

RESEARCH PAPER

Interactions between cell death induced by statins and 7-ketocholesterol in rabbit aorta smooth muscle cells

W Martinet¹, DM Schrijvers¹, J-P Timmermans² and H Bult³

¹Division of Pharmacology, Department of Pharmaceutical Sciences, University of Antwerp, Wilrijk, Belgium; ²Laboratory of Cell Biology & Histology, Department of Veterinary Sciences, University of Antwerp, Antwerp, Belgium and ³Division of Pharmacology, Faculty of Medicine, University of Antwerp, Wilrijk, Belgium

Background and purpose: 7-Ketocholesterol, an oxysterol present in atherosclerotic lesions, induces smooth muscle cell (SMC) death, thereby destabilizing plaques. Statins protect patients from myocardial infarction, though they induce SMC apoptosis. We investigated whether statins and 7-ketocholesterol exerted additive cell death effects.

Experimental approach: Cultured rabbit aorta SMCs (passage 2–6) were exposed to 7-ketocholesterol with or without fluvastatin, simvastatin or pravastatin. Uptake of neutral red (NR), monolayer protein, cleavage of the pan-caspase substrate Asp-Glu-Val-Asp-rhodamine110, cell morphology (light and electron microscopy) and processing of microtubule-associated protein 1 light chain 3 (LC3, immunoblot) were determined.

Key results: NR uptake declined upon 18 h exposure to 25 μM 7-ketocholesterol ($-41 \pm 3\%$, $n = 13$), 100 μM fluvastatin (-59%) or 30–100 μM simvastatin (-28 to -74%). Oxysterol and high statin concentrations exerted additive effects, but lower concentrations (fluvastatin 10–30 μM , simvastatin 1–10 μM) partly reversed viability loss. 7-Ketocholesterol caused intense cytoplasmic vacuolization, processing of LC3-I to LC3-II, but little caspase activation (increase 29.5%). Fluvastatin (10–100 μM , 70–545% increase) and simvastatin (3–100 μM 43–322% increase) induced caspase activation without LC3 processing, but failed to activate caspases in 7-ketocholesterol-treated SMCs. Pravastatin up to 100 μM was always inactive.

Conclusions and implications: 7-Ketocholesterol caused SMC death, mainly via autophagic vesicle formation with LC3 processing, whereas lipophilic statins evoked SMC apoptosis. Cell death following 7-ketocholesterol and low statin concentrations were not additive, presumably because the autophagic process interfered with statin-induced caspase activation. This further illustrates that drug effects in normal SMCs are not necessarily predictive for activities in atherosclerotic settings.

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Keywords: atherosclerosis; autophagy; apoptosis; statins; fluvastatin; pravastatin; simvastatin; vascular smooth muscle cell; viability

Abbreviations: DEVD-R110, Asp-Glu-Val-Asp-rhodamine 110; FBS, fetal bovine serum; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LC3, microtubule-associated protein I light chain 3; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; SMC, smooth muscle cell

Introduction

Atherosclerosis is a protracted inflammatory disease in the intima of large arteries, leading to the formation of atheromatous plaques. The typical lesion shows a necrotic core surrounded by a fibrous cap consisting of smooth muscle cells (SMCs) in a collagen–proteoglycan matrix (Virmani *et al.*, 2000). A thin fibrous plaque atheroma is characterized by trimming of the fibrous cap that is

infiltrated by macrophages and T cells with few SMCs, and an increase of extracellular lipids and necrotic core formation. Such plaques are vulnerable and at risk of rupture, thereby evoking atherothrombosis, myocardial infarction or stroke. Several factors have been proposed to increase the risk of plaque rupture, such as increased collagen degradation by matrix metalloproteinases secreted by macrophages, or decreased collagen biosynthesis because of suppression of SMC activity by interferon- γ , a cytokine produced by T cells.

The disappearance of SMCs from the fibrous cap through apoptosis or other forms of cell death could also jeopardize plaque stability (Clarke *et al.*, 2006). SMC death is seen in the

Correspondence: Professor H Bult, Division of Pharmacology, University of Antwerp, Universiteitsplein 1, Wilrijk, Antwerp B-2610, Belgium.
E-mail: hidde.bult@ua.ac.be

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vicinity of macrophage-rich regions of human atherosclerotic plaques (Kockx *et al.*, 1996, 1998). Factors that are known to kill SMCs can be macrophage derived (nitric oxide (NO), Fas ligand and tumour-necrosis factor- α) or products formed during oxidative modification of lipoproteins or cholesterol. Indeed, oxysterols, such as 7-ketocholesterol, may induce necrosis (Ghelli *et al.*, 2002; Seye *et al.*, 2004), apoptosis or type I cell death (Lizard *et al.*, 1996, 1997; Nishio and Watanabe, 1996; Seye *et al.*, 2004) or autophagy (Martinet *et al.*, 2004) in vascular cells. Autophagic or type II cell death is a caspase-independent form of programmed cell death (Gozuacik and Kimchi, 2004; Ferraro and Cecconi, 2007). Processing of microtubule-associated protein I light chain 3 (LC3) from the cytoplasmic form (LC3-I) to a membrane-associated form (LC3-II) is essential for the formation of autophagic vesicles and leads to increased electrophoretic LC3 mobility on SDS-polyacrylamide gels (Gozuacik and Kimchi, 2004; Ferraro and Cecconi, 2007).

Generation of reactive oxidative species, mitochondrial stress, translocation of the pro-apoptotic protein Bax and release of cytochrome *c* have been implied in 7-ketocholesterol-induced SMC death (Lizard *et al.*, 1998, 2000; Seye *et al.*, 2004). Moreover, oxysterols may also reduce 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity, particularly when culturing mammalian cells with lipoprotein-deficient media (Schroepfer, 2000; Miguet *et al.*, 2001). HMG-CoA reductase is the rate-limiting enzyme in the biosynthesis of cholesterol and of products involved in prenylation or farnesylation of several important membrane-bound proteins.

Statins inhibit HMG-CoA reductase as well, and several large clinical trials have documented that statins reduce death from coronary disease or stroke by 24–42% (Maron *et al.*, 2000; Palinski and Napoli, 2002). In addition to lowering LDL cholesterol, the benefit has been attributed to several other effects of statins, such as improved plaque stability, endothelial cell function or antithrombotic and anti-inflammatory effects. However, lipophilic statins have also been reported to induce apoptotic death of vascular SMCs in culture (Baetta *et al.*, 1997; Guijarro *et al.*, 1998; Werner *et al.*, 2004) and to render cells susceptible to induction of programmed cell death by Fas ligand (Knapp *et al.*, 2000). These pro-apoptotic effects seem paradoxical, as SMC death is likely to increase the risk of plaque rupture. Therefore, this study investigated whether 7-ketocholesterol and statins exerted additive effects on SMC morphology, viability (uptake of neutral red; Babich and Borenfreund, 1990), cell loss (protein), mitochondrial function (reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; MTT), apoptotic processes (annexin V binding and caspase activation) or autophagy (autophagosome formation and conversion of LC3-I to LC3-II).

