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3-Hydroxy-3-methylglutaryl CoA reductase inhibitors reduce serum triglyceride levels through modulation of apolipoprotein C-III and lipoprotein lipase

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Abstract Statins are hypolipidemic drugs which not only improve cholesterol but also triglyceride levels. Whereas their cholesterol-reducing effect involves inhibition of de novo biosynthesis of cellular cholesterol through competitive inhibition of its rate-limiting enzyme 3-hydroxy-3-methylglutaryl CoA reductase, the mechanism by which they lower triglycerides remains unknown and forms the subject of the current study. Treatment of normal rats for 4 days with simvastatin decreased serum triglycerides significantly, whereas it increased high density lipoprotein cholesterol moderately. The decrease in triglyceride concentrations after simvastatin was caused by a reduction in the amount of very low density lipoprotein particles which were of an unchanged lipid composition. Simvastatin administration increased the lipoprotein lipase mRNA and activity in adipose tissue and heart. This effect on lipoprotein lipase was accompanied by decreased mRNA as well as plasma levels of the lipoprotein lipase inhibitor apolipoprotein C-III. These results suggest that the triglyceride-lowering effect of statins involves a stimulation of lipoprotein lipase-mediated clearance of triglyceride-rich lipoproteins.

Key words: Gene regulation; Atherosclerosis; Triglyceride; Hypolipidemic drug; Sterol response element binding protein-1/adipocyte determination and differentiation factor-1

1. Introduction

Intracellular cholesterol levels are tightly maintained within a narrow concentration range by an intricate transcriptional control mechanism, which involves the sterol response element binding proteins (SREBPs) of the helix-loop-helix transcription factor family [1]. Three distinct SREBPs have been identified, designated SREBP-1a and SREBP-1c, derived from the SREBP-1 gene, and SREBP-2, which is encoded by an independent gene. A homologue of SREBP, termed adipocyte determination and differentiation factor-1 (ADD-1) was independently cloned and characterized as implicated in adipocyte differentiation [2]. SREBPs are synthesized as membrane-bound precursors that are localized on the nuclear envelope and the endoplasmic reticulum and become proteolytically activated as the concentration of intracellular sterols decreases. The activated SREBP is subsequently targeted to the nucleus where it transactivates genes involved in the cholesterol synthesis and uptake. These genes include the ones encoding 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase, the low density lipoprotein (LDL) receptor, HMG-CoA reductase, farnesyl diphosphate synthase and squalene synthase [3–8]. Interestingly, SREBP also controls the expression of certain genes involved in fatty acid metabolism, such as acetyl CoA carboxylase [9,10], fatty acid synthase [10–12] and stearoyl-CoA desaturase [13].

SREBPs are not only important for intracellular cholesterol homeostasis, but they also play a role in controlling plasma cholesterol. In fact, the most effective therapy for hypercholesterolemia, a condition associated with atherosclerotic vascular disease, currently consists of the administration of statin lipid-lowering drugs. These compounds block the rate-limiting enzyme of the endogenous cholesterol biosynthesis pathway, HMG-CoA reductase, through competitive inhibition. The resulting decrease in the cellular cholesterol content will subsequently lead to the activation of the SREBPs and the induction of downstream target genes, such as the LDL receptor. On a whole organism level, enhanced LDL receptor clearance will ultimately translate in a significant reduction in circulating cholesterol levels. This reduction in cholesterol levels is often associated with a decrease in triglyceride levels. The aim of the present study was to analyze the mechanisms by which statins achieve this triglyceride-lowering effect. Our results obtained with simvastatin in a rat model demonstrate an important decrease in serum triglyceride-rich lipoproteins without a major effect on high density lipoprotein (HDL) concentrations. This action of simvastatin is mediated by both a decrease in apolipoprotein C-III levels in liver and plasma and a stimulation of lipoprotein lipase (LPL) mRNA and activity levels in heart and adipose tissue, providing a mechanistic basis for its triglyceride-lowering effect.

