Effects of *p*-Chlorophenoxyisobutyric Acid (CPIB) on Adipose Tissue Triglycerides Synthesis, Lipogenesis, and Some Related Enzymes[1] (35929)

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The hypotriglyceridemic effect of CPIB has been demonstrated in man (1) and in the rat (2), but its mechanism of action remains obscure. It has been suggested (3) that CPIB decreases circulating triglyceride levels by working at multiple sites: (a) by depressing hepatic secretion of very low density lipoproteins (3, 4); (b) by reducing free fatty acid (FFA) mobilization from adipose tissue (5), possibly by decreasing sensitivity of fat cells to some lipolytic stimuli (6); (c) by accelerating the rate of incorporation of lipoprotein fatty acids into adipose tissue triglyceride (7); (d) by stimulation of an enhanced activity of adipose tissue lipoprotein lipase (8). Since Nestel and Austin (7) had reported that "a higher proportion of newly synthesized fatty acids was found to be esterified in CPIB-treated rats" we undertook a study of the effect of CPIB treatment on the activity of adipose tissue "esterifying enzymes."

In addition, Greene *et al.* (9) had observed a reduction in adipose tissue adenyl cyclase activity in CPIB-treated rats and since other reports (10, 11) suggested that 3′,5′-cyclic AMP has an inhibitory effect on lipogenesis it seemed possible that there might be an increased incorporation of precursors into fatty acids, as well as a typically hyperlipogenic enzyme profile, in the adipose tissue of CPIB-treated rats. The effect of CPIB treatment on epididymal fat pad lipogenesis *in vitro* and on the activity of the hexose monophosphate shunt dehydrogenases (HMPD) and malic enzyme in adipose tissue were therefore studied.

Materials and Methods. Male Holtzman rats, weighing 130–140 g, were fed diets with or without CPIB[3] at a final concentration of 0.25% (w/w) for 14 days. The composition of the synthetic diets is given in Table I. The rats were killed by decapitation without previous fasting; the epididymal fat pads were removed, placed in closed petri dishes on filter paper moistened with 0.9% saline, weighed and used for study within 5 min.

For enzyme assays, fat pads were immediately chilled; the combined activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (HMPD) and the activity of the NADP-malic enzyme were measured in the 21,000*g* infranatant fraction (*i.e.*, the clear solution between the fat layer and the pellet) of 0.25 *M* sucrose homogenates by method previously described (12, 13). One unit was defined as that amount of the enzyme preparation which reduced 1 μmole of pyridine nucleotide/min at 25° under the assay conditions used. Values reported are referred to infranatant protein assayed by the method of Lowry *et al.* (14).

Fatty acid esterifying activity was assayed as described by Angel and Koncari (15) in the 700*g* infranatants of 0.15 *M* KCl homogenates. Aliquots of these were fortified with ATP, CoASH, Mg^{2+}, F^-, and GSH[4] and incubated at 20° with air as gas phase, in the presence of 1-^{14}C-palmitic acid (250 nmoles, sp act 5 × 10^{-2} μCi/μmole and alpha-

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[3] Sodium salt of *p*-chlorophenoxyisobutyric acid.

[4] Each flask contained the following amounts (μmoles) of cofactors and ions in 3 ml (total volume): ATP, 10; CoASH, 0.25; $MgCl_2$, 6; NaF, 20; GSH, 2.5; KCl, 150; potassium phosphate buffer (pH 7), 140.

TABLE I. Composition of Synthetic Diets.

Component	Sucrose		Glycerol-lard (Gly-lard)	
	% by wt	% by cal	% by wt	% by cal
Sucrose[a]	63	69		
Casein[a] (vitamin free)	28	31	33	34
Vitamin fortification mixture[a]	4		4	
Salt (USP XIV)	5		5	
Lard			9.8	22
Glycerol			43	44
Cellulose	5		5	

[a] Products of Nutritional Biochemical Corp., Cleveland, Ohio.

glycerophosphate (20 μmoles). After a 15 min incubation, the media were extracted by the Folch and co-workers' procedure (16); the chloroform phases were evaporated under a stream of nitrogen, redissolved in petroleum ether, and washed with alkaline ethanol (17). The residual radioactivity was taken to represent newly synthetized triglycerides. Values were corrected for isotope dilution according to the following formula:

$$\text{sp act} = \frac{\text{added } ^{14}\text{C-palmitic acid (dpm)}}{\text{endogenous FFA (nmoles)} + \text{added } ^{14}\text{C-palmitic acid (nmoles)}}$$

and referred to the nitrogen content of infranatants determined by Nesslerization of sulfuric acid digests. Endogenous FFA of infranatants were measured by the colorimetric method of Novak (18). Infranatants were tested for *p*-chlorophenoxyisobutyric acid by a previously described method (8) and none was found.