Methods

Smooth muscle cell culture

The studies were approved by the Ethical Committee of the University of Antwerp. SMCs were isolated from rabbit aortas by collagenase and elastase digestion and cultured in Ham

F10 medium (Gibco Ltd, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 μmL^{-1} , streptomycin 100 $\mu\text{g mL}^{-1}$ and gentamycin 100 $\mu\text{g mL}^{-1}$; Seye *et al.*, 2004). More than 95% of cells were α -SMC actin positive. SMCs (passage 2–6) were seeded in plates (Beckton Dickinson (BD) Biosciences, Erembodegem, Belgium) with 24 wells (50 000 cells) or 6 wells (150 000 cells, flow cytometry) in 3 mL Ham F10 with 10% FBS. After 48 h, the SMCs received 3 mL fresh medium with 2% FBS and 15 μL phosphate-buffered saline, 0.5% ethanol (control) or 6.25, 12.5, 25 or 50 μM 7-ketocholesterol with or without 10^{-8} – 10^{-4} M fluvastatin, simvastatin or pravastatin for 18 or 24 h.

Neutral red assay

Neutral red (300 μL , 0.1%) was added to the well 16 or 22 h after addition of 7-ketocholesterol. Two hours later, cells were examined by light microscopy. Thereafter medium and detached cells were removed, adherent cells were washed twice with phosphate-buffered saline, and neutral red was extracted with 150 μL NaH_2PO_4 (0.05 M) in 50% ethanol to measure absorbance at 540 nm (A540) in duplicate. Monolayer protein was precipitated with 5% trichloroacetic acid, dissolved in 1 mL NaOH (0.1 M) with 0.5% SDS to measure protein content in duplicate (BCA assay; Pierce, Perbio Science Belgium NV/SA, Erembodegem, Belgium).

Within each culture plate, the values of A540 were normalized to control (0 μM 7-ketocholesterol and 0 μM statin) according to the equation: $A540_{\text{sample}}(\%) = A540_{\text{sample}}/A540_{\text{control}} \times 100$. Because the amount of neutral red in monolayers is determined by both cell mass and accumulation in individual cells (Babich and Borenfreund, 1990) (*vide infra*), a viability index was calculated: $\text{Viability} = \text{nmol neutral red} \cdot \text{per } \mu\text{g protein}$, using a calibration curve (0–200 μM neutral red) to convert A540 into nmol neutral red.

Regression analysis showed that intracellular neutral red accumulation (nmol) was a linear function of cell number (range: 10 000–300 000): $\text{Neutral red (nmol)} = 4.1 (3.8\text{--}4.4) n \times 10^{-3} + 8 (-13 \text{ to } 29)$, with n = number of SMCs and (95% confidence intervals). Similarly, protein content of the monolayer was a linear function of SMC number: $\text{Protein } (\mu\text{g}) = 0.57 (0.50\text{--}0.63) n \times 10^{-3} + 0.7 (-4.2 \text{ to } 5.5)$. In contrast, the viability index (nmol \cdot per μg protein) was independent of SMC number.

Mitochondrial function

To assess mitochondrial function, the NADH and NADPH-dependent reduction of MTT to formazan was measured during the final 4 h of the exposure to 7-ketocholesterol using the colorimetric MTT kit (Roche Applied Science 1465007, Vilvoorde, Belgium) (Pirillo *et al.*, 1997). After solubilization of the formazan crystals, absorbance was measured at 540 nm.

Flow cytometry

Cells were collected using a brief trypsin treatment, and transferred into 5 mL FACS tubes in phosphate-buffered

saline with 2% FBS. Apoptosis was detected with an annexin V-FITC and propidium iodide (PI) staining kit (BD Biosciences), always in the presence of 2% FBS. Cell samples (20 000 cells) were analysed on a FACSort cytometer using Cell Quest Pro software (BD Biosciences). All results were confirmed by manual counting of adherent SMCs using fluorescence microscopy.

Caspase assay

The conversion of the pan-caspase substrate Asp-Glu-Val-Asp-rhodamine 110 (DEVD-R110) was measured using the fluorimetric homogeneous caspase assay (Roche Applied Science, category no. 3005372). SMCs (50 000 cells) were seeded in two 24-well plates and exposed to 7-ketocholesterol for 18 h with or without statins. After removal of the medium, 100 μ L lysis buffer containing 50 μ M DEVD-R110 was added. The lysate was incubated at 37 °C and fluorescence of free rhodamine 110 (excitation filter 485 nm, emission filter 518 nm) was measured after 1 and 2 h. The difference between the two readings was converted to nmol rhodamine, using a calibration curve (0.03–1 μ M rhodamine 110). The second 24-well plate was run in parallel to measure adherent SMC protein at 18 h. Caspase activity was expressed as nmol rhodamine per 110 h mg per protein, and compared with a lysate of camptothecin-treated apoptotic U937 cells (provided by the manufacturer).

Transmission electron microscopy

Smooth muscle cells were collected by trypsinization and fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 h, then rinsed (3 \times 10 min) in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5% saccharose and postfixed in 1% OsO₄ solution for 1 h. After dehydration in an ethanol gradient (70% ethanol (20 min), 96% ethanol (20 min) and 100% ethanol (2 \times 20 min)), samples were embedded in Durcupan ACM (EMS, Hatfield, PA, USA). Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM 10 microscope (Philips, Eindhoven, The Netherlands) at 80 kV.

Plasmid construction and transient transfection of LC3

The eGFP coding sequence was excised from plasmid pEGFP-N3 (BD Biosciences Clontech, Palo Alto, CA, USA) by digestion with *Eco*RI and *Not*I. Vector ends were blunted with Klenow polymerase and self-ligated to produce plasmid pN3. Meanwhile, full-length cDNA encoding human LC3 protein was amplified by PCR from HepG2 cells using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) and primers 5'-CAAGATCTCGCGCATGCCCTCMGACCGG-3' and 5'-CAAAGCTTTCAGAAGCCGAAGGTTTCYTGGGAG-3'. The resultant PCR product was *Bgl*II/*Hind*III digested and cloned in the similarly opened pN3, to produce pHuLC3 as a final expression plasmid. Rabbit SMCs were transiently transfected with 5 μ g pHuLC3 via Nucleofection technology (program U-25) using the Human AoSMC Nucleofector kit (Amaxa GmbH, Köln, Germany).

