Role of the intestinal acyl-CoA:cholesterol acyltransferase activity in the hyperresponse of diabetic rats to dietary cholesterol[1]

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Abstract Contrary to normal rats, diabetic rats are known to develop marked hypercholesterolemia when fed a cholesterol-enriched diet. The triggering factor involved in this hyperresponse has not been identified. With the aim of clarifying the role of the intestinal acyl-CoA:cholesterol acyltransferase (ACAT), we studied the effects of a high fat diet and the changes of intestinal ACAT activity during the early development of streptozotocin-diabetes in rats. Feeding diabetic rats with a diet enriched in cholesterol and saturated fat produced an increase in plasma and in tissue cholesterol as early as 3 days after streptozotocin injection in the absence of hyperphagia. Under these experimental conditions, treatment with insulin or with the ACAT inhibitor CL-277082 significantly reduced the plasma cholesterol to levels measured in nondiabetic rats fed the same high fat diet. An increase in [^{14}C]cholesterol in plasma very low density lipoprotein was observed after oral administration of labeled cholesterol to 3-day diabetic rats. In parallel experiments, the direct measurement of small intestine microsomal ACAT activity revealed an increase, averaging 288% in diabetic rats 3 days after diabetes induction. This change in ACAT activity occurred simultaneously with an increase in plasma glucagon and was normalized by insulin treatment. The induction of intestinal ACAT activity in diabetic rats, its modulation by insulin, and the hypocholesterolemic effects of insulin or CL-277082 treatment clearly indicate that ACAT activity plays a major role in the initiation of diabetes-associated hypercholesterolemia.—**Maechler, P., C. B. Wollheim, C. L. Bentzen, and E. Niesor.** Role of the intestinal acyl-CoA:cholesterol acyltransferase activity in the hyperresponse of diabetic rats to dietary cholesterol. *J. Lipid Res.* 1992. **33:** 1475–1484.

Supplementary key words hypercholesterolemia • streptozotocin • CL-277082

Hypercholesterolemia is a feature frequently observed in humans with diabetes mellitus (1–3) and certainly contributes to the high prevalence of atherosclerosis and coronary heart disease associated with this metabolic disorder (4–7). Cholesterol metabolism was also found to be affected in rats with streptozotocin-induced diabetes, as hypercholesterolemia (8–10) and a higher sensitivity to dietary cholesterol (11–13) have been demonstrated. Several studies have shown an increased cholesterol absorption in diabetic rats (8, 14, 15) and more recently, Colca et al. (16) showed that insulin treatment in diabetic rats reduced the percentage of cholesterol absorbed.

The mechanism that may explain the hyperresponsiveness of diabetic rats to a cholesterol-containing diet remains unclear. However, in the established diabetic state, a number of related metabolic changes have been reported. It was observed by Young, Lopez, and McNamara (15) that compared to normal rats whole body cholesterol production was not changed in diabetic rats. These authors concluded that cholesterol absorbed from the diet was the major contributing factor for the hypercholesterolemia of diabetic rats (15, 17). This conclusion is also supported by the fact that hyperphagia (18) and small intestine hypertrophy (19, 20) are present in the established diabetic state. Furthermore, the activity of the intestinal acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26), the rate-limiting enzyme for cholesterol absorption (21, 22), was reported to be increased in 3-week diabetic rats (23). All these changes are certainly implicated in the hyperresponsiveness of diabetic rats to a cholesterol-containing diet, but these parameters have not yet been examined simultaneously and shortly after diabetes induction in order to identify the triggering factor responsible for the diabetes-linked sensitivity to dietary cholesterol.

To elucidate this question, we investigated the effects of feeding a high fat diet to diabetic rats during the 3 days

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CL-277082, N'-(2,4-difluorophenyl)-N-((4-(2,2-dimethylpropyl)phenyl-methyl)-N-heptylurea; STZ, streptozotocin; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TLC, thin-layer chromatography.

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following streptozotocin injection on plasma and tissue lipid levels. Such a short-term protocol offers the advantage of using rats already hyperglycemic, but with organ weight not yet different from the nondiabetic controls and without hyperphagia. In order to study the importance of the intestinal ACAT activity in diabetic rats fed a cholesterol-rich diet for 3 days, the specific ACAT inhibitor CL-277082 (24) or insulin was administered and the hypercholesterolemic response was determined. Furthermore, the activity of intestinal ACAT was directly measured and its modification by insulin treatment was studied.

MATERIALS AND METHODS

Materials

[4-^{14}C]cholesterol and [1-^{14}C]oleoyl-CoA were obtained from Amersham (Zürich, Switzerland); oleoyl-CoA, cholesterol, cholesteryl oleate, and fatty acid-free bovine serum albumin (BSA, 98–99%) were obtained from Sigma (St. Louis, MO); and streptozotocin, phosphatidylcholine, and phosphatidylserine were from Fluka (Buchs, Switzerland). CL-277082 (N'-(2,4-difluorophenyl)-N-((4-(2,2-dimethylpropyl)phenyl-methyl)-N-heptylurea) was synthesized as described (25). Insulin Mixtard® from Novo (Copenhagen, Denmark) was used for insulin treatment.

