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Effect of atorvastatin, simvastatin, and lovastatin on the metabolism of cholesterol and triacylglycerides in HepG2 cells

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Abstract

We evaluated the effects of the hydroxymethylglutaryl coenzyme A reductase inhibitors (HMGRI) atorvastatin, lovastatin, and simvastatin on lipid homeostasis in HepG2 cells. The drugs were almost equally effective in inhibiting cholesterol synthesis and in decreasing cellular cholesterol. Atorvastatin and lovastatin increased low-density lipoprotein receptor mRNA (2.5-fold at 3×10^{-7} M) and the transcription rate at the promoter of the low-density lipoprotein receptor gene (>5-fold at 10^{-6} M). The three compounds enhanced the activity of the low-density lipoprotein receptor at a similar magnitude (1.6–2.1- fold at 10^{-6} M). Atorvastatin and lovastatin increased the nuclear form of sterol regulatory element binding protein (SREBP)-2, but not of SREBP-1. Each of the drugs increased triacylglyceride synthesis (50% at 10^{-7}-10^{-6} M), cellular triacylglyceride content (16% at 10^{-6} M), and expression of fatty acid synthase by reporter gene and Northern blot analysis (2-fold and 2.7-fold at 10^{-6} M and 3×10^{-7} M, respectively). All compounds reduced the secretion of apo B (30% at 3×10^{-7} M). HMGRI decreased the ratio of cholesterol to apo B in newly synthesised apo B containing particles by ~50% and increased the ratio of triacylglycerides to apo B by ~35%. We conclude that regulatory responses to HMGRI are mediated by SREBP-2 rather than by SREBP-1, that HMGRI oppositely affect the cellular cholesterol and triacylglyceride production, that HMGRI moderately decrease the release of apo B containing particles, but profoundly alter their composition, and that atorvastatin does not significantly differ from other HMGRI in these regards.

Keywords: HMG-CoA reductase inhibitors; Cholesterol biosynthesis; Triacylglyceride biosynthesis; Fatty acid synthase; Low-density lipoprotein receptor

1. Introduction

HMGRI† effectively lower the plasma concentrations of atherogenic lipoproteins and reduce morbidity and mortality from coronary artery disease [1–5]. Atorvastatin has been shown to reduce total cholesterol, LDL cholesterol, and apo B more effectively than other HMGRI in man [6–8] and in experimental animals [9–11]. This prompted us to examine whether atorvastatin might be unique among HMGRI in terms of its action on lipid homeostasis. The mechanism whereby HMGRI decrease plasma cholesterol levels is well known. The inhibition of cellular cholesterol biosynthesis results in an increased activity of LDL receptors and in an enhanced uptake of apo B containing lipoproteins [12–14].

The sterol mediated regulation of the LDL receptor gene transcription is accomplished by SREBP-1 and -2. SREBPs are members of the basic-helix-loop-helix-leucine zipper family of DNA binding and transcription regulating proteins [15,16]. SREBPs precursor molecules are anchored to the membrane of the endoplasmic reticulum and the nuclear envelope. When cells starve for cholesterol, SREBPs are proteolytically cleaved [17] and their soluble amino terminal domains translocate to the nucleus where they bind to SRE-1 in target genes, e.g. the genes encoding for LDL receptor and HMG-CoA synthase [15,16]. The transcription of genes encoding for FAS and ACC, two enzymes essential for fatty acid synthesis, is also controlled by sterol regulatory elements [18–21]. In both the promoter of FAS and the

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† *Abbreviations:* ACC, acyl coenzyme A carboxylase; apo, apolipoprotein; BSA, bovine serum albumine; DMEM, Dulbecco's minimal essential medium; FAS, fatty acid synthase; GAPDH, glyceraldehyde-6-phosphate-dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGRI, HMG-CoA reductase inhibitors; LDL, low-density lipoprotein; LPDS, human lipoprotein deficient serum; and SREBP, sterol regulatory element binding protein.

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promoter of ACC one binding site for SREBP is located close to a binding site for the transcription factor Sp1 [18,21]. Sp1 was shown to be essential for the sterol regulation of the LDL receptor promoter [22]. Thus, there is evidence for a cross-talk between the metabolism of cholesterol and fatty acids.

The aim of the present study was to evaluate the effect of atorvastatin in comparison with simvastatin and lovastatin on key steps of the metabolism of cholesterol and triacylglycerides in cultured cells. To investigate the cellular response to the depletion of cholesterol stores caused by inhibition of HMG-CoA reductase, we examined the biosynthesis of lipids, the activity of LDL receptors, the expression of the LDL receptor and FAS, the cellular lipid contents, and the composition of secreted lipoproteins.

2. Materials and methods

2.1. Materials

Lovastatin and simvastatin were from Merck Sharp and Dohme; atorvastatin was from Parke-Davis. The lactone forms of lovastatin and simvastatin were converted into the sodium salt according to the procedure of Cutts *et al.* [23]. Stock solutions containing 10 mM atorvastatin (in DMSO), lovastatin, or simvastatin (both in ethanol/NaOH) were stored at −20° until use. The final concentration of DMSO did not exceed 0.1% (v/v). [2-^{14}C]-acetic acid, sodium salt (58 mCi/mmol), [1,2-^{3}H]-cholesterol (50 Ci/mmol), and Na[^{125}I] (carrier free, 100 mCi/mL) were from Amersham (Piscataway, NJ, USA). The reporter plasmid pLDLR-CAT 1563 was kindly provided by Dr. D. Russell, University of Texas.

2.2. Lipoproteins and lipoprotein-deficient serum

Human LDL (1.030–1.050 kg/L) were isolated from plasma by preparative ultracentrifugation. LDL was iodinated by using iodine-monochloride as oxidizing agent [24]. Specific activities ranged from 80–150 cpm/ng protein. Human LPDS was prepared by ultracentrifugation as described [25] and stored at −25°.

2.3. Cell culture

HepG2 were obtained from the American Type Culture Collection. Cell culture medium, additives (glutamine, penicillin G, streptomycin), and fetal bovine serum were from Seromed (Munich, Germany). The cells were grown in 80-cm^2 polystyrene flasks containing DMEM medium supplemented with penicillin/streptomycin (20,000 units/L) and 10% (v/v) fetal bovine serum in a humidified incubator at 5% (v/v) CO_2 and 37°.