Western blot analysis of LC3

Smooth muscle cells were lysed in an appropriate volume of Laemmli sample buffer (Bio-Rad, Richmond, CA, USA). Cell lysates were then heat denatured for 4 min in boiling water and loaded on an SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA, USA) according to standard procedures. Membranes were blocked in TBS-T (Tris buffered saline containing 0.05% Tween-20) and 5% nonfat dry milk (Bio-Rad) for 1 h. After blocking, membranes were probed overnight at 4 °C with an anti-LC3 polyclonal antibody (raised against the synthetic peptide H₂N-PSDRPFKQRRSFADC-CONH₂; Eurogentec, Seraing, Belgium) in antibody dilution buffer (TBS-T containing 1% nonfat dry milk), followed by 1 h incubation with a peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) at room temperature. Antibody detection was accomplished with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Results are expressed as mean \pm s.e.mean. Treatment effects were evaluated using a factorial ANOVA with two factors: 7-ketocholesterol concentration and drug concentration. When an interaction between both factors was present, drug effects were analysed separately in the presence and absence of 7-ketocholesterol using a repeated measures ANOVA followed by Dunnett's multiple comparison test. A 5% level of significance was selected.

Materials

Fluvastatin was a gift from Novartis AG (Vilvoorde, Belgium), simvastatin from Merck Sharp & Dome (Brussels, Belgium) and pravastatin from Daiichi Sankyo Belgium BV (Louvain La Neuve, Belgium). 7-Ketocholesterol, mevalonolactone, farnesylpyrophosphate and geranylgeranylpyrophosphate were from Sigma (Bornem, Belgium). The lactone rings of simvastatin and mevalonolactone were opened with 1 N NaOH, followed by pH adjustment with HCl. 7-Ketocholesterol and simvastatin were dissolved in ethanol. All concentrations are given as final concentrations.

Results

Light microscopy

Smooth muscle cells showed a flat morphology and accumulated neutral red in cytoplasmic granules following 2 h incubation with the dye. After exposure to 25 μ M 7-ketocholesterol for 18 h, the adherent SMCs contained fewer granules and showed highly variable signs of cell retraction, ranging from elongated to rounded shapes, while some cells were already floating. In globular cells, the granules seemed to merge (Figure 1). After exposure to 10 μ M simvastatin, the density of neutral red granules was unaltered, but more spindle-shaped cells were present. At 100 μ M, simvastatin caused variable degrees of cell detachment, ranging from

