Hypocholesterolaemic effect of $\beta\beta'$ -methyl-substituted hexadecanedioic acid (MEDICA 16) in the male hamster

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Treatment of cholesterol-fed male hamsters kept on a diet of purina chow with $\beta\beta'$ -methyl-substituted hexadecanedioic acid (MEDICA 16) resulted in a progressive hypocholesterolaemic effect, amounting to a 50 % decrease in the cholesterol content of all plasma lipoproteins. The decrease in plasma cholesterol could be accounted for by activation of plasma-cholesterol efflux through the liver into the bile mediated by MEDICA 16-induced (a) increase of the number of liver LDL receptors, (b) activation of liver neutral cholesteryl ester hydrolase with a con-

comitant inhibition of liver acyl-CoA cholesterol acyltransferase, resulting in shifting of the liver cholesteryl ester/free-cholesterol cycle in the direction of free cholesterol, and (c) activation of cholesterol efflux from the liver into the bile. The increase in bile cholesterol output was accompanied by an increase in bile phospholipids but not in bile acids. In contrast with rats, MEDICA 16-treatment of male hamsters did not result in a hypotriacylglycerolaemic effect, inhibition of lipogenesis, nor in a substantial decrease in plasma apolipoprotein C-III content.

INTRODUCTION

Tetramethylhexadecanedioic acid (MEDICA 16) has recently been reported to induce a potent hypolipidaemic effect in the normolipidaemic or nephrotic hyperlipaemic rat [1]. The observed hypolipidaemic effect consisted of a decrease in the plasma triacylglycerol and cholesterol content of chylomicrons and very-low-density lipoproteins (VLDL) with a concomitant increase in the relative abundance of high-density-lipoprotein (HDL) cholesterol [1]. The hypolipidaemic effect, with respect to plasma VLDL, could be partially accounted for by inhibition of synthesis of liver long-chain fatty acid and cholesterol as a result of a reversible inhibition of ATP citrate lyase [2] and acetyl-CoA carboxylase [3] together with a non-reversible inhibition of cholesterol synthesis at a step beyond the hydroxymethylglutaryl (HMG)-CoA reductase [4]. The overall production rate of chylomicrons remained, however, unaffected by MEDICA 16 treatment [5]. The hypolipidaemic effect with respect to both types of triacylglycerol-rich lipoprotein could be further accounted for by an increase in their plasma clearance accompanied by a 10-fold decrease in plasma apolipoprotein (apo) C-III [1,5]. The reduction in plasma apo C-III was proposed to drive premature hepatic uptake of plasma triacylglycerol-rich lipoproteins by de-inhibiting the lipoprotein lipase, hepatic triacylglycerol lipase and receptor-mediated liver uptake of the apo C-III-deficient particles [1,5].

The male hamster may offer a better animal model for examining human plasma lipoprotein profiles, lipoprotein metabolism and liver cholesterol homeostasis than the rat [6–8]. Thus, in contrast with rats, a substantial fraction of plasma cholesterol is carried, in the hamster, by low-density lipoproteins (LDL) and may be further enriched by cholesterol feeding [9]. Moreover, since the rate of rat liver cholesterol synthesis is exceptionally higher than that of other species [7], cholesterol homeostasis in the rat liver as a function of endogenous or exogenous cholesterol availability is maintained in the first

instance by regulating *de novo* cholesterol synthesis [6], and only under conditions where the adaptive synthetic response is blocked or saturated is liver cholesterol homeostasis regulated by receptor-mediated cholesterol uptake [8]. In contrast with rat, the capacity for liver cholesterol synthesis in human or male hamster is limited and may even be further limited by cholesterol feeding, thus allowing for liver cholesterol homeostasis to be mediated by cholesterol influx/efflux rather than *de novo* cholesterol synthesis.

To evaluate the hypolipidaemic potential of MEDICA 16 in an animal model for human lipoproteins, and in light of the above considerations, the effect of MEDICA 16 was studied here in cholesterol-fed male hamsters, where a substantial fraction of plasma cholesterol is carried by LDL and where cholesterol homeostasis may be expected to be accounted for by liver cholesterol traffic rather than *de novo* cholesterol synthesis.

