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Effect of Clofibrate on Lipid Metabolism in Streptozotocin Diabetic Rats[1] (38624)

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Experimental diabetes induced in laboratory animals by alloxan or streptozotocin is accompanied by marked changes in lipid metabolism, notably by an elevation in serum triglycerides (1, 2) and a decrease in hepatic cholesterol synthesis (3, 4). In view of these changes, we considered it of interest to use the streptozotocin-diabetic rat to investigate further the mode(s) of action of the antihyperlipidemic drug clofibrate(ethyl *p*-chlorophenoxyisobutyrate; CPIB).

Materials and Methods. Treatment. Male albino Charles River rats, weighing 110–130 g, were kept under observation for 3 days and used only if body weight gain and food intake were normal. Animals had free access to Purina Laboratory Chow and water throughout the study. The rats were divided in four groups with 18 animals per group; two groups were injected intravenously with 80 mg/kg of streptozotocin (freshly prepared in 0.03 *M* citrate buffer, pH 4.5), while two groups were injected with buffer and served as controls. Seven days after the injection, one diabetic and one control group received by gastric intubation a daily dose of 242 mg/kg (1 mmole/kg) of clofibrate suspended in 2% Tween 80; the two other groups received Tween 80 only. As diabetic rats gained less weight than controls (Table I), and since the dose of clofibrate was based on body weight at the time of administration, the amount of clofibrate administered to diabetic rats was approximately 80% of that given to controls. All animals were decapitated 14 days after the injection of streptozotocin. On the day of killing, food was removed at 8:00 AM; 30 min later, blood was withdrawn from the tail vein, 0.2 ml was added to 1.8 ml physiological saline and the concentration of glucose was determined (5) within 2 hr. Although all streptozotocin-treated rats had elevated serum glucose, only rats with glucose levels at least 250 mg/dl above the average of controls were used for the estimations of cholesterol biosynthesis and free fatty acid release. Animals received the final dose of clofibrate at 9:30 AM and were decapitated at 12:00 noon.

Tissues. Livers and intestines were excised and a portion of the liver was immediately frozen on dry ice for lipid and glycogen determinations. Intestines and the remainder of the liver to be used for the cholesterol biosynthesis studies were immersed in ice-cold saline. Epididymal fat pads were immersed at room temperature in Krebs–Ringer bicarbonate buffer, pH 7.4.

Hepatic and intestinal cholesterol synthesis. Nine rats per group were used. The cholesterogenic activity in the intestines was determined according to Cayen and Dvornik (6). Small intestines (distal 20 cm) were flushed thoroughly with ice-cold Krebs–Ringer bicarbonate buffer (pH 7.4). Sections were prepared and incubated for 1 hr at 37° in buffer containing 10 nmole (0.4 μCi) [2-^{14}C]acetate and 7 nmole (0.2 μCi) [^{3}H]mevalonate (Amersham/Searle Corp., Arlington Heights, IL.). Enzymatic activity was terminated by the addition of KOH pellets. Ethanol, water, and carrier cholesterol (100 mg) were added, the suspension was heated at 75–80° for 1 hr, and the neutral lipids were extracted with *n*-hexane. Cholesterol was isolated, crystallized, and purified as its 5,6-dibromo derivative (7). The cholesterogenic activity in liver homogenates was determined as described by Cayen and Dvornik (8). The homogenates were incubated for 1 hr at 37° in 0.1 *M* potassium phosphate buffer (pH 7.4) con-

[1] Part XLII of a series entitled Agents Affecting Lipid Metabolism. A portion of this study has been reported in abstract form [Circulation (1972) **46**, Suppl. 2, 258].

TABLE I. EFFECT OF CLOFIBRATE (CPIB) ON WEIGHT GAIN, FOOD INTAKE, SERUM GLUCOSE, AND LIVER GLYCOGEN IN STREPTOZOTOCIN-DIABETIC RATS[a]

Group	Daily food intake (g/rat)	Weight gain (g/rat/14 days)	Serum glucose (mg/dl)	Liver glycogen (g/100 g)
Control	9.6	112	112	3.52
	±0.27	±3.0	±1.7	±0.19
CPIB[b]	8.9	100	120	2.74
	±0.12[c]	±3.8[c]	±3.0[c]	±0.19[d]
Diabetic	11.8	35	360	0.39
	±0.74[c]	±4.5[d]	±5.9[d]	±0.07[d]
Diabetic + CPIB[e]	11.8	48	324	0.32
	±0.67	±6.9	±17.4	±0.11

[a] Values are mean ± SE for 18 rats/group.
[b] 242 mg/kg/day p.o. for 7 days.
[c] $P < 0.05$.
[d] $P < 0.001$.
[e] None of the parameters was significantly different from those of diabetic rats.

taining 5 nmole (0.2 μCi) [2-^{14}C]acetate, 5 nmole (0.15 μCi) [^{3}H]mevalonate and the appropriate cofactors.

