely way that elevation in free cytosolic Ca\(^{2+}\) acts to disrupt excitation-contraction coupling in skeletal muscle is via stimulation of certain Ca\(^{2+}\)-activated enzymes such as calpains, phospholipase A\(_2\) or protein kinase C. At this regard, it will be highly interesting to investigate if the statin-induced increase in resting [Ca\(^{2+}\)], may contribute to the decrease of gCl (Pierno et al., 1995; Pierno et al., 2006) through a PKC-dependent CLC-1 inhibition. This may allow a better understanding of the cross-talk mechanism between the two main early targets, cytosolic calcium level and macroscopic gCl, most probably contributing to statin-induced myotoxicity (Pierno et al., 2006).

In conclusion, herein we give the first evidence that an increase of [Ca\(^{2+}\)], in intact tendon to tendon rat skeletal muscle fibers can be obtained upon an in vivo treatment with fluvastatin or atorvastatin. This effect was attributable to a Ca\(^{2+}\) release from SR and mitochondria. Considering the crucial role of resting calcium ions in skeletal muscle function and maintenance, our findings considerably contribute to the understanding of the myopathy mechanism induced by this widely used class of hypolipidemic drugs.
Acknowledgements

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References


Footnotes

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Legends for Figures

Figure 1. Effects of in vivo chronic treatment with fluvastatin on the fatigue-related change of the fore-limb muscle strength. The fore-limb strength has been measured as described in the Method section. In each control and fluvastatin-treated rat the forelimb strength was determined 5 times within 3 min and the difference between the 5th and the 1st measure has been plotted as an index of muscle fatigability. Each bar is the mean value (± S.E.M.) measured from 5 animals of each experimental group (controls, 5 mg/kg fluvastatin and 20 mg/kg fluvastatin) and represents the change of normalized forelimb force measured at different time points (before treatment and after 4-6-8 weeks of treatment). The analysis of variance (ANOVA) test for multiple comparison shows statistical differences in the 20 mg/kg fluvastatin treated group (F= 6.64, P<0.001). Bonferroni’s t-test showed significant differences with respect to the mean value measured before treatment (*P<0.02 or less).

Figure 2. Effect of the in vivo chronic treatment with fluvastatin and atorvastatin on the gross morphologic fibers aspect and on the excitation-calcium release coupling. Top: visible light microscopic representative images of skeletal muscle fibers samples for each indicated group of rats; Bottom: representative traces for each indicated group of rats showing the Ca^{2+} transient induced by the application of depolarizing 100 mM K+ solution.

Figure 3. Resting [Ca^{2+}]_i measured in EDL muscle fibres from rats chronically treated with fluvastatin (5 and 20 mg kg\(^{-1}\) day\(^{-1}\)) and atorvastatin (5 and 10 mg kg\(^{-1}\) day\(^{-1}\)) and from control rats. Each bar is the mean ± SEM from n muscle fibers and N animals (n/N indicated above each bar). Statistical analysis by ANOVA showed significant differences in [Ca^{2+}]_i (F = 22, n fibers – k groups = 602, P <0.005). Bonferroni’s t-test showed significant differences between all treated groups and the control group (P<0.005).
Figure 4. Effect of *in vivo* chronic treatment with fluvastatin and atorvastatin on sarcolemma permeability to calcium ions and on caffeine responsiveness. A) The sarcolemma permeability to Ca\(^{2+}\) was evaluated by measuring the quench rate of fura-2 fluorescence induced by Mn\(^{2+}\) influx. Each square point corresponds to the mean ± SEM value of quench rates measured in the indicated numbers of muscle fibers of at least 3 animals. Statistical analysis by ANOVA showed significant differences in quench rate (F = 7, n fibres – k groups = 309, P <0.005). Bonferroni’s t-test showed a significant difference between the 20 mg kg\(^{-1}\) day\(^{-1}\) fluvastatin treated group and the control group (P<0.005); B) The caffeine responsiveness was determined by evaluating the amplitude of the calcium transient after *in vitro* application of 40mM caffeine for each animal group (25-50 fibers from at least 3 animals). The mean value of the caffeine-induced calcium transient for each treated animal group has been normalized to the correspondent mean value of the control rats. Thus, each bar represents the normalized percent of the amplitude of caffeine-induced calcium transient. Statistical analysis by ANOVA showed significant differences in quench rate (F = 58, n fibres – k groups = 155, P <0.005). Bonferroni’s t-test showed significant differences between the 5 mg kg\(^{-1}\) day\(^{-1}\) fluvastatin treated group and the 20 mg kg\(^{-1}\) day\(^{-1}\) fluvastatin treated group with respect the control group (P<0.005).

