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Effect of clofibrate (ethyl-chlorophenoxyisobutyrate) feeding on glycolytic and lipogenic enzymes and hepatic glycogen synthesis in the rat*

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CLOFIBRATE (ethyl-chlorophenoxyisobutyrate) decreases the plasma triglyceride and cholesterol concentrations in some patients with hyperlipidemia.[1–4] This effect is predominantly on the plasma triglycerides, but the mechanism of this action is understood poorly. Since the plasma triglyceride concentration must be dependent, in part, on the rate of hepatic fatty acid synthesis,[5] clofibrate could decrease the plasma triglyceride concentration by decreasing the activity of liver enzymes which are involved in the conversion of carbohydrates to fatty acids. The activity of both the lipogenic and glycolytic enzymes are important determinants of the rate of hepatic fatty acid synthesis.[6–8] In the rat, clofibrate also decreases the hepatic glycogen concentration.[9] In the present experiments we report data on the effect of clofibrate on the activity of several glycolytic and lipogenic enzymes and on glycogen synthesis in the rat liver.

METHODS

In the studies on glycolytic and lipogenic enzymes, male Holtzman rats weighing 350–500 g were pair-fed diets containing per 100 g: 70 g fructose, 15·5 g casein, 7 g corn oil, 5 g vitamin mixture (Nutritional Biochemical Corp.) and 2·5 g salt mixture (Nutritional Biochemical Corp. No. XIV). In the glycogen synthesis experiments, the diet contained per 100 g: 70 g fructose or glucose, 20 g casein, 4 g corn oil, 3 g vitamin mixture (NBC), 2·5 g salt mixture (NBC XIV), and cystine, 0·3 g. Clofibrate, when added, was present at a level of 1·0 g per 100 g of diet.

In the enzyme studies, all animals had a net loss of a small amount of weight on this diet, but the weight losses were nearly the same in the control and clofibrate-fed rats. Animals were fed these diets for a period of 23–26 days. Two studies were done in the glycogen synthesis experiments. The animals in experiment I were fed *ad lib.*, and in experiment II were pair-fed. The animals were housed in individual cages and had free access to water. Body weight and food consumption were determined weekly in experiment I and daily in experiment II.

The rats were killed by decapitation and the livers were quickly removed. In some animals the entire liver was removed in order to determine the ratio of total liver weight to body weight. In all

* Send requests for reprints to: Robert H. Herman, Colonel, MC, Metabolic Division, U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colo. 80240.

animals a portion of liver was homogenized in 0·15 M KCl, 5 mM EDTA, and 5 mM $MgCl_2$, pH 7·0. Cell-free supernatant was obtained by centrifuging the whole homogenate at 105,000 *g* for 1 hr in a Spinco model L-2 preparative ultracentrifuge. The following enzyme assay methods were used: hexokinase (ATP: D-glucose-6-phosphotransferase; EC 2.7.1.1),[10] fructose-1,6-P aldolase (ketose-1,6-P D-glyceraldehyde-3-P lyase; EC 4.1.2.13),[11] glucose-6-P dehydrogenase (D-glucose-6-P: NADP oxidoreductase; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-P-D-gluconate: NADP oxidoreductase decarboxylating; EC 1.1.1.44),[12, 13] malate dehydrogenase (L-malate: NADP oxidoreductase; EC 1.1.1.40),[14] citrate cleavage enzyme (ATP: citrate oxalacetate-lyase; EC 4.1.3.8),[15] and acetyl-CoA-carboxylase [acetyl-CoA: carbon dioxide ligase (ADP); EC 6.4.1.2].[16] Glucose 6-phosphate dehydrogenase activity was corrected for 6-phosphogluconate dehydrogenase activity present in the same homogenate. Protein was determined by the method of Lowry *et al.*[17]

Liver slices for the studies *in vitro* were prepared from the left lateral lobe with a Stadie–Riggs microtome. All incubations were in calcium-free Krebs–Ringer bicarbonate buffer,[18] pH 7·4, containing the appropriate substrates at a concentration of 5 mM. The procedures used for incubation, isolation and counting of radioactive products have been described previously.[19] Liver glycogen was determined by the anthrone method.[20]

RESULTS

Glycolytic and lipogenic enzymes. Although the food intake and changes in total body weight for the control and clofibrate-treated rats were nearly the same, the ratio of liver weight to whole body weight was greater in the clofibrate-treated than in the control animals (Table 1). This result has been reported by other workers using groups of animals fed *ad lib.*[4] The data in Table 1 also show that the amount of 105,000 *g* supernatant protein per gram of liver was the same in both groups.

