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Effect of hypolipidemic drugs on key enzyme activities related to lipid metabolism in normolipidemic rabbits

Marta Alegret [a], Joan C. Verd [a], Cristina Díaz [b], Gonzalo Hernández [b], Tomás Adzet [a], Rosa M. Sánchez [a], Juan C. Laguna [a,*]

[a] *Unidad de Farmacología y Farmacognosia, Dept. Farmacología y Química Terapéutica, Facultad de Farmacia, Núcleo Universitario de Pedralbes, Barcelona 08028, Spain*

[b] *Departamento I + D, Parke-Davis, Barcelona, Spain*

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Abstract

The effect of atorvastatin (3 mg kg^{-1} day^{-1}), simvastatin (3 mg kg^{-1} day^{-1}) and bezafibrate (100 mg kg^{-1} day^{-1}) administered for 4 weeks to male New Zealand white rabbits on enzyme activities related to lipid metabolism has been studied. Only the statins reduced plasma cholesterol values, while none of the drugs modified plasma triglyceride or high density lipoprotein (HDL)-cholesterol concentrations, nor the activity of enzymes such as hepatic diacylglycerol acyltransferase, lipoprotein lipase or hepatic lipase, directly involved in triglyceride metabolism. Both statins elicited similar increases in the hepatic microsomal 3-hydroxy-3-methyl-glutaryl Coenzyme A (CoA) reductase activity (147 and 109% induction for simvastatin and atorvastatin, respectively), and none of the drugs assayed modified hepatic acyl-coenzyme A:cholesterol acyltransferase activity significantly. Only bezafibrate induced a significant 57% reduction in the activity of hepatic microsomal cholesterol 7α-hydroxylase. Regarding the rate limiting enzyme of phosphatidylcholine biosynthesis, CTP:phosphocholine cytidylyl transferase, atorvastatin and bezafibrate behaved similarly, decreasing the enzyme activity in the liver by 45% and 54%, respectively; simvastatin induced no modification of this activity. The reduction of CTP:phosphocholine cytidylyl transferase activity is not caused by a direct inhibition of the enzyme by bezafibrate and atorvastatin. Further, the inhibitory effect of atorvastatin appears to be unrelated to the inhibition of 3-hydroxy-3-methyl-glutaryl CoA reductase elicited in vivo. © 1998 Elsevier Science B.V.

Keywords: Atorvastatin; Simvastatin; Bezafibrate; Normolipidemic rabbit; CTP:phosphocholine cytidylyl transferase; Cholesterol 7α-hydroxylase

1. Introduction

Statins or vastatins are the most potent drugs available for reducing plasma low density lipoprotein (LDL)-cholesterol concentrations (Larsen and Illingworth, 1994; Havel and Rapaport, 1995). Treatment with the most widely used statins, lovastatin, simvastatin or pravastatin, consistently reduces plasma LDL-cholesterol by 15–40%, either in primary hypercholesterolemic (Weisweiler, 1988; Tikkanen et al., 1991; Hong et al., 1993) or in combined dyslipidemic patients (Nakandakare et al., 1990; Goldberg et al., 1990; Tikkanen et al., 1991). Fibrates are the drugs of choice for controlling plasma triglycerides (Larsen and Illingworth, 1994), achieving impressive reductions of 40–60% in triglyceride values (Klosiewicz-Latoszek and Szostak, 1991). Nevertheless, the picture is not so clear when the effects of these two families of hypolipidemic drugs are assessed on enzyme activities related to lipid biosynthesis and metabolism. Except for the well known inhibitory effect of statins on 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) reductase activity (Chao et al., 1991; Endo, 1992), there is a wealth of even contradictory reports on the effect of statins and fibrates on other key enzyme activities related to hepatic lipid synthesis. Many of these inconsistencies are probably due to high drug dosages, changing experimental conditions (animal species, sex, and tissue, cell cultures, etc.) which makes comparisons difficult.

In order to shed some light on this problem, we have investigated the effects of two widely used representatives

* Corresponding author. Tel.: +34-3-4024531; fax: +34-3-4021886; e-mail: laguna@farmacia.far.ub.es

of both families of hypolipidemic drugs, bezafibrate (Goa et al., 1996) and simvastatin (Plosker and McTavish, 1995), and a new synthetic statin, atorvastatin (Lea and McTavish, 1997), on enzyme activities related to the hepatic metabolism of (i) cholesterol: 3-hydroxy-3-methyl-glutaryl CoA reductase, acyl-coenzyme A:cholesterol acyltransferase, cholesterol 7α-hydroxylase, (ii) triglyceride: diacylglycerol acyltransferase, lipoprotein lipase, hepatic lipase, and (iii) phospholipid: CTP:phosphocholine cytidylyl transferase in male New Zealand white rabbits.