2. Materials and methods

2.1. Materials

Simvastatin was kindly provided by Dr S. Wright from Merck (Rahway, NJ, USA). Carboxymethylcellulose was purchased from Serva (Heidelberg, Germany).

2.2. Animals

10 Weeks old male Sprague-Dawley rats were randomized to treatment groups and treated daily by oral gavage with simvastatin suspended in 1% carboxymethylcellulose at the indicated period of time and doses. Control animals received an equal volume (5 ml/kg/day) of carboxymethylcellulose solution. At the end of the experiments, animals were weighed and killed by exsanguination while under ether anesthesia. Blood was collected and serum was separated and used

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within 1 week for analysis of lipids, lipoproteins and apolipoproteins. Metabolic active tissues, such as liver, muscle, heart and omental fat pads, were removed, weighed and frozen in liquid nitrogen.

2.3. Serum lipid, apolipoprotein and lipoprotein measurements

Serum and lipoprotein lipid (total cholesterol, triglyceride and phospholipid) concentrations were determined by enzymatic assays adapted to microtiter plates using commercially available reagents (Boehringer Mannheim, Germany: for cholesterol and triglycerides; Biomerieux, Marcy l'Etoile, France: for phospholipids). The serum HDL cholesterol content was determined after precipitation of apolipoprotein (apo) B-containing lipoproteins with phosphotungstic acid/Mg (Boehringer Mannheim). Non-HDL cholesterol was calculated as the difference between the total and HDL cholesterol. Serum levels of rat apo A-I and apo A-II were measured by an immunonephelometric assay using specific polyclonal antibodies. Interassay coefficients of variation for lipids and apolipoproteins ranged from 1.5% to 5.3%. The lipoprotein fractions (VLDL+IDL, $d = 1.006$–1.019 g/ml; LDL, $d = 1.019$–1.063 g/ml; HDL_2, $d = 1.063$–1.12 g/ml; HDL_3, $d = 1.12$–1.21 g/ml) were isolated by sequential ultracentrifugation of pooled rat serum [14]. Each fraction was further purified by a second ultracentrifugation at the same density intervals before analysis. After extensive dialysis at 4°C against 10 mM phosphate-buffered saline (PBS) at pH 7.2 containing 10 μmol/l EDTA, the protein concentration of each lipoprotein fraction was determined by the method of Lowry et al. [15]. For fast protein liquid chromatography (FPLC) size fractionation of lipoproteins, 300 μl of serum isolated from individual rats was injected on a Sepharose 6HR 10/30 pre-packed column (Pharmacia, Uppsala, Sweden) and eluted at a constant flow rate of 0.2 ml/min with PBS pH 7.2. The effluent was monitored at 280 nm, collected in 0.3 ml fractions and cholesterol concentrations were determined in 0.1 ml of each fraction. The apolipoprotein composition of isolated lipoproteins was analyzed by polyacrylamide gradient (4–20%) gel electrophoresis in the presence of SDS (SDS-PAGE) using ready to use materials (Novex, San Diego, CA, USA). 15 μg of proteins of each lipoprotein fraction was loaded on the gels. Electrophoresis was performed in 0.025 M Tris, 0.192 M glycine, SDS 0.1%, pH 8.3. The apolipoproteins were identified by their molecular weight determined by the simultaneous migration of standards of a known molecular weight (Low Molecular Weight markers electrophoresis calibration kit, Pharmacia Biotech, Uppsala, Sweden).

2.4. Measurement of tissue LPL activity

LPL enzymatic activity was measured in extracts from heart, muscle and omental adipose tissue according to the procedure of Ramirez [16] with minor modifications. The assay mixture contained 0.6 mM glycerol tri[9,10-(n)-^{3}H]oleate (12 Ci/mol), 50 mM $MgCl_2$, 0,05% fatty acid-free BSA, 3% rat serum (pre-heated 30 min at 56°C), 25 mM PIPES (pH 7.5) and 0.02 ml of sample in a final volume of 0.2 ml. The reaction was stopped and [^{3}H]oleate separated from substrate was quantified. One unit of enzyme activity was defined as the amount of enzyme which releases 1 μmol oleate/min at 25°C.