EXPERIMENTAL

Materials

MEDICA 16 was synthesized as previously described [2]. Triacylglycerol, and total cholesterol were determined using Boehringer kits nos. 701912 and 286691 respectively. [1,2- ^3H]-Cholesterol (60 Ci/mmol), [1- ^{14}C]oleoyl-CoA (60 mCi/mmol) and 3-[glutaryl-3- ^{14}C]hydroxy-3-methylglutaryl-CoA (60 mCi/mmol) were from NEN. Cholesteryl [1- ^{14}C]oleate (60 mCi/mmol) and ^{125}I (15.8 mCi/ μg of iodine) were obtained from Amersham International. $^3\text{H}_2\text{O}$ was from Rotem Industries, Negev, Israel. Alkaline phosphatase (Cat. no. 5130) and 3α -hydroxysteroid dehydrogenase were from Worthington. All other chemicals were from Sigma Chemical Company.

Animals and diets

Male golden Syrian hamsters of the Hebrew University strain weighing 130–150 g were housed in individual cages under

Abbreviations used: MEDICA 16, $\beta\beta'$ -methyl-substituted hexadecanedioic acid; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; HMG-CoA, hydroxymethylglutaryl-CoA; apo, apolipoprotein; LDL, low-density lipoprotein; CETP, cholesteryl ester transfer protein; ACAT, acyl-CoA cholesterol acyltransferase; NCEH, neutral cholesteryl ester hydrolase; PCAT, phosphatidylcholine cholesterol acyltransferase; PMSF, phenylmethanesulphonyl fluoride.

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conditions of alternating periods (12 h) of light (00:00–12:00) and darkness (12:00–00:00). The animals were maintained on a basic diet consisting of 55 % (w/w) carbohydrates, 20 % (w/w) protein, 5 % (w/w) soya oil, 3.4 % (w/w) cellulose, 0.05 % (w/w) cholesterol, 10 % (w/w) moisture and 6.7 % (w/w) salt/vitamin mixture (low-cholesterol group) supplemented with 0.5 % (w/w) cholesterol where indicated (high-cholesterol group). Following 7–14 days of adjustment to the diet, cholesterol-fed animals were either treated for 30 days with 0.07 % (w/w) MEDICA 16 added to the diet or kept untreated. Food consumption/100 g body wt. for MEDICA 16-treated animals was not significantly different from that of non-treated animals. MEDICA 16 treatment of the high-cholesterol groups for one month resulted in a progressive 10 ± 4 % (mean $\pm$ S.D.) loss in weight, while non-treated animals maintained their initial weight throughout the treatment period. Animals were killed in the middle of the dark period. All care and treatment of animals was in conformity with the Animal Care Guidelines of the Israeli Academy of Sciences.

Lipoprotein profiles

Blood samples were collected in a solution of 0.1 % EDTA by heart puncture under ether anaesthesia. Plasma was centrifuged for 20 min at 102 000 *g* in a TST 55.5 rotor and the chylomicrons' free plasma was fractionated into VLDL, LDL and HDL by continuous KBr gradient [10]. Cholesterol and triacylglycerol contents were determined using the respective Boehringer kits. Apolipoproteins were subjected to 11 % (w/v) SDS/PAGE and isoelectric focusing as previously described [5] and their content determined by densitometry of stained gels [5].

Liver lipid content

Liver triacylglycerol and phospholipid contents were determined in liver samples extracted in 20 volumes of chloroform/methanol (2:1, v/v). The dried lipid extract was solubilized in warm 0.4 % SDS. Triacylglycerol was determined using Boehringer kit no. 701912 and phospholipids were determined according to [11]. Liver cholesterol was determined in liver samples ground with anhydrous sodium sulphate [12] and extracted with chloroform/methanol (2:1, v/v). The dried lipid extract was dissolved in propan-2-ol and free cholesterol and cholesteryl ester species were determined by h.p.l.c. [13] using cholesteryl acetate as internal standard. Cholesteryl ester content was calculated by summing up the contents of the three dominant cholesteryl ester species, namely, cholesteryl palmitate, cholesteryl oleate and cholesteryl linoleate. Liver microsomal cholesterol and cholesteryl oleate content were determined in microsomal samples extracted according to [14]. The dried lipid extract was dissolved in acetonitrile and subjected to h.p.l.c. analysis as described above.

Cholesterol in chylomicrons

Cholesterol incorporation into chylomicrons was evaluated in ether-anaesthetized cholesterol-fed animals injected with Triton 1339 (520 mg/kg body wt) into the jugular vein. The anaesthetized animals were bled from the eye choroid plexus at the time of Triton 1339 administration and by heart puncture 1 h later. Blood samples were allowed to clot, and the sera were overlaid with saline and centrifuged in a TST 55.5 rotor at 102 000 *g* for 20 min. The chylomicron fraction was sliced off and

the cholesterol content as a function of time was determined using Boehringer kit no. 286691. Cholesterol incorporation into chylomicrons was found, under these conditions, to be linear for at least 90 min.