Animals

Male Wistar rats (Iffa Credo, Les Oncins, France), weighing 180–200 g were acclimated for 5 days at a controlled temperature of 22–25°C under illumination from 0700 to 1900 h with free access to standard chow (UAR A03, Villemoisson-Sur-Orges, France) and tap water. Diabetes was induced by tail vein injection of streptozotocin at a dose of 80 mg/kg (26). Nondiabetic control rats were injected with buffer only (0.3 ml of 0.05 M citrate buffer, 0.1 M NaCl, pH 4.5). Injections were performed between 1500 and 1600 h at day 0 while the rats were lightly anesthetized with ether. The animals had free access to tap water whereas the daily food rations were controlled (19 g/rat) to ensure equal intake between groups. Nonfasted animals were killed by decapitation under ether anesthesia between 0900 and 1200 h. Blood was collected on EDTA and centrifuged (3000 rpm, 15 min, 4°C) to obtain plasma. Organs were weighed and stored frozen prior to analysis.

Treatments

Where indicated, rats were fed a high-fat diet prepared with standard chow (UAR A03) to which 1% cholesterol plus 1% coconut oil (w/w) was added. Rats were maintained on the specified diets from the day of streptozotocin or buffer injection (day 0) until they were killed. The ACAT inhibitor CL-277082 (24) was dissolved in diethyl ether and mixed with the high-fat diet at a final concentration of 0.06% (w/w) in order to obtain a daily dose of approximately 50 mg/kg. The insulin-treated groups were injected (s.c.) with insulin starting from day 1 at a dose of 10 U/rat per day.

Plasma analysis

Concentrations of glucose, triglycerides, and cholesterol in plasma and lipoprotein fractions were determined enzymatically using kits purchased, respectively, from Ames, Sera-Pak No. 6634 and No. 6687 (Ames, Milano, Italy), and DiaMed (DiaMed, Morat, Switzerland). Insulin and glucagon levels were measured by radioimmunoassay with commercial kits, respectively, from CIS (Gif-sur-Yvette, France) and Medgenix (Sfleurus, Belgium).

Plasma was fractionated by sequential preparative ultracentrifugations (27) in a type 40.3 rotor (Beckman Instrument Inc., Palo Alto, CA) into the following densities: very low density lipoprotein (VLDL), $d < 1.006$ g/ml; low density lipoprotein (LDL), $1.063 > d > 1.006$ g/ml; and high density lipoprotein (HDL), $d > 1.063$ g/ml. It should be noted that in rats the LDL fraction isolated by ultracentrifugation may have been contaminated with apoA- or apoE-containing lipoproteins.

Tissue lipid analysis

For tissue lipid analysis, lipids were extracted from liver, small intestine, and from intestinal microsomes by the method of Folch, Lees, and Sloane Stanley (28). An aliquot of the chloroform extract was evaporated to dryness, and the lipids were redissolved in isopropanol prior to measurement of triglycerides by enzymatic assay. Unesterified cholesterol and cholesteryl esters were separated by silica gel-60 TLC on plates (Merck, Darmstadt, FRG) developed in a petroleum ether–diethyl ether–acetic acid 70:30:0.5. To reveal the spots, plates were immersed for 1 sec in a reagent solution (50 mM $MnCl_2$, 6% H_2SO_4), then heated 15 min at 120°C. The spots of free cholesterol and cholesteryl ester corresponding to the appropriate standards were identified and then quantitated at 380 nm on a computer-controlled Camag TLC scanner II (Muttenz, Switzerland) (29, 30).

[4-^{14}C]cholesterol bolus administration

The third day after diabetes induction, rats fed a standard diet were given a bolus of 1 μCi of [4-^{14}C]cholesterol (sp act 842 mCi/mol) per rat by gastric intubation and killed 2 h later. Plasma was collected and lipoprotein fractions were separated as described under Plasma analysis (see above). The compound CL-277082, dissolved in 20% (w/v) Tween 80, was given intragastrically (50 mg/kg) 1 h before the [4-^{14}C]cholesterol pulse and insulin treatment

was performed as described under Treatments. Under these experimental conditions Tween 80 had no effect on the plasma levels of labeled cholesterol.

Preparation of intestinal microsomes

A 40-cm intestinal segment between 15 cm and 65 cm from the pylorus was used for the preparation. After an extensive wash with chilled saline, the segment was opened longitudinally and the mucosa was scraped off and gently suspended in sucrose solution (0.25 M sucrose, 1 mM EDTA, 0.01 M Tris-HCl, pH 7.4). The suspension was centrifuged at 800 *g* for 10 min and the resulting pellet was resuspended in 10 ml of 0.154 M phosphate buffer (pH 6.2) and homogenized in a motor driven Teflon-glass homogenizer at 4°C. The homogenate was centrifuged at 15000 *g* for 30 min to pellet the nuclei and mitochondria. The resulting supernatant was then centrifuged at 90,000 *g* for 35 min. The pellet (microsomal fraction) was resuspended in 0.154 M phosphate buffer (pH 6.2) for ACAT activity measurements. All centrifugations were performed at 4°C. Protein content of each sample was determined according to Lowry et al. (31).

ACAT assay

ACAT activity was measured essentially as described by Helgerud, Petersen, and Norum (32). The incubation mixture of 0.5 ml of 0.154 M phosphate buffer (pH 7.4) containing 1 mg/ml fatty acid-free BSA, 4 mM reduced gluthatione, and 1 mg protein/ml microsome suspension was preincubated at 37°C for 10 min. The reaction was then started by the addition of 20 nmol [1-^{14}C]oleoyl-CoA (sp act 6.5 mCi/mmol) and stopped 10 min later by the addition of 4 ml chloroform-methanol 2:1 (v/v). Lipids were extracted by the method of Folch et al. (28). The samples were applied on silica gel-60 TLC plates (Merck, Darmstadt, FRG) with 0.1 mg cholesteryl oleate as cold carrier. After development in petroleum ether-diethyl ether-acetic acid 70:30:0.5, the plates were exposed to iodine vapor and the band of cholesteryl oleate was located and scraped into scintillation vials. After the addition of 4 ml of a toluene-Triton X-100 solution, radioactivity was measured in a Beckman LS 7000 liquid scintillation counter (Beckman, Palo Alto, CA). Where mentioned, exogenous cholesterol was provided to the ACAT assay by the addition of liposomes. The liposome suspension had the following composition: phosphatidylcholine-phosphatidylserine-cholesterol 15:5:12 (w/w/w) and was obtained by sonication of the lipid mixture in 0.1 M KH_2PO_4, pH 7.4, with a Branson (Soest, Holland) sonifier for 30 min at 30 W power. Total lipid concentration was 6.1 mg/ml. Aliquots (0.1 ml) of the suspension were preincubated with the remaining components of the ACAT assay, as described above, prior to initiation of the reaction with [1-^{14}C]oleoyl-CoA.