2.5. RNA analysis

RNA was isolated from liver, heart, muscle and omental adipose tissue by the acid guanidinium thiocyanate/phenol/chloroform method [17]. Northern blot analysis of total cellular RNA was performed as described [18]. Rat apo A-I, apo A-II, apo C-III and human LPL cDNA clones were used as probes. The cDNA clone 36B4 encoding the human acidic ribosomal phosphoprotein PO [19] was used as a control probe. All probes were labelled by random-primed labelling (Boehringer Mannheim). Filters were hybridized to 1×10^6 cpm/ml of each probe. They were washed once in 500 ml 75 mmol/l NaCl, 7.5 mmol/l sodium citrate and 0.1% SDS for 10 min at 20°C and twice for 30 min at 65°C and subsequently exposed to X-ray film (X-OMAT-AR, Kodak). Autoradiograms were analyzed by quantitative scanning densitometry (Bio-Rad GS670 Densitometer, Bio-Rad Laboratories, CA, USA) as described [18].

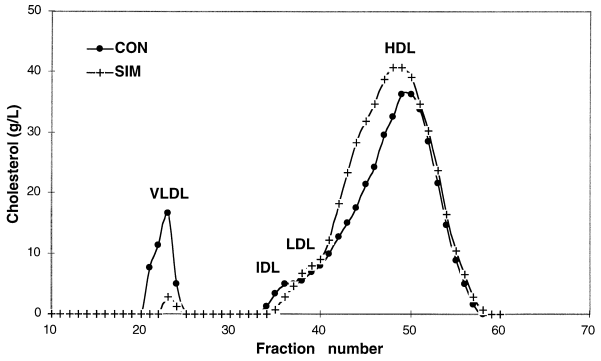

Fig. 1. Effects of simvastatin on serum lipoproteins. A representative profile of the serum cholesterol distribution between lipoproteins from adult male rats treated for 4 days with simvastatin (SIM) at a dose of 120 mg/kg/day or vehicle only (CON) is shown. Equal volumes of serum from control and simvastatin-treated rats were separated by gel filtration chromatography and cholesterol concentrations were measured in the isolated fractions as indicated under Section 2. Elution patterns of VLDL, IDL, LDL and HDL lipoproteins are indicated.

3. Results and discussion

In order to study the effects of statins on serum lipids and lipoproteins, rats were initially treated with simvastatin at a dose corresponding to 120 mg/kg/day. Administration of simvastatin by oral gavage for 4 days did not change body weights (473 ± 35 g in control animals versus 462 ± 35 g in simvastatin treated animals). Although serum total cholesterol levels had a tendency to increase in treated rats, no statistically significant difference with the control animals could be observed. The repartition of cholesterol among the lipoproteins showed slight differences and treated rats displayed higher HDL cholesterol levels ($P < 0.05$) and lower non-HDL cholesterol concentrations than control animals (Table 1). The cholesterol profiles obtained by gel filtration chromatography are depicted in Fig. 1 and confirm the slight increase of HDL cholesterol levels in simvastatin-treated rats measured by the precipitation technique. More particularly, a shift towards larger-sized HDL particles is observed in treated rats. This observation is concordant with the reduction of apo A-I and apo A-II observed in treated rats (Table 1), indicating that administration of simvastatin to rats leads to a more pronounced accumulation of cholesterol-enriched and less dense HDL particles looking like the HDL_2 subspecies. These findings confirm previous observations of poor cholesterol-

Table 1
The influence of simvastatin on serum lipid concentrations and apo A-I and apo A-II levels in rats

