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Reduced cholesterol absorption in hamsters by crilvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor

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Abstract

Crilvastatin, a new drug from the pyrrolidone family, has been previously shown to inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, in vitro and in vivo, to reduce the absorption of dietary cholesterol and to stimulate the activity of cholesterol 7α-hydroxylase in the rat. The aim of this study was to evaluate the effects of crilvastatin on cholesterol and bile acid metabolism in the hamster. In hamsters fed on a lithogenic diet for 8 weeks, crilvastatin treatment (200 mg/day per kg body weight) did not change plasma lipid levels, failed to improve bile parameters and did not prevent gallstone formation. In hamsters fed on a basal cholesterol-rich (0.2%) diet for 8 weeks, crilvastatin at the same dose reduced the cholesterol level in the plasma by 20%, with a decrease of both low-density and high-density lipoprotein cholesterol. The drug did not significantly stimulate the biliary secretion of bile acids but significantly decreased the activity of acyl coenzyme A:cholesterol acyltransferase in the small intestine by 64%. This effect was enhanced when cholestyramine, a bile acid-sequestering resin, was given in combination with crilvastatin. Crilvastatin alone did not change the activity of cholesterol 7α-hydroxylase in the liver, despite the marked reduction in both hepatic cholesterogenesis and intestinal absorption of dietary cholesterol (the absorption coefficient was 44 ± 2% in treated hamsters vs. 61 ± 7% in controls).

Keywords: Cholesterol; Plasma; Liver; Bile; Gallstone; Cholesterol 7α-hydroxylase; Acyl coenzyme A:cholesterol acyltransferase

1. Introduction

Crilvastatin (Pan Medica, Carros, France), a drug from the pyrrolidone family, was shown in the rat to act as a non-competitive in vitro inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key enzyme of cholesterol synthesis (Esnault et al., 1988). In this study, crilvastatin appeared to be more effective than fenofibrate, because a 50% inhibition of enzyme activity was obtained with 1 μM crilvastatin compared with 3 μM fenofibrate. Clerc et al. (1993) have shown that crilvastatin (200 mg/kg per day) has a potent cholesterol-lowering effect in hypercholesterolemic rats fed on a high-fat diet, and stimulates cholesterol 7α-hydroxylase, the limiting enzyme of bile acid synthesis in the liver. In normolipidemic or genetically hypercholesterolemic (RICO) rats fed on a cholesterol-poor diet, we recently observed that the same dose of crilvastatin inhibited in vivo cholesterol synthesis, in the liver as well as in the intestine, and strongly reduced the intestinal absorption of dietary cholesterol (Hajri et al., 1995). The drug, however, had no effect on plasma cholesterol level. Similarly, known statins are reported to be ineffective in reducing plasma cholesterol in rodents (Endo, 1992; Sérougne et al., 1995). This lack of hypocholesterolemic effect of statins has been related to the induction of HMG-CoA reductase synthesis (Kita et al., 1980; Bilhartz et al., 1989) and to the reduced fecal excretion of bile acids (Endo, 1992). Moreover, the hepatic catabolism of lipoproteins is unaffected by statins in rats, whereas the effectiveness of these drugs as cholesterol-lowering agents in humans is partly due to a stimulation of the receptor-mediated uptake of low-density lipoproteins (LDL) (Endo, 1992).

Extensive studies in the hamster showed that the rate of cholesterol conversion into bile acids is relatively limited

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as in humans, in contrast with the rat (Khallou et al., 1991; Dietschy et al., 1993). Moreover, LDL has higher quantities of cholesterol in the hamster than in the rat (Groot et al., 1992; Goulinet and Chapman, 1993). Since the hamster is a suitable model for experimental cholelithiasis (Cohen-Solal et al., 1995), the effects of crilvastatin (200 mg/kg per day) on bile and plasma lipids were studied at first in hamsters fed on a lithogenic diet, which strongly stimulates cholesterol synthesis, for 8 weeks (Khallou et al., 1991). Secondly, crilvastatin was administered to hamsters fed on a semi-purified diet containing 0.2% cholesterol for 8 weeks, in order to study its effect on dietary cholesterol absorption, through the inhibition of cholesterol esterification in enterocytes. Lastly, in order to eventually amplify the action of crilvastatin, the drug was given in combination with cholestyramine, a bile acid-sequestering resin known to inhibit the intestinal absorption of cholesterol and increase bile acid synthesis (Shepherd and Packard, 1988; Suckling, 1991).

2. Materials and methods

2.1. Chemicals and isotopes

Chemicals of the highest purity were obtained from Sigma (St.-Quentin Fallavier, France). Crilvastatin was a gift from Pan Medica Laboratory. Na [1-^{14}C]acetate and [1,2-^{3}H]cholesterol were purchased from CEA (Saclay, France), [^{14}C]β-sitosterol from Amersham (Les Ulis, France) and [1-^{14}C]oleoyl-CoA from NEN (Paris, France).

2.2. Animals

Golden Syrian hamsters (*Mesocricetus auratus*) were raised in our breeding unit. The room temperature was maintained at 23 ± 1°C and lighting conditions were controlled according to a 12-h cycle. Hamsters were fed on a commercial chow diet (CRF20 from UAR, Epinay s/Orge, France) before the experiments, which were carried out with 6-week-old male hamsters.