2. Material and methods

2.1. Materials

3-Hydroxy-3-methyl [3-^{14}C]glutaryl-coenzyme A, *RS*-[2-^{14}C]mevalonic acid lactone, [4-^{14}C]cholesterol, [1-^{14}C]oleoyl-coenzyme A, cholesteryl[1-^{14}C] oleate, phosphoryl [methyl-^{14}C]choline ammonium salt, [1-^{14}C]palmitoyl-coenzyme A and glycerol tri[1-^{14}C]oleate were purchased from Amersham Iberica (Madrid, Spain). Glucose 6-phosphate, NADPH, $NADP^+$, dithiotreitol, glutathion (GSH), 3-hydroxy-3-methyl-glutaryl-coenzyme A, cholesterol, 7-beta-hydroxycholesterol, oleoyl-coenzyme A, palmitoyl-coenzyme A, cholesteryl oleate, ADP, CTP, CDP-choline, phosphorylcholine, phosphatidylcholine, Triton X-100 and fatty acid free bovine serum albumin were from Sigma (Madrid, Spain). Glucose 6-phosphate dehydrogenase and silica gel thin layer chromatography (TLC) plates were from Merck (Barcelona, Spain). Scintillation fluids (Co 136 and Co 36) were purchased from Scharlau (Barcelona, Spain). Other general chemicals were obtained from commercial sources and were of analytical grade.

2.2. Drugs

Atorvastatin calcium (CI-981) was supplied by Parke-Davis (Ann Arbor, MI). Simvastatin was a generous gift from Merck (Barcelona, Spain), and bezafibrate was purchased from Acofarma (Barcelona, Spain).

2.3. Animals and diets

Thirty two male New Zealand white (NZW) rabbits were obtained from Pi Petit (Barcelona, Spain). The animals were housed in individual cages in a room with constant humidity and temperature (22 ± 2°C) under a 12-h light–dark cycle and were fed a standard diet (Mucedola, Milano, Italy). After one week, the animals (average weight 1.93 ± 0.26 kg) were distributed randomly into 4 groups, which were fed, respectively, 120 g of a standard diet or a diet supplemented with bezafibrate (100 mg kg^{-1} day^{-1}), simvastatin (3 mg kg^{-1} day^{-1}) or atorvastatin (3 mg kg^{-1} day^{-1}) for 4 weeks. The diets were prepared as described previously (Alegret et al., 1994). Body weight, plasma total cholesterol, high density lipoprotein (HDL)-cholesterol and triglyceride levels were measured weekly throughout the treatment.

2.4. Experimental

Blood samples for plasma lipid determinations were obtained once a week, after 12 h-fasting, by ear puncture in EDTA-tubes. At the end of the treatment, samples were obtained by the same method, before and 5 min after intravenous heparin injection (100 U kg^{-1}). Plasma was obtained by centrifugation at $3000 \times g$ for 10 min at 4°C. The animals were killed between 8:00 and 9:00 AM to avoid circadian variations in enzyme activity, and the livers were removed and perfused with ice-cold 0.9% NaCl. From each liver, two parts of 6 g were homogenized separately in 6 volumes of buffer A (50 mM KH_2PO_4, pH 7.4, 150 mM NaCl, 1 mM dithiotreitol, 30 mM EDTA, 0.25 M sucrose) or buffer B (100 mM KH_2PO_4, pH 7.4, 30 mM nicotinamide, 1 mM EDTA, 0.25 M sucrose) using a Potter Elvehjem homogenizer with a teflon pestle at low speed (3 strokes). The post-mitochondrial and microsomal fractions were obtained by differential centrifugation as described previously (Montgomery and Cinti, 1977). Microsomes were resuspended in buffer A devoid of sucrose (part A) or in 20 mM KH_2PO_4 pH 7.4, 1 mM EDTA (part B). Part B microsomes were used to prepare a microsomal acetone powder according to Shefer et al. (1981). Protein content of subcellular fractions was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.5. Enzyme assays

2.5.1. 3-Hydroxy-3-methyl-glutaryl CoA reductase (EC 1.1.1.34)

3-Hydroxy-3-methyl-glutaryl CoA reductase (EC 1.1.1.34) activity was determined in liver microsomes by the micro assay described by Shapiro et al. (1974) with some modifications. Incubations were performed in a total volume of 150 μl which contained buffer A, 0.46 mg bovine serum albumin ml^{-1}, 3 mM $NADP^+$, 4.25 mM dithiotreitol, 30 mM glucose 6-phosphate, 0.3 units of glucose 6-phosphate dehydrogenase and 200 μg of microsomal protein. After a preincubation period of 20 min at 37°C, the reaction was carried out with 90 μM hydroxymethyl glutaryl-CoA (HMGCoA) (containing 0.45 μCi of [3-^{14}C]HMGCoA) for 20 min. To stop the reaction, 25 μl of 10 N HCl were added, and the mixture was then lactonized and centrifuged. The supernatant was subjected to TLC and processed as described.

