Delineation of Myotoxicity Induced by 3-Hydroxy-3-
methylglutaryl CoA Reductase Inhibitors in
Human Skeletal Muscle Cells

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ABSTRACT

The 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) are widely used and well tolerated cholesterol-lowering drugs. In rare cases, side effects occur in skeletal muscle, including myositis or even rhabdomyolysis. However, the molecular mechanisms are not well understood that lead to these muscle-specific side effects. Here, we show that statins cause apoptosis in differentiated human skeletal muscle cells. The prototypical representative of statins, simvastatin, triggered sustained intracellular Ca2+ transients, leading to calpain activation. Intracellular chelation of Ca2+ completely abrogated cell death. Moreover, ryanodine also completely prevented the simvastatin-induced calpain activation. Nevertheless, an activation of the ryanodine receptor by simvastatin could not be observed. Downstream of the calpain activation simvastatin led to a translocation of Bax to mitochondria in a caspase 8-independent manner. Consecutive activation of caspase 9 and 3 execute apoptotic cell death that was in part reversed by the coadministration of mevalonic acid. Conversely, the simvastatin-induced activation of calpain was not prevented by mevalonic acid. These data delineate the signaling cascade that leads to muscle injury caused by statins. Our observations also have implications for improving the safety of this important medication and explain to some extent why physical exercise aggravates skeletal muscle side effects.

Statins are reversible and competitive inhibitors of the microsomal enzyme 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. By blocking this rate-limiting step in the mevalonate pathway, endogenous de novo synthesis of cholesterol is prevented and low-density-lipoprotein cholesterol uptake into cells is promoted (Goldstein and Brown, 1990). The efficacy of this therapeutic principle has been confirmed in clinical trials, which showed reduced risk of a recurrent coronary event in patients with established coronary disease treated with simvastatin (S4 group, 1994, 2000; Sacks et al., 1997). It became recently evident that the long-term prevention of cardiovascular events, in part, is also due to the anti-inflammatory and antiproliferatory actions of statins, the so-called pleiotropic effects (Kwak et al., 2000; Weitz-Schmidt et al., 2001). There is accumulating evidence in the literature that statins exert antiproliferative effects and induce cell cycle arrest in various primary cells, like smooth muscle cells or in tumor cells (Negre-Aminou et al., 1997; Rao et al., 1998; Werner et al., 2004).

Usually, statins are well tolerated (S4 group, 1994, 2000; Sacks et al., 1997; Bellosta et al., 2004). However, side effects may arise in skeletal muscle. These may range from transient increases in creatine kinase (CK), muscle pain, and cramps to myositis and potentially life-threatening rhabdomyolysis (Farmer and Torre-Amione, 2000; Bellosta et al., 2004; Rosenson, 2004). Physical exercise predisposes patients who take statins to develop muscle specific side effects (Thompson et al., 1997; Sinzinger and O'Grady, 2004). This is also true for some forms of comedication (Bellosta et al., 2004). The concomitant administration of inhibitors of cytochrome oxidase P450 3A4 (e.g., macrolide antibiotics, cyclosporin A, or azole antimycotics) puts patients at a higher risk (Farmer and Torre-Amione, 2000; Bellosta et al., 2004; Rosenson, 2004). The molecular mechanisms have not yet been identified that underlie statin-induced lysis of skeletal muscle (Thompson et al., 1997; Sinzinger and O’Grady, 2004). It is specifically not clear why skeletal muscle is so...
susceptible to toxicity of statins (Rosenson, 2004). Cardiac muscle, for example, is not adversely affected by statins. To gain mechanistic insight, in the present work we have delineated the signaling pathways that are activated by statins in differentiated primary human skeletal muscle cells. Our experiments show that statins cause sustained increases in cytosolic Ca\(^{2+}\) levels, which are essentially linked to the development of apoptosis in differentiated human skeletal muscle cells.

