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Effects of HMG-CoA reductase inhibitors on growth and differentiation of cultured rat skeletal muscle cells

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Abstract

HMG-CoA reductase inhibitors have been associated with skeletal muscle myopathy, ranging from asymptomatic elevations of serum creatine kinase (CK) activity to rhabdomyolysis. In this study, we assessed the effects of addition of different concentrations of simvastatin and pravastatin on growth and differentiation of cultured primary rat skeletal muscle cells. Protein concentration, CK activity and percentage CK-MM, which is a parameter for maturation, were determined. Effects were generally stronger if inhibitors were added to both growth and differentiation medium rather than only to differentiation medium. Addition of 25 μ M pravastatin caused only a decrease of CK activity. Addition of 1–5 μ M simvastatin resulted in a decrease of protein concentration, CK activity and percentage CK-MM, whereas 25 μ M simvastatin resulted in cell death. Addition of mevalonic acid or cholesterol could not prevent the effects of 1 μ M simvastatin. In addition, 1 μ M simvastatin did not influence the cholesterol and phospholipid content of the cells. Superfusion of cultured cells with simvastatin concentrations of 10 μ M and higher caused a transient increase of the cytoplasmic calcium concentration followed by an apparent second rise and cell puncture. The results indicate that HMG-CoA reductase inhibitors may affect skeletal muscle cell regeneration in vivo by a direct toxic effect on growth and differentiation.

Keywords: HMG-CoA reductase inhibitor; Inhibitor; Growth; Differentiation; (Rat skeletal muscle cell)

1. Introduction

Inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase have gained an important place in the treatment of hypercholesterolaemia. They inhibit the conversion from HMG-CoA into mevalonate, the rate-limiting step in cholesterol synthesis, and thereby reduce the intracellular pool of cholesterol. This leads to an enhanced synthesis of receptors for low density lipoprotein (LDL) cholesterol and increased clearance of these particles from the circulation [1–4]. The current understanding for the mechanism of the hypolipidemic and antiatherosclerotic action of HMG-CoA reductase inhibitors has recently been discussed [5].

Concern has risen about adverse effects on skeletal muscle during treatment with these drugs, ranging from asymptomatic elevations of serum creatine kinase (CK) activity and myopathy to rhabdomyolysis [6–14]. Myopathy (muscle tenderness and CK levels exceeding more than 10 times the upper limit of normal) has been found especially during combination therapy with gemfibrozil [6–9]. Rhabdomyolysis has been observed in combinations with erythromycin, niacin or cyclosporin [10–14]. Cyclosporin increases systemic levels of HMG-CoA reductase inhibitors due to inhibition of their biliary excretion [11]. In animals, severe muscle damage can be induced by HMG-CoA reductase inhibitors [15–18] especially when combined with cyclosporin [15]. The lipophilic HMG-CoA reductase inhibitor simvastatin seems to have more pronounced effects than the hydrophilic compound pravastatin [17].

To evaluate the effects of HMG-CoA reductase inhibitors on skeletal muscle, the influence of hydrophilicity of the drug and the potential mechanism for skeletal muscle damage, we assessed the effects of simvastatin and

Abbreviations: CK, creatine kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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pravastatin on growth and differentiation of cultured rat skeletal muscle cells, and if present, whether these effects are related to inhibition of synthesis of mevalonate and/or cholesterol.

2. Materials and methods

2.1. Materials

Ultrosor G was purchased from Gibco (Brussels, Belgium). Simvastatin and pravastatin were obtained from Merck, Sharp and Dohme and Bristol Myers Squibb, respectively. Mevalonic acid and Fura-2/AM were purchased from Sigma Chemical Co. (St. Louis, MO). Brain extract was prepared from brains of 10-day-old Wistar rats. Homogenates (100 mg/ml Dulbecco's modified Eagle's medium (DMEM)) were centrifuged for 60 min at $100\,000 \times g$ (0–4°C). The supernatant was stored at –70°C until use.