Esterification rates were calculated as pmol of cholesteryl [1-^{14}C]oleate formed per mg microsomal protein per min.

Statistical analysis

Values are expressed as mean ± SEM. Significance of difference between mean values was determined by the *t* test for unpaired data. A value for $P < 0.05$ was considered as significant.

RESULTS

High-fat diet in 3-day diabetic rats

As can be seen in **Table 1,** plasma glucose levels were higher than 300 mg/dl ($P < 0.01$ vs. nondiabetics) 3 days after diabetes induction by streptozotocin. The differences in small intestine weights between diabetic and nondiabetic rats were not statistically significant, and liver weights of diabetic rats were slightly decreased.

Within 3 days of feeding a high-fat diet to normal rats, increased plasma cholesterol (+38%, $P < 0.02$), hypertriglyceridemia (+92%, $P < 0.01$), and a decrease in HDL-cholesterol (−22%, $P < 0.01$) were induced. Liver lipid levels were significantly elevated (**Table 2**).

Diabetic rats fed a standard chow had increased plasma

TABLE 1. Effect of a high-fat diet on plasma glucose, body weight, and organ weights in 3-day diabetic rats

Parameter	Nondiabetic Rats		Diabetic Rats	
	Chow Diet (n = 6)	High-Fat Diet (n = 6)	Chow Diet (n = 6)	High-Fat Diet (n = 5)
Plasma glucose (mg/dl)	183 ± 10	183 ± 8	327 ± 5[a]	332 ± 3[a]
Body weight (g)	224 ± 4	216 ± 6	193 ± 4[a]	193 ± 4[c]
Intestine				
Weight (g)	6.61 ± 0.28	7.03 ± 0.11	6.86 ± 0.21	6.68 ± 0.32
Weight/length ratio (mg/cm)	63.7 ± 2.5	65.6 ± 1.5	63.5 ± 1.7	64.2 ± 2.3
Liver weight (g)	9.63 ± 0.36	10.1 ± 0.46	7.90 ± 0.35[b]	8.50 ± 0.60

Values are the mean ± SEM. The high-fat diet contained 1% cholesterol plus 1% coconut oil.
[a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.02$ versus the appropriate nondiabetic control groups fed the same diet.

TABLE 2. Effect of a high-fat diet on plasma and liver lipid levels in 3-day diabetic rats

Parameter	Nondiabetic Rats		Diabetic Rats	
	Chow Diet (n = 6)	High-Fat Diet (n = 6)	Chow Diet (n = 6)	High-Fat Diet (n = 5)
		mg/dl		
Plasma				
Total cholesterol	55.9 ± 3.0	77.0 ± 5.5[b]	67.9 ± 4.1[e]	130.5 ± 15.5[g,j]
VLDL-cholesterol	10.3 ± 0.9	37.5 ± 4.4[d]	21.9 ± 4.2[g]	79.7 ± 14.4[f,j]
LDL-cholesterol	14.8 ± 1.9	15.6 ± 1.7	15.1 ± 2.1	25.7 ± 2.7[g,i]
HDL-cholesterol	30.8 ± 1.2	24.0 ± 1.0[e]	30.9 ± 2.2	25.2 ± 1.0
Triglyceride	109 ± 25	209 ± 14[e]	289 ± 51[d]	322 ± 59
		mg/g tissue		
Liver				
Free cholesterol	1.53 ± 0.04	2.33 ± 0.07[d]	1.57 ± 0.06	2.24 ± 0.09[k]
Cholesteryl ester	0.12 ± 0.02	3.14 ± 0.38[d]	0.33 ± 0.07[f]	7.50 ± 0.73[h,k]
Triglyceride	4.0 ± 0.3	6.7 ± 1.0[a]	6.2 ± 0.7[e]	12.9 ± 1.6[g,k]

Values are the mean ± SEM. The high-fat diet contained 1% cholesterol plus 1% coconut oil.
[a] $P < 0.05$; [b], $P < 0.02$; [e], $P < 0.01$; [d], $P < 0.001$ versus the nondiabetic chow-fed group.
[e], $P < 0.05$; [f], $P < 0.02$; [g], $P < 0.01$; [h], $P < 0.001$ versus the appropriate nondiabetic controls fed the same diet.
[i], $P < 0.02$; [j], $P < 0.01$; [k], $P < 0.001$ versus the diabetic chow-fed group.

triglycerides (+165%, $P < 0.02$), total plasma cholesterol (+22%, $P < 0.05$) and VLDL-cholesterol (+113%, $P < 0.05$) levels compared to normal rats fed the same diet. Liver cholesteryl ester and triglyceride concentrations were slightly higher (+175%, $P < 0.02$ and +55%, $P < 0.05$, respectively).