Plasma cholesteryl ester transfer protein (CETP) activity

[1,2-³H]Cholesteryl ester-labelled LDL was prepared by incubating human plasma with [1,2-³H]cholesterol (specific activity 60 Ci/mmol) for 18 h [15] followed by five washings with human erythrocytes and isolation of the LDL fraction by KBr-gradient centrifugation. The labelled LDL fraction was dialysed against 5 mM Tris/HCl (pH 7.4) containing 0.15 M NaCl and 0.5 mM EDTA. CETP activity was measured by a modification of [16,17]. [1,2-³H]Cholesteryl ester-labelled human LDL (81 μ g of cholesterol) was incubated with non-labelled human HDL (81 μ g of cholesterol) in 10 mM Tris/HCl (pH 7.4) containing 6 % (w/v) albumin (essentially fatty-acid free), 1.2 mM 5,5'-dithiobis(2-nitrobenzoic acid) and in the presence or absence of added hamster lipoprotein-deficient plasma [*d* > 1.21 g/m (5 mg of protein)] in a total volume of 0.5 ml. After incubation for 3 h at 37 °C, 50 μ l of unlabelled serum was added, LDL was precipitated by heparin-MnCl₂, the HDL-cholesteryl ester was extracted according to [14] and subjected to silicic acid t.l.c. in light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (75:25:1, by vol.). The cholesteryl ester spot was cut off the plate and counted in scintillation fluid.

Output of biliary lipids

The cystic duct of phenobarbital-anaesthetized hamsters was ligated, followed by cannulation of the common bile duct with PE-10 polyethylene tubing (Clay Adams) and collection of bile for 1 h. Bile-acid content was measured enzymically according to [18]. Bile phospholipids were determined according to [11]. Bile cholesterol content was determined using Boehringer kit no. 286691.

Synthesis of liver lipids

Liver cholesterogenesis and lipogenesis rates were determined by following the rate of ³H₂O incorporation into liver cholesterol and long-chain fatty acids. The animals were injected intraperitoneally with 50 mCi of ³H₂O followed 1 h later by quick perfusion of the liver with cold saline [19]. Liver samples were subjected to alkaline hydrolysis, followed by extraction with light petroleum (b.p. 40–60 °C), extensive washings with an ideal upper-phase, digitonin-precipitation of cholesterol, and light petroleum (b.p. 40–60 °C) extraction of the acidified hydrolysate as previously described [2]. Animals perfused for 1 min following the injection of ³H₂O served as controls.

Liver phospholipid synthesis was determined by measuring the incorporation of glycerol into liver lipids 10 min after the injection of 150 μ Ci of [1-(3-³H)]glycerol (specific activity 2.9 Ci/mmol) into the jugular vein [20]. Liver lipids were extracted with chloroform/methanol (2:1, v/v) and phospholipid species were separated by t.l.c. using Kieselgel 60 plates (Merck) eluted with acetone/chloroform/methanol/acetic acid/H₂O (8:6:2:2:1, by vol.). Incorporation of the label under these conditions was found to be linear with time for up to 20 min.

Enzymic assays

Liver microsomal HMG-CoA reductase was measured in alkaline-phosphatase-treated microsomes according to [21]. Liver

microsomal acyl-CoA cholesterol acyltransferase (ACAT) activity was measured according to [22] in unwashed microsomes prepared from liver samples homogenized in 4 vol. of 0.25 M sucrose containing 1 mM EDTA (pH 7.4). Liver cytosolic neutral cholesteryl ester hydrolase (NCEH) activity was measured in the 105000 g supernatant prepared from liver samples homogenized in 0.15 M potassium phosphate buffer (pH 7.4) containing 10% (w/v) glycerol and using cholesteryl [1-¹⁴C]oleate as substrate in a final volume of 0.3 ml [23]. The activity was measured in the presence of 0.5 mg of cytosolic protein and was found to be linear at this range. Concentrations in the range 4–6 mg of cytosolic protein [24] yielded activities non-linear with protein concentrations. The specific activity of the cholesteryl oleate substrate was corrected for the presence of non-labelled cytosolic cholesteryl oleate determined by h.p.l.c. as described above. Activity of liver cholesterol 7- α hydroxylase was measured in washed microsomal preparations according to [25] using endogenous cholesterol as substrate. Plasma phosphatidylcholine cholesterol acyltransferase (PCAT) was measured according to [15].