**Materials and Methods**

**Cell Culture.** All experiments were carried out with differentiated primary human skeletal muscle cells obtained from skeletal muscle biopsies of healthy individuals who underwent diagnostic testing. The leftover material (100–400 mg) was used to isolate satellite cells. The procedure was approved by the local ethic committee of the Medical University of Vienna (Dr. Weigl; Department of Anaesthesiology, General Hospital, Vienna, Austria). Satellite cells were kept in growth medium (Ham’s F-12, 15% fetal calf serum, 50 ng/ml epidermal growth factor, 10 ng/ml insulin, 200 μg/ml dexamethasone, 250 mg/ml fetuin and bovine serum albumin, 78 g/500 ml glucose, 200 mM L-glutamine, 5000 units/ml penicillin G, 5000 μg/ml streptomycin, and 250 μg/ml amphotericin B), and differentiation was initiated by switching to differentiating medium (Dubecco’s modified Eagle’s medium supplemented with 5% horse serum, and 4 mM L-glutamine, 100 ng/ml insulin, and 0.1 μg/ml gentamicin) as described previously (Weigl et al., 2000).

**Caspase Assays.** Differentiated human skeletal muscle cells were maintained in the absence and presence of statins and inhibitors at concentrations and for incubation times indicated in the respective figures. Thereafter, the cells were washed with phosphate-buffered saline and lysed with ice-cold caspase-lysis buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 1 mM EGTA, 5 mM MgCl\(_2\), and 5 mM dithiothreitol) supplemented with protease inhibitors (1.4 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM pefabloc). Microsomal fractions and cytosol were separated by centrifugation at 45,000 g at 4°C for 20 min. The pellet was resuspended in caspase lysis buffer, and samples were stored at −80°C. All steps starting with cell lysis were carried out on ice. Aliquots of the supernatant (10–50 μg) were incubated in reaction buffer (25 mM HEPES, pH 7.4, 6.6% sucrose, 1.4 CHAPS, and 5 mM dithiothreitol) and reaction buffer in the absence of protein.

**Ca\(^{2+}\) Release Experiments.** Differentiated skeletal human muscle cells were loaded with 10 μM fura-2/AM (Molecular Probes) in the presence of 0.025% Pluronic acid for 45 min and prepared for fluorescence photometry in suspension by collecting the cells in Tyrode’s solution to achieve a concentration of 2.5 to 5 × 10\(^6\) cells/ml (Weigl et al., 2003). The fluorescence (excitation at 340 nm and 380 nm and the corresponding emission at 510 nm; 5-nm slits) was continuously recorded with a fluorescence photometer (F-4500; Hitachi, Tokyo, Japan). The ratio of the signals obtained at 340 and 380 nm was used to visualize a proportional value for the intracellular Ca\(^{2+}\) concentration and was done off-line.

**High-Affinity \([\text{H}]\)Ryanodine Binding.** Heavy sarcoplasmic reticulum membranes were prepared from rabbit hind leg and back skeletal muscle as described previously (Klinger et al., 1999). For high-affinity \([\text{H}]\)ryanodine binding, heavy sarcoplasmic reticulum membranes (50 μg) were incubated in 100 μl of binding buffer containing 40 mM HEPES-NaOH, pH 7.4, 200 mM KCl, 15 mM NaCl, 0.5 mM EGTA, 0.45 mM CaCl\(_2\), 20 nM \([\text{H}]\)ryanodine, 1 μM aprotinin, 1 μM leupeptin, 100 μM pefabloc, and the drug concentrations indicated in the figure legends. The samples were incubated for 120 min at 30°C. Nonspecific binding was determined in the presence of 100 μM ryanodine. The reaction was terminated by filtration over glass fiber filters (presoaked in 1% polyethyleneimine) using a Skatron vacuum filtration device. The filters were rinsed with 10 ml of ice-cold 10 mM Tris-HCl, pH 7.4, and 500 mM NaCl, and the remaining radioactivity on the filters was determined by liquid scintillation counting.

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay.** Apoptotic DNA fragmentation was visualized with an in situ cell death detection kit (Roche Diagnostics, Penzberg, Germany). As a negative control, mock incubations with only label solution containing fluorescent-deoxyuridine triphosphate nucleotides were carried out to measure background staining. The
fluorescein labels incorporated in DNA were detected immediately or mounted and analyzed within 48 h. Fluorescence was visualized using a confocal argon laser microscope (LSM410; Carl Zeiss) with an excitation at 488 nm and an emission at 515 nm.