2.2. Rat muscle cell cultures

Satellite cells were isolated from quadriceps muscle of 10-day-old Wistar albino rats. The muscle (about 100 mg) was dissociated according to the dispersion technique of Yasin et al. [19]. Mononuclear cells (5×10^4) were plated in 35-mm plastic tissue culture dishes in DMEM medium containing 20% foetal calf serum, 2% chicken embryo extract, and 4 mM glutamine. Cells were cultured at 37°C in a humidified CO₂ (5%) atmosphere. After one day, non-adhering cells and cell debris were discharged by washing twice with the same medium. Subsequently, cells were cultured for 5–6 days to confluency in DMEM/4 mM glutamine containing 4% Ultrosor G and 10% rat brain extract. Differentiation took place for 6–7 days in

DMEM/4 mM glutamine medium containing 0.4% Ultrosor G and 10% rat brain extract.

Indicated concentrations of pravastatin, simvastatin, mevalonic acid or cholesterol were added at day 1 to growth medium and/or at confluency to differentiation medium.

2.3. Biochemical assays

Protein content was assayed according to Lowry et al. [20] with bovine serum albumin as standard. CK activity was assayed as previously described [21]. For the determination of the percentage CK MM (CK-MM) as a measure of the maturation grade of muscle cells, this isoenzyme was separated from the other CK isoenzymes by anion exchange chromatography and analysed as reported [21]. Lipids were extracted according to Bligh and Dyer [22]. Total cholesterol was determined by an enzymatic method (CHOD-PAP, Sigma, St. Louis). Free and esterified cholesterol were determined as described before [23]. Phospholipids were assayed on the basis of lipid-phosphorus content [24] and phospholipid composition by two-dimensional TLC [25].

2.4. Ca²⁺ homeostasis assays

The free cytosolic Ca²⁺ concentration [Ca²⁺]_i was determined in cultured cells using Fura-2 ratio measurement [26]. Muscle cells were grown on glass coverslips (30 × 10 mm) in sera-containing proliferation and differentiation media [27]. Myotubes were washed with physiological salt solution (PSS, containing in mM: 125 NaCl, 10 NaHCO₃, 1 NaH₂PO₄, 5 KCl, 2 MgSO₄, 1.8 CaCl₂, 10 Hepes and 10 glucose, pH 7.4) and loaded with 5 μM Fura-2/AM in PSS for 60 min at 37°C in the presence of 5% CO₂ and 95% air. Excess of dye was removed by

Table 1
Effects of pravastatin and simvastatin on cultured rat skeletal muscle cells

Addition to:		<i>n</i>	Protein		CK activity		CK-MM
GM	DM		μg/dish	%	U/ mg protein	%	% of total activity
Control							
–	–	8	228 ± 99	100	0.98 ± 0.57	100	73 ± 11
Pravastatin (μM)							
–	25	5	208 ± 89	99 ± 16	0.60 ± 0.37	60 ± 22 ^a	76 ± 10
25	–	4	208 ± 113	95 ± 15	0.83 ± 0.60	76 ± 30	75 ± 13
25	25	5	207 ± 132	91 ± 14	0.43 ± 0.12 ^a	47 ± 21 ^b	69 ± 10
Simvastatin (μM)							
–	0.5	4	196 ± 93	91 ± 7	0.69 ± 0.49	67 ± 32	72 ± 15
0.5	0.5	4	191 ± 94	91 ± 29	0.39 ± 0.13 ^a	48 ± 38 ^a	62 ± 12
–	1	4	194 ± 113	88 ± 11	0.62 ± 0.32	60 ± 20 ^a	72 ± 10
1	1	7	169 ± 107	73 ± 19 ^a	0.36 ± 0.14 ^a	48 ± 27 ^b	54 ± 7 ^b
–	5	4	148 ± 111	62 ± 14 ^a	0.30 ± 0.24 ^a	33 ± 25 ^a	60 ± 17
5	5	4	120 ± 85	51 ± 13 ^b	0.32 ± 0.21 ^a	36 ± 25 ^a	51 ± 14 ^a

Parameters were determined after 6–7 days in differentiation medium. Creatine kinase MM activity is given as a percentage of total CK activity. Values are means ± S.D. of the indicated number (*n*) of independent experiments.