Despite a certain degree of variability, mainly resulting from the heterogeneity of the VLDL fraction, diabetic rats fed a high-fat diet were clearly hypercholesterolemic (131 ± 16 mg/dl) due to increased levels of VLDL (+113%, $P < 0.02$) and LDL-cholesterol (+65%, $P < 0.01$) versus the nondiabetic animals fed similarly. With the high-fat diet, the diabetic state was also associated with a rise in liver cholesteryl ester (+139%, $P < 0.001$) and triglycerides (+93%, $P < 0.01$) (Table 2).

Treatment with insulin of diabetic rats fed the high-fat diet

In this experiment, the streptozotocin-injected rats were divided into an untreated diabetic group and an insulin-treated diabetic group. All groups were fed the high-fat diet including a nondiabetic control group. As shown in **Table 3,** diabetic-control rats were hyperglycemic, whereas insulin-treated diabetic rats had nor-

TABLE 3. Effect of insulin treatment on 3-day diabetic rats fed a high-fat diet

Parameter	Nondiabetic (n = 6)	Diabetic-Control (n = 6)	Diabetic + Insulin (n = 5)
Body weight (g)	223 ± 4	207 ± 4[b]	218 ± 5
Liver weight (g)	10.4 ± 0.2	9.6 ± 0.3	10.8 ± 0.8
Intestine weight (g)	7.81 ± 0.51	7.34 ± 0.17	7.85 ± 0.13[e]
		mg/dl	
Plasma			
Glucose level	184 ± 5	458 ± 43[d]	187 ± 61[b,g]
Cholesterol level	69.2 ± 3.7	116.8 ± 9.8[e]	90.3 ± 2.7[e]
Triglyceride level	151 ± 19	223 ± 57	99 ± 37
		mg/g tissue	
Liver			
Free cholesterol level	2.07 ± 0.04	2.34 ± 0.09[a]	2.00 ± 0.08[f]
Cholesteryl ester level	4.17 ± 0.59	13.68 ± 1.51[d]	9.44 ± 0.85[c,e]
Intestine			
Free cholesterol level	1.49 ± 0.04	1.52 ± 0.02	1.57 ± 0.03
Cholesteryl ester level	0.246 ± 0.026	0.351 ± 0.026[b]	0.170 ± 0.013[a,h]

Values are the mean ± SEM. All groups were fed the high-fat diet (1% cholesterol plus 1% coconut oil).
[a] $P < 0.05$; [b], $P < 0.02$; [c], $P < 0.01$; [d], $P < 0.001$ versus the nondiabetic group.
[e], $P < 0.05$; [f], $P < 0.02$; [g], $P < 0.01$; [h], $P < 0.001$ versus the diabetic-control group.

mal plasma glucose levels. As observed in the previous experiment, diabetic-control rats had elevated plasma cholesterol compared to nondiabetic rats (+69%, $P < 0.01$) despite identical food intake (19 g/rat per day) and similar intestinal weights. In the liver, free cholesterol (+13%, $P < 0.05$) and more markedly cholesteryl ester (+228%, $P < 0.001$) levels were increased, whereas in the intestinal tissue, only the esterified form of cholesterol was significantly elevated (+43%, $P < 0.02$).

In the insulin-treated diabetic group, there was a significant decrease of plasma cholesterol concentrations compared to the untreated diabetic group (−23%, $P < 0.05$). Cholesteryl ester levels were reduced in the liver (−31%, $P < 0.05$) and in the intestine (−52%, $P < 0.001$), whereas a decrease of free cholesterol was observed only in the liver (−15%, $P < 0.02$).

Treatment of high-fat diet-fed diabetic rats with an ACAT inhibitor

Diabetic rats fed a high-fat diet and treated for 3 days with the ACAT inhibitor CL-277082 had neither modified body weights (208 ± 4 vs. 207 ± 3 g) nor plasma glucose concentrations (531 ± 22 vs. 541 ± 16 mg/dl) compared to the untreated diabetic rats. Liver and intestine weights were also similar in treated and untreated diabetic groups (8.70 ± 0.32 vs. 9.18 ± 0.35 g and 8.19 ± 0.29 vs. 8.02 ± 0.23 g, respectively).

As observed in the previous experiment (Table 2), diabetic rats fed a high-fat diet for 3 days displayed a marked increase in plasma cholesterol levels compared to normal rats (176 ± 30 vs. 74 ± 6 mg/dl, $P < 0.01$). Treatment with CL-277082 was effective in preventing this rise (63 ± 5 mg/dl, $P < 0.01$ vs. 176 ± 30 for the untreated diabetic group). **Fig. 1** shows the distribution of cholesterol in lipoprotein fractions. It can be seen that the treatment of diabetic rats with the ACAT inhibitor significantly reduced cholesterol levels in VLDL (20 ± 5 vs. 119 ± 26 mg/dl, $P < 0.01$) and LDL (16.6 ± 1.8 vs. 33.5 ± 3.9 mg/dl, $P < 0.01$) fractions when compared to diabetic control rats, whereas no significant change in HDL-cholesterol levels was observed. Plasma triglyceride levels also tended to be decreased by CL-277082 treatment (180 ± 46 vs. 347 ± 75 mg/dl, NS).