Ligand blotting of LDL receptors

Liver membranes were prepared by Polytron homogenization for 30 s of 2 g of liver pieces suspended in 10 ml of ice-cold buffer, containing 10 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 1 mM CaCl₂ and 1 mM phenylmethanesulphonyl fluoride (PMSF). The homogenate was centrifuged at 500 g for 5 min followed by centrifugation of the supernatant, first at 8000 g for 15 min and finally at 105000 g for 60 min. The membrane pellet was suspended in the homogenization buffer and precipitated again at 105000 g for 60 min. The washed pellets were frozen in liquid nitrogen and stored at -70 °C. For ligand blotting the membranes were solubilized by passing the pellet through 19-gauge and 23-gauge needles using a solubilization buffer containing 125 mM Tris/maleate (pH 6.0), 2 mM CaCl₂, 0.16 M NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, 0.1 mM leupeptin and 1 μ g/ml pepstatin and left on ice for 30 min. Insoluble particles were spun away by centrifugation at 125000 g for 45 min and the protein content was measured using Bradford reagent. Solubilized membranes (150 μ g) were electrophoresed by 7% (w/v) SDS/PAGE and transferred to nitrocellulose paper as described by Semenkovich et al. [26]. The nitrocellulose paper was incubated for 18 h at 4 °C in a blocking buffer containing 50 mM Tris/HCl (pH 8.0), 90 mM NaCl, 5% (w/v) albumin and 2 mM CaCl₂, followed by a 5 h incubation at room temperature in the presence of 5 μ g/ml rabbit ¹²⁵I-labelled β -VLDL (300 c.p.m./ng). Finally, the paper was washed five times with the blocking buffer containing 0.5% albumin at room temperature and subjected to autoradiography. β -VLDL-ligand blotting in the absence of calcium added during pre-incubation, incubation and washings served as control. β -VLDL was prepared by feeding a rabbit 2% (w/w) cholesterol for one week. The animal was starved overnight, blood was collected in 0.1% EDTA solution and the plasma was centrifuged for 23 h at 275000 g in a SW 41 rotor at a KBr density of 1.019 g/ml. The washed β -VLDL fraction was iodinated using iodine monochloride [27].

Statistics

The significance of differences was evaluated using the Mann-Whitney U-test.

RESULTS

Plasma lipoproteins

The hypercholesterolaemic effect of cholesterol feeding in hamsters and the extent of the hypocholesterolaemic effect of MEDICA 16 in cholesterol-fed hamsters were evaluated here compared with non-treated hamsters maintained on a low-cholesterol diet. The time-course of cholesterol feeding and the hypocholesterolaemic effect of MEDICA 16 are shown in Figure 1. While plasma cholesterol content in cholesterol-fed, non-treated animals progressively increased throughout the feeding period approaching a steady level of approx. 300 mg/100 ml,

Figure 1 Time course of the hypocholesterolaemic effect of MEDICA 16

Male hamsters were kept on a high-cholesterol diet for 47 days. After 14 days of adjustment to cholesterol feeding they were either treated with 0.07% (W/W) MEDICA 16 (□) or kept non-treated (◇) for 33 additional days. Values are given as mean $\pm$ S.D. ($n = 6$).

Table 1 Profile of plasma lipoproteins in MEDICA 16-treated hamsters

Male hamsters were kept on low- and high-cholesterol diets in the absence or presence of added 0.07% (w/w) MEDICA 16. After 4 weeks of treatment, the plasma lipoprotein profile was determined as described in the Experimental section. Values are given as means $\pm$ S.D. ($n = 5$) of one representative experiment out of three. * indicates significantly different from the respective value of the cholesterol-fed non-treated group ($P < 0.05$). † indicates significantly different from the respective low-cholesterol value ($P < 0.05$).

Lipoprotein	Low-cholesterol diet	High-cholesterol diet	High-cholesterol diet, MEDICA 16-treated
Cholesterol (mg/100 ml of plasma)			
Total	132 $\pm$ 20	266 $\pm$ 29†	141 $\pm$ 21*
Chylomicrons	4 $\pm$ 2	8 $\pm$ 2	5 $\pm$ 1
VLDL	6 $\pm$ 1	18 $\pm$ 2†	13 $\pm$ 2*
LDL	19 $\pm$ 2	42 $\pm$ 9†	19 $\pm$ 8*
HDL	103 $\pm$ 12	198 $\pm$ 19†	104 $\pm$ 10*
Triacylglycerol (mg/100 ml of plasma)			
Total	91 $\pm$ 23	127 $\pm$ 37	138 $\pm$ 10
Chylomicrons	53 $\pm$ 15	57 $\pm$ 12	57 $\pm$ 10
VLDL	24 $\pm$ 4	45 $\pm$ 4†	55 $\pm$ 3†
LDL	13 $\pm$ 2	18 $\pm$ 2	18 $\pm$ 3
HDL	1 $\pm$ 1	7 $\pm$ 4	8 $\pm$ 6