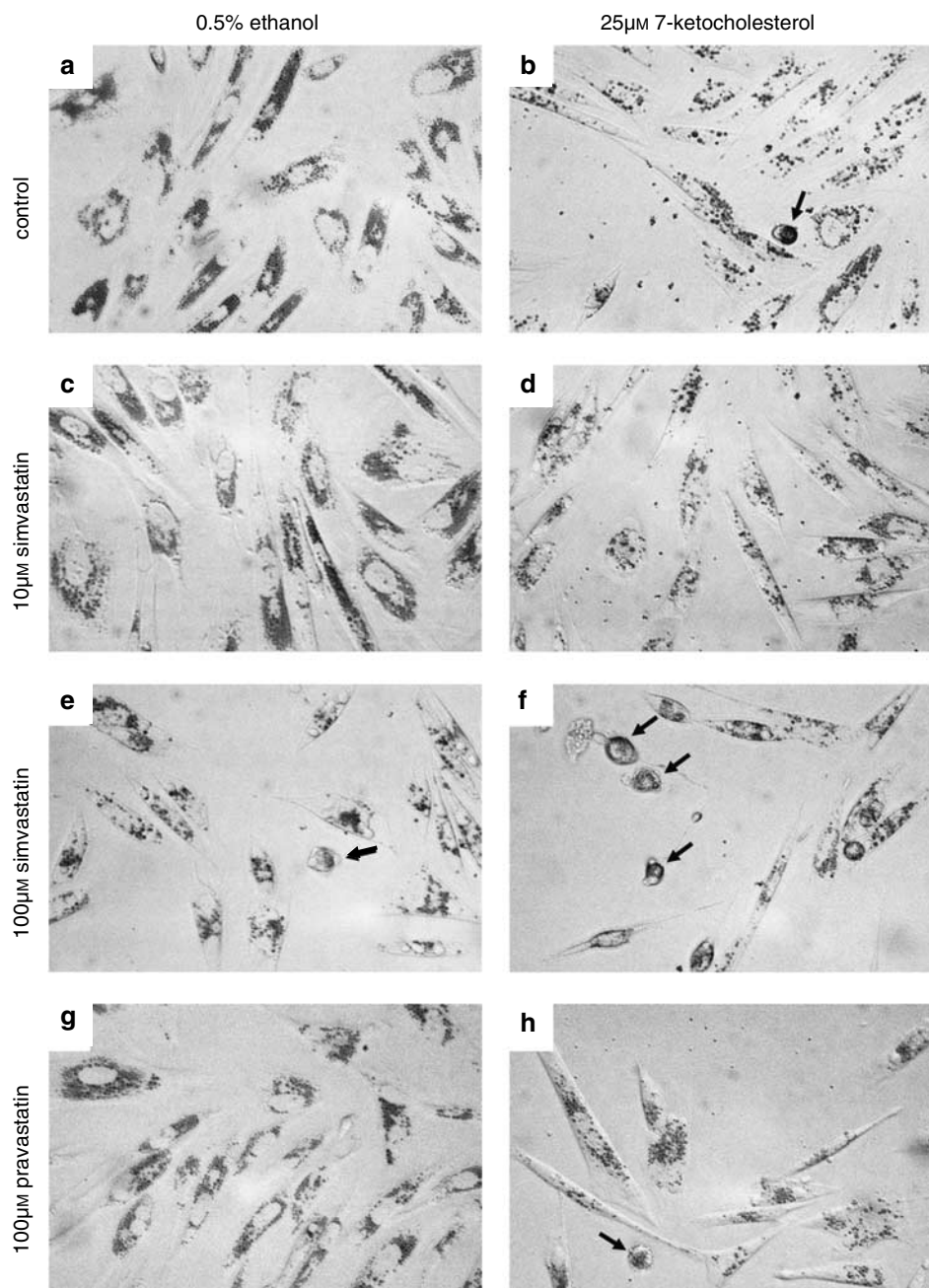


Figure 1 Accumulation of neutral red in smooth muscle cells (SMCs) exposed to (a) 0.5% ethanol, (b) 25 μM 7-ketocholesterol, (c) 10 μM fluvastatin, (d) 7-ketocholesterol and 10 μM fluvastatin, (e) 100 μM simvastatin, (f) 7-ketocholesterol and 100 μM simvastatin, (g) 100 μM pravastatin or (h) 7-ketocholesterol and pravastatin for 18 h. The accumulation of neutral red in cytoplasmic granules (lysosomes) was suppressed by 7-ketocholesterol. The oxysterol-induced variable degrees of cell retraction with formation of spindle-shaped and round cells (arrows), in which granules seemed to merge. SMCs exposed to pravastatin or 10 μM simvastatin were indistinguishable from control, whereas 100 μM simvastatin caused cell retraction, rounding off (arrows) and cell loss. SMCs treated with pravastatin and 7-ketocholesterol were similar to SMCs exposed to 7-ketocholesterol alone, whereas 10 μM simvastatin reduced and 100 μM increased the frequency of rounded SMCs.

elongated to globular cells containing fewer neutral red granules and floating cells. In combination with 7-ketocholesterol, 10 μM simvastatin seemed to attenuate SMC loss and to raise granular staining, whereas 100 μM simvastatin increased cell loss and frequency of spherical cells in comparison with SMCs treated with 7-ketocholesterol alone (Figure 1). The effects of fluvastatin on dye accumulation and SMC morphology were indistinguishable from those of simvastatin (results not shown), whereas even the highest

concentration of pravastatin (100 μM) was without effect apart from increasing the frequency of elongated cells.

Quantification of neutral red and SMC protein

These qualitative observations were confirmed by quantitative assays. The neutral red and—to a lesser extent—protein content of adherent SMCs declined at increasing 7-ketocholesterol concentrations, and the gradual losses became more

pronounced after 24 h exposure (Figure 2). At that time, adherent SMCs showed increased labelling of annexin V (annexin V positive and PI negative $4.6 \pm 0.9\%$) or PI ($13.7 \pm 2.5\%$) at $50 \mu\text{M}$ 7-ketocholesterol. As neutral red accumulation decreased about 50% after 18 h exposure to $25 \mu\text{M}$ 7-ketocholesterol (Figure 2a), those conditions were selected for further experiments. Given that the neutral red content of monolayers was determined by dye content of individual cells and SMC mass (Figure 1), a viability index (neutral red expressed per mg protein) was determined.

Figure 3 shows that none of the variables (neutral red accumulation, protein content or viability index) used to assess cell death were influenced by adding $100 \mu\text{M}$ mevalonic acid, $5 \mu\text{M}$ farnesylpyrophosphate or $5 \mu\text{M}$ geranylgeranylpyrophosphate to the SMCs cultured with 7-ketocholesterol.

Low concentrations of fluvastatin and simvastatin ($1\text{--}30 \mu\text{M}$) caused a gradual decline of neutral red accumulation, which was mainly explained by modest cell loss, as

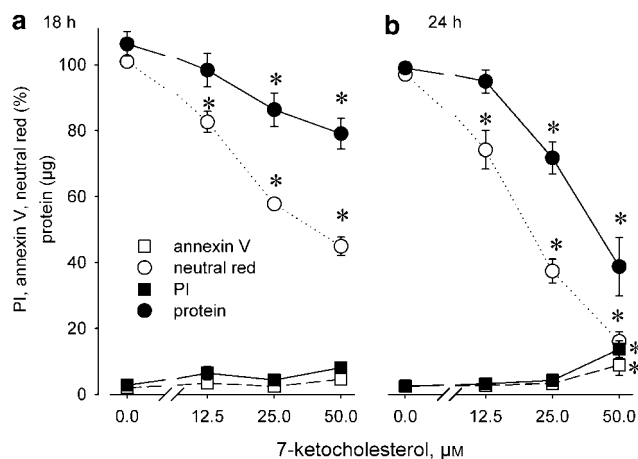


Figure 2 Concentration–response curve of neutral red accumulation (absorbance, %), protein content and percentages of adherent SMCs, which were positive for annexin V and propidium iodide (PI) after exposure to 7-ketocholesterol for (a) 18 h or (b) 24 h. Results shown as means \pm s.e.mean, $n = 4$, $*P < 0.05$ versus $0 \mu\text{M}$, repeated measurements ANOVA, Dunnett's test.

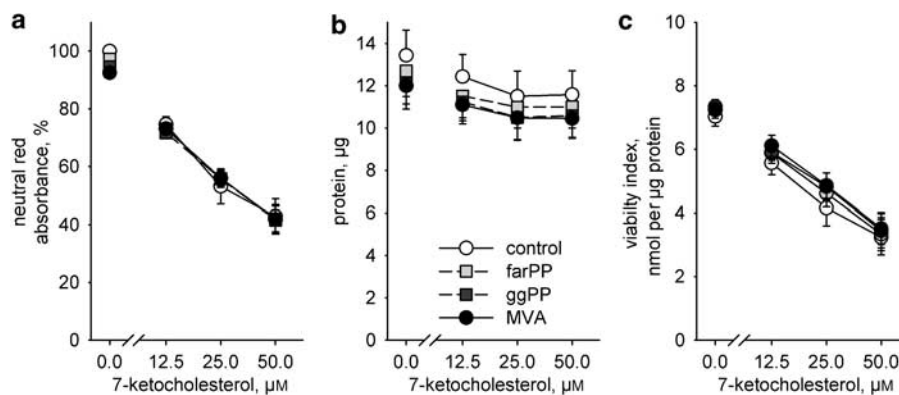


Figure 3 Exposure to 7-ketocholesterol for 18 h caused a concentration-dependent loss of (a) uptake of neutral red ($P = 0.001$), (b) adherent protein ($P = 0.011$) and (c) SMC viability ($P = 0.001$). The effects of mevalonic acid ($100 \mu\text{M}$, MVA), farnesylpyrophosphate ($5 \mu\text{M}$, farPP) and geranylgeranylpyrophosphate (ggPP) or their interactions with 7-ketocholesterol were statistically not significant. Results analysed with a factorial ANOVA and shown as means \pm s.e.mean, $n = 4$.

indicated by the reduction of monolayer protein and unaltered viability index (Figure 4). Higher concentrations of fluvastatin ($100 \mu\text{M}$) and simvastatin ($30\text{--}100 \mu\text{M}$) caused more significant declines of dye accumulation by reducing both viability index and cell mass (protein content).