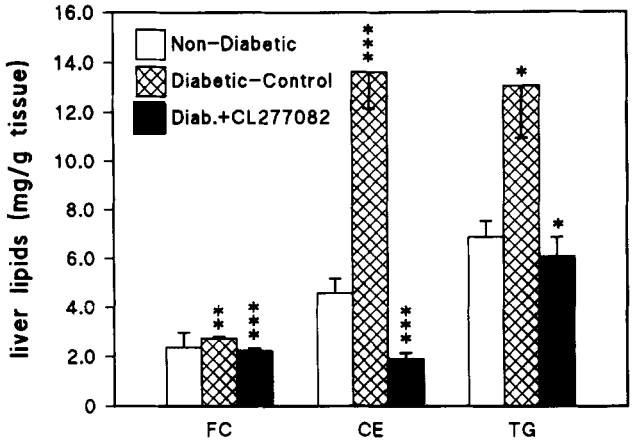

Fig. 2. Effect of the ACAT inhibitor CL-277082 on liver free cholesterol (FC), cholesteryl ester (CE), and triglyceride (TG) levels in 3-day diabetic rats fed a high-fat diet (1% cholesterol plus 1% coconut oil). Values are the mean ± SEM for six rats per group. Groups are compared as follows: Diabetic-Control versus Non-Diabetic, and Diabetic + CL-277082 versus Diabetic-Control; $*P < 0.02$, $**P < 0.01$, $***P < 0.001$.

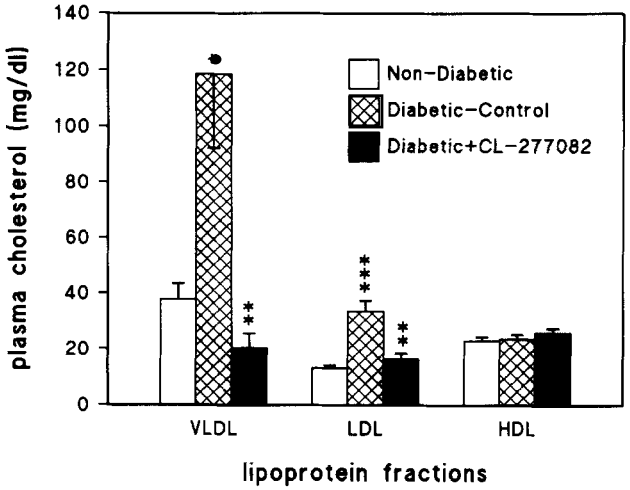

Fig. 1. Effect of the ACAT inhibitor CL-277082 on lipoprotein cholesterol levels in 3-day diabetic rats fed a high-fat diet (1% cholesterol plus 1% coconut oil). Values are the mean ± SEM for six rats per group. Groups are compared as follows: Diabetic-Control versus Non-Diabetic, and Diabetic + CL-277082 versus Diabetic-Controls; $*P < 0.02$, $**P < 0.01$, $***P < 0.001$.

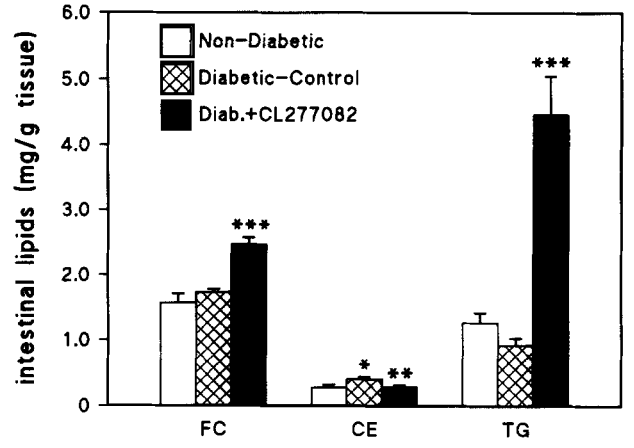

Fig. 3. Effect of the ACAT inhibitor CL-277082 on small intestine free cholesterol (FC), cholesteryl ester (CE), and triglyceride (TG) levels in 3-day diabetic rats fed a high-fat diet (1% cholesterol plus 1% coconut oil). Values are the mean ± SEM for six rats per group. Groups are compared as follows: Diabetic-Control versus Non-Diabetic, and Diabetic + CL-277082 versus Diabetic-Control; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

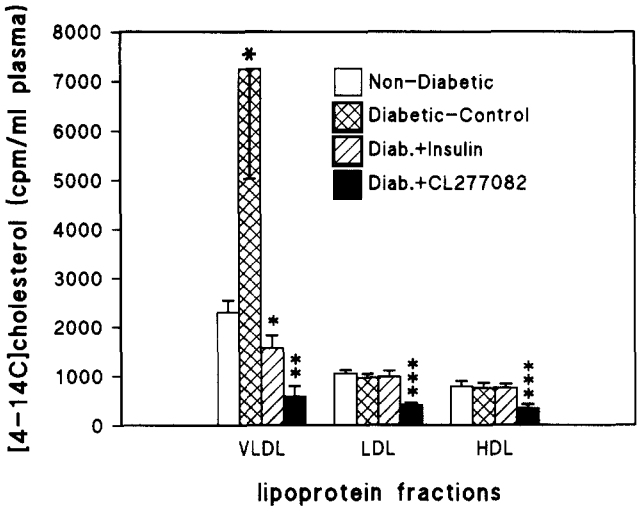

Fig. 4. Effect of treatment with insulin or with the ACAT inhibitor CL-277082 on [4-^{14}C]cholesterol content of lipoprotein fractions in 3-day diabetic rats. A bolus of 1 μCi of [4-^{14}C]cholesterol was given intragastrically 2 h before killing. Values are the mean ± SEM for six rats per group. Groups are compared as follows: Diabetic-Control versus Non-Diabetic and Diabetic-treated (insulin or CL-277082) versus Diabetic-Control; $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$.