GM, growth medium; DM, differentiation medium. Significance: ^a *P* < 0.05, ^b *P* < 0.01 vs control.

washing thrice with PSS. Fluorescence was measured with a Shimadzu RF-5000 spectrofluorophotometer at an emission wavelength of 492 nm and alternating excitation wavelengths of 340 and 380 nm [26]. During the measurement the cells were superfused with PSS (4 ml/min; 37°C) with additions of simvastatin as indicated in Section 3. $[Ca^{2+}]_i$ was calibrated with 4 μ M ionomycin in the presence of 10 mM Ca^{2+} (pH 7.7) or 20 mM EGTA (pH 8.5).

2.5. Statistical analyses

Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test. A two-tailed $P < 0.05$ was considered significant.

3. Results

Optimal conditions for proliferation and differentiation of rat muscle cells were applied as previously described [27]. Relatively large variations are present in the protein content and the CK activity of different experiments due to culture variations and different amounts of contaminating fibroblasts. Therefore we calculated and compared their relative values in the presence of pravastatin, simvastatin and other additions. The percentage of CK-MM, a marker of muscle cell maturation, showed slight variability between the different experiments.

The effects of addition of different concentrations of simvastatin and pravastatin on protein content, CK activity and percentage CK-MM of cultured rat skeletal muscle cells are given in Table 1. In all cases, effects were stronger if inhibitors were added to both growth and differentiation medium, than only to differentiation medium. Pravastatin had only limited effects, even when added in high concentrations (25 μ M). The CK activity was 60% of control when this concentration was present in differentiation medium alone and 47% when present both in growth and differentiation medium. The percentages of CK-MM were unchanged as were the protein yields. In contrast, simvastatin had strong effects. In experiments