2.5.2. Cholesterol 7α-hydroxylase (EC 1.14.13.17)

Cholesterol 7α-hydroxylase (EC 1.14.13.17) activity was determined in microsomal acetone powder according

to Shefer et al. (1981). The incubation mixture contained 100 mM KH_2PO_4 pH 7.4, 5 mM $MgCl_2$, 10 mM NADPH and 250 μg of protein in a total volume of 250 μl. The substrate, [4-^{14}C]cholesterol, was added to the incubation medium as a suspension in Triton X-100. The incubations were carried out in the dark for 20 min at 37°C, and terminated by adding 3.5 ml of dichloromethane/ethanol 5:1 (v/v). Boiled blanks were run along with each experiment. After the addition of 1.5 ml of H_2O, the organic phase was removed, evaporated to dryness, redissolved in acetone, applied to silica gel plates and processed as described in Shefer et al. (1981).

2.5.3. Acyl-coenzyme A:cholesterol acyltransferase (EC 2.3.1.26)

Acyl-coenzyme A:cholesterol acyltransferase (EC 2.3.1.26) was determined using oleoyl-CoA as substrate by the method described by Helgerud et al. (1981) with some modifications. The incubation mixture contained (final volume 250 μl) 100 mM KH_2PO_4 1 mM EDTA buffer pH 7.4, 6 mg bovine serum albumin ml^{-1}, 0.62 mg GSH ml^{-1} and 150 μg of microsomal protein. After a preincubation period of 5 min at 37°C, oleoyl-CoA (containing 0.135 μCi of [1-^{14}C]oleoyl-CoA) was added to a final concentration of 35 μM and samples were incubated for 4 min. The reaction was stopped by adding 5 ml of $CHCl_3$/MeOH (2:1 v/v). 40 μg of unlabeled cholesteryl oleate were added to each tube as a marker. After adding 1 ml of 0.88% KCl pH 3, the organic phase was collected, evaporated to dryness and dissolved in 60 μl of stop solution. The samples were then applied to silica gel TLC plates, which were developed in hexane/diisopropyl ether/acetic acid 10:44:3 (v/v). The spots corresponding to cholesteryl oleate were visualized with iodine vapours, scraped off into scintillation vials and radioactivity was counted in a Beckman LS 1800 liquid scintillation counter.

2.5.4. CTP:phosphocholine cytidylyl transferase (EC 2.7.7.15)

CTP:phosphocholine cytidylyl transferase (EC 2.7.7.15) activity was determined in post-mitochondrial supernatant (30 μg of protein/100 μl final volume of incubation mixture) by measuring the formation of radioactive CDP-choline from phosphoryl[methyl-^{14}C]choline by the charcoal adsorption method, exactly as described by Weinhold and Feldman (1992).

When assays were performed in vitro, drugs were added to the incubation medium 5 min prior to the starting of the enzyme reaction. Simvastatin and atorvastatin stock solutions were prepared in ethanol; the simvastatin-open lactone form was prepared by heating an ethanol solution of the lactone in the presence of 0.1 M NaOH, at 50°C for 2 h. Stock solutions were diluted with assay buffer before the addition to the medium, final ethanol concentration being below 0.05 ppm. Bezafibrate was added from a stock solution adjusted to pH 8–8.5 with 0.1 M NaOH, further diluted with assay buffer, so that the pH of the reaction mixture was not altered (Sánchez et al., 1992). Control incubations were performed by adding to the assay medium enough drug vehicle to obtain the same final concentration of ethanol or NaOH.

2.5.5. Diacylglycerol acyltransferase (EC 2.3.1.20)

Acylation of *sn*-1,2-diacylglycerol with [1-^{14}C]palmitoyl-CoA was determined according to Coleman (1992). The reaction was carried out exactly as described, using liver microsomes (15 μg of protein in a total volume of 200 μl of incubation mixture), for 15 min at 25°C. The reaction was terminated by adding 1.5 ml of 2-propanol/heptane/water (80:20:2, v/v). One ml of heptane and 0.5 ml of water were added and the mixture was vortexed. A total of 2 ml of ethanol/0.5 M NaOH/water (50:10:40, v/v) was added to the top phase and an aliquot of the heptane phase was counted in a Beckman LS 1800 liquid scintillation counter.

2.5.6. Lipoprotein lipase (EC 3.1.1.34) and hepatic lipase (EC 3.1.1.3)

Lipoprotein lipase (EC 3.1.1.34) and hepatic lipase (EC 3.1.1.3) activities were determined in post-heparin plasma samples by using a glycerol tri[1-^{14}C]oleate and phosphatidylcholine emulsion prepared as described by Glaser et al. (1992). For determination of total lipase activity, the emulsion contained inactivated serum as a source of apo C-II, while the emulsion prepared with 2.8 M NaCl and omitting serum was used to assess hepatic lipase activity. Lipoprotein lipase activity was calculated by subtracting hepatic lipase from total lipase activity. The incubations were carried out at 37°C for 30 min with 10 μl of post-heparin plasma and 50 μl of each emulsion (143.3 nmol, 200 000 cpm), as described by Vilaró et al. (1986). The reaction was terminated by addition of 3.5 ml of methanol/chloroform/heptane (141:125:100, v/v). Oleate was extracted by addition of 1 ml of 0.1 M borate/carbonate buffer, pH 10.5, an aliquot of the supernatant was counted in a Beckman LS 1800 liquid scintillation counter.