Dose (mg/kg/day)	Total cholesterol (mg/dl)	HDL Chol (mg/dl)	Non-HDL cholesterol (mg/dl)	TG (mg/dl)	Apo A-I (mg/dl)	Apo A-II (mg/dl)
0	65 ± 10	47 ± 8	19 ± 5	166 ± 39	55 ± 6	26 ± 5
120	80 ± 6	64 ± 3*	17 ± 8	95 ± 16*	50 ± 7	23 ± 5

Adult male rats ($n = 4$/group) received simvastatin intragastrically at the indicated dose for 4 days. Serum total, HDL cholesterol, triglyceride (TG), apo A-I and apo A-II concentrations were measured as described in Section 2. Statistically significant differences (U Mann-Whitney, $P < 0.05$) between control and simvastatin-treated animals are indicated by an asterisk.

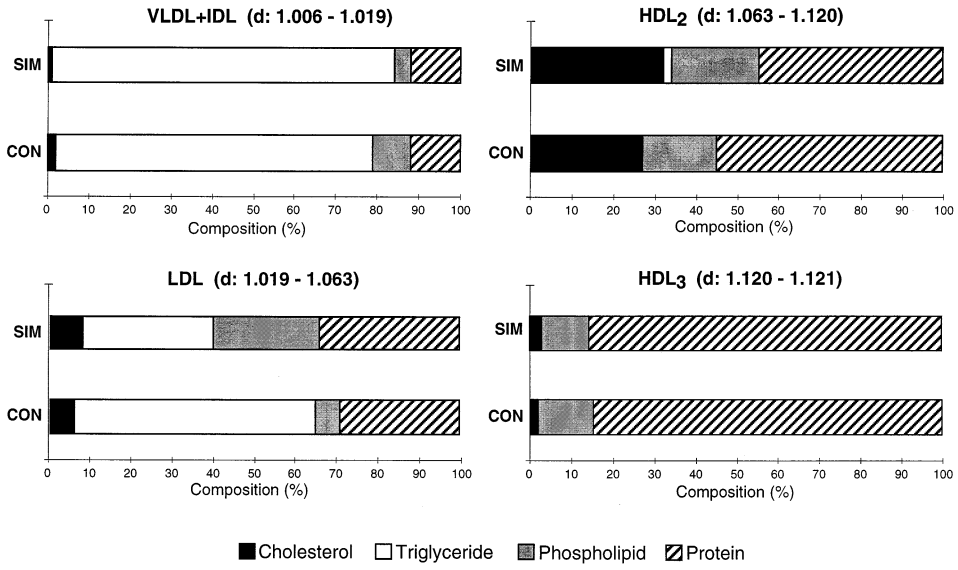

Fig. 2. Adult male rats ($n = 4$/group) were treated intragastrically with simvastatin at a dosis of 120 mg/kg/day for 4 days. Serum lipoproteins VLDL+IDL, LDL, HDL_2 and HDL_3 were isolated by sequential density ultracentrifugation at the indicated density intervals and lipoprotein total cholesterol, triglyceride, phospholipid and protein concentrations were measured and expressed as percentage of the total as described in Section 2.