2.4. Incorporation of acetate into cellular lipids

HepG2 cells were seeded in 6-well-plates and grown to 70% confluence. The monolayers were incubated for 5 h with drugs at the indicated concentrations. Two h before the end of the incubation period, the cells were pulse-labeled with [^{14}C]-acetate at a final concentration of 35 mM (2 mCi/L medium). After incubation, the cells were washed three times with 3 mL of 150 mM NaCl and suspended in 3 mL of n-hexane:isopropanol (3:2 by volume). After adding 0.25 μCi of [1,2-^{3}H]-cholesterol as an internal standard, each monolayer was extracted for 30 min. The resulting suspension was transferred to a glass tube and centrifuged at 3300 *g* for 20 min. The lipid phase was removed, evaporated to dryness under a stream of nitrogen, and resuspended in 1 mL of chloroform:methanol (2:1 by volume). The cell pellet was dissolved in 1 mL of 1 M NaOH and used for the determination of protein. The lipid extracts were subjected to thin-layer chromatography on (aluminium sheet supported) silica gel plates (Merck, West Point, PA, USA). The plates were developed with a solvent of hexane:isopropanol: formic acid (80:30:2 by volume). The spots containing triacylglycerides, nonesterified, and esterified cholesterol were visualized by iodine vapour, cut out, and counted in a scintillation counter. The data were expressed as nmol of [^{14}C]-acetate incorporated per hour and per mg of total cell protein.

2.5. Binding, uptake, and degradation of [^{125}I]-labeled lipoproteins

We followed the procedures described by Goldstein *et al.* [25] with slight modifications [26]. To measure cell surface binding, lipoproteins were incubated with the cells for 1 h at 4° in 1 mL of DMEM containing 10 mM HEPES without serum supplementation. To determine uptake (surface binding *plus* internalization) and degradation, cells were incubated for 4 h at 37° with [^{125}I]-labeled LDL in DMEM containing 24 mM bicarbonate (pH 7.4), also in the absence of serum. The amount of [^{125}I]-labeled material associated with the cells was determined after lysis in 0.3 mM NaOH. Proteolytic degradation was determined as [^{125}I]-labeled trichloroacetic acid-soluble (noniodide) material in the conditioned medium.

2.6. Plasmid construction

The plasmid pLDLR1563 was constructed by subcloning a 1506-bp fragment extending from nucleotide −1563 through −58 relative to the transcription initiation site of the human LDL receptor promoter obtained by *Hind*III digestion of pLDLR-CAT 1563. This fragment was cloned into *Hind*III digested pGL3-Ba basic vector (Promega, Madison, WI, USA) to yield the reporter gene construct pLDLR1563.

A promoter fragment of the human FAS gene 5′-untrans-

lated region from −827 to +134 relative to the transcription start point was amplified from human genomic DNA by polymerase chain reaction using oligonucleotides FAS-Pr1 (CCC *AAG CTT* GGA AGC TGC TAA GGA GGG GC) and FAS-Pr2 (CCC *AAG CTT* GCC GCT GCT CGT ACC TGG TGA GGG), which contain additional bases (underlined sequence) to generate *Hind*III restriction sites [27] (GenBank™ accession number: U52428). This fragment was cloned into the *Hind*III digested pGL3-basic vector (Promega).

2.7. Promoter assay

HepG2 cells were plated in 48-well polystyrene plates. Cells were transiently transfected with the reporter gene constructs (0.5 μg/well) by lipofection (Tfx50™, Promega) for 4 h. To normalize for transfection efficacy, a control plasmid harboring the renilla luciferase gene driven by the simian virus promoter (pRL-SV40, Promega) was cotransfected. Transfected cells were incubated with medium containing LPDS and the drugs at different concentrations for 24 h, lysed, and firefly- and renilla-luciferase activities were determined by using the Dual Luciferase Assay™ (Promega) on a luminometer (Lumat LB9501, EG&G Bertold, Salem, CA, USA).

2.8. Northern blot analysis

HepG2 cells were plated in 25-cm^2 polystyrene flasks. The cells were incubated with medium containing 0.5% (v/v) LPDS and with the drugs at a concentration of 3×10^{-7} M for 24 h. Total RNA was extracted by using the Trizol™ reagent (Life Technologies, Rockville, CA, USA). RNA was electrophoresed (20 μg/lane) in 0.8% agarose/1.8 M formaldehyde gels and transferred onto Hybond-N+™ nylon membranes (Amersham) by capillary blotting. The membranes were hybridized for 1 h at 60° with 1 mg of salmon sperm DNA (Stratagene, La Jolla, CA, USA) in Quick-Hyb® hybridization buffer (Stratagene) and sequentially probed with a polymerase chain reaction-generated cDNA probe corresponding to nucleotides 87–578 of the human LDL-R cDNA, a cDNA probe for FAS, or with a 0.5-kb cDNA probe for human GAPDH, respectively. The probes were labeled by random priming (Redi prime™ II, Amersham) using 30 μCi of [α^{32}P]dCTP (Amersham) and purified by Sephadex G-50 chromatography (Microspin™ G-50 Columns, Amersham). The membranes were exposed to X-ray films (Eastman Kodak, Rochester, NY, USA) for autoradiography at −80°.

2.9. Western blot of SREBP

HepG2 cells plated in 80-cm^2 polystyrene flasks were incubated with medium containing 10% LPDS for 48 h. After stimulation with the drugs at concentrations of 10^{-6} M for 18 h, nuclear extracts were prepared according to the method of Schreiber *et al.* [28] with slight modifications [29]. Aliquots of each sample (40 μg of protein) were separated on a denaturating SDS polyacrylamide gel electrophoresis (4–15%) and blotted to polyvinylidendifluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). Monoclonal antibodies against SREBP-1 (IgG 2A4, 1:1000) and SREBP-2 (IgG 1C6, 1:200, Pharmingen, San Diego, CA, USA), peroxidase-labeled anti-mouse IgG-Fab fragments (1:20,000, Roche Biochemicals, Burlington, NC, USA), and chemiluminescence substrate (SuperSignal™ West Dura Extended Duration Substrate, Pierce, Rockford, IL, USA) were used for Western blot analysis.