2.6. Plasma cholesterol and triglyceride concentration

Plasma cholesterol concentration was determined by the colorimetric test Monotest Cholesterol CHODPAP No. 290319, and triglyceride concentration was assayed by the Peridochrom Triglyceride GPO-PAP No. 701882 test, both from Boehringer Mannheim (Barcelona, Spain). Very low density lipoprotein (VLDL) and LDL from plasma samples were precipitated by using the reagent No. 543004, also

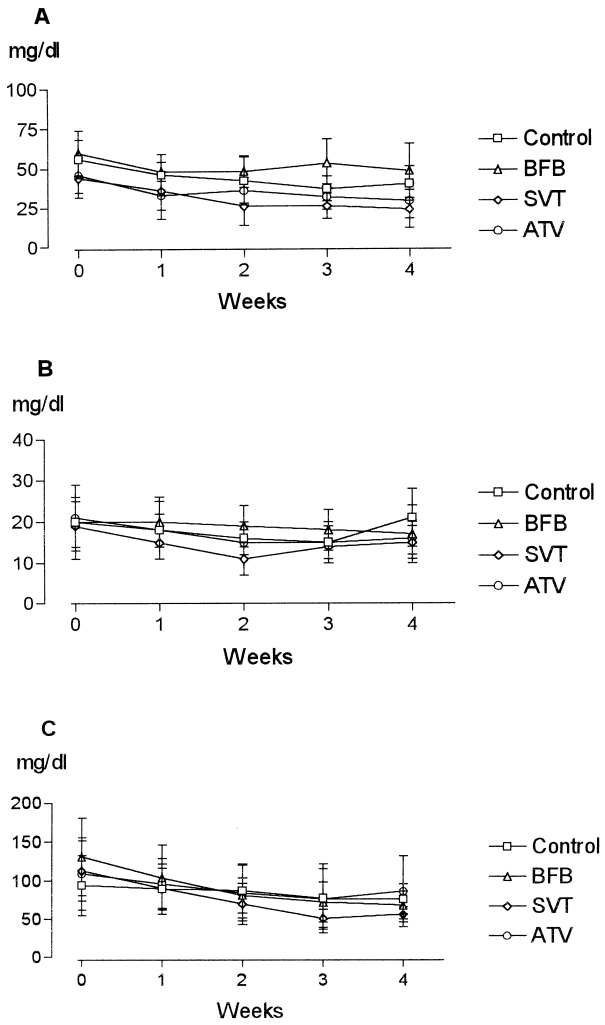

Fig. 1. Effect of bezafibrate (BFB), simvastatin (SVT) and atorvastatin (ATV) on plasma lipid levels. (A) Total cholesterol; (B) HDL-cholesterol; (C) triglyceride. The results are the mean ± standard deviation from 7–8 experiments performed in duplicate.

from Boehringer Mannheim, and HDL-cholesterol concentration was determined in the supernatant.

2.7. Statistics

The results are the mean ± S.D. of n experiments assayed in duplicate. Plasma lipid concentrations are expressed as area under their respective time curves (AUC). Significant differences were established by an analysis of variance test (ANOVA) using the computer programme GraphPad InStat. For CTP:phosphocholine cytidylyl transferase, acyl-CoA:cholesterol acyltransferase and cholesterol 7α-hydroxylase, logarithm of activity was used to calculate statistics, as the variances were not homogeneous. When significant variations were found, the Dunnet multiple comparisons test was performed. Linear correlations between variables were calculated by using the above mentioned computer programme.

3. Results

3.1. Plasma lipid levels

NZW rabbits were fed 120 g day^{-1} of a control diet or a diet containing bezafibrate (100 mg kg^{-1} day^{-1}), simvastatin (3 mg kg^{-1} day^{-1}) or atorvastatin (3 mg kg^{-1} day^{-1}) for 4 weeks. Body weight was measured weekly, and no significant differences were found between control and treated groups (data not shown).

Atorvastatin and simvastatin treatment reduced, at the end of the treatment period, plasma total cholesterol concentrations by 28 and 40%, respectively (Fig. 1A). The decrease in plasma cholesterol levels was mainly due to changes in LDL and VLDL fractions, as plasma HDL-cholesterol levels were not modified by any of the drugs tested (Fig. 1B). In Table 1, cholesterol levels expressed as the area under plasma cholesterol time curves (AUC), show a decrease of 28 and 19% for simvastatin and atorvastatin treatment, respectively (P-values in Table 1). In fact, when AUC for apo-B cholesterol is calculated, a similar reduction is attained with both statins (38 and 34% for simvastatin and atorvastatin, respectively).

Plasma triglyceride concentrations (Fig. 1C) were reduced by 10 and 26%, at the end of the treatment with bezafibrate and simvastatin, respectively. However, when expressed as AUC, triglyceride levels were not significantly modified by drug treatment (Table 1).