2.3. Experimental procedures

2.3.1. First experiment

In the first experiment, the animals were divided into 2 groups ($n = 6$). One group (group L) received for 8 weeks the lithogenic Dam's diet (Dam, 1969), which mainly consisted of 72.5% sucrose, 20% casein, 5% mineral mixture, 2.5% vitamin-cellulose mixture and 0.05% cholesterol. The second group (L + CR) received the lithogenic diet containing crilvastatin at the concentration of 0.3%, which corresponded to a daily oral intake of 200 mg per kg of body weight, as in rats (Esnault et al., 1988; Hajri et al., 1995). On the last day, the hamsters were anesthetized by tiletamine-zolazepam (Virbac, Paris, France), and the common bile duct was cannulated with a polyethylene tube (No. 01015, Biotrol, France) to collect the hepatic bile for 1 h. Gallstones were detected by observation of the gallbladder content. Blood was collected on ethylenediamine tetraacetic acid (EDTA, 1 mg/ml) and monoiodoacetamide, an inhibitor of cholesterol esterification (1 mg/ml). Plasma was separated by centrifugation at 12 000 $\times g$ for 5 min at 4°C. The liver was excised and weighed. Plasma, bile and liver samples were immediately stored at 4°C prior to analysis.

2.3.2. Second experiment

In the second experiment, hamsters were divided into 3 groups ($n = 12$). The first group (C) was fed on a semi-purified diet which mainly consisted of 62.5% sucrose, 20% casein, 9% lard, 0.8% walnut oil, 5% mineral mixture, 2.5% vitamin-cellulose mixture, and was enriched with 0.2% cholesterol. The second group (CR) received the same hypercholesterolemic diet containing 0.3% crilvastatin. The third group (CR-CY) received the same diet, with 0.3% crilvastatin and 2% cholestyramine. After 8 weeks, six animals per group were killed after overnight fasting. Blood was collected on EDTA, and liver and intestine were immediately collected and rinsed with saline. The intestinal mucosa was scrapped off and homogenized in a phosphate buffer (K_2HPO_4, 50 mM; sucrose, 0.1 M; EDTA, 30 mM; KCl, 70 mM; dithiothreitol, 1 mM). Two grams of liver were homogenized in the same buffer. Intestine and liver microsomes were prepared according to the method described by Clerc et al. (1993) and stored at −20°C for later measurement of acyl coenzyme A:cholesterol acyltransferase and cholesterol 7α-hydroxylase activities. In the six other animals, the gallbladder was observed, hepatic bile was collected, as above, and plasma and bile samples were kept at 4°C for further analysis.

2.3.3. Third experiment

The third experiment was carried out under the same conditions as above. The hamsters were also divided into the 3 groups: C, CR and CR-CY ($n = 12$). After 7 weeks, six animals per group were subcutaneously injected at 9.00 a.m. with a solution containing 0.925 MBq of sodium [1-^{14}C]acetate, and killed 70 min later to measure the ^{14}C radioactivity of liver and intestinal sterols, as an index of hepatic cholesterogenesis (Lutton et al., 1990). The six other hamsters received, by stomach intubation after overnight fasting, 0.5 ml of walnut oil containing 370 kBq of [^{3}H]cholesterol and 33 kBq of [^{14}C]β-sitosterol, in order to measure the intestinal absorption of dietary cholesterol (Borgstrom, 1969). Feces were collected daily for 1 week. At the end of the experimental period, the animals were killed after overnight fasting.

2.4. Assays

2.4.1. Plasma and bile

Plasma lipids were assayed by enzymatic methods using commercial kits (total and free cholesterol: CHOD-PAP

method, Boehringer, Rungis, France; phospholipids and triglycerides: Wako tests, Unipath, Meylan, France). Bile samples were previously diluted 10 times with physiological saline before cholesterol and phospholid (Gurantz et al., 1981) assays. Total bile acid concentration was measured enzymatically with 3α-hydroxysteroid dehydrogenase (Turley and Dietschy, 1978). The lithogenic index of bile was calculated (Thomas and Hofmann, 1973).

2.4.2. Lipoproteins

Lipoproteins were isolated from 1 ml plasma (pooled samples from 2 hamsters) by ultracentrifugation on a KBr density gradient at $105\,000 \times g$ for 24 h (Sérougne et al., 1987). Eighteen plasma fractions (0.5 ml each) were collected and pooled according to their density (triglyceride-rich lipoproteins, corresponding to chylomicrons and very low-density lipoproteins, VLDL: $d < 1.010$ g/ml; low-density lipoproteins, LDL: $1.010 < d < 1.063$; high-density lipoproteins, HDL: $1.063 < d < 1.21$). Total and free cholesterol, triglycerides and phospholipids were determined enzymatically in each lipoprotein class by using commercial kits, as above. Proteins were assayed by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard.

Analysis of apolipoproteins was performed using pooled lipoprotein fractions. In regard to the heterogeneity of their apolipoprotein composition, LDL were separated into two subclasses: LDL1, $1.010 < d < 1.040$ and LDL2, $1.040 < d < 1.063$ (Goulinet and Chapman, 1993). Fractions were dialyzed (except for triglyceride-rich particles) and assayed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) without prior delipidation (Connelly and Kuksis, 1982). Apolipoprotein composition was estimated by densitometric analysis of gels stained with Coomassie blue, using a laser densitometer (Ultrascan 2202, LKB, St-Quentin en Yvelines, France) connected to an integrator.