Figure 2 Plasma apolipoproteins profile of cholesterol-fed, MEDICA 16-treated hamsters

Conditions were as described in Table 1. The combined lipoprotein fractions of non-treated (a) and MEDICA 16-treated (b) cholesterol-fed hamsters were subjected to SDS/PAGE as described in the Experimental section. Samples were applied (80 μ g of protein/lane) as follows: 1, VLDL; 2, LDL; 3, HDL. The unidentified protein in the LDL fraction is presumably albumin [28].

Figure 3 Plasma apo Cs of MEDICA 16-treated hamsters

Conditions were as described in Table 1. The combined HDL (a,b) and VLDL (c,d) fractions of MEDICA 16-treated (a,c) and non-treated (b,d) cholesterol-fed animals were subjected to isoelectric focusing as described in the Experimental section. Samples were applied at 80 μ g of protein/lane.

plasma cholesterol content of MEDICA 16-treated cholesterol-fed animals progressively decreased throughout the 1-month-long treatment period, approaching plasma cholesterol levels of animals kept on a low-cholesterol diet (132 ± 20 mg/100 ml).

The lipoprotein profiles induced by cholesterol feeding and

MEDICA 16 treatment are shown in Table 1. It is noteworthy that in the low- as well as high-cholesterol groups most of plasma cholesterol is carried by HDL (74–78 %) and LDL (14–16 %), whereas the contribution made by VLDL- and chylomicron-cholesterol was minimal. The overall hypocholesterolaemic effect induced by MEDICA 16 treatment resulted from a 50 % decrease in the cholesterol content of all of the lipoprotein fractions. However, most of the hypocholesterolaemic effect could be accounted for by that of HDL cholesterol, this being the most dominant plasma cholesterol fraction.

The apolipoprotein profile induced by MEDICA 16 in cholesterol-fed hamsters is shown in Figures 2 and 3. MEDICA 16 treatment resulted in a significant decrease in the apo E content of VLDL and LDL, while apo AI and apo C levels remained essentially unaffected. The presence of apoE in the LDL fraction is noteworthy. This could either reflect contamination of the LDL fraction by VLDL or the authentic composition of hamster's LDL.

The hypocholesterolaemic effect of MEDICA 16 could not be accounted for by a putative decrease in cholesterol incorporation into chylomicrons. Thus, cholesterol incorporation into chylomicrons measured in cholesterol-fed animals injected with Triton 1339 amounted to 126 ± 11 (mean $\pm$ S.D., $n = 9$) and 97 ± 26 ($n = 8$) mg/100 ml of serum per h per kg body wt. in non-treated and MEDICA 16-treated animals respectively (not significantly different at $P = 0.05$).

Plasma PCAT or CETP activities remained unaffected by MEDICA 16 treatment (results not shown).

Liver lipid content

Cholesterol feeding resulted in a > 70-fold increase in liver cholesteryl ester, with only relatively slight changes in liver free cholesterol (Table 2). MEDICA 16 treatment resulted in a pronounced decrease in liver cholesteryl ester while liver free cholesterol content remained essentially unaffected.

Liver microsomal cholesteryl ester and free-cholesterol contents, as a function of cholesterol feeding and MEDICA 16 treatment, reflected total liver cholesterol content as presented above (Table 2). Thus, microsomal cholesteryl oleate content was increased 15-fold by cholesterol feeding and decreased to non-detectable values by MEDICA 16 treatment, while microsomal free cholesterol remained essentially unaffected by either cholesterol feeding or MEDICA 16 treatment.

Liver phospholipid content decreased 20 % in cholesterol-fed animals while being reversed back to normal values in MEDICA 16-treated animals.

Liver triacylglycerol levels remained essentially unaffected by either cholesterol feeding or MEDICA 16 treatment.

Table 2 Lipid content of livers of MEDICA 16-treated hamsters

Male hamsters were kept on low- and high-cholesterol diets in the presence or absence of added 0.07% (w/w) MEDICA 16. After 4 weeks of treatment, liver lipids were analysed as described in the Experimental section. Values were given as means $\pm$ S.D. Values in parentheses indicate no. of animals used. * indicates significantly different from the respective value of the cholesterol-fed non-treated group ($P < 0.05$). † indicates significantly different from the respective low-cholesterol value ($P < 0.05$). Abbreviation: n.d., non-detectable.