2.1.0. Measurement of apo B in cell-culture media

HepG2 cells were seeded in 24-well-plates and grown to 70% confluence. The monolayers received medium containing 1% BSA (Sigma Aldrich, St. Louis, MO, USA) for 24 h. The cells were then incubated with medium containing 1% BSA and 0.8 M oleate (Sigma Aldrich) alone, or together with the drugs (at a concentration of 3×10^{-7} M). Each well was incubated with 1 mL of medium. At the indicated time intervals, 250-μL aliquots were removed from each well and were replaced by an equal amount of fresh medium containing the corresponding compound. The amount of apo B was quantified by using a microwell enzyme immunoassay (AlerCHEK) according to the manufactures instructions.

2.1.1. Measurement of cellular lipids and secreted lipoproteins

HepG2 cells were seeded in 25-cm^2 polystyrene flasks and treated with BSA/oleate as described above. After the medium was removed, the cells were washed three times with 150 mM NaCl and the lipids were extracted with 5 mL of n-hexane:isopropanol (3:2 by volume) for 30 min. After centrifugation, the lipid phase was evaporated to dryness under a stream of nitrogen and resuspended in 160 μL of isopropanol. Cellular triacylglycerides and total cholesterol were measured by using enzymatic reagents and secondary standards from Wako (Richmond, VA, USA). To quantify the lipid composition of secreted lipoproteins, the density of the conditioned media were adjusted to $d < 1.063$ kg/L. After ultracentrifugation at 100,000 *g* for 24 h the top 0.5-mL fractions were removed and the lipids were quantified as described above. Determination of apo B in whole media was performed as described above.

3. Results

3.1. Biosynthesis of sterols and triacylglycerides

We examined the effect of atorvastatin on the biosynthesis of non-esterified cholesterol, esterified cholesterol, and

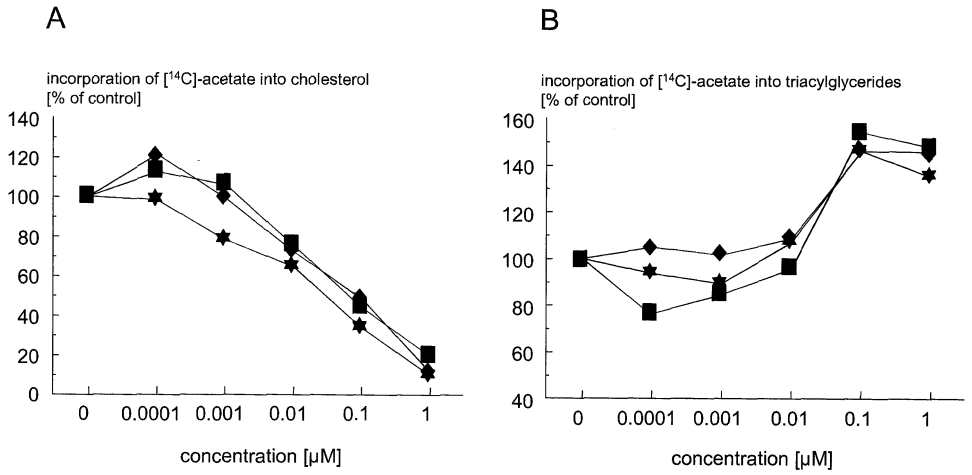

Fig. 1. Effect of atorvastatin, lovastatin, and simvastatin on the incorporation of acetate into *de novo* synthesized lipids. HepG2 cells were grown in DMEM medium supplemented with 10% (v/v) fetal calf serum. Twenty-four h prior to the experiment, the cells were switched to medium containing 10% (v/v) human LPDS. The cells then received atorvastatin (squares), simvastatin (rhombuses), and lovastatin (stars) at the concentrations indicated on the abscissa. Two h prior to the end of the incubation period, the cells were pulse-labeled with [^{14}C]-acetic acid, sodium salt (final concentration 35 mM). The incorporation of acetate into nonesterified cholesterol (panel A) and into triacylglycerides (panel B) was determined as described in Section 2. Results were normalized to the amount of cellular protein and expressed in percent of the respective control incubations. The data are means from triplicates. The standard deviations of the replicates were 11% or less of the respective means. The average 100% of control rates of [^{14}C]-acetate incorporations into nonesterified cholesterol and into triacylglycerides were 2.91 nmol $\times$ h^{-1} $\times$ mg^{-1} and 6.26 nmol $\times$ h^{-1} $\times$ mg^{-1}, respectively.

triacylglycerides from [^{14}C]-acetate in the human hepatoma cell line HepG2 compared to simvastatin and lovastatin. In order to up-regulate the mevalonate pathway, the cells were preincubated with medium containing LPDS. Atorvastatin reduced the production of non-esterified cholesterol in a concentration-dependent fashion similar to lovastatin and simvastatin (Fig. 1a). Significant reductions in cholesterol synthesis were observed at concentrations of 10^{-8}-10^{-6} M. At the highest concentrations analyzed, 10^{-6} M, atorvastatin, simvastatin, and lovastatin inhibited the incorporation of [^{14}C]-acetate into cholesterol by ~85%. The calculated IC_{50} values for the three HMGRI examined only slightly differed from each other (atorvastatin: 68 nM, lovastatin: 31 nM, simvastatin: 93 nM). This is in perfect agreement with data provided by Shaw *et al.* [30] for lovastatin (IC_{50} = 43 nM) and atorvastatin (IC_{50} = 73 nM). Similar results were obtained regarding the *de novo* synthesis of esterified cholesterol (not shown).

In clinical studies, atorvastatin effectively reduced serum triacylglycerides [7,31–33]. There are hardly any data on the effect of HMG-CoA reductase inhibition on the triacylglyceride metabolism in cultured cells. For this reason, we analyzed the incorporation of [^{14}C]-acetate into triacylglycerides. Interestingly, all drugs (at concentrations of 10^{-7}-10^{-6} M) increased the amount of newly synthesized triacylglycerides (Fig. 1b) by ~50%. Again, there were no significant differences between the effects of atorvastatin, lovastatin, and simvastatin.

