Effects of hypothyroidism and high-fat feeding on mRNA concentrations for the low-density-lipoprotein receptor and on acyl-CoA:cholesterol acyltransferase activities in rat liver

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1. Induction of hypothyroidism in rats by feeding propylthiouracil (PTU) significantly increased serum cholesterol concentrations, and the effect was more pronounced for cholesterol in low-density lipoproteins (LDL) rather than high-density lipoproteins (HDL). The concentrations of serum triacylglycerol were decreased in hypothyroidism. These effects on serum lipids were also seen when the normal rats were pair-fed with the PTU-treated group. 2. Feeding a diet rich in saturated fat and cholesterol further increased cholesterol concentrations in LDL and also elevated that in very-low-density lipoprotein (VLDL) of hypothyroid rats. In euthyroid rats such a diet resulted in a relatively small increase in VLDL cholesterol, whereas LDL cholesterol was decreased. 3. Steady-state concentrations of mRNA for the hepatic LDL receptor were significantly decreased in the livers of hypothyroid rats, but were not significantly changed by high-fat feeding in euthyroid or hypothyroid rats. 4. The expression of the LDL receptor in hepatocytes cultured from hypothyroid rats was decreased relative to the euthyroid controls. 5. Whereas the esterification of cholesterol with oleate in hepatocytes cultured from hypothyroid rats was decreased, the activity of acyl-CoA:cholesterol acyltransferase (ACAT) in the livers of these animals was not changed. 6. High-fat feeding increased the hepatic ACAT activity in normal and hypothyroid rats. 7. Incubation of rat hepatocytes with 10 nM-tri-iodothyronine for 4 h increased the relative concentration of the mRNA for the LDL receptor by 25%. 8. It is therefore concluded that thyroid hormones stimulate the synthesis and expression of the hepatic LDL receptor. Elevated cholesterol concentrations in LDL in hypothyroidism probably result from a primary defect in the expression of the hepatic receptor, rather than indirectly via changes in ACAT activity.

INTRODUCTION

Hypothyroidism in humans and animals is frequently associated with raised plasma cholesterol concentrations [1]. In man, this is often accompanied by elevated plasma triacylglycerol [2–4], though normal levels have also been reported [5]. In rats, experimentally induced hypothyroidism is usually associated with normal [6,7] or even decreased [8,9] plasma triacylglycerol concentrations. The hypercholesterolaemia associated with hypothyroidism in both man and animals is largely caused by an increase in cholesterol concentration in LDL, although moderate increases in HDL have also been reported [1,10–12]. It is generally accepted that the increase in LDL is a result of decreased receptor-mediated catabolism of the lipoprotein, primarily in the liver. Our previous studies showing an increase of LDL binding to cultured rat hepatocytes on treatment with T_3 support this view [13].

The precise mechanism whereby thyroid hormones influence LDL-receptor activity are unknown. Experiments with cultured hepatocytes [13] suggest a direct, rather than secondary, effect on the liver. However, it is not known whether this is a primary effect on LDL-receptor gene expression, or whether thyroid hormones act on some other aspect of hepatic cholesterol metabolism which exerts an effect on receptor expression. We

have previously shown that ACAT plays an important role in regulating LDL-receptor expression in rat hepatocytes [14]. Normally, such cells are resistant to down-regulation of LDL receptors by preincubation with LDL [15]. However, if ACAT activity is inhibited, hepatocytes become sensitive to such regulation [14]. In the present study we explore whether thyroid hormones influence LDL-receptor activity through an effect on ACAT.

EXPERIMENTAL

Materials

N-Lauroylsarcosine, diethyl pyrocarbonate, 8-hydroxyquinoline, ethidium bromide, Ficoll 400, polyvinylpyrrolidone, BSA (fraction V), Triton WR 1339, cholesterol, salmon sperm DNA, CoA, T_3 , PTU, pancreatic RNAase 1 and calf thymus DNA were from Sigma Chemical Co., St. Louis, MO, U.S.A. Agarose (electrophoresis grade), Random Primers DNA Labelling System and endonuclease *Eco*R1 were purchased from BRL, Gaithersburg, MD, U.S.A. λ DNA *Hind*III-fragment molecular-size markers were from Promega, Madison, WI, U.S.A. Low-melting-point agarose was from FMC BioProducts, Rockland, ME, U.S.A. Hybond N membranes, [9,10(n)- 3 H]oleic acid

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; PTU, propylthiouracil; T_3 , tri-iodothyronine; VLDL, very-low-density lipoprotein.

