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HEPATIC TRIGLYCERIDE LIPASES—EFFECT OF CLOFIBRATE AND HALOFENATE

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Abstract—Liver lipases could be important for regulating hepatic intracellular triglyceride (TG) concentration and, thus, TG secretion. The possibility that the hypotriglyceridemic drugs clofibrate (CPIB) and halofenate (HFA) reduce hepatic net TG synthesis by stimulating hepatic lipases has been investigated in control and orotic acid fed rats. Liver lipase activities were measured at pH 5, 7.5 and 8.5 in liver homogenates from control and drug-treated animals. The acid lipase was particularly sensitive to changes in hepatic TG concentration, increasing when hepatic TG levels were increased by feeding orotic acid and decreasing after treatment with either HFA of CPIB, drugs which lower hepatic TG levels. Both CPIB and HFA prevented the fatty liver produced by orotic acid. Neither drug increased the activity of the hepatic lipases with triolein as substrate (at an enzyme-saturating concentration) and, thus, the abilities of HFA and CPIB to decrease hepatic net TG synthesis and to prevent orotic acid-induced fatty liver are not related to effects on these liver lipases. Rather, they may be related to reducing the availability of fatty acids for *de novo* TG synthesis. CPIB and HFA produced a 65–75 per cent decrease in plasma free fatty acid (FFA) concentrations and this decrease could be important since circulating free fatty acids are a principal source of the fatty acid needed for hepatic TG synthesis.

Clofibrate (CPIB) and the newer hypotriglyceridemic drug halofenate (HFA) appear to lower plasma triglyceride levels in the rat primarily by decreasing hepatic net triglyceride (TG) synthesis [1, 2]. However, the mechanism of this decrease is yet unclear. A demonstrated ability of both CPIB and HFA to inhibit hepatocyte *de novo* fatty acid synthesis [3] and of CPIB to decrease plasma free fatty acid (FFA) levels [4, 5] could contribute to the observed inhibition of hepatic net TG formation by decreasing the availability of fatty acid substrate. Another possible locus of action, one not yet investigated, is hepatic lipases. Three distinct hepatic lipases have been identified which hydrolyze long-chained fatty acid TG: a lysosomal and a microsomal enzyme of pH optimum 5 and 8.5, respectively, and a heparin-sensitive lipase maximal at pH 7.5 which is localized in the plasma membrane [6]. Goldstein *et al.* [7] recently demonstrated that lysosomal acid lipase is centrally involved in regulating *de novo* cholesterol synthesis of fibroblasts by controlling the intracellular concentration of free cholesterol derived from the cholesterol ester of plasma low density lipoproteins. Thus, it is conceivable that hepatic TG lipase might also be important in the regulation of intracellular TG concentrations and, therefore, TG synthesis and secretion. Drug-induced increases in the activity of these hepatic enzymes could result in an apparent reduction in hepatic net TG synthesis. In the present study, the effect of treating the rat with CPIB or HFA on hepatic TG lipase activities in both normal and orotic acid fed rats was measured; CPIB is reported to prevent orotic acid-induced fatty liver [8]. In addition, the effect of HFA on plasma FFA levels was measured and compared to that produced by CPIB.

MATERIALS AND METHODS

Hepatic lipase measurements. Male Wistar rats (140–160 g) were maintained for 7 days on a fat free diet [9]. Beginning on day 7 and continuing for 14 additional days, the animals received the fat free diet containing either no drug (controls) or 0.10% HFA or 0.25% CPIB. Fat free diets were used in order to investigate endogenous TG metabolism without the complications caused by exogenous fat intake or fasting animals. On day 7, rats were lightly anesthetized with diethylether and 1-ml blood samples were collected by cardiac puncture. On day 21, the rats were again anesthetized, sacrificed by decapitation, and blood samples collected. The livers were quickly removed, chilled, and homogenized in 0.25 M sucrose as described by De Duve *et al.* [10]. Ten per cent liver homogenates were incubated for 1 hr at 37° in sodium cacodylate buffers of pH 5, 7.5 and 7.5 plus 25 i.u. heparin/ml and in Tris–HCl buffer of pH 8.5. The pH levels of the homogenate mixtures after incubation were, respectively, 5.2 to 5.3, 7.0 to 7.2, and 8.0 Incubations were conducted in the absence or presence of purified triolein (100 mM) exactly as described by Guder *et al.* [6] except that the incubated systems also contained 2% fatty acid-poor bovine serum albumin. This was added to maintain a low FFA/albumin molar ratio during the incubation. Aliquots of the suspensions were taken before and after incubation for measurement of FFA concentrations by the automated titration of Lorch and Gey [11]. Lipase activities were expressed as μEq of FFA produced/mg of hepatic protein/hr. Net lipolysis of triolein was calculated by substracting the endogenous FFA production measured at each pH.