3.2. Cellular lipid contents

To examine whether the effect of HMGRI on the *de novo* synthesis of lipids alter their cellular concentration, we determined the contents of cholesterol and triacylglycerides in cellular extracts. After incubation for 24 h with atorvastatin, lovastatin, and simvastatin (10^{-6} M) cellular cholesterol was significantly decreased by more than 30% compared to control (Table 1). The increase in the *de novo* synthesis of triacylglycerides caused by HMGRI resulted in increases of triacylglycerides on average by 16% (Table 1).

3.3. Activities of the LDL receptor and SREBP

To study the influence of atorvastatin on the activity of the LDL receptor, we determined receptor mediated endo-

Table 1
Effect of atorvastatin, lovastatin, and simvastatin on cellular lipid content

	Cholesterol (μg/mg cell protein)	Triacylglycerides (μg/mg cell protein)
Control	18.3 ± 4.5	62.9 ± 8.9
Atorvastatin	11.9 ± 4.3[a]	73.8 ± 9.1[b]
Lovastatin	12.4 ± 3.2[a]	74.1 ± 7.1[a]
Simvastatin	12.7 ± 4.1[b]	71.8 ± 8.8[b]

HepG2 cells were incubated with medium containing 1% BSA for 24 h. Then the cells received medium containing 1% BSA and 0.8 M oleate (Sigma Aldrich) alone, or together with the drugs (at a concentration of 10^{-6} M) for 24 h. After the incubation, the cells were washed and the lipids were extracted from cell monolayers with n-hexane:isopropanol (3:2 by volume). The lipid phase was evaporated to dryness under a stream of nitrogen and resuspended isopropanol. Triacylglycerides and cholesterol were measured by using spectrophotometric assays. The data are reported as the mean and standard deviation of three independent experiments each performed in triplicates.

[a] Indicates $P < 0.01$;

[b] Indicates $P < 0.05$ compared to control.

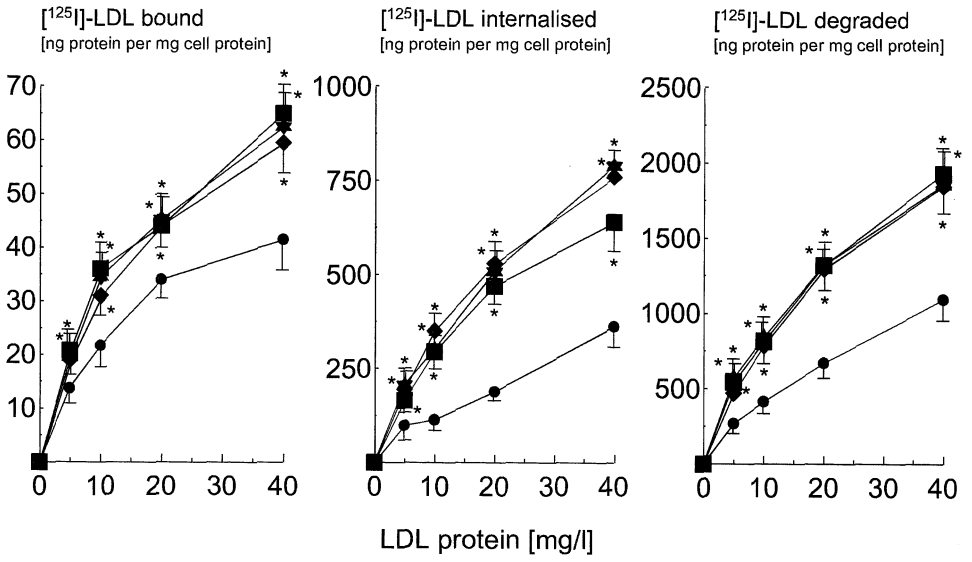

Fig. 2. Effect of atorvastatin on binding, uptake, and degradation of LDL. HepG2 cells were grown in DMEM medium supplemented with 10% (v/v) fetal calf serum. The cells were incubated for 48 h with medium containing 10% (v/v) LPDS alone (circles) or, in addition, with atorvastatin at concentrations of 10^{-8} M (asterisks), 10^{-7} M (rhombuses), or 10^{-6} M (squares), respectively, for 24 h. The cells then received [^{125}I]-labeled LDL (1.030 kg/L $< d <$ 1.050 kg/L) at the concentrations indicated on the abscissa. Binding at 4° (left panel), uptake (center panel), and degradation (right panel) at 37° of [^{125}I]-labeled LDL were determined as described in Section 2. Each data point represents the average from three independent experiments, each performed in triplicates. Data are adjusted for non-specific binding, uptake, and degradation determined in the presence of a 30-fold excess of unlabeled LDL. *$P < 0.05$ vs. the respective control incubations.

cytosis of [^{125}I]-labeled LDL in HepG2 cells. The cells were incubated for 48 h with medium containing LPDS. After 24 h, atorvastatin was added at final concentrations of 10^{-6}-10^{-8} M. Surface binding of labeled LDL was measured after incubation at 4° for 1 h, cellular uptake and degradation were determined after incubation at 37° for 4 h. As shown in Fig. 2, atorvastatin enhanced the receptor-mediated endocytosis of LDL in a concentration-dependent manner. At a concentration of 10^{-6} M surface binding was by ~60% higher compared to control incubations without atorvastatin (Fig. 2). Cellular uptake and degradation increased significantly even at a concentration of 10^{-8} M and were 1.8–2.1-fold higher at 10^{-6} M compared to control incubations (Fig. 2). Simvastatin and lovastatin were as effective as atorvastatin in increasing binding, uptake, and degradation of radiolabeled LDL (data not shown).

3.4. Expression of the LDL receptor gene

Northern blotting was used to determine whether atorvastatin or lovastatin increased the steady-state levels of LDL receptor mRNA. HepG2 cells were incubated for 24 h in medium containing LPDS, and then received either atorvastatin or lovastatin for different time intervals, both at a concentration of 3×10^{-7} M. As shown in Fig. 3, both HMGRI increased the amount of LDL receptor mRNA ~2.5-fold over the level measured in control incubations with LPDS alone. Although the effect of lovastatin occurred earlier compared to atorvastatin, both compounds showed an equivalent enhancement of mRNA, suggesting that atorvastatin would increase the *de novo* synthesis of LDL receptors at a similar magnitude compared with other HMGRI.