2.4.3. Feces

After extraction of fecal lipids with boiling ethanol in a Soxhlet apparatus for 24 h, the extract was saponified in a solution of 2 M potassium hydroxide in 95% ethanol. Neutral sterols were extracted with petroleum ether (Khallou et al., 1991) and ^{14}C and ^{3}H radioactivities were measured by liquid scintillation (Kontron MR 300, Montigny-le-Bretonneux, France). The absorption coefficient (AC) of dietary cholesterol was determined as follows:

$$\mathrm{AC}(\%) = \frac{\text{excreted } ^{3}\mathrm{H} \text{ radioactivity (dpm)}}{\text{excreted } ^{14}\mathrm{C} \text{ radioactivity (dpm)}} \times \frac{\text{ingested } [^{14}\mathrm{C}]\beta\text{-sitosterol (dpm)}}{\text{ingested } [^{3}\mathrm{H}]\text{cholesterol (dpm)}} \times 100$$

The absorption rate of dietary cholesterol was calculated on the basis of daily food intake and cholesterol content of the diet (0.2%).

2.4.4. Liver and intestine

After saponification of lipids in liver or intestine samples with a solution of 2 M potassium hydroxide in 95% ethanol, sterols were extracted with petroleum ether, dried and dissolved in isopropanol to perform the enzymatic assay of total cholesterol, as above. In hamsters injected with sodium [1-^{14}C]acetate, total lipids were extracted from liver and intestine samples with boiling ethanol in a Soxhlet apparatus for 24 h. A fraction was directly saponified to isolate neutral sterols and their radioactivity was measured. Other fractions of liver were used for the separation of esterified and unesterified cholesterol by thin layer chromatography on silica gel plates, after elution with a mixture of 20% ethyl acetate/80% hexane. Each sterol fraction was saponified in the 2 M potassium hydroxide ethanolic solution, extracted with petroleum ether, and enzymatically assayed in isopropanol, as above.

2.4.5. Enzyme activities

Microsomal preparations were used to determine the activities of intestinal and hepatic acyl coenzyme A:cholesterol acyltransferase, and of hepatic cholesterol 7α-hydroxylase. Acyl coenzyme A:cholesterol acyltransferase activity was measured using [^{14}C]oleoyl-CoA as the substrate. The labeled esterified cholesterol produced was then isolated by chromatography on silica gel columns (Chautan et al., 1988). Cholesterol 7α-hydroxylase activity was measured using [^{14}C]cholesterol as the substrate (Clerc et al., 1995). [^{14}C]Cholesterol and [^{14}C]7α-hydroxycholesterol were separated by thin layer chromatography on silica-gel plates, after elution by a mixture of 80% ethyl acetate/20% heptane.

2.4.6. Statistical analysis

Results are expressed as means ± S.E.M. Statistical differences were assessed by a Student's *t*-test or by analysis of variance (ANOVA) and the Neuman-Keuls test. A value of $P < 0.05$ was considered as the criterion of significance.

3. Results

3.1. Effect of 8-week crilvastatin treatment on hamsters fed on a lithogenic diet

Plasma and lipoprotein lipid concentrations in (L) and (L + CR) groups are shown in Table 1. Plasma lipid parameters were not modified by crilvastatin, except the unesterified/total cholesterol ratio in triglyceride-rich lipoproteins, which was significantly reduced by the crilvastatin treatment: 0.55 ± 0.03 in (L + CR) group, vs. 0.65 ± 0.02 in (L) group. The apolipoprotein composition of the various lipoproteins (results only expressed in the text) was similar in the two groups. Triglyceride-rich lipoproteins contained mainly apolipoproteins B100 (40%), E

Table 1
Lipid concentrations in plasma and lipoproteins of hamsters fed on a lithogenic (L) diet or a lithogenic diet supplemented with 0.3% crilvastatin (L + CR), for 8 weeks

Group		L	L + CR
Plasma		*μg / ml*	
	Total cholesterol	971 ± 44	1090 ± 63
	Unesterified cholesterol	364 ± 20	401 ± 21
	Triglycerides	1163 ± 162	1096 ± 124
	Phospholipids	1783 ± 172	1777 ± 94
Lipoproteins (n = 4 per group)		*μg / ml plasma*	
TRL	Total cholesterol	160 ± 21	118 ± 14
	Unesterified cholesterol	104 ± 10	66 ± 12 $^{\alpha}$
	Triglycerides	749 ± 44	657 ± 93
LDL	Total cholesterol	207 ± 11	234 ± 14
	Unesterified cholesterol	120 ± 5	107 ± 7
	Triglycerides	190 ± 9	206 ± 9
HDL	Total cholesterol	615 ± 24	728 ± 48
	Unesterified cholesterol	172 ± 20	227 ± 32
	Triglycerides	129 ± 11	145 ± 15

Mean ± S.E.M ($n = 6$ per group). Differences were analyzed with a Student's *t*-test and are significant at $P < 0.05$: $^{\alpha}$ (L + CR) vs. (L) group. TRL, triglyceride-rich lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

(35–45%) and Cs (15–24%), but the apolipoprotein E/B100 ratio was higher in crilvastatin-treated hamsters (1.13) than in untreated hamsters (0.80). The LDL1 subfraction contained mainly apolipoproteins B100 (81–85%) and E (less than 20%) in the two groups. The LDL2 subfraction, which is a mixture of LDL and HDL particles, contained mainly apolipoproteins AI (50%), B (33%) and E (15%); HDL were rich in apolipoprotein AI (86%), with small proportions of apolipoproteins AIV, E or Cs (less than 6% for each one).