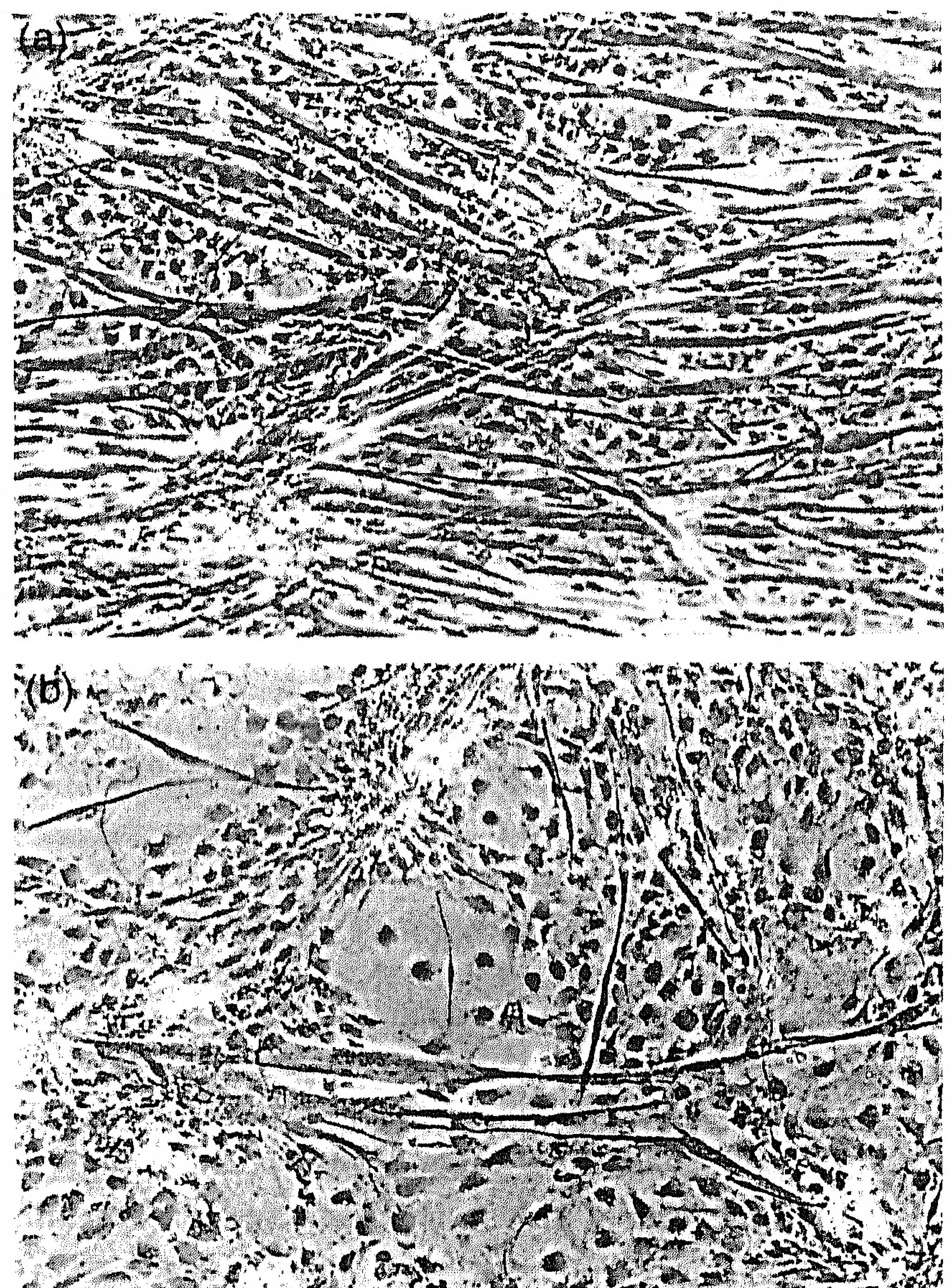


Fig. 1. Morphology of cultured rat skeletal muscle cells after 5 days in growth medium and 6 days in differentiation medium without (a) and with (b) 1 μ M simvastatin. The presence of this compound caused the occurrence of less and shorter myotubes.

using 25 μ M simvastatin, cell growth arrested and all cells detached from the surface. The presence of 1 μ M simvastatin caused a marked inhibition of myotube formation (Fig. 1). The biochemical analyses reflect the morphological observations. Addition of 0.5 μ M simvastatin to both growth and differentiation medium resulted in a significantly decreased CK activity. Concentrations of 1 and 5 μ M simvastatin resulted in significant decreases of protein content and CK activity. The percentage of CK-MM de-

Table 2
Effects of mevalonate and cholesterol on rat skeletal muscle cells cultured in the presence of simvastatin

Addition	Protein		CK activity		CK-MM
	μ g/dish	%	U/ mg protein	%	% of total activity
None	147 \pm 25	100	0.41 \pm 0.22	100	30 \pm 18
10 μ M mevalonate	126 \pm 21	92 \pm 2	0.27 \pm 0.03	94 \pm 2	21 \pm 5
25 μ M mevalonate	114 \pm 12	83 \pm 7	0.28 \pm 0.06	98 \pm 11	21 \pm 8
50 μ M mevalonate	123 \pm 35	84 \pm 18	0.45 \pm 0.32	93 \pm 4	32 \pm 19
10 μ M cholesterol	124 \pm 42	84 \pm 20	0.34 \pm 0.17	82 \pm 5	28 \pm 13

Simvastatin (1 μ M) and indicated concentrations of mevalonate or cholesterol were added to both growth and differentiation medium. Parameters were determined after 6–7 days in differentiation medium. Creatine kinase MM activity is given as a percentage of total CK activity. Values are means \pm S.D. of 3 independent experiments. Values did not change significantly at addition of mevalonate or cholesterol.

Table 3
Effects of simvastatin on lipid content and composition of rat skeletal muscle cells

	Control	Simvastatin
Total cholesterol	79.3 ± 3.2	94.3 ± 14.6
Free cholesterol	58 ± 12	48 ± 7
Esterified cholesterol	42 ± 12	52 ± 7
Total phospholipids	193 ± 25	253 ± 27
Phosphatidylinositol	11 ± 2	10 ± 1
Phosphatidylserine	13 ± 2	11 ± 2
Sphingomyeline	14 ± 1	15 ± 1
Phosphatidylcholine	40 ± 2	40 ± 3
Phosphatidylethanolamine	22 ± 2	24 ± 2

Simvastatin (1 μM) was added to both growth and differentiation medium. Parameters were determined after 6–7 days in differentiation medium. Total cholesterol and phospholipids are given in nmol/mg protein, separate components in % of total. Values are means \pm S.D. of 3 independent experiments and are not significantly different for simvastatin-exposed cells.

creased significantly after addition of 1 and 5 μM simvastatin to both growth and differentiation medium.