For the lipogenesis measurements, 100–200 mg aliquots were removed from the thin segment of fat pads without chilling and incubated for 2 hr in 4 ml of Krebs–Ringer bicarbonate buffer in an atmosphere of 95% O_2–5% CO_2. Precursors were added to the medium as 0.02 *M* U-^{14}C-glucose (0.5 μCi/ml) and tritiated water (0.25 mCi/ml). When insulin was present, its concentration was 0.1 U/ml (in this case each rat served as its own control). At the end of the incubation, the pieces of fat were thoroughly washed with 0.9% saline and saponified with alcoholic sodium hydroxide (1 *N*). The solution was acidified and the fatty acids were extracted with 10 ml of chloroform. Aliquots of the chloroform extract were transferred to counting vials, evaporated to dryness, and the residue was dissolved in Liquifluor[5] for counting. Radioactive glycerol was counted in aliquots of the aqueous phase, using Scintisol-GP (Isolab) as solubilizer.

When oxidation of 0.02 *M* U-^{14}C-glucose was measured, the $^{14}CO_2$ released in the rubber-capped incubation vessels by acidifying the medium (19) was absorbed in NCS solution[5] injected into the center well. Isotope counting was performed in a Mark I,[5] with Liquifluor as the counting medium.

Results. The effect of CPIB feeding on palmitate esterification by adipose tissue is shown in Table II. The CPIB-treated rat preparations show an increased activity of esterifying enzymes whether related to nitrogen of the infranatant fraction, to adipose tissue wet weight, or to body weight.

In glycerol-lard fed rat adipose tissue incorporation of precursors into fatty acids (Table III) in the presence of glucose and insulin is also increased with CPIB treatment. A significant increase in incorporation of ^{3}H from water into fatty acids was also found in the absence of insulin. The incorporation of glucose carbon into triglyceride glycerol and into CO_2 (not shown) was the same for control and CPIB-treated groups. As shown in Table IV, there are increases of HMPD and NADP-malic enzyme activities in the adipose tissue of CPIB-treated rats, a finding consistent with an increased need for reducing equivalents for lipogenesis.

[5] Products of Nuclear Chicago.

TABLE II. Effect of Dietary Administration of CPIB on Fatty Acid Esterification in Adipose Tissue.[a]

Diet	CPIB, 0.25%	Final body wt (g)	Epididymal fat (g/100 g of body wt)	Infranatant		^{14}C-Palmitate in TG in 15 min	
				Nitrogen (mg/ml)	Free fatty acids (ng moles/ml)	(ng moles/100 mg of adipose tissue)	(ng moles/mg of N)
Gly-lard (6)	0	243 ± 3	1.2 ± 0.1	0.31 ± 0.01	2.13 ± 16	42.2 ± 2.0	383 ± 12
		NS	NS	NS	NS	$p < 0.02$	$p < 0.02$
Gly-lard (6)	+	229 ± 6	1.0 ± 0.1	0.33 ± 0.02	249 ± 37	66.9 ± 11.2	543 ± 67

[a] Values are means ± SEM; NS = not significant (*i.e.*, $p > .05$); number of animals per group are in parentheses.