Figure 5. Mechanical threshold for contraction (MT) of EDL muscle fibres from untreated rats before and after *in vitro* application of 200\(\mu\)M fluvastatin. Each point, expressed as mean value ± s.e.m from 34 to 61 fibers of 3 rats, shows the voltage potential for fibre contraction at each pulse duration. The curves fitting the experimental points have been obtained using the equation described in Methods section. * Significantly more negative with respect to control value (by Student’s t-test, P < 0.05).
Figure 6. Effect of *in vitro* application of fluvastatin on $[\text{Ca}^{2+}]_i$ of EDL muscle fibers of untreated rats. A) Superimposed traces showing the effect of the application of 200 µM fluvastatin in control condition (black trace), in presence of the ryanodine receptor antagonist ruthenium red (RR) (grey trace), or in presence of the mitochondrial permeability transition pore (PTP) inhibitor cyclosporine A (CsA) (light grey trace). Particularly, muscle fibres were incubated with RR for 5 min and with CsA for 10 min prior to fluvastatin application; B) Dose-response relationship for the effect of fluvastatin on $[\text{Ca}^{2+}]_i$. Data are shown as mean ± s.e.m and each point is representative of 15-24 fibers.

Figure 7. A) Effect of *in vitro* application of fluvastatin on store-operated calcium entry. Traces represent fura-2 fluorescence quench associated with Mn$^{2+}$ influx in skeletal muscle fibers following depletion of SR Ca$^{2+}$ stores induced by thapsigargin and caffeine (see Methods) in absence and presence of fluvastatin. Muscle fibers were treated with thapsigargin/caffeine in zero calcium solution for 15 min in absence or in presence of fluvastatin, followed by Mn$^{2+}$ addition to the perfusate. B) Investigation of the calcium source responsible of the fluvastatin-induced increase of $[\text{Ca}^{2+}]_i$. The increase in $[\text{Ca}^{2+}]_i$ induced by 200 µM fluvastatin measured in presence of external calcium (control, n=16), in absence of external calcium (Ø Ca$^{2+}$, n=15), in presence of ruthenium red (RR, n= 17) or cyclosporine A (CsA, n=15) was reported. Each bar represent the mean ± s.e.m. * Significantly different with respect to control value with P < 0.05 (by Student’s t-test).
Figure 1

Fatigue-related change in forelimb strength (g/g)

control  fluvastatin  fluvastatin
5mg/kg   20mg/kg

before treatment  4 weeks  6 weeks  8 weeks

*
Figure 2

Control

Fluvastatin 5 mg Kg\textsuperscript{-1}

Fluvastatin 20 mg Kg\textsuperscript{-1}

Atorvastatin 5 mg Kg\textsuperscript{-1}

Atorvastatin 10 mg Kg\textsuperscript{-1}
Figure 3

The figure shows the effects of different doses of Fluvastatin and Atorvastatin on the intracellular calcium concentration ([Ca^{2+}]) in (nM). The control group is compared to the treated groups at two different Fluvastatin doses (5 mg Kg^{-1} and 20 mg Kg^{-1}) and two different Atorvastatin doses (5 mg Kg^{-1} and 10 mg Kg^{-1}).

- Control group: [Ca^{2+}] = (112/13) nM
- Fluvastatin 5 mg Kg^{-1}: [Ca^{2+}] = (162/9) nM
- Fluvastatin 20 mg Kg^{-1}: [Ca^{2+}] = (53/6) nM
- Atorvastatin 5 mg Kg^{-1}: [Ca^{2+}] = (48/6) nM
- Atorvastatin 10 mg Kg^{-1}: [Ca^{2+}] = (232/10) nM
Figure 4

A

<table>
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<th>Treatment</th>
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</tr>
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B

<table>
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Concentration (µM) Fluvastatin-induced increase in [Ca²⁺]ᵢ (nM)

Figure 6

A

Time (s)

B

Fluvastatin-induced increase in [Ca²⁺]ᵢ (nM)

Concentration (µM)
Figure 7

A

Mn

Fluorescence Intensity at 360nM (% of initial)

Time (s)

with fluvastatin

without fluvastatin

B

200μM Fluvastatin-induced increase in [Ca²⁺]i (nM)

control Ø [Ca²⁺]o RR CsA

*