As shown in Table 2, the effects of addition of 1 μM simvastatin to growth and differentiation medium could not be reversed by concomitant addition of either mevalonic acid or cholesterol. In Table 3, the effects of addition of 1 μM simvastatin to growth and differentiation medium on lipid content of rat muscle cells are given. No indications for a decreased cholesterol content or changes in the ratio free/esterified cholesterol were found. The content of total phospholipid was not decreased either, nor were significant differences observed in the phospholipid composition and the cholesterol/phospholipid ratio.

The presence of brain extract in the medium was not the cause of the absence of counteracting effects of mevalonate and cholesterol. Omission of brain extract from growth and differentiation medium caused a decrease of protein concentration, CK activity and CK-MM percentage of control and simvastatin-treated cells. However, simvastatin addition had a comparable effect as on cells cultured with brain extract, which could not be restored by addition

Table 4
Effects of simvastatin on the cytoplasmic calcium concentration of cultured rat skeletal muscle cells

Concentration simvastatin (μM)	Extracellular calcium	
	1.8 mM	absent
0	132 ± 18 (5)	128 ± 14 (3)
1	126 ± 19 (4)	134 ± 15 (3)
10	369 ± 51 (4) ^a	133 ± 22 (3)
50	487 ± 63 (5) ^a	131 ± 17 (3)

Cultured cells (after 6–7 days of differentiation) were superfused with PSS containing the indicated simvastatin concentration. In superfusions without extracellular calcium, 1 mM EGTA was present. The $[\text{Ca}^{2+}]_i$ was measured by the Fura-2 ratio method. Values of $[\text{Ca}^{2+}]_i$ (in nM) are means \pm S.D. of the number of cultures given between parentheses. Significance: ^a $P < 0.01$ vs 0 μM simvastatin.