3.2. Effects of treatment on enzyme activities involved in cholesterol metabolism

Fig. 2 shows the effect of the treatments on 3-hydroxy-3-methyl-glutaryl CoA reductase (HMG-CoA reductase)

Table 1
Effect of bezafibrate (BFB), simvastatin (SVT) and atorvastatin (ATV) on plasma lipid levels

	CTRL	BFB	SVT	ATV
Total cholesterol	1165.3 ± 213.4	1321.6 ± 235.1	821.3 ± 256.1[b]	915.9 ± 181.9[a]
HDL-cholesterol	440.9 ± 100.9	497.1 ± 99.8	377.3 ± 62.5	444.7 ± 108.7
Apo B-cholesterol	724.4 ± 204.0	824.4 ± 264.5	446.1 ± 237.7[a]	479.9 ± 189.7[a]
Triglyceride	2400.6 ± 652.0	2356.9 ± 751.7	1940.9 ± 368.8	2310.9 ± 881.2

Data are expressed as area under the curve (AUC) in mg day dl^{-1}, and are the mean ± standard deviation of 7–8 experiments performed in duplicate. [a]$P < 0.1$; [b]$P < 0.05$.

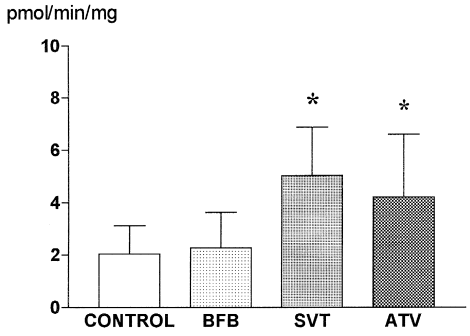

Fig. 2. Effect of bezafibrate (BFB), simvastatin (SVT) and atorvastatin (ATV) on 3-hydroxy-3-methyl-glutaryl-CoA reductase activity. The results are the mean ± standard deviation from 7–8 experiments performed in duplicate. * Values significantly different from control ($P < 0.05$).

activity, the rate limiting enzyme in the synthesis of cholesterol in the liver. This enzyme activity was unaffected by bezafibrate treatment, but significantly increased by simvastatin (147%) and atorvastatin (109%).

In Fig. 3, the activity of cholesterol 7α-hydroxylase, the rate limiting enzyme in the synthesis of bile acids from cholesterol, is shown in control and treated animals. The method used to determine this enzyme activity (Shefer et al., 1981) ensured the removal of endogenous cholesterol, so that the cholesterol used as a substrate was not diluted within the endogenous pool. Bezafibrate treatment caused a 57% reduction in cholesterol 7α-hydroxylase activity, while neither simvastatin nor atorvastatin had any significant effect on this enzyme activity. On the other hand, no drug treatment was able to modify acyl-coenzyme A:cholesterol acyltransferase activity (Table 2).

3.3. Effects of treatment on enzyme activities related to triglyceride metabolism

The activity of diacylglycerol acyltransferase, one of the key enzymes regulating the synthesis of triglycerides, was not affected by any of the drugs assayed (Table 2). Further, hepatic lipase and lipoprotein lipase, two enzyme activities that are involved in the catabolism of triglyceride-rich lipoproteins, were also unaffected (Table 2).

Table 2
Effect of bezafibrate (BFB), simvastatin (SVT) and atorvastatin (ATV) on lipid metabolism enzymes

	CTRL	BFB	SVT	ATV
$ACAT_a$	11.85 ± 5.4	16.60 ± 6.37	10.67 ± 5.45	8.38 ± 1.68
LPL_b	53.03 ± 24.91	65.88 ± 28.48	64.28 ± 21.56	66.16 ± 26.63
HL_b	7.04 ± 2.55	7.14 ± 3.85	7.10 ± 1.88	9.35 ± 2.81
$DGAT_c$	2.92 ± 0.94	3.31 ± 0.98	3.11 ± 1.23	2.99 ± 1.53

ACAT: acyl-coenzyme A:cholesterol acyltransferase. LPL: lipoprotein lipase. HL: hepatic lipase; DGAT: diacylglycerol acyltransferase. Data are expressed in a: pmol/min per mg protein, b: nmol/min per ml plasma and c: nmol/min per mg protein, and are the mean ± standard deviation of 7–8 experiments performed in duplicate.

3.4. Effects of treatment on enzyme activities involved in the synthesis of phospholipids

Both bezafibrate and atorvastatin caused a significant ($P < 0.05$) reduction (45 and 54%, respectively) of CTP:phosphocholine cytidylyl transferase activity, whereas simvastatin had no effect on this enzyme activity (Fig. 4).