The incidence of gallstones was 6/6 in both groups at the end of the experiment. No difference appeared in the biliary parameters between the two groups (Table 2), except for the bile flow, which was higher with the drug, while the cholesterol concentration in the bile tended to be lowered.

Table 2
Bile secretion in hamsters fed on a lithogenic diet (L) or a lithogenic diet supplemented with 0.3% crilvastatin (L + CR), for 8 weeks

Group	L	L + CR
	ml / h	
Bile flow	0.32 ± 0.01	0.40 ± 0.04 $^{\alpha}$
Biliary secretion	*μmol / h per 100 g body weight*	
Bile acids	5.05 ± 0.44	5.13 ± 0.30
Phospholipids	1.17 ± 0.07	1.01 ± 0.09
Cholesterol	0.83 ± 0.08	0.72 ± 0.09
Lithogenic index	1.27 ± 0.07	1.19 ± 0.05

Mean ± S.E.M. ($n = 6$ per group). Differences were analyzed with a Student's *t*-test and are significant at $P < 0.05$: $^{\alpha}$ (L + CR) vs. (L) group.

Table 3
Lipid concentration in plasma and lipoproteins of hamsters fed on a cholesterol-rich (0.2%) diet (C) or a cholesterol-rich diet supplemented with crilvastatin (CR) or a combination of crilvastatin-cholestyramine (CR-CY), for 8 weeks

Group		C	CR	CR-CY
Plasma		*μg / ml*		
	Total cholesterol	2201 ± 70 ^{c}	1753 ± 64 ^{b}	1163 ± 77 ^{a}
	Unesterified cholesterol	718 ± 11 ^{c}	603 ± 23 ^{b}	385 ± 29 ^{a}
	Triglycerides	1557 ± 284	1581 ± 334	2290 ± 134 $^{\alpha}$
	Phospholipids	2403 ± 204	2290 ± 134	1630 ± 250
Lipoproteins (n = 4 per group)		*μg / ml plasma*		
TRL	Total cholesterol	225 ± 70	212 ± 15	116 ± 18 $^{\alpha}$
	Unesterified cholesterol	85 ± 14 ^{b}	92 ± 11 ^{b}	44 ± 7 ^{a}
	Triglycerides	448 ± 142	557 ± 127	464 ± 137
LDL	Total cholesterol	696 ± 48 ^{c}	459 ± 20 ^{b}	330 ± 46 ^{a}
	Unesterified cholesterol	285 ± 22 ^{b}	216 ± 20 ^{a}	138 ± 22 ^{a}
	Triglycerides	332 ± 80	268 ± 38	229 ± 51
HDL	Total cholesterol	1455 ± 73 ^{c}	1041 ± 78 ^{b}	707 ± 71 ^{a}
	Unesterified cholesterol	245 ± 37	234 ± 10	164 ± 32
	Triglycerides	211 ± 70	120 ± 27	82 ± 25

Mean ± S.E.M. ($n = 9$ per group). Differences were analyzed by analysis of variance (ANOVA) and the Neuman-Keuls test: groups with a different letter superscript are significantly different at $P < 0.05$; $^{\alpha}$ significantly different from (C) group with Student's *t*-test. TRL, triglyceride-rich lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

3.2. Effect of 8-week crilvastatin treatment in normal hamsters fed a cholesterol-rich diet

Food intake, body weights and weights of livers and small intestines were similar in (C), (CR) and (CR-CY) groups. Plasma lipid concentrations and lipoprotein cholesterol distribution are shown in Table 3. Crilvastatin treatment reduced the plasma level of cholesterol by 20% without changing the other parameters. Cholestyramine administered in combination with the drug markedly reduced the level of cholesterol (by 50%), phospholipids (by 32%), and triglycerides (by 24%), as compared to (C) group. Crilvastatin alone lowered the cholesterol content of both LDL (by 34%) and HDL (by 28%) and slightly increased the triglyceride/esterified cholesterol ratio in triglyceride-rich lipoproteins (4.59 vs. 3.17) and LDL (1.11 vs. 0.80). Cholestyramine associated with the drug decreased the cholesterol content of all lipoproteins by about 50%.

The absorption coefficient and daily absorption rate of dietary cholesterol are shown in Table 4. These two parameters were markedly lowered by crilvastatin alone (about 30%) and were further lowered by cholestyramine associated with the drug (55%).