As depicted in **Fig. 2,** the elevated liver free cholesterol, cholesteryl ester, and triglyceride levels in the diabetic rats were significantly reduced to the range measured in nondiabetic rats after treatment with CL-277082. In addition, cholesteryl ester levels were decreased below the value of nondiabetic rats (1.92 ± 0.23 vs. 4.58 ± 0.59 mg/g tissue, $P < 0.01$). **Fig. 3** shows the lipid levels in the small intestine. There was no significant change in free cholesterol and triglyceride concentrations associated with the 3 days of diabetes, but a 47% ($P < 0.05$) increase in cholesteryl ester levels was observed. The response to the ACAT inhibitor treatment, compared to the diabetic group, comprised: lowered levels of cholesteryl ester (−28%, $P < 0.01$) and marked rises of free cholesterol (+43%, $P < 0.001$) and triglyceride (+385%, $P < 0.001$) concentrations.

Similar results were obtained when this experimental protocol was repeated with another selective inhibitor of the ACAT enzyme, the Sandoz compound 58-035 (33), (data not shown).

Plasma [4-^{14}C]cholesterol levels after bolus administration

When 3-day diabetic rats were given a bolus of [4-^{14}C]cholesterol and killed 2 h later, there was a significant increase in VLDL -[4-^{14}C]cholesterol concentration compared to normal rats (7249 ± 2221 vs. 2304 ± 243 cpm/ml, $P < 0.05$). These values were reversed to normal or even subnormal levels in diabetic rats treated with insulin or CL-277082 (1568 ± 260, $P < 0.05$ and 591 ± 206, $P < 0.02$ cpm/ml respectively vs. diabetic-control rats, **Fig. 4**). In the liver, there were no significant changes of [4-^{14}C]cholesterol concentrations between the groups, except a slight decrease in the CL-277082-treated group (data not shown).

Time course of the effect of streptozotocin injection in rats

Table 4 shows the effect of diabetes induction on glycemia, plasma insulin, and glucagon levels in rats during the 3 days after streptozotocin injection compared to the buffer-injected animals. At day 1, glycemia was already elevated (+64%, $P < 0.001$) and plasma insulin levels were decreased (−44%, $P < 0.05$). On the third day, streptozotocin-injected rats were hyperglycemic (+77%, $P < 0.01$), hypoinsulinemic (−52%, $P < 0.05$), and displayed a slight reduction in body weight (−8%, $P < 0.01$). Plasma glucagon concentrations at day 1 and day 2 were not significantly changed but on day 3 a rise of +92% ($P < 0.05$) was observed.

Compared to control rats, intestinal ACAT activity of streptozotocin-injected rats was slightly decreased at day 1 (2.31 ± 0.08 vs. 2.60 ± 0.09 pmol/mg protein per min, $P < 0.05$), was not significantly changed at day 2 (4.59 ± 0.98 vs. 2.80 ± 0.09 pmol/mg protein per min,

TABLE 4. Effects of diabetes in rats during the first 3 days after streptozotocin injection

Parameter	Day 1		Day 2		Day 3	
	Nondiabetic	Diabetic	Nondiabetic	Diabetic	Nondiabetic	Diabetic
Body weight (g)	233 ± 2	227 ± 1	237 ± 1	214 ± 1[d]	228 ± 4	209 ± 3[c]
Liver weight (g)	11.1 ± 0.4	10.0 ± 0.5	10.2 ± 0.3	8.6 ± 0.4[b]	10.5 ± 1.2	8.9 ± 0.3[c]
Intestine weight (g)	8.2 ± 0.2	8.3 ± 0.6	7.4 ± 0.2	8.1 ± 0.4	7.7 ± 0.2	7.9 ± 0.2
Plasma glucose level (mg/dl)	190 ± 5	312 ± 6[d]	191 ± 3	309 ± 3[d]	179 ± 2	316 ± 3[d]
Plasma insulin level (μU/ml)	30.5 ± 5.0	17.0 ± 3.0[a]	27.0 ± 3.0	4.5 ± 0.4[d]	24.4 ± 3.1	11.7 ± 3.5[a]
Plasma glucagon level (pg/ml)	175 ± 40	191 ± 59	292 ± 28	363 ± 17	154 ± 32	297 ± 46[a]
Intestinal microsome: free cholesterol (μg/mg protein)	3.96 ± 3.6	29.2 ± 0.9[a]	28.3 ± 0.3	26.7 ± 2.3	38.6 ± 0.4	42.8 ± 0.8

Values are the mean ± SEM for four rats per group. All groups were fed the standard diet.
[a], $P < 0.05$; [b], $P < 0.02$; [c], $P < 0.01$; [d], $P < 0.001$ versus the nondiabetic group.

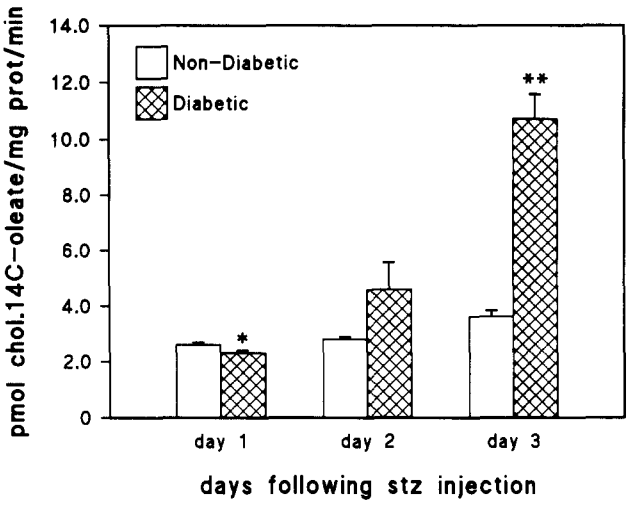

Fig. 5. Time course of the effect of diabetes induction with streptozotocin (80 mg/kg) on intestinal ACAT activity in rats. All groups were fed the standard diet. Values are the mean ± SEM for four rats per group; $^{*}P < 0.05$, $^{**}P < 0.001$.