Analytical measurements. Liver samples were saponified in alcoholic KOH and, after addition of an equal volume of water, the mixture was extracted with hexane. An aliquot of the hexane extract was evaporated to dryness, the residue taken up in 2 ml of isopropanol and the cholesterol content determined by the method of Zlatkis *et al.* (9) as modified for the Autoanalyzer (method Np-24). Phospholipids and triglycerides were determined by the semiautomated methods of Kraml (10), and of Kraml and Cosyns (11), respectively. Serum lipoproteins were separated into fractions of low (LDL) and high density (HDL) with dextran sulfate as described by Walton and Scott (12). Since VLDL are precipitated with LDL by this procedure, reference to LDL in this report also includes VLDL. The cholesterol and phospholipid contents of the LDL and HDL fractions were measured directly. Unfractionated serum was used for triglyceride determination.

Liver glycogen was measured by the fluorimetric method of Beyer (13). Total nitrogen in liver homogenates and intestinal sections was determined by the Kjeldahl digestion procedure as adapted for the Autoanalyzer (14).

Free fatty acid release from adipose tissue. Free fatty acids (FFA) released from epididymal fat pads were determined by the semiautomated procedure of Kraml (15) based on the method of Duncombe (16) as modified by Itaya and Ui (17). Fat pads from six rats per group were weighed and individually incubated at 37° for 30 min. Contralateral fat pads from each rat were incubated in the presence of norepinephrine (final concentration, 1×10^{-5} *M*). After acidification, the color was developed in the chloroform extract by the addition of copper nitrate–triethanolamine and sodium diethyldithiocarbamate solution, and read at 440 nm.

Results. The diabetic state induced in rats with streptozotocin was characterized by increased food intake and elevated serum glucose, and by a decrease in body weight gain, liver weight, and liver glycogen. Treatment with clofibrate did not alter these changes (Table I).

Cholesterol and phospholipid levels in serum LDL fractions were markedly elevated in diabetic rats (Table II); slight increases were also observed in the HDL. CPIB decreased cholesterol in the LDL of both normal and diabetic rats; decreases in the HDL fraction were found only in normal rats. CPIB also diminished the pronounced hypertriglyceridemia in diabetic rats. Liver lipid concentrations were virtually unaltered (Table III). However, since diabetic rats had markedly decreased liver weight, the total liver content of cholesterol, phospholipids, and triglycerides was significantly lower.

As demonstrated in previous studies (18), the incorporation of acetate into cholesterol by liver homogenates from normal rats treated with clofibrate was inhibited by 90% (Table IV). A similar degree of suppression was observed in livers from diabetic rats; treatment of these rats with CPIB suppressed the remaining cholesterogenic activity below the limit of sensitivity. In diabetic rats, the rate of mevalonate conversion to cholesterol was also diminished. In contrast, intestinal cholesterol synthesis in diabetic rats remained unchanged; an in-

TABLE II. EFFECT OF CLOFIBRATE (CPIB) ON SERUM LIPID LEVELS IN STREPTOZOTOCIN-DIABETIC RATS[a]

Group	Cholesterol (mg/dl)			Phospholipid phosphorus (mg/dl)			Triglyceride glycerol (mg/dl)
	LDL	HDL	Total	LDL	HDL	Total	
Control	20.9 ±0.78	34.0 ±0.88	54.9 ±1.30	1.17 ±0.06	3.09 ±0.06	4.26 ±0.09	7.96 ±0.53
CPIB	13.5 ±0.87[b]	23.9 ±1.05[b]	37.4 ±1.46[b]	0.89 ±0.05[c]	2.43 ±0.07[b]	3.29 ±0.11[b]	3.25 ±0.15[b]
Diabetic	35.5 ±3.63[b]	37.7 ±1.51[d]	72.6 ±3.00[b]	2.76 ±0.23[b]	3.42 ±0.13[d]	6.18 ±0.32[b]	30.00 ±3.43[b]
Diabetic + CPIB[e]	22.6 ±2.78[c]	35.3 ±2.41	57.9 ±4.19[d]	1.91 ±0.22[d]	3.05 ±0.14	4.95 ±0.33[d]	16.50 ±2.87[c]

[a] Values are mean ± SE for 18 rats/group.
[b] $P < 0.001$.
[c] $P < 0.01$.
[d] $P < 0.05$.
[e] Significance level compared to diabetic rats.