Miscellaneous Procedures. All experiments were carried out in triplicate, and each experiment was repeated at least two times. All data are presented as mean ± S.D., if not otherwise stated. The data of concentration-response curves were fitted by nonlinear least-squares regression to the Hill equation. Statistical significance (p < 0.05) was determined with Student’s t test and for multiple comparison with ANOVA and post hoc Scheffe’s test. The protein concentration was determined with the Bio-Rad Coomassie Blue kit (Bio Rad, Munich, Germany) or the bicinchoninic acid assay (Micro-BCA; Pierce Chemical) using bovine serum albumin as a protein standard.

Results

Simvastatin-Induced Apoptosis. Differentiated primary human skeletal muscle cells underwent apoptosis when exposed to simvastatin. Simvastatin (Fig. 1A), and to a similar extent lovastatin (data not shown), reduced the number of viable cells in a time- and concentration-dependent manner. The reduction in cell number was prevented in part by coadministration of mevalonic acid (Fig. 1B). Even a mevalonic acid concentration of 10 mM could not overcome the simvastatin-induced cell death. This indicates that a mechanism independent of the mevalonate pathway contributes to cell toxicity (Fig. 1B). This conclusion is based on the following observations.

Differentiated skeletal muscle cells do not proliferate; the reduction in cell number is thus due to a loss of viable cells. We searched for the hallmarks of apoptosis by TUNEL staining for fragmented DNA. Nuclear DNA fragmentation increased significantly after simvastatin treatment from 12.0 ± 2.4% (n = 4) to 50.6 ± 11.0% (n = 7; p < 0.03) of total cells (cf. Fig. 1C). The breakdown of the nuclear envelope is another hallmark of apoptosis (Ferri and Kroemer, 2001). We therefore quantified the level of LAP2α, a typical nuclear envelope protein (Gotzmann et al., 2000). As expected, LAP2β was recovered in the particulate fraction and migrated as a doublet (Fig. 1D). The two bands represent the nonphosphorylated and phosphorylated form of LAP2β (Gotzmann et al., 2000). In the presence of 30 μM simvastatin (Fig. 1D, lanes 30S), LAP2β immunoreactivity was reduced in the particulate fraction, and there was no concomitant increase in the cytosolic fraction. This indicates that 30 μM simvastatin (but not 1 μM simvastatin) caused a significant degradation of LAP2β (p < 0.0001) and presumably other nuclear envelope proteins within 24 h.

To address the mechanism by which simvastatin induces apoptosis, we analyzed the kinetics of caspase activation, by using selective fluorogenic substrates (Fig. 2). Caspase 3 is an effector caspase (Ferri and Kroemer, 2001). After 8 h of simvastatin exposure, caspase 3 activation was detectable (Fig. 2A) and sustained up to 30 h. Caspase 3 activation coincided with the sharp rise in caspase 9 activity (Fig. 2B), but the peak activation in caspase 9 activity occurred clearly before that of caspase 3. To confirm the conjecture that statins trigger apoptosis only via a caspase 9/caspase 3 cascade, we also measured caspase 8 activity. Within 36 h of simvastatin exposure, caspase 8 activity remained undetectable (Fig. 2C). Tumor necrosis factor-α is known to trigger caspase 8 activity in myoblasts and was therefore used as a positive control (Stewart et al., 2004).

Moreover, caspase 9 and 3 activation was concentration-dependent for both simvastatin and lovastatin (Fig. 2, D and E). Of utmost importance is the observation that caspase 9 as well as caspase 3 activities were almost completely prevented by the coapplication of mevalonic acid. These data strongly argue against a pathway mediated by a death receptor. In skeletal muscle, the Ca2+-activated cysteine protease calpain may play an important regulatory role in the initiation of statin-induced apoptosis. As depicted in Fig. 2F, simvastatin and lovastatin induced calpain activation.