Interestingly, highly significant interactions were seen between effects of 7-ketocholesterol and those of fluvastatin or simvastatin. Lower concentrations of both statins did not reduce the dye or protein content of the monolayer any further and even raised the viability index. In contrast, at high concentrations both statins exerted additive effects with 7-ketocholesterol by further reducing dye accumulation, monolayer protein and viability index. Below $1 \mu\text{M}$ ($0.01\text{--}1 \mu\text{M}$), neither fluvastatin nor simvastatin influenced viability with or without oxysterol (results not shown). Pravastatin was without effect on viability in either control or oxysterol-treated SMCs (Figure 4).

Mitochondrial function

To test whether the differential effects of statins in control or 7-ketocholesterol-treated cells were due to an interference with mitochondrial function, the reduction of MTT by succinate dehydrogenase was assessed (Figure 5). Exposure to 7-ketocholesterol for 5 h led to a concentration-dependent decline of mitochondrial activity. At $100 \mu\text{M}$, fluvastatin and simvastatin suppressed MTT reduction; their effect was additive with that of 7-ketocholesterol. Both statins were without effect at 1 or $10 \mu\text{M}$, whereas pravastatin was inactive (results not shown).

Caspase activation

Conversion of the pan-caspase substrate DEVD-R110 increased slightly (29.5%) after 18 h exposure to 7-ketocholesterol ($158 \pm 7 \text{ nmol per mg protein per h}$, $n = 12$) compared with SMCs exposed to 0.5% ethanol ($122 \pm 12 \text{ nmol per mg protein per h}$, $n = 12$, $P = 0.015$, paired Student's *t*-test). Fluvastatin ($\geq 10 \mu\text{M}$) and simvastatin ($\geq 3 \mu\text{M}$) raised hydrolysis of DEVD-R110 more significantly (Figure 6), although maximum activity was modest (18% ,

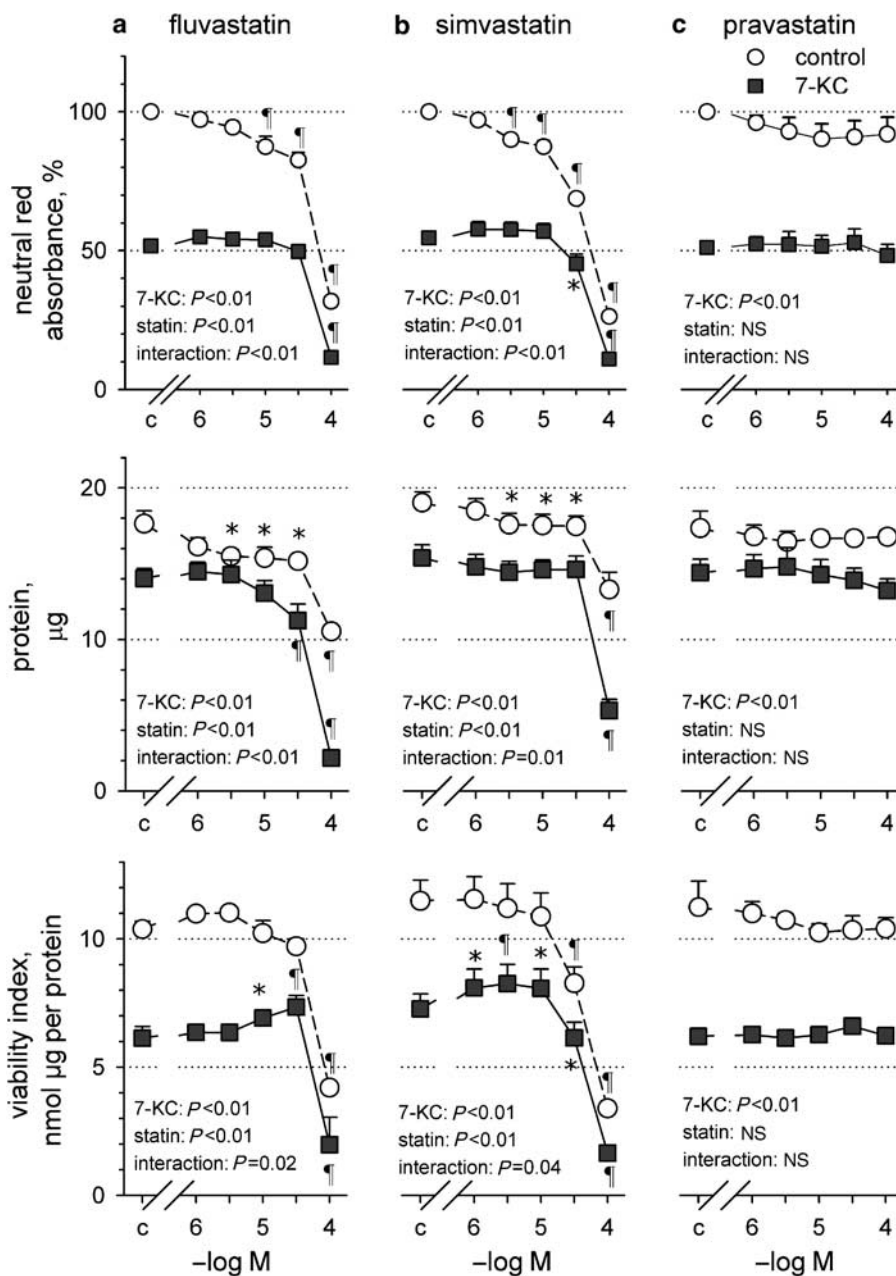


Figure 4 Effects of increasing concentrations of fluvastatin (a), simvastatin (b) and pravastatin (c) on neutral red absorbance, protein content and viability index of adherent SMCs exposed to 0.5% ethanol (control) or 25 µM 7-ketocholesterol (7-KC) for 18 h. Results representative of three independent trials, shown as mean ± s.e.mean, with fluvastatin *n* = 4, simvastatin *n* = 6 and pravastatin *n* = 4. Significance of the effects of 7-ketocholesterol, statin and their interaction in a factorial ANOVA is shown. **P* < 0.05, †*P* < 0.01, different from corresponding control without statin, repeated measurements ANOVA and Dunnett's test.

range: 4–34%) compared with the positive control of the assay (lysate of apoptotic U937 cells). Surprisingly, fluvastatin or simvastatin failed to activate caspases in SMCs exposed to 7-ketocholesterol. Pravastatin was inactive in either condition.