lowering by statins in rodents, an effect mainly attributed to an increased hepatic induction of the HMG-CoA reductase mass and activity that compensates for the enzymatic inhibitory effect of simvastatin [20–22]. Despite the absence of a significant cholesterol-lowering effect of simvastatin in rats, which is in sharp contrast to the situation observed in man and other animal models, our results unequivocally show that treatment of normal rats with simvastatin results in a major decrease in serum triglyceride concentrations (Table 1), attributed essentially to a robust decrease in VLDL concentrations in serum (Fig. 1). In addition, a moderate decrease in IDL was observed in treated rats whereas the LDL fraction was slightly increased (Fig. 1). To analyze whether the effects of simvastatin on the lipoprotein levels of treated rats (i.e. a slight increase in HDL and LDL, a moderate decrease in IDL and a strong reduction of VLDL) were associated with alterations in the biochemical composition of the lipoprotein fractions, lipoproteins were isolated by sequential ultracentrifugation and their chemical composition determined (Fig. 2). Compared to control rats, particles flotating between 1.006 and 1.019 (VLDL+IDL) isolated from rats treated with simvastatin displayed basically a similar pattern with an unchanged protein and cholesterol composition and minor variations regarding the triglyceride and phospholipid content, indicating therefore that the decrease in triglyceride concentrations observed in treated rats is rather due to a decrease in the amount of lipid particles than to changes in the lipid composition. In contrast, the relative triglyceride content of particles in the LDL density range (1.019–1.063) decreased by 27% after simvastatin (Fig. 2). The relative decrease in the triglyceride content of the LDL particles was compensated by an enrichment in phospholipids and, to a lesser extent, in proteins (Fig. 2). These relative changes in LDL composition after simvastatin treatment are indicative of an increased lipolysis of triglycerides in plasma lipoproteins leading to an accelerated conversion into LDL particles, which is consistent with the cholesterol profile of the different lipoprotein fractions outlined in Fig. 1. Finally, the overall composition of HDL, isolated as particles in the 1.063–1.12 (HDL_2) and in the 1.12–1.21 (HDL_3) density range, was not significantly affected by simvastatin treatment (Fig. 2), reinforcing the hypothesis that, most likely, the number of HDL_2 particles is increased in simvastatin-treated animals.

In order to define the mechanism by which simvastatin affects the lipid and lipoprotein metabolism, we studied its effect on some key apolipoproteins and lipases involved in triglyceride metabolism, such as apo C-III, LPL and hepatic lipase (HL). Whereas LPL and HL promote the hydrolysis and removal of triglyceride-rich lipoproteins from plasma, apo C-III antagonizes the clearance of triglycerides by inhibiting the plasma LPL activity. Therefore, the apolipoprotein composition of the different lipoprotein fractions was first

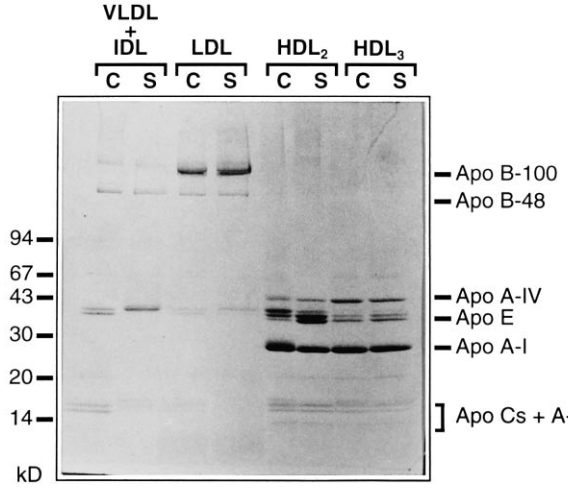

Fig. 3. The influence of simvastatin treatment on the apolipoprotein composition of serum lipoproteins. Rats were treated daily for 4 days with simvastatin (S) at a dosis of 120 mg/kg/day or vehicle only (C). Serum was pooled ($n = 4$/treatment group), lipoproteins were isolated by sequential ultracentrifugation and the apolipoprotein composition was analyzed by non-reducing SDS-PAGE as described under Section 2.