Simvastatin Triggers the Mitochondrial Pathway of Apoptosis. The role of calpain in apoptosis is a matter of debate; calpain was proposed to be both upstream and downstream of the caspase cascade (for review, see Goll et al., 2003). We therefore focused first on the activation of the mitochondrial pathway of apoptosis. Simvastatin caused activation that was in magnitude comparable with the effect of staurosporine (Fig. 3B, right-hand column). If the proapoptotic action of simvastatin depends on the mitochondrial pathway, its effect ought to be abolished by an inhibitor of the mitochondrial transition pore. We verified this prediction by using bongkrekic acid, an inhibitor of the mitochondrial adenine transporter, which is an essential component of the mitochondrial transition pore (Gross et al., 1999). The addition of bongkrekic acid suppressed the activation of caspase 3 (Fig. 3A) and 9 (Fig. 3B) by 90 and 80%, respectively. In a similar manner, the simvastatin-induced activation of caspase 3 and 9 was abrogated by the simultaneous application of a cell-permeable pan-caspase inhibitor.

There is a general consensus that the translocation of Bax from the cytosol to the mitochondrial membrane represents a critical control point in apoptosis (Wolter et al., 1997; Ferri and Kroemer, 2001; Danial and Korsmeyer, 2004). One would expect that Bax accumulates in the membrane fraction of skeletal muscle cells when simvastatin is applied. This was clearly the case (Fig. 3C). Furthermore, the translocation of Bax was accompanied by a decrease of Bax in the cytosolic fraction. Interestingly, if mevalonic acid was added back to simvastatin-treated cells, the accumulation of Bax in the membrane fraction was not prevented (Fig. 3C, lanes 30S+ m.a.). We corroborated these observations in part by immunocytochemistry (Fig. 3D). In the absence of any stimulus, Bax was speckle-distributed over the cell. But in the presence of simvastatin, Bax accumulated in the mitochondrial membrane. This is to some extent in contradiction to the results obtained in the Western blot experiments, where Bax is still in the particular fraction (Fig. 3C, lanes 30S+ m.a.). A possible explanation could be that in the presence of simvastatin and mevalonic acid Bax may be localized to the endoplasmatic reticulum, which has been recently described to be related to Ca2+- release mechanisms (Zong et al., 2003; Oakes et al., 2005). Nevertheless, in the presence of simvastatin (Fig. 3D, 30S) skeletal muscle cells get rounded, the nuclei are more pronounced and the cytosol condensed, which is typical for apoptotic cells. In comparison, control cells or cells treated with simvastatin and mevalonic acid do not undergo such morphological changes.
Fig. 1. Simvastatin-induced apoptosis in differentiated human skeletal muscle cells. 

A, differentiated human skeletal muscle cells were treated with 1 (open triangle), 3 (filled circle), 30 (filled square), and 100 μM (open diamond) simvastatin or the carryover of 1% DMSO (open circle) for 24 h. Four phase contrast pictures were collected from each time point and sample treatment. The number of cells in four areas of equal size was counted from each picture, and the mean of the cell numbers was plotted against time. 

B, differentiated human skeletal muscle cells were treated for 24 h in the absence (CTL) and presence of 30 μM simvastatin (30S) or a combination of 30 μM simvastatin and 0.1, 1, or 10 mM mevalonic acid (30S+ m.a.) or mevalonic acid alone at 0.1, 1, or 10 mM (0.1m.a., 1m.a., and 10m.a.). Trypan blue-stained cells were counted in four phase contrast pictures of equal size and subtracted from the total number of cells. The columns represent the mean ± S.D. 

C, TUNEL assay was carried out with differentiated human skeletal muscle cells that had been incubated in the absence (CTL) and presence of 30 μM simvastatin (30S) for 24 h. Pictures were generated at a magnification of 10× for CTL and 20× for simvastatin (SIM) by phase contrast and confocal fluorescence microscopy. 

D, differentiated human muscle cells were incubated in the absence (CTL) and presence of 1 μM (1S) or 30 μM simvastatin (30S) for 24 h. LAP2β and actin were visualized in the cytosolic (10 μg; SUPERNATANT) and membrane (20 μg; PELLET) fractions. The intensity of the protein bands was quantified using the Scion Image software. The columns represent the mean ± S.E.M. 