NS) but was markedly increased at day 3 (10.72 ± 0.87 vs. 3.61 ± 0.23 pmol/mg protein per min, $P < 0.001$), as illustrated in **Fig. 5.** The intestinal microsome concentrations of free cholesterol were not consistently affected by diabetes induction, except for a slight decrease at day 1 (−26%, $P < 0.05$), see Table 4.

In order to ensure that the amount of microsomal cholesterol was not rate limiting, exogenous cholesterol was provided in the ACAT assay at day 3 by the addition of liposomes. As expected, ACAT activities were increased under these experimental conditions, but the difference between diabetic and nondiabetic rats remained in the same range (18.20 ± 3.82 vs. 4.91 ± 0.40 pmol/mg protein per min, respectively, $P < 0.01$).

Effect of insulin treatment

Insulin treatment of diabetic rats for 3 days maintained body and liver weights at normal values and corrected glycemia (93 ± 4 mg/dl) and glucagon concentrations (**Table 5**). Since rats are known to be relatively resistant to the hypoglycemic effect of exogenous insulin, the daily dose of insulin injected was large (10 U/day), resulting in high plasma insulin levels (126.4 ± 3.2 μU/ml). In order to ensure that the increase in plasma glucagon levels observed in diabetic animals (Tables 4 and 5) was consistent despite an inter-experiment spread, a third 3-day experiment was performed. The results of this experiment and those of Tables 4 and 5 gave the following mean values of plasma glucagon in pg/ml: 299 ± 30 for nondiabetic rats (n = 15) versus 464 ± 32 for diabetic rats (n = 16), $P < 0.001$.

As depicted in **Fig. 6,** intestinal ACAT activity, which was markedly elevated in the 3-day diabetic rats compared to normal rats (10.91 ± 1.90 vs. 2.28 ± 0.24 pmol/mg protein per min, $P < 0.01$) was significantly lowered by insulin treatment (2.19 ± 0.42 pmol/mg protein per min, $P < 0.01$).

DISCUSSION

In the present study we used short term diabetic rats, i.e., during the 3 days following streptozotocin injection, to demonstrate the role of the intestinal ACAT in the induction of the diabetes-linked hypercholesterolemia independently of intestinal hypertrophy or hyperphagia which are factors implicated in the hyperresponse of diabetic rats to cholesterol feeding (18–20).

Our results show that hyperphagia and intestinal hypertrophy can be excluded as triggering factors for the hyperresponsiveness of diabetic rats to cholesterol feeding. As early as 3 days after streptozotocin injection, a marked hypercholesterolemia was measured in diabetic rats fed a high-fat diet compared either to diabetic rats fed a standard diet or to normal rats fed the high-fat diet. These results are in contradiction with conclusions made from earlier work (17, 18), where hyperphagia was thought to underlie the hypercholesterolemia.

Diabetic rats fed a high-fat diet and treated simultaneously with insulin or with the ACAT inhibitor CL-277082

TABLE 5. Effect of insulin treatment on 3-day diabetic rats

Parameter	Nondiabetic	Diabetic-Control	Diabetic + Insulin
Body weight (g)	234 ± 3	208 ± 3[b]	232 ± 4[c]
Liver weight (g)	10.9 ± 0.3	8.7 ± 0.4[b]	11.9 ± 0.6[c]
Intestine weight (g)	8.25 ± 0.29	8.27 ± 0.15	9.02 ± 0.17
Plasma glucose level (mg/dl)	165 ± 1	540 ± 25[b]	93 ± 4[b,c]
Plasma insulin level (μU/ml)	38.0 ± 7.5	7.8 ± 1.3[a]	126.4 ± 3.2[b,c]
Plasma glucagon level (pg/ml)	330 ± 36	545 ± 28[b]	312 ± 8[c]
Intestinal microsome: free cholesterol (μg/mg protein)	49.0 ± 5.5	34.0 ± 5.0	46.7 ± 8.3

Values are the mean ± SEM for six rats per group. All groups were fed the standard diet.
[a], $P < 0.01$; [b], $P < 0.001$ versus the nondiabetic group.
[c], $P < 0.001$ versus the diabetic-control group.

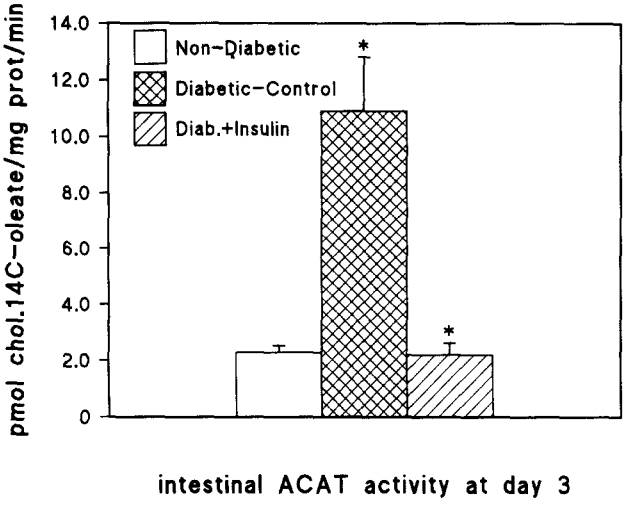

Fig. 6. Effect of insulin treatment on the intestinal ACAT activity in 3-day diabetic rats. All groups were fed the standard diet. Values are the mean ± SEM for six rats per group. Groups are compared as follows: Diabetic-Control versus Non-Diabetic and Diabetic + Insulin versus Diabetic-Control; $^*P < 0.01$.

revealed the role played by the intestinal ACAT in the hyperresponse of 3-day diabetic rats to a high-fat diet. The untreated diabetic rats were hypercholesterolemic and liver cholesteryl ester concentrations were markedly increased compared to nondiabetic animals fed the same diet. Insulin treatment significantly reduced the hypercholesterolemia resulting in plasma cholesterol levels comparable to those of nondiabetic rats. Tissue cholesterol levels were also significantly reduced. When treated with the ACAT inhibitor, diabetic rats fed the high-fat diet normalized their plasma cholesterol to levels comparable to those of nondiabetic rats fed the same diet, and liver cholesteryl ester concentrations were even below the value of normal rats.