Electron microscopy

Transmission electron microscopy showed intense vacuolization in the cytoplasm of SMCs treated with 7-ketocholesterol alone or in combination with fluvastatin, pravastatin

(Figure 7) or simvastatin for 18 h. Some of those cytoplasmic vesicles were filled with intracellular organelles. In contrast, fluvastatin, pravastatin (Figure 7) or simvastatin induced very little cytoplasmic vacuolization when compared with control cells.

LC3 processing

Recruitment of LC3 to autophagic membranes is the biochemical marker for autophagic vesicle formation. An increased electrophoretic mobility from the nonautophagic

(LC3-I) form to the membrane recruited (LC3-II) form of LC3 was evident in SMCs transiently transfected with human LC3 and then exposed to 7-ketocholesterol in the absence or presence of fluvastatin or pravastatin, but not in SMCs treated with fluvastatin or pravastatin alone (Figure 7).

Discussion

The current study addressed the hypothesis that statins and 7-ketocholesterol, which are both known to induce SMC death, would exert additive cell death effects. Indeed, additive effects were seen with 7-ketocholesterol and high concentrations of fluvastatin and simvastatin. However, a

more important finding was that, at lower and clinically more relevant concentrations, both statins did not increase oxysterol-induced SMC loss (protein) and even improved cell viability (uptake of neutral red). This was apparently explained by interactions between the different cell death pathways that were activated by 7-ketocholesterol (autophagy) and statins (apoptosis), as explained below.

7-Ketocholesterol-induced cell death in SMCs

7-Ketocholesterol caused early mitochondrial dysfunction, as indicated by decreased succinate dehydrogenase activity 6 h after exposure to the oxysterol. This was in line with previous data showing a reversible mitochondrial dysfunction with cytochrome *c* release in SMCs (Seye *et al.*, 2004) and monocytes (Lizard *et al.*, 2000; Miguet *et al.*, 2001). The mitochondrial dysfunction was followed by decreased lysosomal accumulation of neutral red (viability index), followed by gradual cell retraction, rounding off and ultimately cell detachment (protein loss). Light microscopy revealed that the sensitivity of individual SMCs to the cytotoxic effects of 7-ketocholesterol was highly variable. The oxysterol-induced cytotoxicity was not due to suppression of HMG-CoA reductase activity in the current experimental setup, in which 2% FBS served as a source of cholesterol. Oxysterols may reduce HMG-CoA reductase activity under certain conditions, although 7-ketocholesterol and other major oxysterols present in oxidized LDL, showed only a low potency (Schroepfer, 2000). Indeed, intermediates of isoprenoid biosynthesis (mevalonic acid, farnesylpyrophosphate or geranylgeranylpyrophosphate) failed to improve the mitochondrial dysfunction or the viability loss in 7-ketocholesterol-treated SMCs.

Flow cytometry showed that a small percentage of the SMCs showed characteristics of necrosis (uptake of PI) or of apoptosis (annexin V labelling). The latter finding is in accordance with the observation that 4–5% of the adherent SMCs showed DNA fragmentation (TUNEL labelling) after 24 h exposure to 7-ketocholesterol (Seye *et al.*, 2004). The

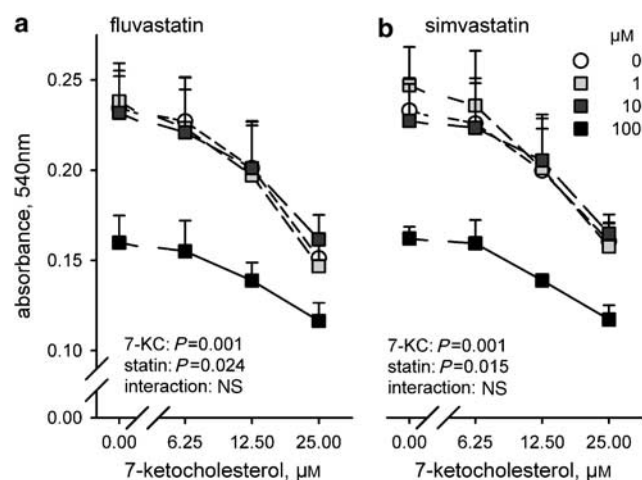


Figure 5 Reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) in SMCs is suppressed by increasing concentrations of 7-ketocholesterol (7-KC) and by 100 μM fluvastatin (a) or 100 μM simvastatin (b). Results shown as mean absorbance ± s.e.mean, $n=9-10$, except for 100 μM $n=3$. Significance of the effects of 7-ketocholesterol, statin and their interaction in a factorial ANOVA is shown.

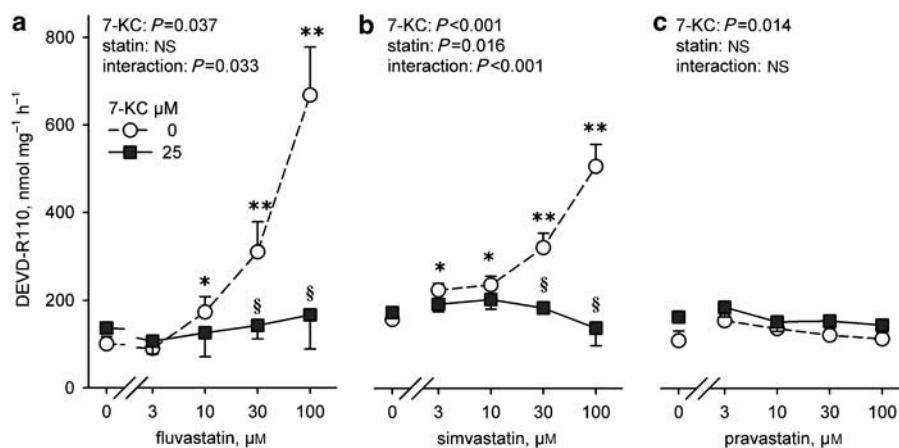


Figure 6 Conversion of the pan-caspase substrate Asp-Glu-Val-Asp-rhodamine 110 (DEVD-R110) by adherent SMCs after 18 h exposure to fluvastatin, simvastatin or pravastatin with or without 25 μM 7-ketocholesterol (7-KC). Results shown as mean ± s.e.mean, $n=4$. * $P<0.05$, ** $P<0.01$ versus 0 μM statin, § $P<0.05$ different from 0 μM 7-ketocholesterol, repeated measurements ANOVA and Dunnett's test of logarithmically transformed values.