Statistical significance for multiple comparison was calculated with ANOVA and post hoc Scheffe’s test.
Fig. 2. Caspase and calpain activation by simvastatin in differentiated skeletal muscle cells. Differentiated human skeletal muscle cells were harvested at the indicated time points, and the cytosolic fraction (50 μg) was incubated with the 50 μM of the substrate for caspase 3 (A), caspase 9 (B), or caspase 8 (C) in caspase reaction buffer for 90 min at 37°C in the dark. The skeletal muscle cells were incubated in the absence (filled circle) and presence of 10 μM simvastatin (open triangles) or 10 μM simvastatin plus 1 mM mevalonic acid (filled square). After 36 h, cells treated with 0.1% DMSO (open diamond) were also prepared to control for the solvent carryover in the simvastatin sample. The filled triangle (B and C) shows the reaction buffer plus caspase substrate in the absence of cellular lysate. Caspase 8 activity was triggered with 10 ng/ml tumor necrosis factor-α (filled diamond) (C). The data represent mean values ± S.E.M. obtained from two independent experiments in triplicates that were repeated twice. Differentiated human skeletal muscle cells were also exposed to increasing concentrations (1, 10, and 30 μM) of simvastatin (SIM) and lovastatin (LOV) for 24 h and compared with control (CTL). Caspase 3 (D), caspase 9 (E), and calpain (F) activity were measured in the cytosolic fraction (50 μg) as described above. The columns represent mean values ± S.E.M. obtained from three to 12 independent experiments carried out in duplicates. Statistical significance for multiple comparison was calculated with ANOVA and post hoc Scheffe’s test (*, p < 0.05; **, p < 0.01; ***, p < 0.005; *****, p < 0.0001).
Simvastatin-Triggered Ca\textsuperscript{2+} Release Activates Calpain.

The proteolytic activity of calpain is activated by a long-lasting increase in the intracellular Ca\textsuperscript{2+} concentration (Goll et al., 2003). Therefore, we monitored the intracellular Ca\textsuperscript{2+} concentration in mass suspension. Under these conditions simvastatin triggered a sustained rise in the intracellular Ca\textsuperscript{2+} concentration (Fig. 4A). This amplitude of the Ca\textsuperscript{2+} response was comparable with that in the presence of extracellular EGTA, indicating that an intracellular Ca\textsuperscript{2+} release is triggered (Fig. 4B, open triangles). The simvastatin-triggered Ca\textsuperscript{2+} release did not return to basal resting Ca\textsuperscript{2+} concentrations but remained elevated. The amplitude of the Ca\textsuperscript{2+} transient was not attributable to an effect of the solvent, because dimethyl sulfoxide (DMSO) alone had no effect below 0.3%. Nevertheless, between 0.3 and 1.0% DMSO, a slight increase in the Ca\textsuperscript{2+} concentration was observed, which was subtracted from the Ca\textsuperscript{2+} release amplitudes at the respective simvastatin concentrations (Fig. 4B).

Concentrations of simvastatin above 10 \(\mu\)M immediately led to an elevation of the intracellular Ca\textsuperscript{2+} concentration. This may represent the prerequisite for the activation of calpain. Simvastatin-induced calpain activity was neither reversed by mevalonic acid nor by bongkrekic acid (Fig. 4C). In contrast, the simvastatin-induced calpain activity was prevented by various calpain inhibitors. This is exemplified for calpain inhibitor I in Fig. 4D. If a simvastatin-triggered Ca\textsuperscript{2+} transient initiated calpain activation, buffering of the intracellular Ca\textsuperscript{2+} elevation must have cytoprotective effects.