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(2–10 Ci/nmol) were from Amersham Corp. Arlington Heights, IL, U.S.A., and [α - 32 P]dCTP [tetra(triethylammonium) salt, 9300 Ci/nmol] was from Dupont Canada Inc., Lachine, Quebec, Canada. [3 H]Oleoyl-CoA (91 Ci/mol) was prepared by the method of Martin-Sanz *et al.* [16].

The rat LDL-receptor cDNA clone (pLDL-1) was a 2.3 kb insert expressed in pGEM Blue expressed in DH5 α , which was kindly provided by Dr. R. Tanaka, Squibb Corp., Princeton, NJ, U.S.A. Plasmid DNA (4.8 kb) carrying the insert (2.3 kb) was digested with *Eco*R1 and applied in 0.04 % Bromophenol Blue, 0.04 % xylene cyanol, 5 % (v/v) glycerol and 5 μ g of ethidium bromide/ml to an agarose gel prepared with two parallel wells. The gels were then electrophoresed at 100 V in 50 mM-Tris/50 mM-borate/1 mM-EDTA adjusted to pH 8. Progression of the DNA fragments was monitored under u.v. illumination, and the cDNA fragment (2.3 kb, as assessed by λ DNA *Hind*III-fragment markers) for the LDL receptor was harvested as it reached the second well. The volume of buffer collected was decreased by shaking with butan-1-ol, the DNA was extracted with phenol/chloroform (1:1, v/v), and DNA was finally precipitated with ethanol. The human γ -actin cDNA in pUC13 was given by Dr. L. Kedes, Department of Biochemistry, University of South California, Pasadena, CA 90033, U.S.A. The inserts were labelled with [α - 32 P]dCTP by using the Random Primers DNA-labelling system as described in the manufacturers' instructions.

Treatment of rats

Feeding trial 1. For this, 12 male Wistar rats, which were from the University of Nottingham Joint Animal Breeding Unit and which weighed about 200 g, were housed individually and were divided into three groups of four animals. Group A was fed *ad libitum* on normal 41B laboratory diet, which was ground to a meal and which consisted of about 14 % of digestible protein, 45 % carbohydrate and 3 % of fat, by wt. Group B were fed on the same diet containing 0.1 % (w/w) PTU. Group C were paired on the normal diet with the same quantity of food consumed on the previous day by group B. All rats were given free access to water.

On the evening of day 13 (groups A and B) or day 14 (group C), all food was removed and rats were fasted until the following morning. Rats were then anaesthetized with 1 ml of Sagatal (60 mg of sodium pentobarbitone/ml)/kg body wt, and approx. 5 ml of blood was collected into glass tubes by cardiac puncture. Blood was allowed to clot, and serum was collected by low-speed centrifugation at 4 °C. Total serum cholesterol and triacylglycerol concentrations were determined by using commercially available enzymic kits from Wako Chemicals, Neuss, West Germany. VLDL and LDL were precipitated from 250 μ l of serum with sodium phosphotungstate [17], and the cholesterol content of the remaining HDL fraction was determined. Serum (2–2.5 ml) from each of the rats in groups B and C was used for the separation of lipoprotein fractions by sequential preparative ultracentrifugation [18]. Lipoproteins were isolated within the following density ranges: 1.006–1.02 g/ml (VLDL + IDL), 1.02–1.06 (LDL) and > 1.06 (HDL).

Feeding trial 2. Male Wistar rats (approx. 200 g), from Charles River (Quebec City, Quebec, Canada) were housed three to a cage and were divided into four groups of six animals each. Group I was fed on normal rat diet (Wayne Lab Blox, from Continental Grain Co., Chicago, IL, U.S.A.) *ad libitum*, group II was fed *ad libitum* on the same diet but supplemented with 30 % lard and 1 % cholesterol (w/w), group III was given normal diet *ad libitum*, but this contained 0.1 % PTU, and group IV was fed on group II diet, but including 0.1 % PTU. Cholesterol was first dissolved in the melted lard, which was then mixed, with or

without PTU, with the ground diet. The mixture was then reformed into pellets before feeding. At the end of the 14 days, animals were bled and lipoproteins isolated as described above. The liver was removed from each rat and quickly frozen in liquid N₂ and stored at –70 °C for further analysis (see below).