To discard a direct effect of the drugs on CTP:phosphocholine cytidylyl transferase activity, in vitro assays were performed. The addition of bezafibrate to the assay medium showed a slight reduction of 7% in CTP:phosphocholine cytidylyl transferase activity at 0.25 mM, which was not apparent at 1 mM. When we tested the effect of the statins at 10 μM in vitro, a decrease of 18, 9 and 25% in CTP:phosphocholine cytidylyl transferase activity was obtained for atorvastatin, simvastatin and simvastatin open lactone form, respectively (control activity: 1070 ± 131 pmol min^{-1} mg^{-1} of protein). However, the inhibitory effect was not dose-dependent, as it was not maintained at 50 μM.

The changes in HMG-CoA reductase and CTP:phosphocholine cytidylyl transferase activities elicited by atorvastatin treatment, were further analyzed and compared to

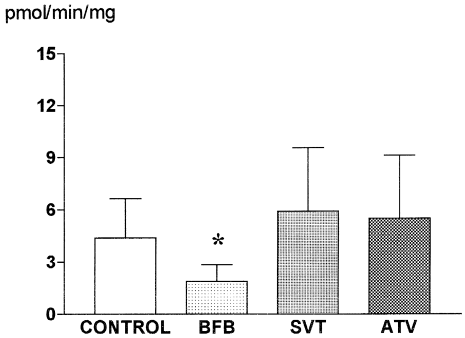

Fig. 3. Effect of bezafibrate (BFB), simvastatin (SVT) and atorvastatin (ATV) on cholesterol 7-α hydroxylase activity. The results are the mean ± standard deviation from 7–8 experiments performed in duplicate. * Values significantly different from control ($P < 0.05$).

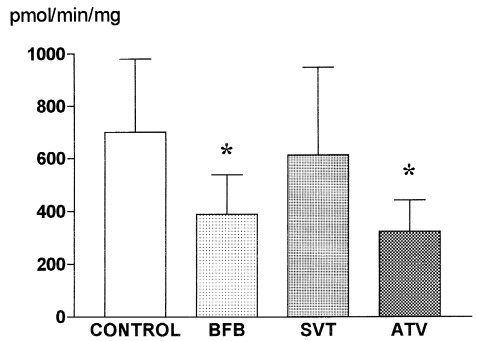

Fig. 4. Effect of bezafibrate (BFB), simvastatin (SVT) and atorvastatin (ATV) on CTP:phosphocholine cytidylyl transferase activity. The results are the mean ± standard deviation from 8 experiments performed in duplicate. * Values significantly different from control ($P < 0.05$).

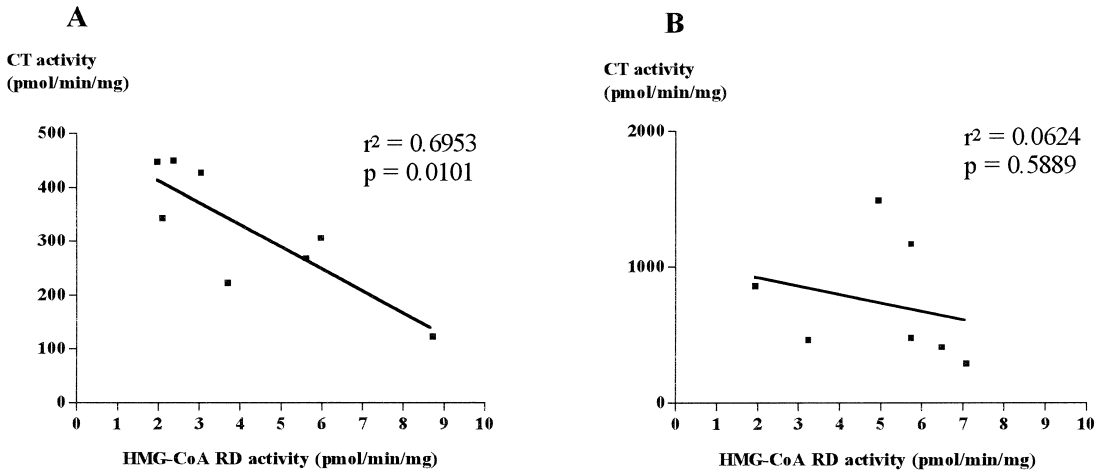

Fig. 5. Correlation between 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA RD) and CTP: phosphocholine cytidylyl transferase (CT) activities. (A) Atorvastatin-treated rabbits ($n = 8$). (B) Simvastatin-treated rabbits ($n = 7$).

data obtained from simvastatin group. There was a correlation of $r = 0.834$ ($r^2 = 0.695$, $P = 0.0101$) for atorvastatin, but not for simvastatin-treated animals (Fig. 5).

4. Discussion

4.1. Enzyme activities involved in cholesterol metabolism

Treatment of rabbits with both statins, simvastatin and atorvastatin, induced a significant increase in the microsomal HMGCoA reductase activity (Fig. 2) due to a compensatory response of the organism, which increases the enzyme mass to overcome the inhibition caused by the statins. Directly related to the inhibitory effect, there is also an increased expression of LDL receptors in liver hepatocytes responsible for plasma cholesterol reduction (Qin et al., 1992). Thus, for the statin-treated animals, there is a high inverse correlation ($r^2 = 0.669$, $P < 0.0004$) between the corresponding values of plasma cholesterol and HMG-CoA reductase activity. Regarding the effect of fibrate administration on HMG-CoA reductase activity conflicting results have been reported (Stahlberg et al., 1989, 1991, 1995; Stange et al., 1991; Qin et al., 1992). In the present study, bezafibrate had no effect on HMG-CoA reductase activity and did not reduce plasma cholesterol.