Treatment	Liver free cholesterol (mg/g of liver)	Liver cholesteryl ester (mg/g of liver)	Microsomal free cholesterol (μ g/mg of protein)	Microsomal cholesteryl oleate (μ g/mg of protein)	Liver triacylglycerol (mg/g of liver)	Liver phospholipids (μ mol/g of liver)
Low-cholesterol diet	1.4 ± 0.3 (3)	n.d. (6)	12.0 ± 5.4 (3)	0.6 ± 0.2 (3)	5.90 ± 1.30 (8)	41.3 ± 2.4 (5)
High-cholesterol diet	3.3 ± 0.9 † (6)	68.1 ± 20.0 † (6)	13.5 ± 4.0 (3)	8.2 ± 5.2 † (3)	4.02 ± 0.89 (3)	32.9 ± 1.7 † (3)
High-cholesterol diet, MEDICA 16-treated	2.4 ± 0.5 (6)	10.9 ± 3.5 *† (6)	12.7 ± 2.3 (3)	n.d.* (3)	5.00 ± 0.52 (6)	42.5 ± 1.4 * (3)

Figure 4 Liver LDL receptors in MEDICA 16-treated hamsters

Conditions were as described in Table 2. LDL receptors were determined by β -VLDL blotting as described in the Experimental section. Lane A, high-cholesterol diet, MEDICA 16-treated; lane B, high-cholesterol diet only; lane C, low-cholesterol diet only. Results are from one out of three experiments.

Liver activities

Liver LDL-receptor activity, as a function of cholesterol feeding and MEDICA 16 treatment, was evaluated by β -VLDL ligand blotting (Figure 4). Liver LDL-receptor activity was substantially reduced in cholesterol-fed animals [0.67 ± 0.18 relative densitometric units ($n = 3$)] and was increased by MEDICA 16 treatment [1.26 ± 0.42 relative densitometric units ($n = 3$)] to a

level similar to that observed in the cholesterol-deficient group [1.0 ± 0.0 relative densitometric units ($n = 3$)].

Liver cytosolic NCEH and microsomal ACAT activities were significantly reduced and increased respectively by cholesterol feeding (Table 3) and were found to be extensively affected by MEDICA 16 treatment of cholesterol-fed animals. Thus, MEDICA 16 was found to inhibit ACAT activity 3.5-fold, while activating NCEH activity 3-fold. The overall effect of MEDICA 16 thus appears to induce liver cholesterol ester conversion into free cholesterol while inhibiting cholesterol's esterification back into cholesterol ester, thus antagonizing the cholesterol flux induced by cholesterol feeding.

HMG-CoA reductase was found to respond to the cholesterol status of the liver, being 3-fold suppressed by cholesterol feeding while becoming up-regulated by MEDICA 16 treatment (Table 3). Down-regulation of the HMG-CoA reductase activity by cholesterol feeding resulted in a 4-fold inhibition of cholesterol synthesis in cholesterol-fed animals, as deduced from the incorporation of $^3\text{H}_2\text{O}$ into liver cholesterol *in vivo* (Table 3). However, up-regulation of the HMG-CoA reductase activity by MEDICA 16 treatment did not result in activation of liver cholesterol synthesis, indicating perhaps that similarly to rats, cholesterol synthesis was inhibited in hamsters by MEDICA 16 at a step beyond the HMG-CoA reductase [4].

Liver phospholipid synthesis was assessed by following the incorporation of radioactive glycerol into liver lipids 10 min after injection of the label into the jugular vein, assuming similar specific activities of hepatic glycerol 3-phosphate following glycerol injection into treated and non-treated animals. Incorporation of the glycerol label into liver phosphatidylcholine was found to be increased 4.3-fold following MEDICA 16 treatment. MEDICA 16 treatment did not result, however, in

Table 3 Liver enzyme activities of MEDICA 16-treated hamsters

Conditions were as described in Table 2. Liver activities were determined as described in the Experimental section. Values are given as means $\pm$ S.D. ($n = 3$) of one representative experiment out of three. * indicates significantly different value from the respective value of the cholesterol-fed non-treated group ($P < 0.05$). † indicates significantly different value from the respective low-cholesterol value ($P < 0.05$).