Isolation of hepatic RNA

RNA was isolated from samples of liver by a modification of the method described by Chomczynski & Sacchi [19]. Samples of liver (500 mg) were homogenized in 10 ml of denaturing solution containing 4 M-guanidinium thiocyanate, 25 mM-sodium citrate, pH 7.0, 0.5 % sodium lauryl sarcosinate and 0.1 M-mercapto-ethanol. Portions (0.5 ml) were then transferred to 1.5 ml micro-centrifuge tubes, and to each was added 50 μ l of 2 M-sodium acetate, pH 4.0, 0.5 ml of water-saturated phenol and 100 μ l of chloroform/3-methylbutan-1-ol (49:1, v/v). After shaking vigorously, tubes were left on ice for 15 min. Tubes were then centrifuged in a micro-centrifuge at full speed for 10 min. The upper aqueous phase was transferred to a new micro-centrifuge tube, and an equal volume of propan-2-ol was added. This was left for 10 min in liquid N₂ and then re-centrifuged for 20 min as above. The supernatant was removed, the walls of the tube were blotted dry and the pellet was resuspended in 300 μ l of denaturing solution. To this solution 300 μ l of propan-2-ol was added, and after 10 min in liquid N₂ the samples were re-centrifuged. The supernatant was removed and the pellets were resuspended in 75 % (v/v) ethanol. After centrifugation, the ethanol was removed and the final pellet dissolved in 25 μ l of 0.5 % (w/v) SDS. After heating at 65 °C for 10 min, RNA samples were stored at –70 °C until used for analysis.

Determination of total RNA and the mRNA concentrations for the LDL receptor and actin

RNA concentrations were measured by using the difference in fluorescence with ethidium bromide in samples before and after treatment with RNAase [20].

The relative amounts of mRNA for the LDL receptor and for γ -actin were first determined by Northern-blot analysis. Electrophoresis of the total RNA on 0.8 % agarose gel was performed, and the RNA was transferred to Hybond N membranes [21]. The membranes were prehybridized overnight at 42 °C in 6 $\times$ SSPE (1 $\times$ SSPE is 8.77 g of NaCl, 1.38 g of NaH₂PO₄·H₂O and 0.37 g of EDTA in 1 litre of water adjusted to pH 7.4 with NaOH), 5 $\times$ Denhardt's solution [1 $\times$ Denhardt's solution contains 0.2 % (w/v) each of Ficoll, polyvinylpyrrolidone and BSA], 0.5 % SDS, 100 μ g of heat-denatured salmon sperm DNA and 50 % (v/v) formamide in the solution. Hybridization was then performed overnight at 42 °C with the same solution as used for prehybridization, except that Denhardt's solution was omitted and (2–8) $\times 10^6$ c.p.m. of 32 P-labelled DNA (about 50 ng) hybridization probe was added. Probes were labelled by the Random Primer Method. The membranes were then washed twice at room temp. for 30 min in 2 $\times$ SSC (0.3 M-NaCl/0.03 M-sodium citrate) containing 0.1 % SDS and then washed once at 65 °C with 0.1 $\times$ SSC containing 0.1 % SDS for 30 min. The damp membranes were then subjected to autoradiography by using preflashed Kodak XAR-5 film and an intensifying screen at –70 °C.

The validity of the Northern-blot analyses was checked in a rat treated for 5 days with 5 mg of 17- α -ethynylloestradiol/kg. The treatment resulted in an average increase of 2.5-fold in the expression of the mRNA for the LDL receptor compared with the control when these were measured at five different concentrations of RNA for the Northern blots. The mean increase was 2.8-fold when expressed relative to the mRNA for γ -actin. These results are in agreement with previous observations [22].

Routine quantification of relative mRNA levels was performed by slot-blot analysis on Hybond N membranes by using probes labelled as above. The RNA samples were mixed with 2.5 vol. of 37% (v/v) formaldehyde and 9 vol. of $20\times$ SSC, and then applied to the slots, which were washed once with $20\times$ SSC before fixing with u.v. light. The prehybridization, hybridization and washing conditions were the same as described for Northern-blot analysis. Relative mRNA concentrations were determined by quantitative scanning densitometry using a Camag t.l.c. Scanner II in transmission mode.

Measurement of ACAT activity

This activity was determined in homogenates of rat liver by a method based on that of Lichtenstein & Brecher [23]. The measurements were made in either the absence or the presence of exogenous cholesterol. Dispersions of cholesterol were made [23] in 0.1 M-potassium phosphate buffer, pH 7.4 by mixing cholesterol with Triton WR1339 in the ratio 1:30 (w/w). The Triton WR1339 was prepared in 10% (v/v) acetone, and when added to cholesterol it was warmed to aid the solubilization of the cholesterol. A solution of 0.1 M-potassium phosphate buffer, pH 7.4, was added at 60 °C, the mixture was shaken and the acetone was removed with a stream of N_2 . The resulting solution contained 200 μ g of cholesterol/ml, and this was used immediately for measuring ACAT activities in an assay volume of 0.2 ml, which contained 0.1 mM-potassium phosphate buffer, pH 7.4, 0.3 mg of fatty-acid-poor BSA and 11–44 μ g of homogenate protein. The assays also contained 129 μ M-cholesterol dispersed with Triton WR1339, or the equivalent amount of Triton WR1339. The reaction was started by adding 50 μ M- $[^3H]$ oleoyl-CoA (91 Ci/mol), and after 5 min at 37 °C the reaction was stopped by adding 1.88 ml of chloroform/methanol (1:2, v/v) plus 0.3 ml of water. The lipids were extracted by the method of Bligh & Dyer [24] and cholesterol esters were purified on t.l.c. plates of silica gel G (Merck) by using light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (90:10:1, by vol.) for development. The incorporation of $[^3H]$ oleic acid into cholesterol esters was determined by liquid-scintillation counting.