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The effect of CPIB and HFA on orotic acid's production of fatty liver was determined by feeding rats for 1 week the fat free diet containing 2% orotic acid or orotic acid plus either 0.10% HFA or 0.25% CPIB. Hepatic total lipid, neutral lipid and triglyceride concentrations were determined as described before [2], and hepatic lipase activities were measured as described above.

Measurement of plasma FFA. Plasma FFA levels cannot be determined directly on the blood plasma from drug-treated rats, since the circulating chemical forms of CPIB and HFA are lipid-soluble, carboxylic acids which could be extracted and titrated along with the plasma FFA. A washing procedure previously developed in our laboratory [4] to remove CPIB from the plasma FFA extract is ineffective for HFA, and thus, in the present study both CPIB and HFA were separated from FFA by thin-layer chromatography. Plasma samples collected at days 7 and 21 from both control and treated rats were extracted into 20 vol. of 2:1 chloroform–methanol (v/v). Total lipid was recovered and chromatographed on thin-layer chromatographic plates (Silica gel G) in a solvent of *n*-hexane–diethylether–glacial acetic acid (68:30:2). Standards of linoleic acid, CPIB (free acid), and HFA (free acid) migrate with respective R_f values of about 0.40, 0.25 and 0.20. The Silica gel area containing the FFA was recovered free of CPIB and HFA, transferred to small columns, and eluted with about 10 ml of 2:1 chloroform–methanol. The FFA residue recovered after evaporation of the solvent was dissolved in *n*-heptane and the FFA titrated by the automated method. In several experiments, the areas of the plate corresponding to CPIB and HFA were recovered, eluted with chloroform–methanol (2:1), and the drugs quantitated by gas–liquid chromatography (g.l.c.) as we have done before [12].

Measurement of plasma CPIB and HFA. When HFA was to be measured, the g.l.c. was run isothermal at 180° for 5 min, increased to 200° (at 20°/min) and then maintained at 200° for 35 min. The retention time of HFA was about 32 min, and that of CPIB 4 min. Plasma levels of CPIB were 6.74 ± 0.49 (S. E.) mg/100 ml (average four rats), and the HFA levels measured in two rats were 20.3 and 24.9 mg/100 ml. Although the CPIB was given at twice the dose of HFA, the higher plasma levels of HFA can perhaps be explained by the observation that albumin binds HFA much more strongly than CPIB [13].

RESULTS

Hepatic lipase activities (absence of orotic acid). Hepatic lipolysis of endogenous substrate increased with increasing pH (Table 1). As compared with livers of control rats, liver of animals treated with either HFA or CPIB possessed significantly lower levels of endogenous lipase activity at acid and, to a lesser extent, neutral pH. Both HFA and CPIB decrease liver TG concentrations in rats fed this fat free diet [2]. HFA did not alter lipolysis of triolein at any of the pH levels investigated; however, treatment with CPIB increased lipase activity against triolein at neutral pH in the presence of heparin and decreased hydrolysis of triolein at pH 5 and 8.5.

Hepatic lipase activities (presence of orotic acid). The triglyceride concentration of the liver increased 20-fold when rats were fed the free diet containing 2% orotic acid (Table 2) for 7 days. Including either 0.10% HFA or 0.25% CPIB with the orotic acid completely suppressed development of fatty liver.

Treatment with orotic acid resulted in a 3- to 4-fold increase in lipase activity with endogenous substrate at pH 5 but not at pH 7.5 (Table 3). Including either HFA or CPIB with the orotic acid prevented this increase. Treatment with orotic acid alone or in combination with HFA or CPIB produced no clear changes in hepatic triolein lipase activity at either pH 5 or 7.5.

Table 1. Hepatic lipase activities in control, HFA- and CPIB-treated rats

	Hepatic lipase activity (μEq FFA produced/mg protein/hr × 10^{-2})†			
	Endogenous substrate			
Treatment*	pH 5	pH 7.5	pH 8.5	
Control	3.42 ± 0.24	8.23 ± 0.72	12.6 ± 1.1	
HFA (0.1%)	1.84 ± 0.21‡	5.78 ± 0.33§	10.8 ± 0.4	
CPIB (0.25%)	1.00 ± 0.12‡	6.22 ± 0.34‖	10.6 ± 0.5	
	Triolein substrate¶			
	pH 5	pH 7.5	pH 7.5+ heparin (25 i.u./ml)	pH 8.5
Control	10.6 ± 0.7	19.9 ± 1.6	19.1 ± 0.8	18.2 ± 1.2
HFA (0.1%)	10.1 ± 0.6	18.7 ± 1.2	19.0 ± 0.9	16.3 ± 0.9
CPIB (0.25%)	7.8 ± 0.4§	22.3 ± 1.1	23.6 ± 0.7**	15.3 ± 0.5‖

* Rats were fed a fat free diet for 14 days containing no drug (control) or 0.10% halofenate (HFA) or 0.25% clofibrate (CPIB).