Approximately 40% of total cholesterol removal occurs via its degradation to bile acids in the liver, a process whose limiting step is catalyzed by cholesterol 7α-hydroxylase (Vlahcevic et al., 1991). Only bezafibrate modified cholesterol 7α-hydroxylase activity, with a 57% reduction respect to control values (Fig. 3). This reduction is in accordance with results from other authors obtained in laboratory animals and humans (Leiss et al., 1986; Stahlberg et al., 1991, 1995; Bertolotti et al., 1991, 1995).

In our study, no drug significantly modified microsomal acyl-CoA:cholesterol acyltransferase activity. It has been pointed to species dissimilarities in the acyl-CoA:cholesterol acyltransferase response to hypolipidemic drugs. Stahlberg et al. (1989, 1991, 1995) reported a marked reduction of this enzyme activity in rat liver microsomes by bezafibrate, while humans were not responsive at all. Similarly, simvastatin reduced hepatic acyl-CoA:cholesterol acyltransferase activity in male rats (Shand and West, 1995), while this enzyme activity was not affected in normal-fed rabbits (Ishida et al., 1989) or humans (Owens et al., 1991). Thus, rabbit hepatic acyl-CoA:cholesterol acyltransferase activity seems to behave similar to the human enzyme regarding its responsiveness to hypolipidemic drugs.

4.2. Enzyme activities involved in triglyceride metabolism

Neither the activity of one of the rate limiting enzymes in triacylglycerol synthesis, diacylglycerol acyltransferase, nor the lipolitic activities of those enzymes involved in triglyceride-rich lipoprotein catabolism, lipoprotein lipase and hepatic lipase, were modified by any drug (Table 2). Although fibrates have been described as inducers of lipoprotein lipase activity (Vessby and Lithell, 1990; Schoonjans et al., 1996), this is not always apparent from experimental results (Simsolo et al., 1993), as in our case.

The differences in the effect of fibrates on lipoprotein lipase activity could be ascribed to selective modification of specific pools of lipoprotein lipase that can be masked when the whole heparin releasable lipoprotein lipase activity is determined (Vessby and Lithell, 1990) or, more important, to the different species evaluated. In rats, liver lipoprotein lipase is regulated in response to fibrates via peroxisomal proliferator activated receptor alpha (PPARα) activation (Staels and Auwerx, 1992), resulting in a marked hypotriglyceridemic effect. As rabbit is far less sensitive than rat to peroxisomal proliferation (Lake and Gray, 1985), increased lipoprotein lipase expression via PPARα

activation by bezafibrate can be undetectable in short treatments. This could explain the lack of response of rabbit lipoprotein lipase to bezafibrate treatment in our study. With respect to simvastatin, although Sato et al. (1991), and Benhizia et al. (1994) have reported increases in lipoprotein lipase and hepatic lipase activities, respectively, these studies were performed in rats treated with very high doses, not comparable to the one used in here.

4.3. Enzyme activities involved in phospholipid synthesis

Although phosphatidylcholine may be synthesized through the phosphatidylserine/phosphatidyletanolamine pathway, most of the phosphatidylcholine for lipoprotein synthesis is derived from the CDP-choline pathway (Vance, 1990). Accordingly, CTP:phosphocholine cytidylyl transferase activity, as it is the rate-limiting enzyme of the CDP-choline pathway (Boggs et al., 1995), becomes crucial for the regulation of phosphatidylcholine synthesis. To our knowledge, there are only a few reports on the effect of hypolipidemic drugs on CTP:phosphocholine cytidylyl transferase activity (none in humans), and the results are conflicting.

An increase in CTP:phosphocholine cytidylyl transferase activity has been reported in gemfibrozil-treated rat hepatocytes (Lamb et al., 1993), although at very high millimolar concentrations. Nevertheless, fibrates are strong peroxisome proliferators in rats (Alegret et al., 1994; Schoonjans et al., 1996), increasing the cellular requirement of structural phospholipids for membrane biosynthesis (Kawashima et al., 1990). A similar response should not be expected in a species such as rabbit, considered almost non-responsive to the peroxisome proliferation phenomena (Lake and Gray, 1985). When we assayed CTP:phosphocholine cytidylyl transferase activity from livers of control rabbits in the presence of exogenously added bezafibrate (0.25 and 1 mM), no significant modification of the enzyme activity was observed. Thus, the observed reduction of rabbit CTP:phosphocholine cytidylyl transferase activity after bezafibrate treatment seems to be produced by an indirect mechanism, attributable either to a bezafibrate metabolite or to a disturbance introduced by the drug in the metabolic pathways involved in CTP:phosphocholine cytidylyl transferase activity.