Studies on monolayer cultures of hepatocytes

Rats were maintained on either normal rat diet (41B) or 41B diet plus 0.1% PTU for 14–28 days before hepatocyte preparation. Hepatocytes from a control and a PTU-treated rat were prepared on the same day and cultured on collagen-coated tissue-culture dishes in modified Leibovitz L-15 medium by procedures previously described [15,25]. The cells were maintained for 20 h in Leibovitz L-15 medium containing 10% (v/v) newborn-calf serum before the experiments. Specific dextran

sulphate-releasable binding of ^{125}I -labelled human LDL [25] to hepatocytes and the incorporation of $[^3H]$ oleate in cholesterol ester and triacylglycerol were measured as described elsewhere [14].

In other experiments, hepatocytes from euthyroid rats were maintained in culture for 24 h and then incubated in medium containing 2 g of fatty-acid-poor BSA and in the absence or the presence of an optimum concentration [13] of 10 nM- T_3 . The relative concentrations of mRNA for the LDL receptor and γ -actin were then measured by slot-blot analysis.

RESULTS

Effects of PTU treatment on body weights, food intake and serum lipids in rats

PTU-treated animals consumed approx. 25% less food after 14 days than did controls fed *ad libitum* (Table 1). This resulted in the PTU-treated animals remaining at the same weight as at the beginning of the trial, whereas the controls fed *ad libitum* gained an average of 25 g. The pair-fed controls, restricted to the same food intake as the PTU-treated animals, lost weight over the trial, finishing at a mean of 15 g below starting weight.

Compared with controls either fed *ad libitum* or pair-fed, PTU treatment resulted in increases in total serum cholesterol and decreases in triacylglycerol (Table 1). The magnitude of the change in cholesterol was not affected by pair-feeding the rats, but, although still significant, the effect on triacylglycerol was less pronounced. HDL cholesterol as measured after phosphotungstate precipitation was not significantly different between the groups, suggesting that most of the increase was in the less-dense fractions. Serum from control and pair-fed animals was subjected to sequential ultracentrifugation to separate the lipoprotein fractions. Table 2 shows that the most pronounced effect occurs in the LDL fraction, where there was a 4-fold increase in cholesterol concentration. The fall in total serum triacylglycerol can be seen to be primarily as a result of a decrease in the VLDL+IDL fraction.

The results of Trial 1 suggest that the effect of PTU feeding on LDL cholesterol concentrations cannot be attributed to decreased food consumption. It was therefore decided in the second trial to compare control and PTU-treated animals fed *ad libitum* and to determine the effect of high-fat feeding. As in Trial 1, PTU treatment resulted in increased plasma cholesterol and decreased triacylglycerols (Table 3). The high-fat high-cholesterol diet increased VLDL+IDL cholesterol, but decreased LDL cholesterol in control animals, while dramatically increasing the cholesterol concentration of both fractions in the PTU-treated

Table 1. Characteristics of PTU-treated and control (*ad libitum* and pair-fed) rats

Rats were fed for 14 days on normal laboratory diet or on the same diet supplemented with 0.1% PTU as indicated. The concentrations of triacylglycerol and cholesterol were measured in the blood serum. Results are means $\pm$ S.D. for four rats per group: * indicates a significant difference at $P < 0.01$.

		Weight change (g)	Food intake (g)	Total triacylglycerol (mm)	Cholesterol (mm)	
					Total	HDL
I.	Control (<i>ad libitum</i>)	25 $\pm$ 4	252 $\pm$ 27	0.26 $\pm$ 0.03	1.31 $\pm$ 0.25	0.91 $\pm$ 0.16
	I versus II*		I versus II*	I versus II*	I versus III*	
II.	PTU-treated	-1 $\pm$ 3	177 $\pm$ 7	0.09 $\pm$ 0.02	2.11 $\pm$ 0.29	1.09 $\pm$ 0.14
	II versus III*			II versus III*	II versus III*	
III.	Control (pair-fed)	-15 $\pm$ 4	177 $\pm$ 7	0.18 $\pm$ 0.01	1.36 $\pm$ 0.13	0.91 $\pm$ 0.09
	I versus III*		I versus III*	I versus III*		

Table 2. Lipoprotein cholesterol and triacylglycerol concentrations in PTU-treated and pair-fed control rats

Rats were fed for 14 days on the normal laboratory diet supplemented with 0.1 % PTU or fed with the equivalent weight of untreated food. Serum lipoprotein fractions were then prepared by sequential ultracentrifugation. Results are means $\pm$ s.d. for four rats per group; significant differences between the groups are indicated by ** $P < 0.01$ and * $P < 0.05$.