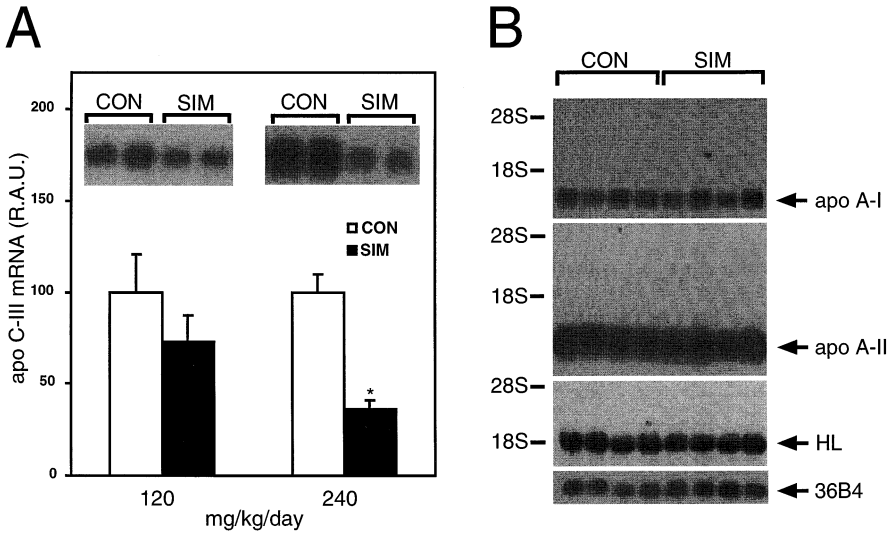

Fig. 4. The influence of simvastatin treatment on liver apo C-III (A), apo A-I, apo A-II and HL mRNA levels (B). (A) Rats ($n = 4$/group) were treated for 4 days with simvastatin at two different doses corresponding to 120 mg/kg/day and 250 mg/kg/day. Liver apo C-III mRNA levels were measured at the end of the treatment period as described in Section 2. RNA values are expressed in relative absorbance units (R.A.U.) taking the control values as 100%. Statistically significant differences ($P < 0.05$ with the U Mann-Whitney test) are indicated by an asterisk. (B) Liver RNA samples from controls and rats treated for 4 days with simvastatin at a dosis corresponding to 120 mg/kg/day were blotted on a nylon membrane and hybridized to apo A-I, apo A-II, HL and 36B4 cDNAs.

analyzed by SDS-PAGE. Compared to control, treatment with simvastatin did induce a drastic decrease in the apo Cs and apo E content in VLDL-IDL particles (Fig. 3, lane 1 versus lane 2). In contrast, the apolipoprotein distribution in LDL, HDL_2 and HDL_3 was not really affected by simvastatin treatment, except for a slight increase of the apo E content in the HDL_2 particles derived from treated rats (Fig. 3, lanes 5 and 7 versus lanes 6 and 8, respectively). Since apo C-III is a major determinant of serum triglyceride levels [23,24], we next determined the steady state levels of its mRNA in the liver of control and treated animals. Consistent with the decreased apo C-III protein content in the VLDL-IDL fraction in treated rats, administration of simvastatin reduced also apo C-III mRNA levels by 30% compared to control animals ($P = 0.08$; Fig. 4A). The reducing effect of simvastatin on apo C-III mRNA expression was even more pronounced (approximately 70%) when rats were treated at a dose of 250 mg/kg/day (Fig. 4A). The decrease of apo C-III mRNA expression seemed specific, since no significant changes in liver HL, apo A-I, and A-II mRNA levels were observed (Fig. 4B).

In adipose tissue, treatment with simvastatin increased LPL mRNA levels by 2-fold in comparison to controls (Fig. 5A), although no statistically significance could be reached. This increase in LPL gene expression after simvastatin was paralleled by a comparable statistically significant increase in adipose tissue LPL activity (2-fold increase; $P < 0.01$; Fig. 5B). In heart, comparable increases in LPL mRNA and activity were detected (Fig. 5A and B). Interestingly, LPL mRNA and activity levels in skeletal muscle, another site of LPL production, did not change after simvastatin treatment (Fig. 5A and B). These observations in rat of an enhanced LPL activity in heart and adipose tissue together with the observed shift towards HDL_2 particles after simvastatin treatment are in agreement with previous studies in man demonstrating a positive correlation between HDL_2 levels and the LPL activity or triglyceride clearance rate (for review, see [25]).