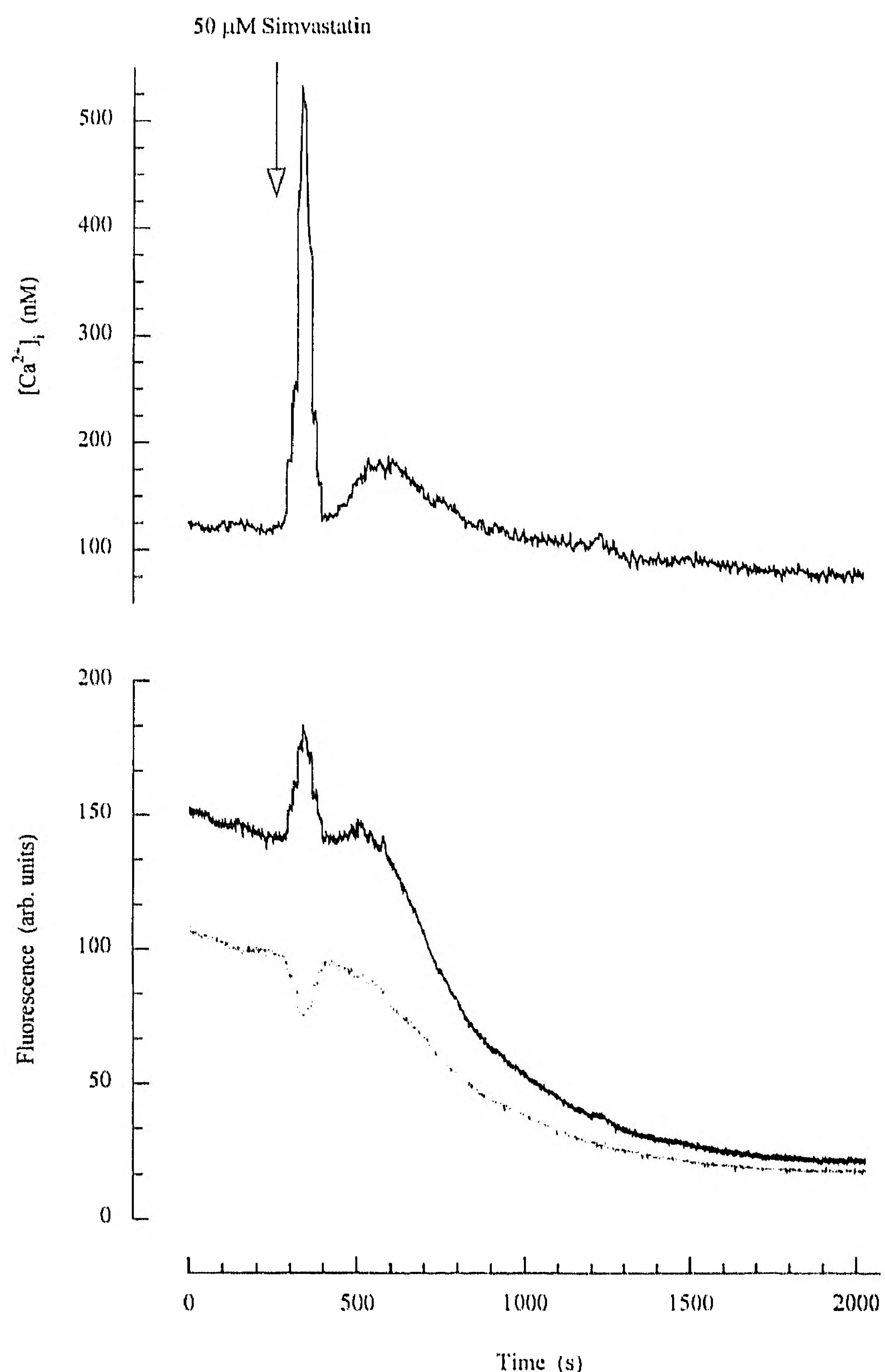


Fig. 2. Effect of superfusion of cultured rat skeletal muscle cells with 50 μM simvastatin in PSS. Above: the cytoplasmic calcium concentration $[\text{Ca}^{2+}]_i$ measured with the Fura-2 ratio method. Below: the fluorescence of Fura-2 at the two excitation wavelengths 340 nm (fixed line) and 380 nm (dotted line). Two apparent peaks of transient increase of $[\text{Ca}^{2+}]_i$ are visible. The first is a specific increase, the second is caused by leaking Fura-2. The simultaneous decrease of the fluorescence at both wavelengths after about 600 s indicates cell damage and leakage of Fura-2 which disappears at superfusion.

of 50 μM mevalonate or 10 μM cholesterol to both media (data not shown). Higher concentrations of mevalonate or cholesterol were not tested. The Ultrosor G, indispensable for culturing, contributes to the growth and differentiation medium 25 μM and 2.5 μM cholesterol, respectively.

The effect of simvastatin on the cytoplasmic calcium concentration $[\text{Ca}^{2+}]_i$ and cell damage was studied in cultured cells using Fura-2 ratio measurement. Addition of 1 μM simvastatin had no effect on $[\text{Ca}^{2+}]_i$, but 10–50 μM caused a dose-dependent transient increase (Table 4). In contrast to physiological stimulation with acetylcholine [26], this increase did not occur in the absence of extracellular Ca^{2+} and presence of 1 mM EGTA and is thus from extracellular origin. If cells were maintained in the presence of 10–50 μM simvastatin the $[\text{Ca}^{2+}]_i$ increased a

second time and decreased subsequently, but the measurement of Fura fluorescence showed a decrease at both wavelengths (340 and 380 nm), indicating the leakage of Fura through the cell membrane (Fig. 2). The time interval between the two peaks of Ca^{2+} depended on the simvastatin concentration. Similar results were obtained with cultured human skeletal muscle cells as with those from rat.

4. Discussion

The present study was performed to get insight into the effects of HMG-CoA reductase inhibitors during growth and proliferation of skeletal muscle cells. The lipophilic compound simvastatin had substantial effects on protein yield, CK activity and percentage of CK-MM. In high concentration, simvastatin caused cell puncture and in culture cell detachment and death. In contrast, pravastatin had only a limited effect. Exposure of 8-day-old cultures of rat myotubes for 48 h to lovastatin, simvastatin or pravastatin also showed the lowest myotoxicity of the last, hydrophilic drug [28]. Pravastatin was also much less inhibitory in various other types of human extrahepatic cells [29–32]. These differences are related to the active uptake mechanism of that drug into hepatocytes in contrast to into other cell types. The 48-h exposure of 8-day-old cultures to simvastatin at a concentration of $4.8 \mu\text{M}$ or higher caused morphologic injury, including vacuolization, changes in cell contour and disappearance of myotubes [28]. Protein synthesis and intracellular ATP concentration were reduced to 50% of control values at 2 and $4 \mu\text{M}$ simvastatin, but CK activity at $> 24 \mu\text{M}$ and cholesterol synthesis at $0.1 \mu\text{M}$. Mevalonate ($19\text{--}100 \mu\text{M}$) reversed the inhibition of protein synthesis at $2 \mu\text{M}$ simvastatin. Although other parameters were assayed, effects of simvastatin are much larger in our muscle cells, especially if the drug was present during both proliferation and differentiation for 11–13 days. The presence of $0.5\text{--}1 \mu\text{M}$ simvastatin already caused an inhibition by 52% of CK activity. The addition of $10\text{--}50 \mu\text{M}$ mevalonate did not reverse the decrease of CK activity and of CK-MM percentage.