Cholesterol concentrations in the liver and intestine, sterol radioactivities and activities of cholesterol 7α-hydroxylase and acyl coenzyme A:cholesterol acyltransferase are shown in Table 5. No difference was observed between the values of unesterified hepatic cholesterol in the 3

Table 4
Absorption of dietary cholesterol in hamsters fed on a cholesterol-rich (0.2%) diet (C) or a cholesterol-rich diet supplemented with crilvastatin (CR) or a combination of crilvastatin-cholestyramine (CR-CY), for 8 weeks

Group	C	CR	CR-CY
	Percent		
Absorption coefficient	61 ± 3^{c}	44 ± 2^{b}	27 ± 0.3^{a}
	mg / day		
Absorption rate	7.9 ± 0.5^{b}	$5.4 \pm 1.1^{a,c}$	3.3 ± 0.2^{a}

Mean ± S.E.M. ($n = 5$ per group). Differences were analyzed by ANOVA and the Neuman-Keuls test: groups with a different letter superscript are significantly different at $P < 0.05$.

groups. The concentration of esterified cholesterol was decreased 2-fold by crilvastatin, and 9-fold by the combination crilvastatin-cholestyramine. Compared with the value in the (C) group, the radioactivity incorporated into liver sterols decreased by 30% with crilvastatin, whereas it increased by 144% with the cholestyramine-crilvastatin combination. The radioactivity of intestinal sterols decreased by 14% with crilvastatin, while the combined treatment restored the value observed in untreated hamsters. The hepatic activity of cholesterol 7α-hydroxylase was not changed by crilvastatin alone, and was markedly stimulated (2.8-fold) by the combined treatment. In the liver, acyl coenzyme A:cholesterol acyltransferase activity was lowered by crilvastatin alone (42%), and even more by the combined treatment, despite no significant intergroup difference. In the intestine, acyl coenzyme A:cholesterol acyltransferase activity was obviously inhibited (64%) by crilvastatin alone, and even more (87%) by the combined treatment.

Table 5
Cholesterol concentration, sterol radioactivity and enzyme activities in liver or intestine of hamsters fed on a cholesterol-rich diet (C) or a cholesterol-rich diet supplemented with crilvastatin (CR) or a combination of crilvastatin-cholestyramine (CR-CY), for 8 weeks

Group	C	CR	CR-CY
Cholesterol concentration	*mg / g*		
Liver			
Esterified	16.4 ± 0.4^{c}	8.1 ± 0.6^{b}	1.8 ± 0.2^{a}
Unesterified	2.4 ± 0.3	2.1 ± 0.2	1.9 ± 0.2
Intestine			
Total	2.3 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
Sterol radioactivity	*dpm per 3.7 MBq of injected [^{14}C]acetate*		
Liver	8050 ± 219^{b}	5612 ± 484^{a}	11603 ± 791^{c}
Intestine	163497 ± 5672	139999 ± 5736	160401 ± 9055
Activity	*pmol / min per mg protein*		
Cholesterol 7α-OHase	17.3 ± 3.3^{a}	19.4 ± 3.3^{a}	48.0 ± 4.0^{b}
ACAT			
Liver	55.6 ± 14.4	32.0 ± 5.8	25.0 ± 7.0
Intestine	69.0 ± 15.0^{b}	$25.0 \pm 7.0^{a,c}$	8.5 ± 1.5^{c}

Mean ± S.E.M. ($n = 5$ per group). Differences were analyzed by ANOVA and Neuman-Keuls test: groups with a different letter superscript are significantly different at $P < 0.05$.

Table 6
Bile secretion and lithogenic index of hamsters fed on a cholesterol-rich diet (C) or a cholesterol-rich diet supplemented with crilvastatin (CR) or a combination of crilvastatin-cholestyramine (CR-CY), for 8 weeks

Group	C	CR	CR-CY
	ml / h		
Bile flow	0.28 ± 0.04	0.36 ± 0.07	0.28 ± 0.02
Biliary secretion rate	*μmol / h per 100 g body weight*		
Bile acids	4.90 ± 0.75	$6.70 \pm 0.29^{\alpha}$	5.37 ± 0.70
Phospholipids	0.70 ± 0.10	1.03 ± 0.13	0.78 ± 0.13
Cholesterol	0.39 ± 0.05	0.50 ± 0.12	0.48 ± 0.17
Lithogenic index	0.82 ± 0.05	0.75 ± 0.02	0.72 ± 0.10

Mean ± S.E.M. ($n = 5$ per group). No differences were found with ANOVA and the Neuman-Keuls test. $^{\alpha}$ (CR) group significantly different from (C) group by Student's *t*-test.

The biliary parameters are shown in Table 6. Crilvastatin led to a non-significant increase in bile flow and phospholipid and cholesterol secretion rates, and to a significant increase in bile acid secretion. However, the lithogenic index was not significantly lowered by crilvastatin. The combination of cholestyramine with the drug did not change biliary parameters, as compared to those of untreated hamsters.