The enzyme data in Table 2 are presented as millimicromoles of substrate metabolized per min per milligram of protein. Since the clofibrate-fed animals had a greater liver weight in relation to body weight than did the controls (Table 1), clofibrate could decrease the specific activity of any enzyme to 66 per cent of the control value without lowering the total activity per 100 g of body weight.

TABLE 1. EFFECT OF CLOFIBRATE FEEDING ON LIVER WEIGHT AND 105,000 *g* SUPERNATANT PROTEIN CONTENT

Experimental group	Liver wt. (g/100 g body wt.)	105,000 *g* Protein (mg/g liver)
Control	3·27 ± 0·09 (7)*	63·8 ± 4·24 (7)
Clofibrate-fed	4·99 ± 0·11 (8)	62·1 ± 0·62 (8)

* Mean ± S.E. Figure in parentheses indicates number of livers tested.

TABLE 2. EFFECT OF CLOFIBRATE FEEDING ON THE ACTIVITY OF HEPATIC GLYCOLYTIC AND LIPOGENIC ENZYMES*

Enzyme	Controls	Clofibrate-fed
Hexokinase	1·22 ± 0·17 (10)†	1·19 ± 0·20 (11)‡
Fructose-1,6-P aldolase	69·8 ± 3·8 (11)	22·0 ± 2·6 (11)
Glucose-6-P dehydrogenase	12·6 ± 2·6 (7)	0·98 ± 0·38 (8)
6-Phosphogluconic dehydrogenase	7·1 ± 0·81 (7)	5·83 ± 0·51 (8)‡
NADP-malate dehydrogenase	78·0 ± 10·9 (7)	133·0 ± 16·7 (6)
Citrate cleavage enzyme	12·58 ± 1·92 (7)	8·40 ± 1·28 (8)‡
Acetyl-CoA-carboxylase	9·53 ± 0·47 (7)	3·27 ± 0·38 (8)

* Activities are mμmoles substrate metabolized/min/mg protein.
† Mean ± S.E. Figure in parentheses indicates number of livers tested.
‡ P value for control vs. clofibrate-fed is not significant.

The specific activity of hexokinase was not changed by clofibrate feeding. In contrast, the activity of fructose-1,6-diphosphate aldolase was depressed in the clofibrate-treated rats. The activity of glucose-6-P dehydrogenase fell in the clofibrate-treated animals as compared to the controls, whereas the activity of 6-phosphogluconate dehydrogenase per 100 g of body weight was actually slightly greater in the clofibrate-treated animals. Clofibrate feeding enhanced the activity of NADP malate dehydrogenase. The activity of the citrate cleavage enzyme was lower after clofibrate feeding, but this difference was not statistically significant. Acetyl-CoA-carboxylase activity was decreased to $\frac{1}{3}$ of control activity by clofibrate feeding. Addition of liver homogenate from clofibrate-treated rats to the assay medium of controls did not decrease the activity of glucose-6-P dehydrogenase or acetyl-CoA-carboxylase (Table 3).

TABLE 3. EFFECT OF LIVER HOMOGENATE (105,000 *g* SUPERNATANT) FROM CLOFIBRATE-TREATED RATS ON CONTROL LIVER GLUCOSE-6-*P* DEHYDROGENASE AND ACETYL-CoA-CARBOXYLASE ACTIVITIES

Enzyme	% Predicted activity control plus clofibrate homogenate
Glucose-6-P dehydrogenase	98·5 ± 4·4 (4)*
Acetyl-CoA-carboxylase	95·0 ± 10·1 (5)

* Mean ± S.E. Figure in parentheses indicates number of assays.

TABLE 4. EFFECT OF CLOFIBRATE TREATMENT ON THE HEPATIC GLYCOGEN CONCENTRATION IN RATS FED *AD LIB.*

Diet*	Clofibrate	Initial body wt. (g)	Final body wt. (g)	Hepatic glycogen (g/100 g liver wet wt.)
Fructose	−	405 ± 8·0†	394 ± 7·3	8·9 ± 0·5
Fructose	+	391 ± 2·8	337 ± 6·1‡	4·0 ± 0·5§
Glucose	−	403 ± 5·7	397 ± 5·7	6·2 ± 0·2
Glucose	+	403 ± 8·2	354 ± 11·8‡	2·3 ± 0·3§

* Rats were fed the experimental diets for 11 days.
† Mean ± S.E. for six rats.
‡ $P < 0·01$ vs. control.
§ $P < 0·001$ vs. control.