HMG-CoA reductase inhibitors as simvastatin, fluvastatin and lovastatin inhibit proliferation of arterial wall smooth muscle cells, glioma cells and mamma tumor cells [29–35]. These effects could be prevented or restored by addition of mevalonate, but not by cholesterol. Mevalonate is not only a precursor for cholesterol, but also delivers isoprenoid groups for synthesis of farnesyl- and geranyl-pyrophosphate. These molecules are substrates for post-translational modification of proteins (as, e.g., Ras proteins, nuclear lamins and G-proteins, and for synthesis of dolichol and ubiquinone, that have important regulatory functions in cell metabolism and growth [36–38].

Lovastatin and mevastatin inhibited the fusion of L6 rat myoblasts and the concurrent increase of CK activity [39]. A cause could be the lack of dolichol phosphate oligosaccharide for glycoprotein synthesis [39]. The decreased isoprenylation of proteins and the decrease of coenzyme Q could be other mechanisms of interference with cell growth [38,40]. In addition, shortage of cholesterol could affect cell membrane stability. However, in the present study, the additions of mevalonate and cholesterol were not able to prevent the effects of simvastatin on the cultured skeletal muscle cells. Moreover, addition of simvastatin to the medium did not change the cholesterol and phospholipid content of the cells. Apparently, another mechanism, not directly coupled to inhibition of mevalonate formation, plays a role. Recently it has been suggested that disruption of early events in insulin signalling is a potential mechanism for the antimitogenic activity of lovastatin [41]. The concentration of lovastatin necessary to induce growth inhibition and apoptosis is a thousand-fold higher in glioma cells than in normal glial cells [42]. So a specific sensitivity of cells seems to be involved. Lack of products from HMG-CoA may partially be compensated in our cultured cells by the addition of brain extract to the medium, but also in its absence the same changes were observed.

Plasma cholesterol-lowering drugs as clofibrate and 20,25-diazcholesterol have myotoxic effects as well, and induce myotonic discharges [43,44], related to a decreased chloride conductance of the muscle cell membrane [45]. Simvastatin also induced myotonia in rabbits due to a decreased chloride conductance [46]. High concentrations of simvastatin caused a transient increase in $[\text{Ca}^{2+}]_i$ of L6 rat myoblasts followed by cell puncture [47] like we found in short-time experiments and in culture on primary rat myotubes. Pravastatin and lower concentrations of simvastatin had no measurable effect. Cardiomyocytes showed a similar acute increase of $[\text{Ca}^{2+}]_i$ at $20\text{--}50 \mu\text{M}$ simvastatin [48]. Lovostatin ($1 \mu\text{M}$) in the medium depressed Ca^{2+} channel activity in cardiac cells in culture and uncoupled excitation from contraction [49]. Thus addition of simvastatin to the culture medium causes dose-dependent effects on $[\text{Ca}^{2+}]_i$ which influence cellular growth and function.

In conclusion, the present study demonstrates the inhibitory effects of lipophilic HMG-CoA reductase inhibitors on growth and differentiation of cultured muscle cells. The absence of a preventive effect of mevalonate and the unchanged lipid composition suggest that other mechanisms than inhibition of HMG-CoA reductase play a role. In usual situations of patients, systemic plasma concentrations of HMG-CoA reductase inhibitors are considerably lower than the concentrations used in the present study [50,51], and may not lead to muscle damage. Increased systemic concentrations, as during combination therapy with cyclosporin may especially affect regeneration of muscle cells after exercise-induced damage and in this way lead to myopathy.

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