4. Discussion

In the hamster, the essential fatty acid deficiency induced by the Dam diet is associated with a high frequency of cholesterol gallstones (Dam, 1969). We had previously observed that cholesterolemia was lower (30%) in hamsters fed on this lithogenic diet than in hamsters fed on a control diet (Khallou et al., 1991). In the present study, the plasma lipid parameters of lithiasic hamsters, previously fed on the Dam diet, agree with our previous data. Crilvastatin, at a dose of 200 mg/day per kg of body weight, does not induce changes in plasma lipid level or cholesterol distribution among the lipoproteins. Compared with humans, hamsters are characterized by VLDL and LDL deficient in esterified cholesterol, and by lipoproteins enriched in apo-E, like other species such as the dog or rat (Goulinet and Chapman, 1993). In untreated hamsters fed on the lithogenic diet (Table 1), the unesterified/total cholesterol ratio in triglyceride-rich lipoproteins and LDL was abnormally high, and even higher than in non-lithiasic hamsters fed on the cholesterol-enriched diet (Table 3). This difference may reflect the poor esterification process in the liver in cholelithiasis (Smith et al., 1990). Crilvastatin decreased the unesterified/total cholesterol and the triglyceride/esterified cholesterol ratios in triglyceride-rich lipoproteins and LDL, and increased the proportion of apolipoprotein E in triglyceride-rich lipoproteins, an effect

which stimulates the plasma turnover of these lipoproteins (Yamada et al., 1989). Nevertheless, the incidence of gallstones and the major biliary parameters were not changed by crilvastatin. These results are similar to those of a previous study carried out on hamsters fed on the same lithogenic diet for 2 months and treated with fenofibrate (personal data). Furthermore, pravastatin, a powerful inhibitor of cholesterol synthesis, did not prevent gallstone formation and was not effective in the treatment of cholesterol metabolism disorders induced by the Dam diet in hamsters (Koide et al., 1989). In hamsters, the lithogenic Dam diet led to an excessively high concentration of cholesterol in bile and to a very low concentration of bile acids (Khallou et al., 1991). Thus, crilvastatin or other drugs cannot really improve the process of bile lipid secretion to a great extent.

Therefore, another experiment was done with hamsters fed on a basal semi-synthetic diet moderately enriched with cholesterol. Compared to a similar diet without cholesterol added, this diet produced a marked increase in cholesterolemia (50%), due to higher cholesterol levels in the three major lipoprotein classes (Khallou et al., 1991). In hamsters fed on this hypercholesterolemic diet, crilvastatin thus prevented the 'exogenous' hypercholesterolemia due to an excess of dietary cholesterol. Similar results were obtained with crilvastatin in male Wistar rats also fed on a cholesterol-rich diet (Clerc et al., 1993), while no effect was observed in normocholesterolemic or in genetically hypercholesterolemic (RICO) rats receiving a cholesterol-poor diet (Hajri et al., 1995). Moreover, it has recently been demonstrated that crilvastatin enhances the hepatic uptake of LDL-cholesterol in the rat (Clerc et al., 1995). Therefore, in cholesterol-fed hamsters as well as in rats, the reduction in the LDL-cholesterol level by crilvastatin could be due to a stimulation of LDL-receptors after inhibition of cholesterol synthesis, this mechanism being observed with statins (Endo, 1988).

The most relevant observation concerns the strong inhibition of intestinal acyl coenzyme A:cholesterol acyltransferase by crilvastatin, in cholesterol-fed hamsters. This effect could represent the first mode of action of the drug during the digestive process. It is noteworthy that simvastatin was shown to inhibit intestinal acyl coenzyme A:cholesterol acyltransferase in rabbits (Ishida et al., 1989). Previous studies with various drugs (Heider et al., 1983; Clarck and Tercyak, 1984; Schnitzer-Polokoff et al., 1991; Kusunoki et al., 1995) showed that intestinal acyl coenzyme A:cholesterol acyltransferase plays a major role in cholesterol absorption. Indeed, the intestine secretes chylomicrons, which bear newly absorbed cholesterol mainly in the esterified form, and are rapidly metabolized by the liver (Redgrave and Vakakis, 1976). Therefore, inhibition of cholesterol esterification in the intestine by crilvastatin explains the low rate of dietary cholesterol absorption in treated hamsters fed on the cholesterol-enriched diet. As a result, the storage of esterified cholesterol in the liver, established at a high level in untreated hypercholesterolemic hamsters, was reduced by 50% under crilvastatin treatment. A similar effect of this drug on cholesterol absorption has recently been reported in genetically hypercholesterolemic RICO rats fed on a cholesterol-poor diet (Hajri et al., 1995).

The activity of acyl coenzyme A:cholesterol acyltransferase, in the liver of cholesterol-fed hamsters, was not significantly lowered by crilvastatin, despite a strong reduction in hepatic cholesterol storage. In fact, the drug produces two major effects in the liver, which contribute to the reduction in cholesterol delivery to this organ. First, the uptake of newly absorbed cholesterol is reduced, via the inhibition of intestinal acyl coenzyme A:cholesterol acyltransferase. Second, cholesterol synthesis is reduced, via the inhibition of HMG-CoA reductase, as reflected also by the reduced incorporation of exogenous radioactive acetate into liver sterols. Nevertheless, the drug did not change the activity of hepatic cholesterol 7α-hydroxylase.

In the bile of untreated hypercholesterolemic hamsters, the bile acid/cholesterol ratio was about 13, which agrees with results reported in hamsters also fed on a 0.2% cholesterol-enriched diet (Liu et al., 1991). This parameter was not changed by crilvastatin alone, which only produced a modest enhancement in the secretion of bile acids without any significant change in that of cholesterol, as observed with simvastatin (personal observations, and Loria et al., 1994). Therefore, in treated cholesterol-fed hamsters, the bile flow maintained a sufficient cholesterol secretion, leading to the elimination of cholesterol from the hepatocyte, and to a reduction in hepatic cholesterol storage.