One potential candidate involved in translating the effects of simvastatin on gene expression is the SREBP family of transcription factors, which have been shown to become proteolytically activated by reduced intracellular cholesterol levels after HMG-CoA reductase inhibitors (see Section 1). Consistent with a role for SREBP in controlling LPL expression, it was recently shown that LPL mRNA levels were induced by ectopic overexpression of SREBP [10,12]. Furthermore, we recently identified a functional sterol responsive element for SREBP in the human LPL promoter (unpublished data). In contrast to the regulatory effects on LPL, which are most likely mediated via interaction of the SREBPs with the LPL promoter, it is at present unclear how simvastatin affects apo C-III expression. Studies addressing this issue are currently underway in our laboratory.

Altogether, our data suggest that in a rat model, simvasta-

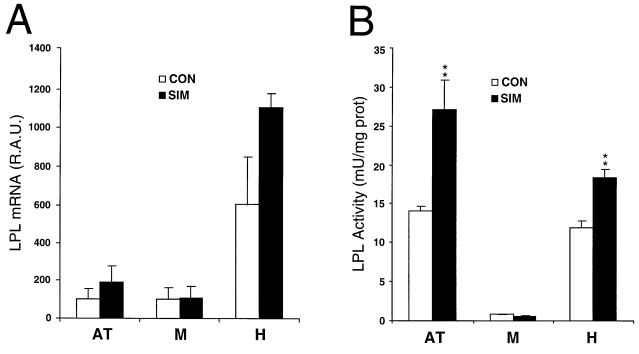

Fig. 5. The influence of simvastatin treatment on LPL mRNA levels (A) and activity (B) in rat adipose tissue, heart and skeletal muscle. (A) Adult male rats were treated daily for 4 days with simvastatin at a dosis of 120 mg/kg/day. Total RNA was extracted and LPL and 36B4 mRNA levels were measured as described in Section 2. LPL mRNA values (mean ± S.D.) are expressed relative to 36B4 mRNA levels and represented in relative absorbance units (R.A.U.). (B) Tissue LPL activity was measured as described in Section 2. Statistically significant differences ($P < 0.01$, U Mann-Whitney test) are indicated by two asterisks.

tin increases lipolysis of triglycerides rather than causing a reduction in hepatic VLDL production. This is in contrast to the triglyceride-lowering induced by activators of the peroxisome proliferator-activated receptor (PPAR) α, such as fibrates, which in general is associated with a reduced hepatic VLDL secretion [26–29]. Furthermore, fibrate treatment does not change the lipid composition of LDL lipoproteins (data not shown), suggesting that these drugs have rather minor effects on VLDL lipolysis. The triglyceride-reducing effect of statins appears therefore more similar to the effects observed with thiazolidinedione PPARγ agonists, which in contrast to PPARα agonists, mainly affect the peripheral LPL-mediated lipolysis of triglycerides. These differences are indicatives that the same triglyceride-lowering effect of different lipid-lowering agents, such as fibrates, thiazolidinediones and statins, are achieved through distinct mechanisms.

The statins currently form the cornerstone for the clinical management of hypercholesterolemia. Administration of these drugs to subjects with hypercholesterolemia results in a significant reduction of cardiovascular events and a decrease in mortality [30]. The present data obtained in a rat model underscore the fact that these compounds not only lower cholesterol levels, but also significantly reduce the concentration of triglycerides, through an LPL-mediated clearance of triglyceride-rich lipoproteins. It is very likely that the combination of cholesterol-lowering with triglyceride-lowering underlies the remarkable efficacy of statins in subjects with combined hyperlipidemia syndromes, as often seen in obesity and type 2 diabetes mellitus [31]. Consistent with this hypothesis, subjects with type 2 diabetes would benefit most from intervention with this class of drugs.

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