† The values are means ± one S. E. of six rats/group.

‡ P(t) of difference from control is <0.001 (Student's t-test).

§ P(t) < 0.02.

‖ P(t) < 0.05.

¶ Lipase activities with triolein substrate are net lipolytic rates corrected for endogenous lipolysis.

** P(t) < 0.005.

Table 2. Effect of HFA and CPIB on orotic acid-induced fatty liver

Treatment*	No. of rats	Lipids and triglyceride in liver (wet weight)† (mg/g)		
		Total lipid	Neutral lipid	Triglyceride
Control	4	43.2 ± 1.9	3.68 ± 0.61	1.31 ± 0.38
Orotic acid (2%)	4	88.8 ± 12.4‡	32.5 ± 9.0‡	26.7 ± 8.1‡
HFA (0.1%)+ orotic acid (2%)	4	55.0 ± 1.3§	5.19 ± 0.19	1.53 ± 0.50
CPIB (0.25%)+ orotic acid (2%)	4	49.0 ± 0.9‖	4.10 ± 0.39	0.64 ± 0.19

* Rats were fed the fat free diet for 1 week containing either no drug (control), 2% orotic acid, 0.1% HFA plus 2% orotic acid, or 0.25% CPIB plus 2% orotic acid.

† Values are means ± one S. E. Total lipids and total neutral lipids were determined gravimetrically and triglycerides colorimetrically.

‡ $P(t)$ of difference from control < 0.02.

§ $P(t) < 0.005$.

‖ $P(t) < 0.05$.

Plasma free fatty acid levels. HFA at 0.10% of the diet and CPIB at 0.25% of the diet produced 65–75 per cent reductions in the circulating levels of FFA after 2 weeks of treatment. The decrease due to clofibrate was somewhat greater than that produced by HFA (Table 4). Over the same period, the plasma FFA levels of control fed rats (plain fat free diet) did not change significantly.

DISCUSSION

Of the various hepatic TG lipases studied, the activity of the acid lipase was particularly sensitive to changes in intracellular TG concentration. Guder *et al.* [6] demonstrated that hepatic acid lipase activity is substrate dependent up to about 60 mM triolein. Triolein is present at 100 mM in the lipase assay used in their laboratory and our laboratory. The endogenous concentration of TG in rat liver is about 0.002 mM. In the present study, the activity of acid lipase decreased when liver TG concentration was lowered by treatment with HFA or CPIB and sharply increased upon raising the hepatic endogenous TG level by feeding orotic acid. Feeding HFA at 0.1% or CPIB at 0.25% of the fat free diet for 2 weeks results in respective 50 and 30 per cent decreases of liver TG concentration [2], and orotic acid treatment for 1 week increases hepatic TG levels by 20-fold. Since neither HFA nor orotic acid significantly affected the acid lipase activity against exogenous triolein (at an enzyme-saturating substrate concentration), the changes in activity against endogenous substrate are probably due to the changes in hepatic TG levels rather than to direct drug effects on this enzyme. Thus, hepatic acid lipase activity appears directly responsive to intracellular TG levels and this

Table 3. Effect of HFA and CPIB upon hepatic lipase activity in orotic acid-treated rats

Treatment*	Hepatic lipase activity (μEq FFA produced/mg protein/hr × 10^{-2})†			
	Endogenous substrate			
	pH 5		pH 7.5	
	Expt 1	Expt 2	Expt 1	Expt 2
Control	2.17	3.17	9.27	9.19
Orotic acid (2%)	8.25	11.0	7.61	11.5
HFA (0.1%)+ orotic acid (2%)	1.70	1.66	9.03	9.51
CPIB (0.25%)+ orotic acid (2%)	1.68	1.34	7.75	6.83
	Triolein substrate‡			
	6.54	6.72	14.3	17.1
Orotic acid (2%)	11.3	6.83	13.9	11.5
HFA (0.1%)+ orotic acid (2%)	5.02	5.97	12.5	11.4
CPIB (0.25%)+ orotic acid (2%)	4.29	5.83	14.2	15.1

* Rats were treated for 7 days with fat free diet containing either no drug (control), 2% orotic acid, 0.1% HFA plus 2% orotic acid, or 0.25% CPIB plus 2% orotic acid.