Group	Lipid	Concn. (mm)		
		VLDL+IDL	LDL	HDL
1. Control	Cholesterol	0.028 $\pm$ 0.005	0.089 $\pm$ 0.020	0.901 $\pm$ 0.091
	Triacylglycerol	0.070 $\pm$ 0.012	0.019 $\pm$ 0.002	0.099 $\pm$ 0.005
2. PTU-treated	Cholesterol	0.019 $\pm$ 0.004*	0.321 $\pm$ 0.023**	1.115 $\pm$ 0.176**
	Triacylglycerol	0.015 $\pm$ 0.006**	0.020 $\pm$ 0.005	0.076 $\pm$ 0.010**

Table 3. Effects of PTU treatment and high-fat diets on the lipid composition of serum lipoproteins

Rats were fed *ad libitum* for 14 days on normal diet or high-fat, high-cholesterol diet with or without addition of 0.1 % PTU as indicated. Serum lipoproteins were then prepared by sequential ultracentrifugation, and cholesterol (CHOL) and triacylglycerol (TAG) concentrations were measured. Results are means $\pm$ s.d. for the numbers of rats indicated in parentheses. Significant differences between groups are indicated by: ^asignificantly different from control (VLDL CHOL, I versus II and I versus IV, $P < 0.01$; VLDL TAG, I versus IV, $P < 0.01$; LDL CHOL, I versus II, $P < 0.05$, I versus III, $P < 0.01$, and I versus IV, $P < 0.001$; LDL TAG, I versus II and I versus IV, $P < 0.01$; HDL CHOL, I versus II, $P < 0.01$, I versus III, $P < 0.05$); ^bsignificantly different from high-fat (VLDL CHOL and TAG and HDL CHOL, II versus IV, $P < 0.01$; LDL CHOL, II versus III, $P < 0.001$); ^csignificantly different from PTU (VLDL CHOL, III versus IV, $P < 0.001$; LDL CHOL and TAG, III versus IV, $P < 0.05$; HDL CHOL, III versus IV, $P < 0.01$).

			Concn. (mm)		
			VLDL (+IDL)	LDL	HDL
I. Control (5)	CHOL		0.088 $\pm$ 0.035	0.161 $\pm$ 0.047	1.248 $\pm$ 0.259
	TAG		0.135 $\pm$ 0.054	0.029 $\pm$ 0.007	0.056 $\pm$ 0.009
II. High-fat (6)	CHOL		0.163 $\pm$ 0.035 ^a	0.098 $\pm$ 0.038 ^a	0.712 $\pm$ 0.147 ^a
	TAG		0.071 $\pm$ 0.033	0.015 $\pm$ 0.003 ^a	0.056 $\pm$ 0.019
III. PTU (6)	CHOL		0.061 $\pm$ 0.032	0.550 $\pm$ 0.193 ^a	1.675 $\pm$ 0.304 ^a
	TAG		0.071 $\pm$ 0.046	0.059 $\pm$ 0.037	0.049 $\pm$ 0.011
IV. PTU + high fat (6)	CHOL		0.848 $\pm$ 0.411 ^{abc}	0.944 $\pm$ 0.138 ^{abc}	1.075 $\pm$ 0.168 ^{ab}
	TAG		0.062 $\pm$ 0.026 ^{ab}	0.013 $\pm$ 0.005 ^{ac}	0.057 $\pm$ 0.0174

animals. The high-fat diet decreased HDL cholesterol in both control and PTU-treated animals.

Effects of PTU treatment on the relative concentrations of mRNA for the hepatic LDL receptor

To investigate whether the above changes in serum LDL cholesterol concentrations were associated with changes in the expression of hepatic LDL receptors, the concentration of LDL receptor mRNA was measured. Initial analysis of total rat liver RNA by Northern blotting, followed by hybridization against the LDL-receptor cDNA probe, revealed the presence of a single complementary RNA species, which migrated on agarose-gel electrophoresis in a similar region to 28 S rRNA (Fig. 1). The mRNA for the LDL receptor was decreased in rats treated with PTU and maintained on either the control or high-fat diets. Further quantification of LDL-receptor mRNA was performed on total liver RNA by slot-blot analysis. Fig. 2(a) shows that hypothyroidism caused a marked (approx. 50 %) decrease ($P < 0.005$) in hepatic LDL-receptor mRNA concentrations for rats fed on the normal or high-fat diets compared with rats fed on the normal diet. Feeding a high-fat high-cholesterol diet had no significant effect on LDL-receptor mRNA concentrations in normal or hypothyroid rats. As a control we also measured mRNA concentrations for γ -actin (Figs. 1b and 2b). Although there was some indication of higher concentrations of mRNA for actin in the two groups fed on the high-fat diet, no statistically