TABLE III. EFFECT OF CLOFIBRATE (CPIB) ON LIVER LIPID LEVELS IN STREPTOZOTOCIN-DIABETIC RATS[a]

Group	Liver weight (g)	Liver lipid levels					
		Cholesterol		Phospholipid phosphorus		Triglyceride glycerol	
		mg/100 g	mg/liver	mg/100 g	mg/liver	mg/100 g	mg/liver
Control	10.2±0.22	220±2.7	22.4±1.6	141±2.8	14.4±3.31	83±2.1	8.47±0.18
CPIB	12.2±0.37[b]	203±5.3[b]	24.8±2.0	148±3.7	18.1±0.44[c]	92±3.0[d]	11.22±0.28[c]
Diabetic	6.8±0.20[c]	230±5.6	15.6±0.8[c]	141±3.4	9.6±0.28[c]	78±4.1	5.30±0.30[c]
Diabetic + CPIB[e]	8.0±0.38[d]	217±4.5	17.4±1.0	142±1.9	11.4±0.15[c]	77±5.2	6.16±0.36

[a] Values are mean ± SE for 18 rats/group.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] $P < 0.05$.
[e] Significance level compared to diabetic rats.

crease was noted on treatment with CPIB (Table IV).

Data on free fatty acid release from epididymal fat pads are presented in Table V. In diabetic rats, the weight of fat pads was greatly reduced; hence, FFA release per fat pad was decreased, but increased when expressed per unit weight of tissue. In all groups addition of norepinephrine to the contralateral fat pad resulted in increased FFA release. CPIB treatment had no effect on basal or norepinephrine-induced FFA generation in fat pads from normal or diabetic rats.

Discussion. The effects of experimentally induced diabetes on serum and liver lipids and on hepatic cholesterol synthesis have been previously investigated in rats. It has been reported that, in rats with diabetes induced by β-cytotoxic agents and fed a stock diet, the rate of hepatic cholesterol synthesis is decreased (3, 4). The decrease was ascribed to suppression of β-hydroxymethylglutaryl-CoA reductase (19). In accordance with that conclusion, in the present study cholesterol synthesis in streptozotocin-induced diabetic rats was suppressed at a step before the formation of mevalonate; an additional suppression was also noted after mevalonate. Unchanged intestinal cho-

TABLE IV. EFFECT OF CLOFIBRATE (CPIB) ON HEPATIC AND INTESTINAL CHOLESTEROL SYNTHESIS IN STREPTOZOTOCIN-DIABETIC RATS[a]

Group	Liver				Intestine
	[2-^{14}C]Acetate		[^{3}H]Mevalonate		[2-^{14}C]Acetate[b]
	dpm/mg N[c]	dpm/liver	dpm/mg N[c]	dpm/liver	dpm/mg N
Control	418 ± 90	76,800 ± 15,760	6,350 ± 822	1,184,000 ± 144,000	1,080 ± 180
CPIB	44 ± 2[d]	8,000 ± 400[d]	4,420 ± 316[e]	1,040,000 ± 96,000	1,700 ± 140[f]
Diabetic	33 ± 2[d]	4,000 ± 280[d]	1,740 ± 450[d]	220,000 ± 68,000[d]	920 ± 90
Diabetic + CPIB[h]	(0)[g]	(0)[g]	920 ± 265	140,000 ± 36,000	2,280 ± 190[d]

[a] Values are mean ± SE for nine rats/group.
[b] [^{3}H]Mevalonate data are not presented because of the limited incorporation of mevalonate into cholesterol by rat intestine (6).
[c] Dpm calculated as cholesterol/mg N in liver homogenate.
[d] $P < 0.001$.
[e] $P < 0.01$.
[f] $P < 0.05$.
[g] Values below limit of sensitivity, i.e., 15 dpm/mg N, or approximately 2000 dpm/liver.
[h] Significance level compared to diabetic rats.

lesterol synthesis in diabetic rats has been reported previously (20).

Treatment of diabetic rats with clofibrate virtually abolished the biosynthesis of liver cholesterol from acetate; the observed decrease in synthesis from mevalonate was not statistically significant. The finding of somewhat enhanced acetate incorporation into intestinal cholesterol of both normal and diabetic CPIB-treated rats was unexpected, since in earlier studies with clofibrate we found no effect on intestinal cholesterol synthesis in rats (21).

Regarding serum lipid levels in diabetic rats, cholesterol has been reported to be elevated in animals fed a diet high in cholesterol but unaffected in those fed stock diet (22–24). In contrast to the latter, our diabetic rats fed Purina chow had marked increase in serum cholesterol, mainly in the LDL fraction. This was associated with a comparable increase in phospholipid levels. Although the cholesterol concentration of liver was unchanged in diabetic rats, total liver cholesterol was markedly lower due to decreased liver weight (Table III). Therefore, the increase in serum cholesterol coupled with the decrease in hepatic cholesterol synthesis found in diabetic rats can be explained by a redistribution of cholesterol from liver to blood and by impaired catabolism and removal of cholesterol from the body (24).