As expected, the treatment with the ACAT inhibitor produced low levels of cholesteryl ester and elevated levels of free cholesterol in the small intestine tissue. Under these conditions, it is noteworthy that the triglyceride concentration was increased. This accumulation of intestinal triglycerides as well as the low level of plasma VLDL cholesterol suggest that the intestinal secretion of lipoproteins is impaired in the CL-277082-treated diabetic rats. This is in accordance with the observation reported previously by Kam et al. (34) who showed that inhibition of ACAT activity results in intracellular triglyceride accumulation and altered lipoprotein production by the human intestinal cell line CaCo-2. It can be hypothesized that cholesteryl ester, as a core component of the lipoprotein particle, is necessary for the assembly and secretion of lipoproteins by intestinal cells. Thus, the inhibition of ACAT activity in the intestinal tissue of diabetic rats might have produced low rates of cholesteryl ester formation, resulting in a reduced production of triglyceride-rich lipoproteins. As a consequence, CL-277082-treated diabetic rats had low plasma and liver lipid levels.

In order to ensure that the diabetes-linked hypercholesterolemia resulted from exogenous cholesterol uptake, we measured the VLDL-[4-^{14}C]cholesterol concentrations 2 h after an intragastric pulse in 3-day diabetic rats with or without insulin treatment. The marked increase observed in the untreated diabetic rats compared to normal rats and the low levels in the insulin-treated diabetic rats suggest a relationship between the administration of insulin and the appearance of exogenous cholesterol in plasma lipoproteins, which is in accordance with the observations of Colca et al. (16). The majority of labeled cholesterol was most likely in chylomicrons which are isolated in the same lipoprotein fraction as VLDL. It has been demonstrated that chylomicron cholesterol clearance is not significantly altered in diabetic rats (35, 36), which suggests that this increase in labeled cholesterol in the circulation is due to increased absorption rather than slowed clearance. The very low concentrations of VLDL-[4-^{14}C]cholesterol measured in diabetic rats treated with CL-277082 emphasize the role of ACAT in the diabetes-linked hyperabsorption.

The direct measurement of ACAT activity revealed an increase of 197% 3 days after diabetes induction. Thus, a critical factor for cholesterol absorption, intestinal ACAT activity, is enhanced very early in the course of diabetes suggesting that this enzyme plays a major role in the diabetes-linked hypercholesterolemia. This significant change was not observed on days 1 and 2 at which time the streptozotocin-injected rats were already hyperglycemic and hypoinsulinemic but not hyperglucagonemic.

The marked increase of intestinal ACAT activity observed in diabetic rats was completely prevented by insulin treatment, and other parameters such as body and liver weights, glycemia and plasma glucagon levels were also corrected by insulin treatment. The onset of the elevated intestinal ACAT activity in diabetic rats occurred simultaneously with the rise of plasma glucagon levels and was not directly correlated with hypoinsulinemia which was already observed at day 1. These results suggest that glucagon rather than insulin might be implicated in the diabetes-linked changes of ACAT activity or that there is a delay for the insulin deficiency to be manifest. The first hypothesis is supported by the findings of Müller et al. (37) and Unger et al. (38), who demonstrated that the severity of the metabolic derangement of clinical diabetes mellitus is more directly related to the hyperglucagonemia than to the decrease in plasma insulin levels. On the other hand, the observations of Jiao et al. (39) suggesting a direct relationship between the presence of insulin and the ACAT activity on the human intestinal cell line CaCo-2 are not consistent with our results in which hypoinsulinemia can be dissociated from the rise in ACAT activity.

It should be noted that an increase of intestinal ACAT activity in diabetic rats was not observed by Scott and Tomkin (40), probably due to the experimental conditions used by the authors and already criticized by Jiao et al. (23).

Taken together these results clearly indicate that the diabetic state is characterized by a rapid rise in intestinal ACAT activity leading to a higher rate of dietary cholesterol absorption and/or biliary cholesterol reabsorption. Therefore, the modulation of ACAT activity plays a major role in plasma cholesterol levels especially evident in diabetic rats fed a high cholesterol diet. However, in diabetic animals fed a low-cholesterol diet, the increase in intestinal cholesterol synthesis resulting from hyperphagia has been demonstrated to play a key role in the hypercholesterolemia of diabetes (41–43). Thus, in the established diabetic state, both a higher rate of intestinal cholesterol synthesis and of cholesterol absorption are probably present.

It is clearly established that animals fed a normal diet do not respond to ACAT inhibitors (44, 45). As expected from the animal data, a recent clinical study confirmed the lack of hypocholesterolemic activity of the ACAT inhibitor CL-277082 (46) on nondiabetic normocholesterolemic men. In diabetic patients a number of questions remain to be answered: are they more sensitive to dietary cholesterol? do they have a higher intestinal ACAT activity? As the alterations observed in diabetic rats are amenable to down-regulation by insulin treatment or by treatment with ACAT inhibitors, the potential beneficial effect of these agents should be investigated in humans.

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