Treatment	Enzyme activity (pmol/min per mg of protein)				Cholesterol synthesis (nmol of $^3\text{H}_2\text{O}$ incorporated/h per g of wet tissue)	Fatty-acid synthesis (nmol of $^3\text{H}_2\text{O}$ incorporated/h per g of wet tissue)
	ACAT	NCEH	Cholesterol 7- α hydroxylase	HMG-CoA reductase		
Low-cholesterol diet	12.9 ± 3.6	32.1 ± 7.3	—	9.0 ± 2.0	123 ± 40	10914 ± 1915
High-cholesterol diet	$44.6 \pm 6.3^\dagger$	$15.8 \pm 6.0^\dagger$	4.6 ± 0.2	$3.0 \pm 0.5^\dagger$	$36 \pm 8^\dagger$	14193 ± 2369
High-cholesterol diet, MEDICA 16-treated	$12.8 \pm 6.7^*$	$41.5 \pm 11.0^*$	$1.6 \pm 0.6^*$	$8.2 \pm 1.5^*$	$34 \pm 5^\dagger$	13935 ± 4065

Table 4 Biliary-lipid output and content of MEDICA 16-treated hamsters

Male hamsters were kept on a high-cholesterol diet in the absence or presence of added 0.07% MEDICA 16. After 4 weeks of treatment, the bile secretion rate and bile-lipid content were determined as described in the Experimental section. Values are given as mean $\pm$ S.D. ($n = 7$). * indicates value is significantly different from the respective non-treated value ($P < 0.05$). Results are from one representative experiment out of two.

	Bile lipids ($\mu\text{mol/ml}$)			Bile secretion (ml/kg body wt. per h)	Biliary-lipid output ($\mu\text{mol/kg body wt. per h}$)			Cholesterol mole fraction (%)
	Bile acids	Phospholipids	Cholesterol		Bile acids	Phospholipids	Cholesterol	
Non-treated	12.9 ± 5.6	1.8 ± 0.5	0.18 ± 0.1	1.40 ± 0.73	16.5 ± 7.3	2.3 ± 1.0	0.36 ± 0.16	1.2 ± 0.2
MEDICA 16-treated	7.5 ± 1.6	1.7 ± 0.4	$0.35 \pm 0.1^*$	$2.97 \pm 0.79^*$	22.0 ± 7.2	$5.0 \pm 1.7^*$	$0.98 \pm 0.37^*$	$3.6 \pm 1.1^*$

changes in the flux of liver lipogenesis, as deduced from the incorporation of $^3\text{H}_2\text{O}$ into total liver fatty acids (Table 3).

Biliary lipid output

Biliary lipid output, as a function of MEDICA 16 treatment, is shown in Table 4. MEDICA 16 treatment resulted in 2.7- and 2.1-fold increases in biliary cholesterol and phospholipid output respectively, while bile-acid output was insignificantly increased. The change observed in biliary lipid output was accompanied by a 2.1-fold increase in the bile secretion rate, thus resulting in a decrease in bile-acid concentration but a concomitant 2.4-fold increase in cholesterol (mol fraction).

DISCUSSION

The overall hypocholesterolaemic effect of MEDICA 16 in cholesterol-fed hamsters consists of increasing the cholesterol flux from the plasma compartment into the liver and from the liver into the bile. Since the steady-state levels of free and esterified cholesterol, both in plasma and liver, were reduced by MEDICA 16 treatment, the increased plasma to liver and liver to bile cholesterol fluxes are not accounted for by cholesterol mass action, but presumably reflect MEDICA 16-induced activation of steps controlling the influx and efflux of cholesterol into and out of the liver.

The decrease in plasma cholesterol may indeed be ascribed to a MEDICA 16-induced increase in liver apo B,E receptor activity (Figure 4), resulting in an increased hepatic uptake of apo B,E-containing plasma lipoproteins. The hypocholesterolaemic effect with respect to HDL cholesterol could then result from a concomitant transfer of HDL cholesteryl ester into VLDL and LDL catalysed by CETP present in hamster plasma [16,29]. Alternatively, the decrease in plasma HDL cholesteryl ester could reflect a direct increase in the hepatic uptake of HDL cholesterol mediated by putative HDL receptors or catalysed perhaps by hepatic lipase. The role played by the direct and indirect effects of MEDICA 16 on plasma HDL cholesterol is now being investigated in rats which lack cholesteryl ester transfer activity in their plasma.