Glycogen synthesis. Table 4 shows that the animals fed fructose or glucose plus clofibrate lost more weight than those fed the sugar alone. In addition, the clofibrate groups had significantly lower hepatic glycogen concentrations than did the controls. These differences could result in part from different food intakes by the animals in the different experimental groups: 16·04 and 19·6 g per day per rat consumed by fructose- and glucose-fed groups as compared to 12·7 and 15·1 g per day per rat by the groups fed fructose and glucose plus clofibrate respectively. In order to investigate this possibility, the control animals were pair-fed with the clofibrate-treated group in a separate experiment. When this was done, the control and clofibrate-treated groups lost a similar amount of weight and had nearly identical liver glycogen concentrations (Table 5). The glycogen content of the pair-fed fructose control rats (Table 5) was essentially the same as the value in the fructose plus clofibrate-treated group in the non-pair-fed experiment (Table 4). This also suggests that the reduced food intake associated with clofibrate administration is responsible for the lowered hepatic glycogen concentration in rats fed *ad libitum*.

In the non-pair-fed experiment (experiment I), clofibrate decreased the conversion of [$^{14}C_6$]-glucose and [$^{14}C_6$]-fructose to glycogen in liver slices (Table 6). The difference between the conversion of

TABLE 5. EFFECT OF CLOFIBRATE TREATMENT ON THE HEPATIC GLYCOGEN CONCENTRATION OF PAIR-FED RATS

Diet*	Clofibrate	Initial body wt. (g)	Final body wt. (g)	Hepatic glycogen (g/100 g liver wet wt.)
Fructose	—	398 ± 8·3†	381 ± 7·6	3·8 ± 0·6
Fructose	+	434 ± 6·0‡	409 ± 12·0	4·4 ± 0·4

* Rats were fed the experimental diets for 22 days.
† Mean ± S.E. for seven rats.
‡ Mean ± S.E. for eight rats.

TABLE 6. EFFECT OF CLOFIBRATE ON THE CONVERSION OF [$^{14}C_6$]-GLUCOSE (5mM) AND [$^{14}C_6$]-FRUCTOSE (5 mM) TO GLYCOGEN AND CO_2 IN RAT LIVER SLICES*

			Glycogen		CO_2	
Rats	Diet	Clofibrate	[$^{14}C_6$]-glucose	[$^{14}C_6$]-fructose	[$^{14}C_6$]-glucose	[$^{14}C_6$]-fructose
Fed *ad lib.*	Fructose	—	13·0 ± 3·6	40·8 ± 15·4	89·3 ± 6·0	432·5 ± 28·0
	Fructose	+	1·8 ± 0·1†	2·8 ± 0·3‡	104·2 ± 8·1	419·4 ± 17·2
	Glucose	—	7·7 ± 1·8	21·7 ± 5·5	169·3 ± 13·4	459·1 ± 33·3
	Glucose	+	3·8 ± 0·6	1·8 ± 0·3§	194·6 ± 13·7	527·3 ± 13·9
Pair-fed	Fructose	—	10·9 ± 4·4	26·9 ± 5·3	111·4 ± 5·9	567·2 ± 32·0
	Fructose	+	2·7 ± 0·2‖	4·4 ± 0·3‖	106·9 ± 8·0	509·1 ± 33·3

* Results are expressed as mμmoles substrate recovered as indicated metabolite/100 mg slices/3 hr of incubation. Mean ± S.E. for at least six rats.
† $P < 0·025$ vs. control.
‡ $P < 0·05$ vs. control.
§ $P < 0·005$ vs. control.
‖ $P < 0·001$ vs. control.

[$^{14}C_6$]-fructose and [$^{14}C_6$]-glucose to glycogen was less in the liver slices from the clofibrate-treated than in those from the control rats. In contrast to the effect of pair feeding on body weight and hepatic glycogen concentration, clofibrate decreased glycogen synthesis from [$^{14}C_6$]-glucose and [$^{14}C_6$]-fructose in the liver slices from the pair-fed rats (experiment II, Table 5). Clofibrate had no effect on $^{14}CO_2$ production in any experiment (Table 6).

DISCUSSION

The present studies demonstrate that clofibrate decreases the activity of several enzymes in rat liver. This effect seems to be a specific one, since fructose-1,6-diphosphate aldolase, glucose-6-*P* dehydrogenase, and acetyl-CoA-carboxylase activities were decreased whereas hexokinase, 6-phosphogluconate dehydrogenase, and citrate cleavage enzyme activities were not. The fact that the NADP-malate dehydrogenase activity was higher in the clofibrate-treated animals than in the controls indicates that clofibrate may specifically increase the activities of certain enzymes while it decreases the activity of others.