† Each value is the lipase activity for homogenates consisting of equal quantities of liver pooled from two rats.

‡ Lipase activities with triolein substrate are net lipolytic rates corrected for endogenous lipolysis.

Table 4. Effect of HFA and CPIB on plasma FFA levels

Treatment*	No. of rats	(μEq FFA/liter plasma)† Pre-treatment	Post-treatment	%Δ	P(t) of Δ‡
Control	6	392 ± 67	369 ± 24	−6	NS
HFA (0.1%)	8	240 ± 72	81 ± 29	−66	<0.03
CPIB (0.25%)	8	332 ± 41	82 ± 20	−75	<0.005

* Rats were fed a fat free diet for 14 days containing no drug (control) or 0.10% HFA or 0.25% CPIB. FFA levels were determined for each individual rat before and after treatment.

† Values are means ± one S. E.

‡ P(t) of the differences between pre- and post-treatment levels was determined by the paired *t*-test. NS = not significant.

enzyme could be important in regulating intracellular TG concentration in a way similar to the importance of cholesterol ester-acid-hydrolase (lipase) in regulating intracellular cholesterol ester levels [7]. Both of these enzymes are of lysosomal origin [6, 14]. Deficiencies in lysosomal cholesterol ester-acid-lipase have recently been shown to explain human cholesterol ester storage disease [7]. Futher, we recently demonstrated that rat cerebral cortex cholesterol ester-acid-lipase activity is increased in cobalt-induced epilepsy, a condition where cortex cholesterol ester levels increase by more than 20-fold.* Studies of the possible involvement of TG acid lipase in clinical problems of TG metabolism such as fatty liver and hypertriglyceridemia could be warranted.

Neither the effects of CPIB nor HFA on hepatic lipase activities would appear to contribute to their hypotriglyceridemic properties or explain their ability to prevent the fatty liver induced by orotic acid. Although CPIB significantly increased lipolysis of triolein in the presence of heparin, it is uncertain that this actually represents an increase of hepatic lipoprotein lipase, since the supernatant fraction of rat whole liver homogenates is reported to contain an inhibitor of heparin action [6].

CPIB and HFA must lower plasma triglycerides basically either by increasing clearance of triglyceride from the circulation and/or decreasing secretion of TG from the liver and intestines. These drugs appear to work in the rat primarily by producing a net decrease of hepatic TG formation, since both CPIB and HFA markedly decrease the incorporation *in vivo* of [2-^{3}H]-, [1(3)-^{3}H] and [U-^{14}C]glycerol into hepatic TG without simultaneously increasing the serum fractional turnover rates of labeled TG released to the circulation [2]. The fatty liver preventing and hypotriglyceridemic properties of CPIB and HFA are probably due to decreased *de novo* formation of hepatic triglycerides. *De novo* TG synthesis could be decreased by direct effects on the enzymes of TG formation, such as the glycerol-phosphate acyltransferases or the fatty acid CoA synthetases, or by decreasing the concentration of fatty acid substrate available for esterification of glycerol. The importance of direct drug effects upon the enzymes of TG synthesis is presently uncertain [15–17].

In contrast to drug effects on the enzymes of TG synthesis, there appears to be good agreement that CPIB and HFA can reduce fatty acid availability by inhibiting *de novo* fatty acid synthesis [3, 18–21] and lowering the circulating levels of FFA [4, 5]. The ability of HFA to lower plasma FFA levels was demonstrated in the present study. Circulating free fatty acids are a main source of fatty acid needed for hepatic TG formation. The TG content of the liver has been shown to be directly proportional to the concentration of FFA perfusing it both *in vivo* [22] and *in vitro* [23].

In summary, treatment of the rat with either CPIB or HFA did not increase hepatic lipase activities in the presence of an enzyme-saturating concentration of triolein substrate. Thus, these drugs do not decrease hepatic net TG synthesis by stimulating catabolism of TG within the liver. However, treatment with these drugs did significantly reduce lipolysis of hepatic endogenous TG at acid pH. This effect is probably due to the decrease in hepatic TG concentrations produced by CPIB and HFA. The activity of the hepatic acid lipase is apparently dependent upon the concentration of available TG. This conclusion is further supported by finding a marked increase in hepatic acid lipase activity against endogenous substrate when liver TG levels were greatly increased after treatment with orotic acid. The present study also demonstrated that HFA, as was shown before for CPIB, can decrease circulating levels of FFA. Thus, it is possible that these drugs reduce hepatic net TG synthesis by decreasing the availability of fatty acids for *de novo* TG synthesis.

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