Regarding statins, simvastatin has been described to decrease CTP:phosphocholine cytidylyl transferase activity by 30% in HepG2 cells, a hepatoma cell line of human origin (Yanagita et al., 1994), while lovastatin increased CTP:phosphocholine cytidylyl transferase activity 2.5 fold in hepatic microsomes from treated rats (Linscheer et al., 1995). Our results (Fig. 4) show that the administration of atorvastatin to normolipidemic rabbits caused a marked reduction in CTP:phosphocholine cytidylyl transferase activity (54%), while simvastatin was ineffective. Given the reported long half-life of the atorvastatin molecule, 14 h in humans, (Lea and McTavish, 1997), it can be argued that enough drug could be found in our hepatic postmitochondrial samples to cause a direct CTP:phosphocholine cytidylyl transferase inhibition; as simvastatin has a shorter half-life (1.4 h) (Desager et al., 1996), no such an effect should be observed with this statin. When a possible direct inhibitory effect of both statins was assayed, a slight inhibition was observed at 10 μM concentration that was not maintained at a higher concentration (50 μM). Although there are no pharmacokinetic data for atorvastatin in rabbits, in male rats receiving 20 mg kg^{-1} of oral atorvastatin for 15 weeks, Dostal et al. (1996) reported a C_{max} value for atorvastatin around 0.08 μM. If we assume for rabbits a behaviour similar to that reported for rats, it would be very difficult, at the dose we have been working (3 mg kg^{-1} day^{-1}, 4 weeks), to have enough drug in our samples to attain even the 18% inhibition of CTP:phosphocholine cytidylyl transferase activity elicited by atorvastatin 10 μM in the in vitro assay. Therefore, the reduction of CTP:phosphocholine cytidylyl transferase activity caused by atorvastatin does not appear to be related to a direct molecular interaction between the drug and the enzyme. To elucidate whether the effect on CTP:phosphocholine cytidylyl transferase activity was related to HMG-CoA reductase inhibition by the statins, we studied the correlation between both activities. When data from all animals were included, no correlation between CTP:phosphocholine cytidylyl transferase and HMG-CoA reductase was obtained ($n = 31$; $r = -0.141$; $P = 0.450$). In contrast, when each group was studied separately we obtained a high negative correlation for atorvastatin treated-rabbits ($r^2 = 0.695$, $P < 0.01$), as shown in Fig. 5A, but not for simvastatin treatment (Fig. 5B). A priori, these results suggest that atorvastatin is not likely to act on CTP:phosphocholine cytidylyl transferase through HMG-CoA reductase inhibition. Since atorvastatin has at least two active metabolites able to inhibit HMG-CoA reductase (Lea and McTavish, 1997), the high correlation between CTP:phosphocholine cytidylyl transferase and HMG-CoA reductase activities for atorvastatin-treated rabbits could be explained as the result of the direct inhibition of both HMG-CoA reductase and CTP:phosphocholine cytidylyl transferase by any of these metabolites. However, the results from Fig. 5 do not allow definite conclusions, as we cannot exclude that the high biological variance within the simvastatin group could be responsible for the lack of correlation between CTP:phosphocholine cytidylyl transferase and HMG-CoA reductase activities. Other authors (Bjorkhem and Berglund, 1987; Humble et al., 1994) have described parallel changes in HMG-CoA reductase activity and other enzyme activities involved in glycerolipid metabolism, such as phosphatidic acid phosphatase in rats after 3 days' statin treatment. It is well known that CTP:phosphocholine cytidylyl transferase and phosphatidic acid phosphatase are both involved in the phosphatidylcholine cycle (Tronchère et al., 1994), CTP:phosphocholine cytidylyl transferase as the rate-limiting step in phosphatidylcholine synthesis, and

phosphatidic acid phosphatase as a branching point enzyme controlling the direction of de novo glycerolipid biosynthesis, either to neutral triglycerides and diglycerides or to phospholipids. Thus, depending on the species or treatment period used, there could be a statin-related effect either on phospholipid or on triglyceride. Nevertheless, it has been reported that lovastatin treatment in rats does not affect the synthesis of triglyceride or phospholipid, and that the output of VLDL is altered by causing changes in the putative metabolic pool of cholesterol (Khan et al., 1990). Obviously, more studies are required to clarify the role of phospholipids in VLDL secretion and the link between triglyceride, phospholipid and cholesterol regulation.

In conclusion, we have studied the effects of atorvastatin, simvastatin and bezafibrate administration on enzymes related to lipid metabolism in normolipidemic rabbits. Besides the modification of HMG-CoA reductase by statins, the most remarkable finding was the decrease in CTP:phosphocholine cytidylyl transferase activity elicited by atorvastatin and bezafibrate treatment. There is probably no relation between the inhibitory mechanism of each drug, but in both cases a direct interaction of the drugs with the enzyme can be excluded. Moreover, the inhibition caused by atorvastatin seems to be independent on its ability to inhibit HMG-CoA reductase.

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