Fig. 1. Northern-blot analysis of hepatic mRNA for (a) the LDL receptor and (b) γ -actin of rats treated with PTU or a high-fat diet

The Figure shows typical Northern blots obtained with 10 μ g of total RNA from livers of rats treated as described in Table 3. The position of 18 S and 28 S rRNA is indicated. The conclusions from this analysis are confirmed by slot-blot analysis as shown in Fig. 2.

Fig. 2. Slot-blot analysis of hepatic mRNA for (a) the LDL receptor and (b) γ -actin of rats treated with PTU or a high-fat diet

The Figure shows the relative concentrations of mRNA for the LDL receptor and γ -actin for the groups of rats described in Table 1. Results are means $\pm$ S.D. for the groups of five or six rats. Statistical analysis by ANOVA showed a significant difference ($P < 0.005$) among the groups for the mRNA for the LDL receptor. The differences between groups from an unpaired t test were: I versus IV, $P < 0.0005$; I versus III, $P < 0.005$; II versus III, $P < 0.005$; II versus IV, $P < 0.025$. The analysis that is shown was performed with 10 μ g of total RNA from each liver. Essentially similar results were obtained by using 2.5, 5, or 20 μ g of RNA for the analysis (results not shown).

significant differences were found. There were also no significant differences between the control and hypothyroid groups.

If the results for the expression of the mRNA concentrations for the LDL receptor are expressed relative to that for γ -actin, the decreases ($P < 0.05$) with PTU treatment are still observed. However, we consider that this calculation may not be entirely valid, because of what appears to be a possible effect of high-fat feeding on the γ -actin mRNA.

Binding of LDL to hepatocytes

Fig. 3 shows the specific dextran sulphate-releasable binding of LDL hepatocytes isolated from control and PTU-treated rats

Fig. 3. Effects of PTU treatment *in vivo* on the subsequent expression of the LDL receptor in cultured hepatocytes

Rats were treated with PTU (■) so that the effect on the expression of the LDL receptor in hepatocyte cultures could be compared with that of control animals (□) fed *ad libitum*. The incubations contained either (a) 10% (v/v) newborn-calf serum, or (b) 0.2% fatty-acid-free BSA. Results are means $\pm$ S.D. of duplicate determinations and were confirmed in an independent experiment.

Fig. 4. Effects of PTU treatment on the ACAT activity of rats treated with PTU or a high-fat diet

Rats were treated as described in Table 1, and ACAT activities were measured in liver homogenates in the presence of Triton WR 1339 in the absence (a) or the presence (b) of saturating amounts of exogenous cholesterol. Results are means $\pm$ S.D. for the groups of five or six rats. The significance of the difference between groups was as follows: (a) I versus II, $P < 0.0005$; I versus IV, $P < 0.0005$; II versus III, $P < 0.005$; III versus IV, $P < 0.05$; and (b) I versus II, $P < 0.0005$; I versus IV, $P < 0.005$; II versus III, $P < 0.005$; III versus IV, $P < 0.025$.

as a function of time in culture. As we have previously shown [15,25], binding increased markedly with time in culture in the presence of 10% newborn-calf serum. The magnitude of this increase was approximately twice as great in control hepatocytes as in those from the PTU-treated rat. If cells were placed in serum-free medium, supplemented with 2 g of fatty-acid-free BSA/litre, binding increased 4-fold between 2 and 44 h for control cells, but remained unchanged in cells from PTU-treated rats.

Hepatic ACAT activity and cholesterol esterification in hepatocytes

Feeding a high-saturated-fat high-cholesterol diet significantly increased ($P < 0.005$) hepatic ACAT activity in liver homogen-

Fig. 5. Effects of PTU treatment *in vivo* on the subsequent esterification of oleate by hepatocytes in culture

Rats were treated with PTU (■) and the effect on the incorporation of oleate into (a) triacylglycerol and (b) cholesterol ester was compared to those for hepatocytes cultured from control rats (□) fed *ad libitum*. Results are means $\pm$ S.D. of duplicate determinations and were confirmed in two further experiments (see text).