TABLE III. Effects of Dietary Administration of CPIB on Adipose Tissue Lipogenesis.[a]

	CPIB. 0.25%	Final body wt (g)	Epididymal fat (g/100 g of body wt)	Epididymal fat nitrogen (mg/100 mg of wet wt)	Incubation insulin	U-^{14}C-Glucose in fatty acids (μg-atoms/mg of N)	^{3}H-Water in fatty acids (μg-atoms/mg of N)
Gly-lard (5)	0	227 ± 3	1.0 ± 0.1	0.213 ± 0.01	0	1.2 ± 0.12	2.3 ± 0.2
					+	17.4 ± 1.80	19.2 ± 2.0
Gly-lard (5)	+	228 ± 5	1.0 ± 0.1	0.245 ± 0.02	0	2.4 ± 0.48	3.7[b] ± 0.6
					+	33.0[c] ± 2.64	33.1[c] ± 1.2
Sucrose (6)	0	218 ± 3	0.9 ± 0.03	0.299 ± 0.03	0	6.0 ± 1.2	8.4 ± 1.1
					+	54.0 ± 3.6	57.7 ± 3.9
Sucrose (6)	+	215 ± 4	0.8 ± 0.04	0.386[b] ± 0.01	0	4.2 ± 0.6	7.0 ± 0.9
					+	50.4 ± 2.4	56.9 ± 2.9

[a] Values are means ± SEM. Number of animals per group are in parentheses. Incubations were done for 2 hr in bicarbonate buffer with 0.02 *M* U-^{14}C-glucose, tritiated water 250 μCi/ml and ± insulin 0.1 U/ml.

[b] Significance, p values of CPIB group versus respective controls: $<.0.05$; [c] < 0.01; other differences are not significant ($p > .05$).

TABLE IV. Effect of Dietary Administration of CPIB on Adipose Tissue Enzymes.[a]

Diet	CPIB, 0.25%	Final body wt (g)	Epididymal fat (g/100 g of body wt)	Infranatant proteins	HMPD (mU/mg of protein)	Malic enzyme (mU/mg of protein)
Gly-lard (6)	0	215 ± 3	0.96 ± 0.03	3.6 ± 0.4	65 ± 6	33 ± 4
Gly-lard (6)	+	225[c] ± 1	1.14[c] ± 0.03	3.6 ± 0.1	116[d] ± 13	87[e] ± 4
Sucrose (5)	0	232 ± 5	1.01 ± 0.04	3.5 ± 0.4	164 ± 11	172 ± 14
Sucrose (6)	+	227 ± 5	0.93 ± 0.04	3.8 ± 0.3	193 ± 13	228[b] ± 17

[a] Values are means ± SEM. Number of animals per group are in parentheses.

[b] p values versus respective controls: [b] < 0.05; [c] < 0.01; [d] < 0.005; [e] < 0.001. Other differences are not significant ($p > .05$).

Studies in rats fed the sucrose diet showed a trend toward higher HMPD activity of fat tissue and a significant increase in NADP-malic enzyme activity. However, no effect on lipogenesis was found.

Discussion. The results of these experiments are consistent with the suggestions of Greene *et al.* (9) that some of the effects of CPIB may be explained by its inhibition of the activity of adenyl cyclase. They pointed out that the reciprocal effects of the drug on adipose tissue lipoprotein lipase and hormone-sensitive lipase might result from a reduced cyclic AMP formation. The increased lipogenesis we observed in CPIB-treated rats fed the glycerol-lard diet could also be related to decreased CAMP availability. Increased activity of triglyceride synthesizing enzymes has been reported to accompany hyperlipogenesis in rats refed after a fast (15) and the CPIB effect on this activity, as well as on HMPD and NADP-malic enzyme, may be secondary to the increased lipogenesis.

It is probable that the tissues of rats fed the high sucrose diet are under marked insulin influence. Since insulin has been shown to decrease the concentration of cyclic AMP in adipose tissue (20, 21) an effect of CPIB on adenyl cyclase would be expected to be more difficult to demonstrate under these circumstances. This may explain the relative lack of effect of CPIB in rats fed the sucrose diet. The contribution of CPIB effects on adipose tissue to its plasma triglyceride lowering action is difficult to assess. It seems likely, however, that the increase in lipoprotein lipase shown earlier (8) and the increased lipogenesis and triglyceride synthesis reported here could be important components of the total effect of the drug.

Summary. The addition of 0.25% CPIB to a glycerol-lard diet fed to rats resulted in increased adipose tissue activities of triglyceride synthesizing enzymes, hexose monophosphate shunt dehydrogenases, and NADP-malic enzyme. Incorporation of ^{14}C-glucose and ^{3}H from water into long chain fatty acids by fat pads *in vitro* was also greater in CPIB-treated rats. The drug had only small or no such effects when added to a fat-free high sucrose diet.

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