To confirm this, we examined the effect of HMGRI on the transcription of the promoter of the LDL receptor gene.

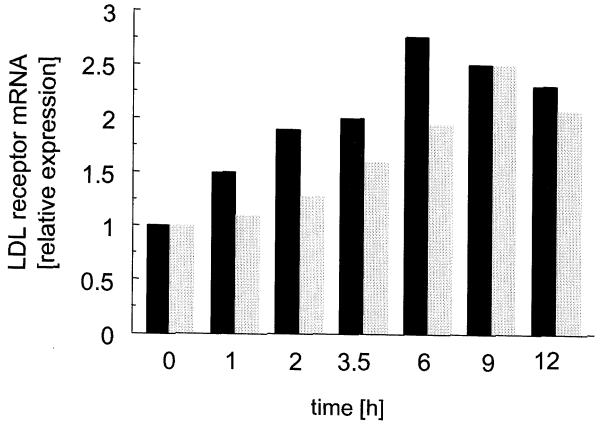

Fig. 3. Effect of atorvastatin and lovastatin on the LDL receptor mRNA. HepG2 cells were incubated for 24 h with DMEM medium containing 0.5% (v/v) LPDS or, in addition, with atorvastatin (grey bars), or lovastatin (black bars) at a concentration of 3×10^{-7} M for the indicated time periods (0–12 h). Total cellular RNA was isolated, subjected to electrophoresis, and blotted to nitro-cellulose. The membrane was hybridized with [α^{32}P]-dCTP labeled cDNA-probes for the LDL-receptor and for GAPDH (internal control) and exposed to autoradiography. The autoradiographs were scanned on a laser densitometer. The results were normalized to the amount of GAPDH, and expressed as percentage of the respective control incubations. Each bar represents the average from two independent experiments.

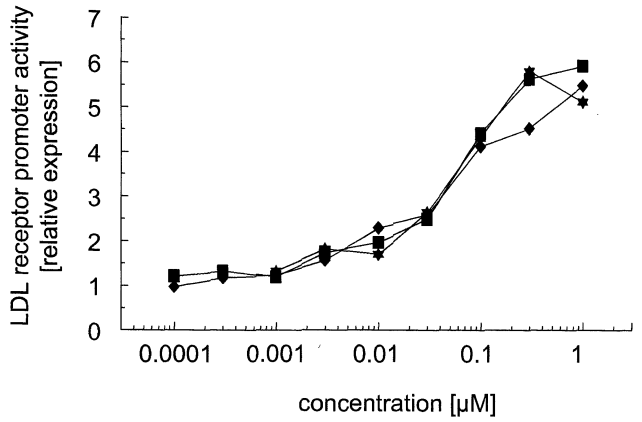

Fig. 4. Effect of atorvastatin, lovastatin, and simvastatin on the activity of the LDL receptor promoter. HepG2 cells were incubated for 24 h with DMEM medium containing 10% (v/v) LPDS. The cells were transiently transfected with a reporter gene construct containing LDL receptor promoter and firefly luciferase (pLDLR1563) and were then incubated for 24 h with atorvastatin (squares), simvastatin (rhombuses), or lovastatin (asterisks), respectively, at the concentrations indicated on the abscissa. A plasmid coding for renilla luciferase was used as control for transfection efficacy. Luciferase activities were determined as described in Section 2. The results were normalised to transfection efficacy and expressed as percentages of the respective control incubations (medium containing 10% [v/v] LPDS alone). Each data point represents the average of two experiments, each performed in quadruplicates. The standard deviations of the replicates were 18% or less of the respective means.

HepG2 cells were transiently transfected with a plasmid coding for the LDL receptor promoter (pLDLR1563) and firefly-luciferase. After stimulation with medium containing LDPS, the cells were treated with atorvastatin, lovastatin, and simvastatin (10^{-10}-10^{-6} M) for 24 h. As shown in Fig. 4, treatment of HepG2 cells with HMGRI resulted in a concentration-dependent induction of LDL receptor promoter activity. A concentration of 10^{-8} M was sufficient to obtain a significant increase, drug concentrations of 10^{-6} M resulted in transcription rates more than 5-fold higher than control incubations. All compounds analyzed produced similar enhancement of the promoter activity.

3.5. Activation of SREBPS

The enhancement of the LDL receptor promoter activity upon incubation with HMGRI was paralleled by an increase of SREBP-2 protein in nuclear cell extracts. As detected by Western blotting, atorvastatin and lovastatin substantially increased the amount of the mature form of SREBP-2 (Fig. 5A), whereas the amount of mature SREBP-1 was not affected (Fig. 5B).

3.6. Expression of the FAS gene

Depletion of the cellular sterol content leads to a coordinated upregulation of proteins essential for both cholesterol and fatty acid synthesis mediated by SREBPs [15–18, 21]. We wanted to find out whether the inhibition of cholesterol synthesis caused by HMGRI results in modifications of the fatty acid synthesis. Treatment of the cells with either atorvastatin or lovastatin (3×10^{-7} M) for up to 12 h resulted in an increase of FAS mRNA (Fig. 6). In parallel to the behavior of the mRNA for the LDL receptor, marked enhancement was observed as early as after 1 h of incubation suggesting a coordinated upregulation of the mRNAs for the LDL receptor and FAS.

These results are in good agreement with those of the transfection experiments in which we measured the activity of the FAS promoter by using the plasmid pFAS-GL3. Atorvastatin, as well as lovastatin, enhanced the transcription of the FAS promoter concentration-dependently (Fig. 7). At 10^{-6} M, the activity of the FAS promoter was more than 2-fold that of control incubations. This was confirmed when we analysed the time course of the promoter activation (not shown). Both, the enhancement in LDL receptor promoter and FAS promoter activities followed identical time kinetics reaching saturation after 18 h of incubation with HMGRI.