Fig. 6. Effects of T_3 on the relative concentrations of mRNA for the LDL receptor in cultured rat hepatocytes

Rat hepatocytes were incubated for the time indicated with 10 nM- T_3 . The values show the relative concentration of the mRNA for the LDL receptor relative to that for γ -actin. Control values for each time period are given the value of 100. Results are means $\pm$ S.D. for five independent experiments at 4 and 8 h, and four experiments at 16 h. The significance of the differences at 4 and 8 h between the control and T_3 -treated hepatocytes were calculated by using a paired *t* test and are indicated by: * $P < 0.025$ and ** $P < 0.005$.

ates, and this was independent of whether the enzyme activity was measured in the absence (Fig. 4a) or the presence (Fig. 4b) of exogenous cholesterol. Hypothyroidism itself had no significant effect on hepatic ACAT activity, but it decreased ($P < 0.025$) the magnitude of the increase brought about by high-fat feeding.

Cholesterol esterification was also monitored in hepatocytes prepared from normal and PTU-treated rats. PTU treatment had no significant effect on the incorporation of [3 H]oleate into triacylglycerol, but there was a marked decrease in cholesterol esterification (Fig. 5). In three independent experiments the mean rate of cholesterol esterification was 0.694 ± 0.192 nmol of oleate incorporated/h per mg of cell protein in control cells, compared with 0.369 ± 0.125 ($P < 0.05$ by paired *t* test) in those isolated from PTU-treated animals.

Effects of T_3 on the relative concentrations of mRNA for the LDL receptor in cultured rat hepatocytes

Rat hepatocytes were incubated with 10 nM- T_3 . This caused significant increases after 4 and 8 h, of 25 and 15% respectively, in the steady-state concentration of the mRNA for the LDL receptor relative to that for γ -actin (Fig. 6). There was no significant difference with T_3 after 16 h of incubation.

DISCUSSION

At the completion of this study, there was no other work to our knowledge to demonstrate that the decreased catabolism of LDL by the liver in hypothyroidism might be mediated by a

decreased expression of mRNA for the LDL receptor. However, Staels *et al.* [26] have recently demonstrated a decrease in the steady-state concentration of the mRNA for the LDL receptor in livers of hypothyroid rats, and an increase in hyperthyroid rats. Our results (Figs. 1 and 2) agree with the former observation. However, results from studies *in vivo* do not establish that the expression of the mRNA for the receptor is directly regulated by thyroid hormones. For example, the changes could arise indirectly by alterations in the actions of other hormones, or by differences in the availability of metabolites, e.g. cholesterol. We therefore investigated further the mechanisms for the changes in mRNA concentration by determining the interactions of hypothyroidism with high-fat high-cholesterol diets, and the effects of these on ACAT activities and the concentrations of circulating lipoproteins. In addition, we checked whether T_3 could directly alter the steady-state concentration of mRNA for the LDL receptor in the cultured hepatocytes, and whether this is translated into differences in the expression of the LDL receptor at the cell surface.

The results from Trial 1 agree with those of several other workers, in that PTU treatment of rats results in an increase in plasma cholesterol [1,27] and a decrease in plasma triacylglycerol [8,9]. Pair-feeding showed that the change in cholesterol concentration was independent of food intake, whereas the decrease in triacylglycerol in fasting could be only partly attributed to the decreased food intake of the PTU-treated animals. The cholesterol content of all the lipoprotein classes increased after PTU treatment, but the most dramatic effect was in the LDL fraction, where a 4-fold increase was observed. Also, when HDL cholesterol was measured after precipitation of other lipoproteins with sodium phosphotungstate, there was no significant change in the PTU-treated rats, whereas that isolated ultracentrifugally was significantly increased. This and other work [27] suggests that the increase in HDL might be primarily due to an increase in the number of apolipoprotein-E-containing particles, which are known to be precipitated by phosphotungstate [17]. This is supported by the findings of Davidson *et al.* [28] that hepatic apolipoprotein E synthesis is increased in PTU-treated rats. The fall in serum triacylglycerol concentrations after PTU treatment was primarily due to a decrease in the VLDL fraction. The resulting relative enrichment of the VLDL fraction with cholesterol ester has been described elsewhere [29].

The rate of incorporation of oleate into triacylglycerol was similar in hepatocytes isolated from control and PTU-treated rats, suggesting no difference in the synthetic capabilities of the two sets of cells (Fig. 5a). Thus it seems more likely that PTU alters fatty acid synthesis (or fatty acid availability), and decreases the secretion of triacylglycerol within VLDL. Support for the latter possibility has been provided by Davidson *et al.* [28]. Furthermore, lipoprotein lipase activity, and hence triacyl-

glycerol catabolism, may be enhanced. It is, however, important to note that in human myxoedema most workers report elevated plasma triacylglycerol concentrations [1], and the decreased concentrations found in some PTU-treated rats ([1]; Tables 1–3) may be species-specific and not a result of hypothyroidism itself. Staels *et al.* [26] did not observe a decrease in serum triacylglycerols in PTU-treated rats.