When cholestyramine, which is an anti-micellar agent, was associated with the drug, cholesterol absorption decreased even more and cholesterol turnover strikingly increased. Compared with hamsters treated with crilvastatin alone, the incorporation of [^{14}C]acetate into liver sterols doubled and the activity of cholesterol 7α-hydroxylase was stimulated 2.5-fold. As a result, hepatic cholesterol storage was further reduced, but the apparent drop in hepatic acyl coenzyme A:cholesterol acyltransferase activity (by more than 50%, compared with that of untreated hamsters) failed to be significant. Again, biliary parameters were not modified, particularly bile acid secretion, since the stimulation of bile acid synthesis by cholestyramine conterbalanced the reduced contribution of reabsorbed bile acids to global bile acid secretion.

In conclusion, the mode of action of crilvastatin remains unclear. The potent inhibition of intestinal acyl coenzyme A:cholesterol acyltransferase could be due to a direct effect of the drug on the enzyme or to an indirect effect, subsequent to the impairment of cholesterol absorption in the lumen (Clarck and Tercyak, 1984). The fact that the activity of intestinal acyl coenzyme A:cholesterol acyltransferase was further decreased when cholestyramine was associated with crilvastatin favors this last hypothesis.

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References

Bilhartz, L.E., D.K. Spady and J.M. Dietschy, 1989, Inappropriate hepatic cholesterol synthesis expands the cellular pool of sterol available for recruitment by bile acids in the rat, J. Clin. Invest. 84, 1181.

Borgstrom, B., 1969, Quantification of cholesterol absorption in man by fecal analysis after the feeding of a single isotope-labelled meal, J. Lipid Res. 10, 331.

Chautan, M., E. Termine, G. Nalbone and H. Lafont, 1988, Acylcoenzyme A-cholesterol acyltransferase assay: silica gel column separation of reaction products, Anal. Biochem. 173, 436.

Clarck, S.B. and A.M. Tercyak, 1984, Reduced cholesterol transmucosal transport in rats inhibed mucosal acyl CoA:cholesterol acyltransferase and normal pancreatic function, J. Lipid Res. 25, 148.

Clerc, T., M. Jomier, M. Chautan, H. Portugal, M. Senft, A.M. Pauli, C. Laruelle, O. Morel, H. Lafont and F. Chanussot, 1993, Mechanisms of action in the liver of crilvastatin, a new hydroxymethylglutaryl-coenzyme A reductase inhibitor, Eur. J. Pharmacol. 235, 59.

Clerc, T., V. Sbarra, N. Diaconescu, H. Lafont, G. Jadot, C. Laruelle and F. Chanussot, 1995, Effect of crilvastatin, a new cholesterol lowering agent, on unesterified LDL-cholesterol metabolism into bile salts by isolated rat hepatocytes, Br. J. Pharmacol. 114, 624.

Cohen-Solal, C., M. Parquet, B. Tiffon, A. Volk, M. Laurent and C. Lutton, 1995, Magnetic resonance imaging for the visualization of cholesterol gallstones in hamster fed a new high sucrose lithogenic diet, J. Hepatol. 22, 486.

Connelly, P.W. and A. Kuksis, 1982, SDS-glycerol, polyacrylamide gel electrophoresis of plasma lipoproteins, Biochim. Biophys. Acta 711, 245.

Dam, H., 1969, Nutritional aspects of gallstones formation with particular reference to alimentary production of gallstones in laboratory animals, World Rev. Nutr. Diet 11, 199.

Dietschy, J.M., S.D. Turley and D.K. Spady, 1993, Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans, J. Lipid Res. 34, 1637.

Endo, A., 1988, Chemistry, biochemistry, and pharmacology of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, Klin. Wochenschr. 66, 421.

Endo, A., 1992, The discovery and development of HMG-CoA reductase inhibitors, J. Lipid Res. 33, 1569.

Esnault, C., H. Lafont, F. Chanussot, M. Chautan, J. Hauton and C. Laruelle, 1988, Inhibition of hepatic HMG-CoA reductase activity by two new hypocholesterolemic drugs, in: Liver Cells and Drugs, Colloque INSERM 164, ed. A. Guillouzou (INSERM, Paris) p. 99.

Goulinet, S. and M.J. Chapman, 1993, Plasma lipoproteins in the golden syrian hamster (*Mesocricetus auratus*): heterogeneity of apo B- and apo AI-containing particles, J. Lipid Res. 34, 943.

Groot, P.H.E., N.J. Pearce, K.E. Suckling and S. Eisenberg, 1992, Effects of cholestyramine in lipoprotein levels and metabolism in syrian hamsters, Biochim. Biophys. Acta 1123, 76.

Gurantz, D., M.F. Laker and A.F. Hofmann, 1981, Enzymatic measurements of choline-containing phospholipids in bile, J. Lipid Res. 22, 373.

Hajri, T., J. Férézou, C. Laruelle and C. Lutton, 1995, Crilvastatin, a new hydroxymethylglutaryl-CoA reductase inhibitor, inhibits cholesterol absorption in genetically hypercholesterolemic rats, Eur. J. Pharmacol. 286, 131.