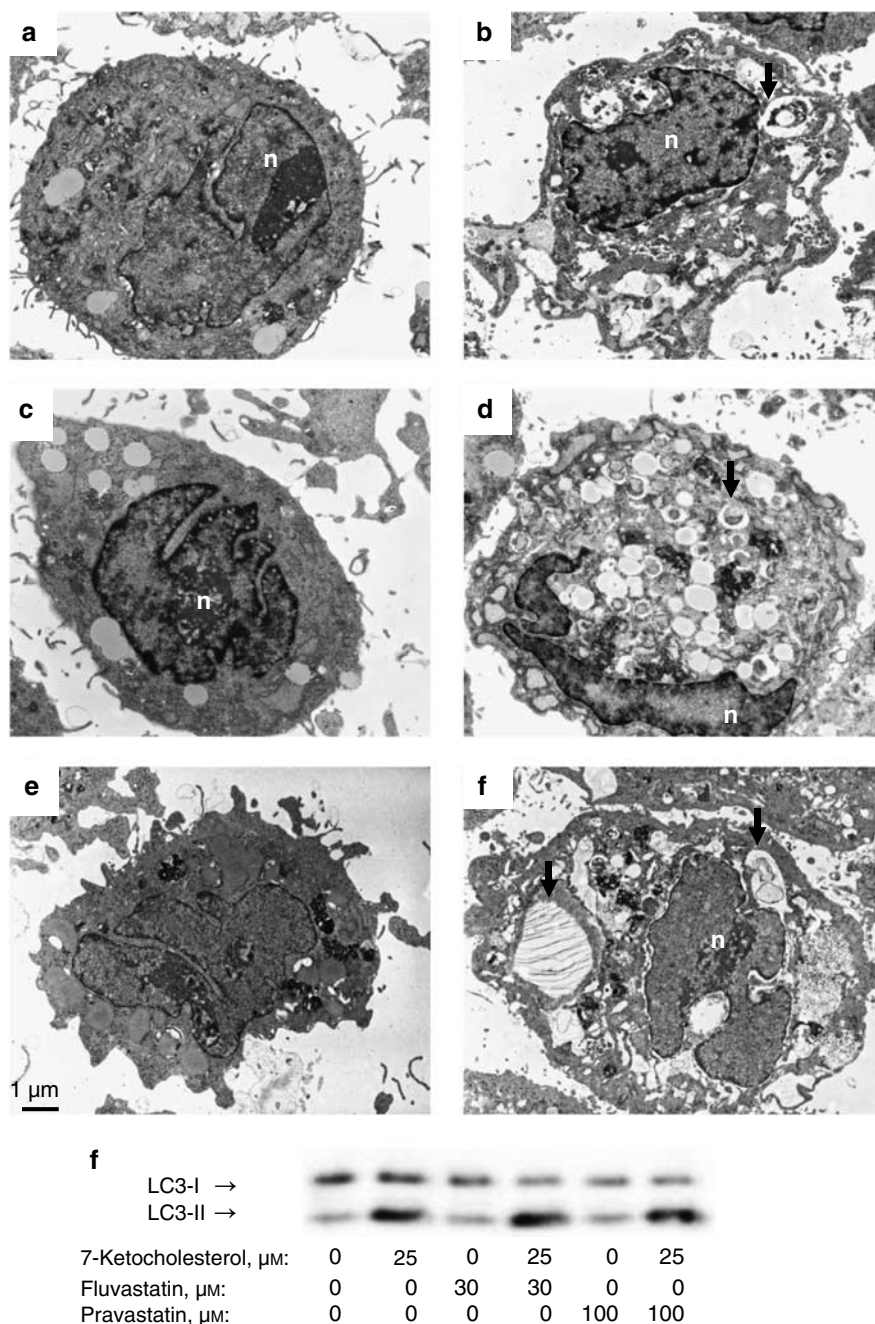


Figure 7 Transmission electron micrographs of SMCs exposed to (a) 0.5% ethanol, (b) 25 μM 7-ketocholesterol, (c) 30 μM fluvastatin, (d) the combination of 7-ketocholesterol and fluvastatin, (e) 100 μM pravastatin or (f) the combination of 7-ketocholesterol and pravastatin for 18 h. Cytoplasm of SMCs exposed to 7-ketocholesterol without, or with fluvastatin or pravastatin showed intense vacuolization with vesicles containing cytoplasmic material (arrows). Magnification, $\times 6610$; n = nucleus. (g) Immunoblot analysis of conversion of LC3-I to LC3-II in SMCs exposed to 0 or 25 μM 7-ketocholesterol without, or with 30 μM fluvastatin or 100 μM pravastatin for 18 h. LC3, microtubule-associated protein I light chain 3.

modest increase of caspase activity also indicated that some SMCs were committed to undergo apoptotic or type I cell death.

However, when examined by transmission electron microscopy, many SMCs showed intense vacuolization, with appearance of double-membrane cytoplasmic vesicles, as shown previously in human vascular SMCs exposed to 7-ketocholesterol (Martinet *et al.*, 2004). Formation of double-membrane cytoplasmic vesicles engulfing cytoplasm

and/or cytoplasmic organelles such as mitochondria and endoplasmic reticulum is a hallmark of autophagy (Gozuacik and Kimchi, 2004; Ferraro and Cecconi, 2007). These autophagic vesicles and their contents are destroyed by the lysosomal system of the same cell. On light microscopic inspection of SMCs stained with neutral red, this was evident from the rarefaction and enlargement of the lysosomes, particularly in globular cells. Autophagy has multiple physiological functions, including protein degradation and

organelle turnover and provision of amino acids and macromolecules necessary for survival under stress conditions. Increasing lines of evidence indicate that the same molecular mechanisms may be recruited by an alternative, caspase-independent form of programmed cell death, named autophagic type II cell death (Gozuacik and Kimchi, 2004; Ferraro and Cecconi, 2007). The microtubule-associated LC3 is essential for autophagic vesicle formation. When LC3 is cleaved at its C terminus, it is subsequently conjugated via an amide bond to the amino group of phosphatidylethanolamine. This conjugation converts the otherwise cytoplasmic form (LC3-I) to a tightly membrane-associated form (LC3-II), which is essential for autophagic vesicle formation. LC3 processing from LC3-I to LC3-II is thus a marker for autophagic vesicle formation (Gozuacik and Kimchi, 2004; Ferraro and Cecconi, 2007). Immunoblot analysis of the LC3-I to LC3-II conversion in SMCs exposed to 7-ketocholesterol confirmed the formation of autophagosomes. Taken together, the findings indicate that 7-ketocholesterol induces mitochondrial stress in rabbit aorta SMCs, gradually leading to autophagic (type II) cell death in most cells and apoptotic (type I) cell death in a minority of the cells.