3.7. Composition of secreted lipoproteins

The influence of HMGRI on the assembly and secretion of apo B containing lipoproteins has been examined *in vivo* and *in vitro* [34]. An extracellular source of fatty acids may be required to stimulate apo B secretion in HepG2 cells [35,36]. Following preincubation with oleate, we treated HepG2 cells with atorvastatin, lovastatin, and simvastatin at a concentration of 3×10^{-7} M each. After incubation for different time intervals we measured the concentration of apo B secreted into the media by using an enzyme immunoassay. The three compounds reduced the secretion of apo B significantly by about 30% ($P < 0.05$) compared to oleate-treated controls (Fig. 8).

To further elucidate the effect on lipoprotein production, we measured the composition of secreted lipoproteins in the conditioned media of HepG2 cells after treatment with HMGRI (10^{-6} M) for 24 h. The amount of cholesterol in the density fraction $d < 1.063$ kg/L was significantly reduced by about 60%, whereas the concentration of apo B decreased by 25–29% only (Table 2). Hence, the cholesterol to apo B ratio of the secreted lipoprotein was reduced. The decrease of cholesterol content was accompanied by an increase of triacylglycerides by 30–40% (Table 2).

4. Discussion

Inhibition of HMG-CoA reductase, the rate-limiting enzyme of sterol biosynthesis, almost completely suppresses the incorporation of [^{14}C]-acetate into newly synthesized sterols [30,36–38]. We compared the efficacy of atorvastatin, lovastatin, and simvastatin in affecting lipid biosynthesis in HepG2 cells. Atorvastatin produced a concentration-dependent reduction in cholesterol synthesis at a magnitude

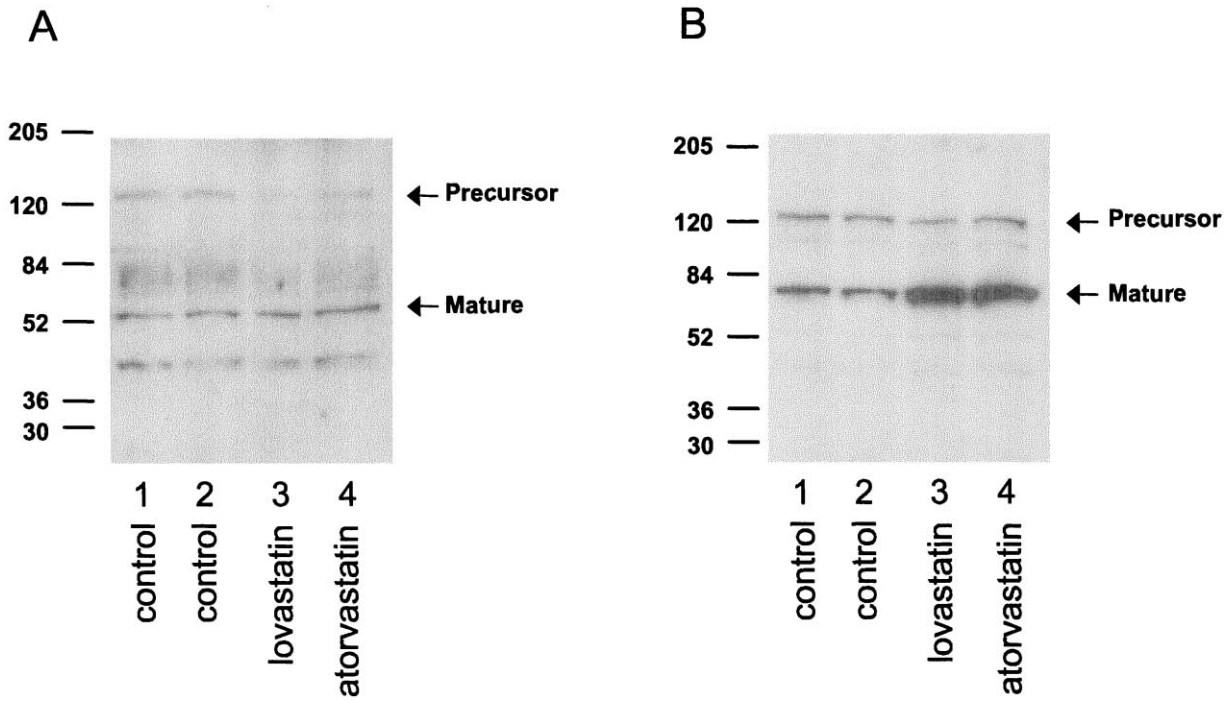

Fig. 5. Effect of atorvastatin and lovastatin on the activation of SREBPs. HepG2 cells were incubated with DMEM containing 10% LPDS for 48 h. After stimulation with the drugs at concentrations of 10^{-6} M for 18 h, nuclear extracts were prepared as described in Section 2. Aliquots of the extracts (40 μg of protein) were separated on a denaturating SDS polyacrylamide gel electrophoresis (4–15%) and blotted on PVDF membranes. SREBPs were detected using monoclonal antibodies against SREBP-1 (IgG 2A4, 1:1000; panel A) and SREBP-2 (IgG 1C6, 1:200; panel B). Lane 1 and lane 2, controls with medium alone; lane 3, lovastatin 10^{-6} M; lane 4, atorvastatin 10^{-6} M. Position of molecular size markers are as indicated.

similar to lovastatin and simvastatin; the IC_{50} values for the three compounds were in the range of 25–100 nM. These results are in agreement with data provided by Bergstrom *et al.* [39] who studied the effects of HMG-CoA reductase inhibition in rat liver and in HepG2 cells. In humans identical daily doses of atorvastatin have been more potent in lowering LDL cholesterol than lovastatin or simvastatin [6–8]. Together, our results and those of previous work suggests [30,39] that the greater efficacy of atorvastatin is not explained by a greater ability to inhibit cholesterol biosynthesis. The major difference between atorvastatin and the other HMGRI may thus lie in the pharmacokinetic properties. The mean terminal elimination half-life ($t_{1/2\beta}$) of most HMGRI ranges between 1 and 4 h. The mean half-life of atorvastatin in plasma is about 14 h, and enzyme inhibition lasts for 20–30 h due to active metabolites [40–42] so that sustained inhibition of the mevalonate occurs *in vivo* [43].