Chelation of the intracellular Ca\textsuperscript{2+} concentration with BAPTA-AM was indeed sufficient to prevent a reduction of cell number in the presence of simvastatin (Fig. 5A). In line with this finding, the simvastatin-induced activation of caspase 3, caspase 9, and calpain was abrogated by the co-administration of BAPTA-AM (Fig. 5, B–D). Interestingly, coadministration of ryanodine was also capable of preventing activation of the apoptotic-signaling cascade. However, it is unlikely that simvastatin directly activates the ryanodine receptor. The protective action of ryanodine is rather related to a block of the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release that amplifies the simvastatin-induced Ca\textsuperscript{2+} transient. We measured high-
affinity \[^{[3]H}\text{ryanodine binding, which is proportional to the opening of the ryanodine receptor and that is sensitive to ryanodine receptor channel openers (Hohenegger et al., 1996; Klinger et al., 1999). We did not detect any significant alteration in \[^{[3]H}\text{ryanodine binding by simvastatin or lovastatin at concentrations up to 300 \(\mu M\) (Fig. 6). Conversely, caffeine, a ryanodine receptor agonist, gave a significant stimulation of \[^{[3]H}\text{ryanodine binding. The activation of caspase 3 and caspase 9 by simvastatin was also prevented by the calpain inhibitor I (Fig. 5, B and C) or by a combination of calpain inhibitors I and II (data not shown). Together, these data show that the simvastatin-evoked elevation of intracellular \(Ca^{2+}\) levels suffices to activate calpain, which is upstream of the mitochondrial pathway of apoptosis.

**Discussion**

Statins are well tolerated, cholesterol-lowering drugs. Because of the high prevalence of hypercholesterolemia and because of their demonstrated efficacy, statins became one of the most widely prescribed classes of medications. However, in rare cases, myopathy, myositis, or rhabdomyolysis occurs during therapy (Bellosta et al., 2004; Rosenson, 2004). The incidence of skeletal muscle side effects is significantly increased by elevated dose regimes or by the concomitant administration of drugs that block CYP3A4 and thereby the degradation of some statins. These circumstances have led to the withdrawal of cerivastatin from the international market in 2001 (Griffin, 2001). The incidence of myopathies may be also facilitated by preexisting conditions such as diabetes mellitus, hypothyroidism, surgery, trauma, or excessive alcohol intake (Bellosta et al., 2004; Rosenson, 2004). A genetic linkage of individuals susceptible for statin induced myopathies has not been investigated. Interestingly, physical exercise is also an aggravating factor for the development of muscle pain, myositis, and CK elevation (Sinzinger and O’Grady, 2004). However, CK elevation is an unreliable parameter for the development and severity of a myopathy (Dujovne, 2002; Phillips et al., 2002; Rosenson, 2004). Patients have been described who generate symptoms of severe muscle pain and restriction in mobility despite normal serum CK levels (Phillips et al., 2002; Bellosta et al., 2004).