Heider, J.C., C.E. Peckens and L.A. Kelly, 1983, Role of acylCoA:cholesterol acyltransferase in cholesterol absorption and its inhibition by 57-118 in the rabbit, J. Lipid Res. 24, 1127.

Ishida, F., A. Sato, Y. Iizuka, K. Kitani, Y. Sawasaki and T. Kamei, 1989, Effects of MK-733 (simvastatin), an inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A reductase, on intestinal acylcoenzyme A:cholesterol acyltransferase activity in rabbits, Biochim. Biophys. Acta 1004, 117.

Khallou, J., M. Riottot, M. Parquet, C. Verneau and C. Lutton, 1991, Biodynamics of cholesterol and bile acids in the lithiasic hamster, Br. J. Nutr. 66, 479.

Kita, T., M.S. Brown and J.L. Goldstein, 1980, Feedback regulation of 3-hydroxy 3-methyl glutaryl coenzyme A reductase in livers of mice treated with mevilonin, a competitive inhibitor of the reductase, J. Clin. Invest. 66, 1094.

Koide, K., K. Hayashi, I. Horiuchi and G. Kajiyama, 1989, Effect of CS-514, a competitive inhibitor of hydroxymethylglutaryl coenzyme A reductase, on cholesterol gallstone formation in hamsters, Biochim. Biophys. Acta 1005, 65.

Kusunoki, J., K. Aragane, T. Kitamine, S. Higashinakagawa, N. Kase, T. Yamaura and H. Ohnishi, 1995, Hypocholesterolemic action and prevention of cholesterol absorption via the gut by F-1394, a potent acyl-coA:cholesterol acyltransferase (ACAT) inhibitor, in cholesterol diet-fed rats, Jpn. J. Pharmacol. 69, 53.

Liu, G.L., L.M. Fan and R.N. Redinger, 1991, The association of hepatic apoprotein and lipid metabolism in hamsters and rats, Br. Comp. Biochem. Physiol. 99A, 223.

Loria, P., M. Bertolotti, M.T. Cassinadri, M.A. Dilengite, M. Bozzoli, F. Carubbi, M. Concari, M.E. Guicciardi and N. Carulli, 1994, Short term effects of simvastatin on bile acid synthesis and bile lipid secretion in human subjects, Hepatology 19, 882.

Lowry, O.H., H.J. Rosebrough, A. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Lutton, C., J. Férézou, C. Sérougne, C. Verneau, G. Champarnaud, T. Magot, D. Mathé and J.C. Sulpice, 1990, Critical analysis of the use of ^{14}C-acetate for measuring in vivo rat cholesterol synthesis, Reprod. Nutr. Dev. 30, 71.

Redgrave, T.G. and N. Vakakis, 1976, Hepatic vitamin A fat storage cells and metabolism of chylomicron cholesterol, Aust. J. Exp. Biol. Med. Sci. 54, 519.

Schnitzer-Polokoff, R., D. Compton, G.B. Brykow, H. Davis and R. Burrier, 1991, Effects of acylCoA:cholesterol acyltransferase inhibition on cholesterol absorption and plasma lipoprotein composition in hamsters, Comp. Biochem. Physiol. 99A, 665.

Sérougne, C., J. Férézou and A. Rukaj, 1987, A new relationship between cholesterolemia and cholesterol synthesis determined in rats fed an excess of cystine, Biochim. Biophys. Acta 921, 522.

Sérougne, C., D. Mathé, J. Férézou, C. Bertin, M. Riottot, C. Lutton and B. Jacotot, 1995, Effects of simvastatin on plasma lipoprotein concentrations and very-low-density lipoprotein secretion in the rat, J. Clin. Biochem. Nutr. 18, 55.

Shepherd, J. and C.J. Packard, 1988, Pharmacologic control of plasma cholesterol: mechanisms of action of hypocholesterolemic agents, in: Atherosclerosis Reviews, Hypercholesterolemias: Clinical and Therapeutic Implications, Vol. 18, eds. J. Stokes III and M. Mancini (Raven Press, New York, NY) p. 109.

Smith, J.L., I.R. Hardie, S.P. Pillay and J. De Jersey, 1990, Hepatic acyl-coenzyme A:cholesterol acyltransferase activity is decreased in patients with cholesterol gallstones, J. Lipid Res. 31, 1993.

Suckling, K.E., 1991, Drugs working on the intestine, Curr. Opin. Lipidol. 2, 31.

Thomas, P.J. and A.F. Hofmann, 1973, A simple calculation of the lithogenic index of bile: expressing biliary lipid composition on rectangular coordinates, Gastroenterology 65, 698.

Turley, S.D. and J.M. Dietschy, 1978, Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile, J. Lipid Res. 19, 924.

Yamada, N., H. Shimano, H. Mokuno, S. Ishibashi, T. Gotohda, M. Kawakami, Y. Watanabe, Y. Akanuma, T. Murase and F. Takaku, 1989, Increase clearance of plasma cholesterol after injection of apo E into Watanabe heritable hyperlipidemic rabbits, Proc. Natl. Acad. Sci. USA 86, 665.