Fasting and high carbohydrate diets lead to parallel changes in the activity of glucose-6-*P* dehydrogenase, NADP-malic enzyme and citrate cleavage enzyme.[21] The addition of clofibrate to the diet, however, leads to non-parallel changes in the activity of these enzymes. Since our clofibrate studies were carried out in animals fed large amounts of fructose, it is possible that changes in the activities of these enzymes would be smaller if animals were fed diets containing different amounts and types of carbohydrates.

It is not possible at this time to state the relationship of the effect of clofibrate on hepatic enzyme activity to the plasma lipid lowering action of this drug. Clofibrate seems to increase hepatic fatty acid synthesis from [^{14}C]-acetate,[22] though the present studies show that the drug decreases acetyl-CoA-carboxylase activity.

One postulated mechanism of clofibrate action is that the drug displaces the protein binding of thyroid hormone, thereby leading to an increased tissue effect of thyroid hormone.[9] However, the effect of clofibrate on the hepatic activity of glucose-6-*P* dehydrogenase and 6-phosphogluconate dehydrogenase (Table 2) is different from that of thyroid hormone administration.[23]

Although clofibrate itself may have no direct effect on the hepatic glycogen concentration, the administration of this drug decreased glycogen synthesis from [$^{14}C_6$]-glucose and [$^{14}C_6$]-fructose in rat liver slices. Over relatively long periods of time there may be counterbalancing regulatory mechanisms for maintaining a constant hepatic glycogen concentration despite variations in the rate of glycogen synthesis. The data in the pair-fed, clofibrate-treated animals suggest that such a mechanism must exist in the intact rat.

We can only speculate as to the mechanism of the clofibrate effect on glycogen synthesis. Clofibrate decreases hepatic cholesterol synthesis[22] and we have now demonstrated that clofibrate decreases the activity of a variety of hepatic enzymes in the rat. Thus, it is possible that clofibrate decreases the activity of glycogen synthetase or the enzymes involved in its activation. Most important, these experiments indicate that clofibrate may exert its therapeutic effects because of its ability to alter enzyme activities in a specific manner.

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Metabolic Division,
U. S. Army Medical Research and Nutrition Laboratory,
Fitzsimons General Hospital,
Denver, Colo. 80240, *U.S.A.*

DAVID ZAKIM*
RONALD S. PARADINI†
ROBERT H. HERMAN

* Present address: Metabolic Unit, Department of Medicine, Veterans Administration Hospital, San Francisco, Calif. 94121.
† Present address: Department of Chemistry, University of Nevada, Reno, Nev.

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The anti-diuretic action of L-azaserine, as compared with ω-diazoacetophenone

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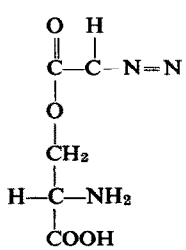
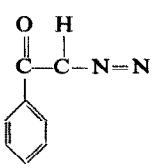
WHEN mice were injected i.p. with the anti-mitotic, *O*-diazoacetyl-L-serine, (azaserine),[1] it was observed that they drank less water than a control group of animals, maintained under the same conditions, and injected with an equivalent volume of saline.

The anti-neoplastic action of azaserine is well known,[2] and it has been used clinically against several types of tumours.[3, 4] Levenberg *et al.*,[5] showed that L-azaserine blocked *de-novo* purine biosynthesis, by irreversibly combining with the enzyme, α-*N* Formyl-glycinamide ribotide amidotransferase (EC 6.3.5.3), in the absence of L-glutamine. We can find no reports of the action of L-azaserine, or diazo compounds in general, on water balance in experimental animals. In an attempt to determine whether the effect on water balance was due to the active diazo group of L-azaserine, or to the molecule as a whole, we have compared the action of L-azaserine with that of another diazo compound, ω-diazoacetophenone[6] (Fig. 1).



L-azaserine



ω-diazoacetophenone

FIG. 1. A comparison of the structure of L-azaserine and ω-diazoacetophenone.

The diuretic activity of groups of twenty male, Hornes mice of body weight 27–32 g, was investigated by the method of Heller and Blackmore.[7] The animals were given three water loads, by stomach tube, 1·0 ml/20 g body weight, at hourly intervals. Immediately after the last water load, the test group of mice was given L-azaserine, 1·0 mg/kg, in a volume of normal saline of 0·2 ml/30 g body weight, by the i.p. route. Controls were injected with an equivalent volume of saline. The ω-diazoacetophenone was given at the same dose, in the same manner.

The urine output of the animals was recorded, after the last water load, at 10-min intervals, for 70 or 80 min.

The results are shown in Figs. 2 and 3, they suggest that at the dose and both L-azaserine, and ω-diazoacetophenone cause a fall in urine production.