Depletion of the regulatory cholesterol pool below a certain threshold elicits feedback regulation of pathways involved in cholesterol homeostasis. We examined the LDL receptor pathway at different molecular levels. HMGRI enhanced the amount of mature SREBP-2, the steady-state levels of LDL receptor mRNA, the transcription rate at the LDL receptor promoter and the activity of the LDL receptor on the cell surface. Atorvastatin, lovastatin, and simvastatin proved to be equally effective in stimulating the LDL receptor pathway.

Each of the HMGRI examined increased the production and contents of triacylglycerides at in HepG2 cells at a similar magnitude. At drug concentrations at which LDL receptor expression was induced, the expression of the FAS

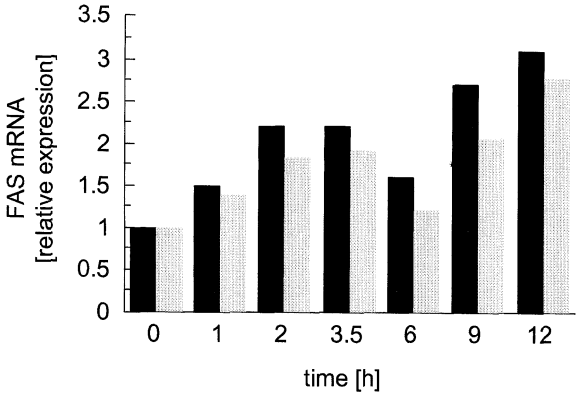

Fig. 6. Effect of atorvastatin and lovastatin on FAS mRNA. HepG2 cells were incubated for 24 h with DMEM medium containing 0.5% (v/v) LPDS or, in addition, with atorvastatin (grey bars), or lovastatin (black bars) at a concentration of 3×10^{-7} M for the indicated time periods (0–12 h). As described in Section 2 total cellular RNA was isolated, subjected to electrophoresis, and blotted to nitro-cellulose. The membrane was hybridised with [α^{32}P]-dCTP labeled cDNA-probes for FAS and for GAPDH (internal control) and exposed to autoradiography. The autoradiographs were scanned on a laser densitometer. The results were normalized to the amount of GAPDH, and expressed as relative values of the control incubations (panel C). Each bar represents the average from two independent experiments.

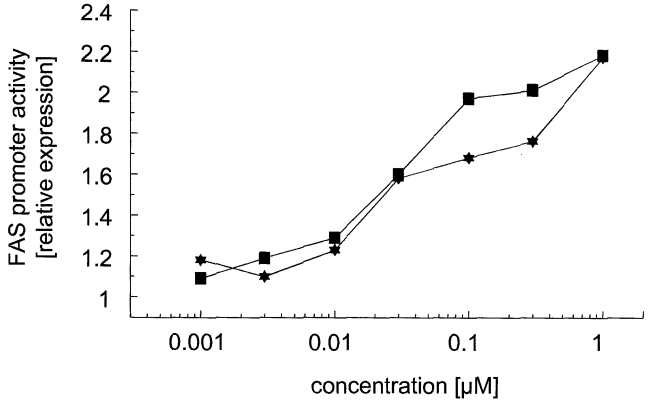

Fig. 7. Effect of atorvastatin and lovastatin on the expression of the FAS promoter. HepG2 cells were incubated for 24 h with DMEM medium containing 0.5% (v/v) LPDS. Following the transient transfection with a reporter gene construct containing the FAS promoter and firefly luciferase, the cells were stimulated for 24 h with atorvastatin (squares) or lovastatin (stars) at the concentrations indicated on the abscissa. The results were normalised to transfection efficacy and expressed as relative values of the respective control incubations (medium containing 0.5% (v/v) LPDS alone). Each data point represents the average of two experiments, each performed in quadruplicates. The standard deviations of the replicates were 20% or less of the respective means.

gene was stimulated, and the changes in lipid biosynthesis translated into altered cellular lipid content: cellular cholesterol decreased, whereas triacylglycerides increased. These findings were unexpected as HMGRI have been shown to lower triacylglycerides in clinical studies [1,4,7,31] and in experimental animals [9,11]. According to clinical experience, however, changes in triacylglyceride levels during

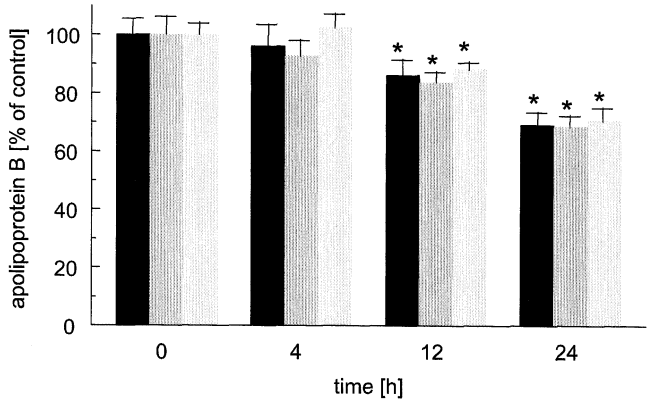

Fig. 8. Effect of atorvastatin, lovastatin, and simvastatin on the secretion of apo B. HepG2 cells were grown in DMEM medium supplemented with 10% (v/v) fetal calf serum. Twenty-four h prior to the experiment, the cells were switched to medium containing 1% BSA. The cells were then stimulated for 4, 12, or 24 h with medium containing 1% BSA and 0.8 M oleate alone or, in addition, atorvastatin (light grey bars), simvastatin (dark grey bars), and lovastatin (black bars) at a concentration of 3×10^{-7} M. Apo B in the media was determined by using an enzyme immunoassay and the amounts of apo B secreted from the cells were calculated. Results are expressed in percent of the respective control incubations. The results are means of two independent experiments, each performed in duplicates. *$P < 0.05$ versus the respective control incubations.