SMC death induced by statins

In accordance with previous reports, fluvastatin- and simvastatin-induced cell death in SMCs (Baetta *et al.*, 1997; Guijarro *et al.*, 1998; Knapp *et al.*, 2000; Sindermann *et al.*, 2000). In agreement with those publications, SMC loss was mainly due to apoptosis, as indicated by the concentration-dependent activation of the caspase family of cysteine proteases. Owing to the highly variable sensitivity of individual SMCs (Figure 1), the activation occurred gradually in particular cells. The absence of autophagic vesicle formation (transmission electromicroscopy) and lack of LC3 processing (immunoblot) showed that autophagic cell death was a rare event, if occurring at all. Because the experiments were performed in the presence of 2% bovine fetal serum containing lipoproteins, it is unlikely that the statins caused cholesterol deprivation. Indeed, previous studies indicated that statin-induced apoptosis is most likely linked to suppression of prenylation of p21-Rho B or other proteins (Guijarro *et al.*, 1998; Knapp *et al.*, 2000; Takahashi *et al.*, 2002), whereas high concentrations may evoke mitochondrial stress (Werner *et al.*, 2004) and changes in calcium homeostasis (Teschfamiar *et al.*, 1999).

In contrast to fluvastatin and simvastatin, pravastatin induced neither caspase activation nor changes in SMC morphology or viability. The lack of effect of pravastatin is in accordance with most studies on induction of apoptotic cell death by statins in vascular SMCs (Baetta *et al.*, 1997; Guijarro *et al.*, 1998; Knapp *et al.*, 2000; Sindermann *et al.*, 2000). Owing to its hydrophilic nature, pravastatin is a weak inhibitor of HMG-CoA reductase activity in rat (IC_{50} 195 μ M) or human (IC_{50} 15 μ M) vascular SMCs (Corsini *et al.*, 1993; Bellosa *et al.*, 1998). Biological effects of various statins in vascular SMCs are directly proportional to the IC_{50} of HMG-CoA reductase, but require 10–20-fold higher concentrations to suppress isoprenoid biosynthesis by more than 90% (Corsini *et al.*, 1993; Bellosa *et al.*, 1998). Hence, the highest

concentration of pravastatin (100 μ M) was apparently insufficient to affect viability of rabbit aorta SMCs.

Interactions between 7-ketocholesterol and statins

Interestingly, SMC death induced by 7-ketocholesterol was not raised further and the viability index was even improved by low statin concentrations that induced SMC loss by themselves. In previous (Knapp *et al.*, 2000) and the present experiments, pravastatin was without effect, but a similar protection against 7-ketocholesterol-induced cell death has been described for pravastatin in human SMCs (Miyashita *et al.*, 2002). The difference is not entirely clear, but different experimental conditions (10% FBS, prolonged exposure leading to more intracellular diffusion of the hydrophilic drug), another cell enumeration technique or greater sensitivity of human vascular SMCs to HMG-CoA inhibition by statins in comparison with rodent or rabbit cells (Corsini *et al.*, 1993; Bellosa *et al.*, 1998) could possibly explain the discrepancy.

7-Ketocholesterol and statins could interact at the level of the mitochondria, but early oxysterol-induced mitochondrial dysfunction, assessed by MTT reduction, was neither attenuated nor worsened by 1–10 μ M fluvastatin or simvastatin. A striking finding was that fluvastatin and simvastatin failed to raise caspase activity in SMCs treated with 7-ketocholesterol, suggesting that activation of autophagic cell death processes interfered with the statin-induced apoptotic pathway. Indeed, recent lines of evidence indicate that interactions between both cell death programmes exist (Gozuacik and Kimchi, 2004). A study in breast cancer cells showed that autophagy delays apoptosis following DNA damage (Abedin *et al.*, 2007). The mechanisms of this suppression are not yet understood, but it has been proposed that the engulfment of defective mitochondria by autophagosomes limits the release of pro-apoptotic factors, such as cytochrome *c* and apoptosis-inducing factor into the cytosol (Gozuacik and Kimchi, 2004; Ferraro and Cecconi, 2007). These findings confirm the general assumption that autophagy is an adaptive survival strategy that permits the cell to eliminate unwanted or unnecessary organelles and to recycle cellular components for reuse (Debnath *et al.*, 2005). Only when autophagy is extensively triggered, will the cell die due to progressive degradation of cytoplasmic components.

At high concentrations, both statins and 7-ketocholesterol exerted additive effects on SMC death. This could be due to combined induction of mitochondrial dysfunction, as indicated by the assay of succinate dehydrogenase activity, which did not occur at lower statin concentrations. Additive effects have previously been reported for statins and apoptosis-inducing agents. In human rhabdomyosarcoma cells, high concentrations (≥ 30 μ M) of simvastatin induce Bax translocation and mitochondrial stress, followed by activation of caspases 9 and 3 and ultimately apoptotic cell death. In accordance with the present data on 7-ketocholesterol, these high statin concentrations exerted additive effects with doxorubicin-induced apoptosis in those cells (Werner *et al.*, 2004). In addition, the SMC apoptosis induced by activation of cell death receptors by Fas ligand or cytokines (Knapp *et al.*, 2000) was increased by a 24-h

pretreatment with pharmacological concentrations of simvastatin or lovastatin, whereas pravastatin was inactive. The mechanisms were not completely understood, but upregulation of cell surface expression of Fas or the involvement of geranylgeranylated proteins in the assembly of the intracellular death-inducing signalling complex were proposed (Knapp *et al.*, 2000). The main difference between those studies and the current data is that 7-ketocholesterol appeared to induce predominantly caspase-independent cell death pathways in rabbit (this study) and human vascular SMCs (Miguet-Alfonsi *et al.*, 2002; Martinet *et al.*, 2004).

In conclusion, our main finding is that vascular SMC death induced by an oxysterol, which was studied as a model for SMCs in an atherogenic setting, and cell death induced by relatively low concentrations of statins were not additive. This could be explained by the observation that autophagy, induced by 7-ketocholesterol, suppressed caspase activation leading to apoptotic cell death in statin-treated cells. The data further show that cell death processes induced by drugs in SMCs under standard conditions are not necessarily predictive for their effects on SMCs in an atherogenic setting.

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Conflict of interest

The authors state no conflict of interest.

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