Trial 2 showed that feeding a diet high in saturated fat (lard contains approx. 38% saturated fatty acids) and cholesterol resulted in a modest increase in VLDL cholesterol and a decrease in LDL cholesterol in normal rats. By contrast, as previously reported [30], in hypothyroidism there was a dramatic increase in both VLDL and LDL cholesterol. There were, however, no further significant decreases in the hepatic mRNA concentrations for the LDL receptor. This, together with the increased cholesterol in VLDL as well as LDL, suggests that there might have been an increased production of VLDL cholesterol. However, it is also possible that LDL-receptor activity may be affected without changes in steady-state mRNA concentrations. Changes in plasma-membrane fatty acyl composition have recently been shown to change LDL uptake and metabolism in the human hepatocarcinoma cell line HepG2, without changing gene expression [31]. HDL cholesterol decreased in both groups fed on the high-fat diets. No significant effect on serum triacylglycerol was seen, in either normal or hypothyroid groups, on feeding the high-fat diet (Table 3).

We have previously shown that cholesterol esterification plays a major role in regulating LDL-receptor activity in cultured rat hepatocytes [14]. The relative inability of LDL to decrease the activity of the LDL receptor in rat hepatocytes [15] was attributed to the high ACAT activity, which could prevent an increase in the regulatory pool of cholesterol (or oxysterol). The observed increase in ACAT activity in the livers of rats fed on the high-fat high-cholesterol diet may further help to prevent changes in the regulatory pool of cholesterol. This, together with the known decrease in hepatic cholesterol synthesis that occurs on high-cholesterol feeding [32], probably combine to prevent any changes in LDL-receptor expression. In hypothyroid rats the high-fat high-cholesterol diet also increased ACAT activity. It is not known what happens to hepatic cholesterol synthesis in such animals. However, the fact that LDL-receptor mRNA concentrations are not further decreased suggests that either there is no change in the regulatory pool of cholesterol or the cells are unable to respond to such a change. Again, the possibility of changes in LDL-receptor activity not associated with receptor synthesis cannot be ruled out.

Although we failed to show any decrease in ACAT activity in the livers of hypothyroid rats (Fig. 4), we did find a decrease in cholesterol esterification in hepatocytes isolated from them (Fig. 5). The most likely explanation for this is a decreased availability of metabolically active cholesterol, owing to a decreased uptake of LDL, or decreased hepatic cholesterologenesis, or both. A further possibility is that differences in the phosphorylation state of the enzyme may mean that, although total ACAT activity may not change, levels of the active enzyme may still be lower in the livers of hypothyroid animals. However, although it has been shown that ACAT activity *in vitro* can be stimulated by an ATP-dependent process, it has been questioned whether this involves a phosphorylation of the enzyme [33]. Furthermore, this mechanism presupposes that the phosphorylation state of the enzyme is changed during homogenization of the livers during the assay. We have no evidence as to whether this actually occurs. If changes in phosphorylation were changing the activity of ACAT in hypothyroid animals, then it is possible that changes in LDL-receptor gene expression are secondary to this.

We also show that this decrease in receptor expression persists

when hepatocytes from PTU-treated rats are maintained in culture. The fact that both normal- and hypothyroid-rat hepatocytes express similar levels of binding when freshly isolated may suggest that receptors could be damaged or destroyed on collagenase digestion of the liver. The increase in binding seen at 44 h of culture in normal cells in serum-free medium probably represents synthesis of new receptors coded for by existing mRNA (Fig. 3).

The relative lack of response in cells from PTU-treated rats could therefore result from the decreased mRNA levels for the receptor in these cells. The further stimulation of binding in the presence of newborn-calf serum probably involves new synthesis of receptor mRNA, partly as a result of HDL₃ in the serum [15]. This may cause cholesterol depletion in the hepatocytes. An increase was also observed in the hepatocytes from the hypothyroid rats, but there was again a lower increase in LDL compared with controls.

Further work with isolated hepatocytes demonstrated that T₃ can directly increase the steady-state concentrations of the mRNA for the LDL receptor by 25% after 4 h (Fig. 6). This could then account for the 30% increase in the expression of the LDL receptor that occurs on the surface of hepatocytes incubated for 9 h with T₃ [34]. It is therefore concluded that thyroid hormones play a direct role in stimulating the synthesis and expression of the hepatic LDL receptor. The defect that occurs in these processes in hypothyroidism leads to decreased LDL catabolism and a hypercholesterolaemia that is aggravated by a high-fat diet.

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