HMGRI therapy are heterogeneous, ranging between an effective reduction and, in some cases, distinct increases. Some patients with isolated hypertriacylglyceridemia at baseline paradoxically respond to HMGRI with increases of triacylglycerides [44]. A possible reason for this may be that the genes encoding ACC and FAS, both enzymes essential for fatty acid synthesis, are linked to the cellular level of sterols [18,21]. SREBP binding sites in the promoters of ACC and FAS mediate the activation of gene transcription similar to genes that encode proteins involved in cholesterol metabolism [18,19,21]. Here, atorvastatin and lovastatin increased nuclear SREBP-2, but not SREBP-1. This is essentially consistent with previous work [45] showing that treatment of hamsters with colestipol, a bile acid-binding resin, and the HMGRI mevinolin increased the nuclear form of SREBP-2, while the nuclear form of SREBP-1 decreased. Both SREBP-1 and SREBP-2 coordinately enhance the expression of enzymes involved in the production of free fatty acids and sterols. The activity of the transcription factors towards the two pathways, however, is not identical. In transgenic mice over-expressing SREBP-1, production of sterols and of free fatty acids was enhanced 5-fold and 26-fold, respectively, compared to wild-type animals. In contrast, over-expression of SREBP-2 resulted in 28-fold and 4-fold increases in sterol production and free fatty acid production [46]. Hence, SREBP-1 may be 5-fold more effective toward triacylglyceride than toward sterol synthesis, whereas the inverse may be true for SREBP-2. Nevertheless, we believe that activation of SREBP-2 provides a sufficient explanation for the upregulation of triacylglyceride synthesis seen in our study. In mice overexpressing soluble SREBP-2, fatty acid production was still as much as 4-fold higher than in wild-type mice, and hepatocytes of the transgenic animals contained significantly elevated amounts of triacylglycerides.

HMGRI thus appear to affect the biosynthesis of sterols and triacylglycerides in an opposite fashion. These two major lipid classes are essential for the assembly and secretion of apo B containing lipoproteins [47,48]. Studies in experimental animals support the hypothesis that HMGRI reduce hepatic apo B production [9,11,49,50]. In humans, the role of statins in the regulation of apo B secretion has not been clearly established. Turnover studies in humans treated with HMGRI revealed inconsistent results [51–54]. In this investigation, HMGRI decreased the release of apo B from HepG2 cells. These data, however, need to be interpreted cautiously. HepG2 cells are thought to be defective in mobilization of triacylglycerides for lipoprotein assembly and secretion [55,56]. The majority of *de novo* synthesised triacylglycerides is shifted to a cytoplasmic pool not linked to the regulatory microsomal pool. HepG2 cells do not assemble triacylglyceride-rich lipoproteins; the secreted apo B is mainly associated with particles with a density of LDL [57]. Thus, HepG2 cells may not be an entirely representative model for very low-density lipoprotein production in the liver. Nevertheless, a number of researchers have used

Table 2
Effect of atorvastatin, lovastatin, and simvastatin on the compositions of secreted lipoproteins

	Cholesterol (μg/mg cell protein)	Triacylglycerides (μg/mg cell protein)	Apo B (μg/mg cell protein)	Cholesterol/Apo B (molar ratio)	Triacylglycerides/Apo B (molar ratio)
Control	1.03 ± 0.12	1.12 ± 0.23	0.83 ± 0.09	1765	853
Atorvastatin	0.35 ± 0.09[a]	1.59 ± 0.29[b]	0.61 ± 0.11[a]	816	1648
Lovastatin	0.38 ± 0.11[a]	1.47 ± 0.31[c]	0.63 ± 0.09[a]	858	1475
Simvastatin	0.41 ± 0.12[a]	1.49 ± 0.27[b]	0.59 ± 0.10[a]	988	1597

HepG2 cells were incubated with medium containing 1% BSA for 24 h. Then the cells received medium containing 1% BSA and 0.8 M oleate alone, or together with the drugs (at a concentration of 10^{-6} M) for 24 h. After incubation, the media were removed and the fractions $d < 1.063$ kg/L were isolated by ultracentrifugation at 100,000 g for 24 h. Triacylglycerides and cholesterol were measured in the top 0.5 ml fractions as described in Section 2, apo B was determined in the whole media. The data are reported as means and SDs of three independent experiment, each performed in triplicates.

[a] Indicates $P < 0.001$;

[b] Indicates $P < 0.01$;

[c] Indicates $P < 0.05$ compared to control.

this cell line to investigate the effect of HMGRI on the secretion apo B. Atorvastatin has been shown to reduce apo B secreted from oleate-loaded cells [58,59], an effect not observed with lovastatin [58] or simvastatin [59]. Consistent with others [35,36], we show that lovastatin, simvastatin, and atorvastatin were able to decrease apo B secretion in HepG2 cells. The mechanisms by which HMGRI might modulate apo B secretion are not clearly understood. The rate of cholesterol synthesis may affect the rates of apo B degradation [60,61] or translocation [62] across the endoplasmic reticulum membrane. Further, there is evidence that the expression of microsomal triacylglyceride transfer protein expression is modified by sterol-sensitive pathways [63–65]. Finally, a decrease in free cholesterol might limit the synthesis of cholesteryl esters by acyl-coenzymeA:cholesterol acyltransferase, and consequentially reduce apo B secretion [50,66]. In this study, the effects of HMGRI on apo B secretion were smaller than anticipated from their impact on the synthesis and the secretion of cholesterol. An attractive explanation for this would be that the decrease of the cholesterol content in newly secreted lipoproteins was accompanied by an enrichment of the secreted particles with triacylglycerides. The net effect of these changes would be that the reduction of cholesteryl esters as core components of the secreted lipoprotein particles was at least partially compensated by the relative enrichment with triacylglycerides.

Taken together, this research provides a comprehensive picture of the actions of HMGRI on lipid homeostasis in a hepatoma cell line. HMGRI enhanced the activity of the LDL receptor pathway at multiple levels. In this respect, atorvastatin was equally effective compared to lovastatin and simvastatin, suggesting that the greater efficacy of this compound to lower LDL cholesterol in humans is most likely due to its unique pharmacokinetic properties.The significance to human metabolism of our unexpected finding that HMGRI increase hepatic triglyceride production and the triglyceride content of newly released apo B containing lipoproteins is currently difficult to determine. Overt hypertriacylglyceridemia develops in a small proportion only of patients receiving HMGRI. Two compensatory mechanisms may be responsible for this. First, the rate of assembly of apo B containing particles might by modulated by SREBP-mediated down-regulation of microsomal triacylglyceride transfer protein [63]. Second, accumulation of the triacylglyceride-enriched particles might be prevented by clearance of these particles through up-regulated LDL receptors before or shortly after secretion from hepatocytes [46].

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