TABLE V. EFFECT OF CLOFIBRATE (CPIB) ON FREE FATTY ACID (FFA) RELEASE BY EPIDIDYMAL FAT PADS FROM STREPTOZOTOCIN-DIABETIC RATS[a]

Group	Fat pad weight (mg)	FFA released (μmole/g)	
		Basal	Norepinephrine ($1 \times 10^{-5}M$)
Control	501±32	1.22±0.19	3.08±0.19
CPIB	400±22	1.15±0.12	4.51±0.22
Diabetic	50±6[b]	2.88±0.34[c]	9.00±1.82[d]
Diabetic + CPIB[e]	52±8	3.37±0.38	10.20±1.66

[a] Values are mean ± SE for six samples/group.
[b] $P < 0.001$.
[c] $P < 0.01$.
[d] $P < 0.05$.
[e] None of the parameters was significantly different from those of diabetic rats.

The hypertriglyceridemia observed in diabetic rats in the present study is in accordance with previous reports (1, 2). As found with cholesterol, liver triglyceride concentration was unchanged, but total hepatic triglyceride levels were lower in diabetic rats.

The clofibrate-induced reduction of serum

lipid levels in normal rats (Table II) was similar to that reported earlier (25). In diabetic rats, oral administration of clofibrate lowered the elevated serum lipids; the extent of this antihyperlipidemic effect was comparable to the hypolipidemic effect produced by clofibrate in normal rats. The opposite effects of diabetes and clofibrate on serum triglycerides in rats may be due to opposite effects on α-glycerophosphate dehydrogenase (α-GPD). This suggestion is based on reports that experimental diabetes increases hepatic triglyceride synthesis by decreasing the activity of α-GPD (4), while clofibrate increases α-GPD activity thus reducing the rate of triglyceride biosynthesis (26). Furthermore, since the activity of lipoprotein lipase is decreased in diabetic rats (27) but increased after clofibrate treatment (28), these opposing effects on the removal of triglycerides from serum may provide an additional contribution to the antihypertriglyceridemic activity of clofibrate in diabetic rats.

An interesting finding was the clofibrate-induced decrease of serum cholesterol levels in diabetic rats whose rate of hepatic cholesterol synthesis was already suppressed to less than 1/10 of that found in normal rats. Thus, the antihypercholesterolemic effect of clofibrate in diabetic rats cannot be ascribed to suppression of cholesterol synthesis. This finding suggests that the hypocholesterolemic activity of clofibrate in normal rats is not due to inhibition of hepatic cholesterol synthesis.[2]

Our investigation of FFA release in fat pads from diabetic rats was prompted by the finding of increased levels of circulating fatty acids in streptozotocin-diabetic rats (1). On a weight basis, the capacity to generate FFA was increased in fat pads from diabetic rats; this activity was unaffected by treatment with clofibrate. The finding suggests that the antihypertriglyceridemic activity of clofibrate in diabetic rats is not due to an effect on the FFA release from adipose tissue.

Summary. The effect of clofibrate (CPIB) on lipid metabolism was studied in male rats rendered diabetic by intravenous injection of 80 mg/kg of streptozotocin. After 1 wk, the rats received by gastric intubation 242 mg/kg/day of CPIB for 7 days. Liver lipid concentration remained unchanged in experimental diabetes and after treatment with CPIB; however, due to decreased liver weight, total liver lipids were lower in diabetic rats. Elevation of cholesterol, phospholipids, and triglycerides in the serum of diabetic rats was reversed by CPIB treatment. Hepatic cholesterol synthesis in diabetic rats was suppressed to approximately 1/10 of that in normal rats. Treatment with CPIB abolished this residual cholesterogenic activity. Diabetes had no effect on intestinal cholesterol synthesis; a slight increase was noted after CPIB treatment. Basal and norepinephrine-induced lipolysis in fat pads was elevated in diabetic rats; CPIB had no effect on these changes.

The data show that the elevated serum lipids in diabetic rats are lowered by treatment with CPIB. It was concluded that the hypocholesterolemic activity of clofibrate in rats is not caused by its suppression of hepatic cholesterol synthesis.

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[2] Unpublished observations from our laboratory lend support to this conclusion. In cholesterol-fed rats treated with clofibrate, the elevated liver cholesterol levels remained unchanged, while serum cholesterol was reduced by 30%. Since hepatic cholesterol synthesis was already suppressed by cholesterol feeding, any further suppression of biosynthesis by clofibrate would be insignificant. Therefore, the fall in serum cholesterol levels induced by clofibrate could not have been caused by such minimal additional suppression of hepatic cholesterol synthesis.

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