The putative increase in plasma cholesterol influx into the liver induced in cholesterol-fed hamsters by MEDICA 16 treatment did not result in flooding of the liver with cholesteryl esters. In fact, liver cholesteryl ester content was found to be dramatically reduced in MEDICA 16-treated animals. The decrease in liver cholesteryl ester may be ascribed to activation of NCEH together with inhibition of liver ACAT activity, resulting in shifting of the cholesteryl ester/free-cholesterol cycle towards free cholesterol. Moreover, MEDICA 16 treatment was found to induce a 3-fold increase in biliary cholesterol output (Table 4), thus pointing to activation of free-cholesterol efflux from the liver. This increase in biliary cholesterol output could not be accounted for by a respective increase in bile-acid output with a concomitant increase in bile-acid-induced cholesterol extraction. MEDICA 16-induced increase of biliary cholesterol output was, however, accompanied by an induced increase in liver phospholipid content, as well as in bile phospholipid output, indicating that most of the cholesterol efflux into bile which was induced by MEDICA 16 treatment was perhaps mediated by vesicular cholesterol transport [30]. Three essential steps are thus proposed to mediate the overall hypocholesterolaemic effect of MEDICA 16 in cholesterol-fed hamsters: an increase in liver LDL-receptor activities resulting in plasma cholesterol influx into the liver, pulling the liver cholesteryl ester/free-cholesterol cycle towards free cholesterol as a result of inhibition and activation of liver

ACAT and NCEH respectively, and finally an increase in biliary cholesterol output mediated presumably by vesicular cholesterol transport.

The effect exerted by MEDICA 16 on the various steps involved in plasma and liver cholesterol metabolism in the male hamster may be dissected into steps primarily affected by the drug, and others which presumably respond secondarily to the induced decrease in liver cholesterol. Activation of biliary cholesterol output by MEDICA 16 under conditions where the steady-state level of liver free-cholesterol remained unaffected (Table 2), may indeed be considered as a primary effect of the drug and, as pointed out above, could have resulted from an increase in liver phospholipid synthesis induced by MEDICA 16. MEDICA 16 treatment was reported previously to induce in rats an absolute increase in fatty-acid esterification into liver phospholipids compared with that esterified into liver triacylglycerol [31]. Some other steps, involved in plasma and liver cholesterol metabolism and affected by MEDICA 16, may be considered as responding to the liver cholesterol status rather than being primarily affected by the drug. Liver HMG-CoA reductase, LDL receptors and NCEH were indeed observed to be decreased in this system by cholesterol feeding as previously reported [9,21,24] and increased by MEDICA 16 treatment, thus responding to the liver cholesterol status. Similarly, plasma apo E and liver ACAT were found to be decreased by MEDICA 16, in line with previous reports indicating that these activities are positively correlated with liver cholesterol content [22,32]. It should be pointed out, however, that assuming secondary effects reflecting liver cholesterol status does not rule out a possible primary effect of MEDICA 16 on any of the various liver activities found to be affected by the drug.

The hypolipidaemic effect induced by MEDICA 16 in cholesterol-fed hamsters is different from that previously reported in normo- or hyper-lipidaemic rats, both with respect to the phenomenology observed as well as the respective underlying modes of action. Some of the differences are worth noting. The hypolipidaemic effect in normo- or hyper-lipidaemic rats consisted of a decrease in plasma cholesterol as well as plasma triacylglycerol [1], while MEDICA 16 treatment of cholesterol-fed hamsters did not result in a triacylglycerolaemic effect. The inefficacy of MEDICA 16 as a hypotriacylglycerolaemic agent in cholesterol-fed hamsters could not be accounted for by cholesterol feeding since it could still be observed in cholesterol-fed rats but not in hamsters maintained on a low-cholesterol diet (N. Mayorek, unpublished). It may be accounted for, however, by the inefficacy of the drug as an inhibitor of the lipogenic pathway (Table 3), or as an effector of plasma apo C-III content (Figures 2 and 3) in hamsters. Finally, it is worth noting that the hypocholesterolaemic effect of MEDICA 16, with respect to HDL cholesterol in hamsters (Table 1), may be related to transfer of cholesteryl ester from HDL to apo B-containing lipoproteins catalysed by CETP, an activity that is lacking in the rat. The differences between rats and hamsters with respect to the hypolipidaemic effect exerted by MEDICA 16 may point to the importance of species-specific factors in defining the overall effect of an hypolipidaemic drug.

MEDICA 16 has been reported previously to act as an adipose-reducing agent in lean rats [33] as well as in animal models for obesity and obesity-induced diabetes [31,34]. The adipose-reductive effect of MEDICA 16 was accounted for by activation of lipolysis in adipose tissue accompanied by increased oxygen consumption [33]. The progressive 10% decrease in weight induced by MEDICA 16 in hamsters after 1 month of treatment may indicate that MEDICA 16 may act as an anti-obesity agent in hamsters as well. In a similar way to adipose reduction in rats,

that observed in cholesterol-fed hamsters was not accounted for by an anorectic effect of the drug.

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