Here, we present a signaling cascade in differentiated human skeletal muscle cells that is triggered by simvastatin-induced \(Ca^{2+}\) transients and is capable of explaining the phenomena described above. This evidence is based on the following observations. 1) Simvastatin and lovastatin induced apoptosis in human skeletal muscle cells in a concentration- and time-dependent manner (Figs. 1 and 2). Whereas a high simvastatin concentration of 10 \(\mu M\) triggered the mitochondrial pathway within 24 h, similar effects were seen with lower concentrations only after 36 or 48 h (data not shown). 2) The statin-induced reduction in cell number is in part abrogated by mevalonic acid. The fact that only 30 to 50% of the reduction in viable cells was due to inhibition of HMG-CoA reductase inhibition is indicative of an additional, mevalonate-independent mechanism underlying simvastatin induced cell death. 3) The application of simvastatin to fura-2-loaded skeletal muscle cells initiated a long-lasting \(Ca^{2+}\) transient that led to activation of calpain. 4) Abrogation of the intracellular \(Ca^{2+}\) elevation was sufficient to prevent activation of calpain and caspases 9 and 3. Moreover, the chelation of intracellular \(Ca^{2+}\) completely rescued skeletal muscle cells from simvastatin-induced cell death. A rough estimate of the \(EC_{50}\) value for simvastatin and lovastatin triggered caspase activity is in the range of 1 to 5 \(\mu M\) (Fig. 2, D and C). An earlier observation in neonatal rat skeletal myocytes shows that cell viability, monitored by mitochondrial dehydrogenase activity, is inhibited half-maximally at 21.2 \(\mu M\) simvastatin and 20.2 \(\mu M\) lovastatin, whereas the \(IC_{50}\) for simvastatin and lovastatin to inhibit the HMG-CoA reductase was between 0.09 and 0.12 \(\mu M\) (Mas-
Statin-induced apoptosis via the activation of calpain is clearly in line with previous observations that the Ca\(^{2+}\)-dependent protease calpain may trigger apoptosis (Goll et al., 2003). In the case of differentiated human skeletal muscle cells, calpain is clearly upstream of the mitochondrial pathway. A cell-permeable calpain inhibitor was able to overcome the activation of calpain and the mitochondrial pathway of apoptosis (Fig. 5). The chelation of intracellular Ca\(^{2+}\) was also sufficient to prevent the simvastatin-induced calpain activation as well as activation of the caspases 9 and 3. Strikingly, such a treatment kept the number of viable cells identical to that of the control (Fig. 5). Conversely, coadministration of mevalonic acid was not capable of abrogating simvastatin-induced reduction in cell numbers (Fig. 1B) and calpain activation. A possible target of such a mevalonate-independent trigger mechanism of apoptosis is via the Ca\(^{2+}\) release channel of the sarcoplasmic reticulum, the ryanodine receptor. This interpretation is strengthened by the finding that in the absence of extracellular Ca\(^{2+}\), the amplitude of the simvastatin induced Ca\(^{2+}\) release is comparable with that in the presence of extracellular Ca\(^{2+}\) (Fig. 4B). Moreover, the plant alkaloid ryanodine could block the simvastatin-induced activation of calpain. Nevertheless, a direct activation of the ryanodine receptor by simvastatin is unlikely for the following reasons. 1) High-affinity \(^{3}H\)ryanodine binding to sarcoplasmic reticulum fractions revealed no activation of the ryanodine receptor by simvastatin (Fig. 6). Possibly, simvastatin may bind to a cytoplasmic target protein that then may activate the ryanodine receptor. In such a case, an activation in the \(^{3}H\)ryanodine binding would not be seen because we used sarcoplasmic reticulum membranes. 2) It also has to be mentioned that due to limited human cell material, the above-described binding studies have been done with rabbit skeletal muscle. However, it cannot be excluded that species differences may account for the absence of a statin effect on the \(^{3}H\)ryanodine binding. 3) Elevation of the cytoplasmic Ca\(^{2+}\) concentration could be triggered by a statin-induced inhibition of the Ca\(^{2+}\) ATPase of the sarcoplasmic reticulum. Such a scenario, however, does not explain the protective feature of ryanodine to prevent calpain and caspase activation by simvastatin (Fig. 5). It is therefore clear that intensive effort has to be undertaken to address the exact target of simvastatin induced Ca\(^{2+}\) release. Nakahara and colleagues investigated in L6 rat myoblasts simvastatin induced Ca\(^{2+}\) transients. A target for the described Ca\(^{2+}\) fluxes has not been elucidated. Besides an intracellular Ca\(^{2+}\) release mechanism, they also described a Ca\(^{2+}\) influx, which together may contribute to cellular damage (Nakahara...
et al., 1994). In human skeletal muscle cells, a Ca\(^{2+}\) influx plays only a minor role, because we readily can detect a simvastatin-induced Ca\(^{2+}\) release in the presence of extra-cellular EGTA (Fig. 4B). The amplitudes of the Ca\(^{2+}\) was determined with Student's test of three experiments carried out in duplicates. Statistical significance was determined with Student's t test.

The therapeutic administration of 40 mg of simvastatin per day has been summarized to give peak plasma levels of 10 to 34 ng/ml (~25–80 nM) (Bellosta et al., 2004). Due to CYP3A4 inhibition, these plasma levels may increase by a factor of 10 to 15 and may reach at maximum 1.2 μM simvastatin. Interestingly, in a phase I dose finding study lovastatin was administrated to give plasma concentrations of up to 3.9 μM to treat cancer patients (Thibault et al., 1996). The authors report that myopathy was the dose-limiting factor. Interestingly, the reduction of ubiquinone was transient and not dose-dependent, but substitution with ubiquinone reduced and rescued the individuals from muscular side effects. The elevated statin concentrations described here are close to the concentration range where we observed half-maximum caspase and calpain activation. Moreover, caspase 3, caspase 9, and calpain were already activated by 1 μM simvastatin significantly; thus, underlining the clinical relevance of this study (Fig. 2, D–F).

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