The Effect of Clofibrate on Rat Tissue Adenyl Cyclase (34938)

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Clofibrate (ethyl-*p*-chlorophenoxyisobutyrate) reduces elevated serum triglyceride levels in man (1) and in the rat (2). Its mechanism of action is unknown, although previous investigations with this drug have shown that it affects the activity of several lipogenic and glycolytic enzymes in rat liver and human intestine (3, 4). Maragoudakis showed that clofibrate inhibits chicken liver acetyl-CoA carboxylase and suggested that the triglyceride-lowering action of clofibrate might be partly through decreased fatty acid synthesis (5).

Wing and Robinson have shown that clearing factor lipase or lipoprotein lipase (LPL) is inhibited by a cyclic adenosine monophosphate analogue, 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic) AMP, in epididymal fat bodies (6), and Tolman *et al.* found increased LPL activity in rat epididymal fat after clofibrate ingestion (7). Furthermore, in the isolated, profused rat liver, clofibrate causes increased hepatic uptake of labeled triglycerides (8). Therefore, the removal of lipoprotein from plasma might be mediated by a hepatic (9) and adipose lipoprotein lipase whose activity is increased by clofibrate administration.

Havel suggested that increased amounts of plasma free fatty acids recirculate from fat tissue to the liver and reform triglycerides which are reincorporated into pre-beta lipoprotein (10). Cyclic AMP apparently mediates the breakdown of triglyceride to free fatty acids (11, 12) and Hunninghake *et al.* have demonstrated that the epinephrine-induced increase in plasma free fatty acids in man is inhibited by clofibrate (13). These findings suggest a relationship between clofibrate and cyclic AMP concentration, and pre-beta lipoprotein and serum triglyceride concentrations.

Therefore, we undertook to determine the affect of clofibrate on the adenyl cyclase activity in rat epididymal fat, liver, and intestine.

Methods. Male Holtzman rats, weighing 350–500 g, were fed diets containing 70% of calories as fructose, 15% as casein, 15% as corn oil, 5% (w/w) vitamin mixture (Nutritional Biochemicals Corp., vitamin diet fortification mixture) and 2.5% (w/w) salt mixture (Nutritional Biochemicals Corp. No. XIV). The diet fed to the clofibrate-treated animals contained the drug at a level of 1% (w/w).

Animals were fed this diet for 23–25 days. The clofibrate-treated animals consumed an average of 5 g of food per day and their weight declined an average of 2 g per day. The control rats consumed an average of 10 g per day and gained an average of 3 g per day. Because of the discrepancy between the two groups in dietary intake and weight gain, the experiment was repeated using pair-fed animals. Each group consisted of 20 rats, except for the pair-fed group which had liver adenyl cyclase measured on 18 rats.

The rats were killed by cervical fracture, the tissue removed immediately and homogenized in approximately 20 vol of 0.25 *M* sucrose. The three tissues assayed were prepared slightly differently for the assay procedure.

The liver sample weighed 40–50 mg and was carefully homogenized by hand in a Potter Teflon homogenizer, and centrifuged at 2000*g* for 20 min (4°). The supernatant fraction was discarded, and the pellet was homogenized with 1 ml of glycylglycine buffer, 0.02 *M*, pH 7.8, containing magnesi-

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um sulfate, 10^{-3} *M*. The homogenate was centrifuged at 2000*g* for 20 min, the pellet mixed thoroughly with 0.4 ml of the glycylglycine buffer, and centrifuged at 2000*g* for 20 min. The remaining supernatant fluid was clear of any gross evidence of fat or opalescence by this washing procedure. The remaining pellet was used for the assay of adenyl cyclase activity.

To measure intestinal adenyl cyclase activity approximately 3 cm of jejunum was removed, flushed with 15 ml of 0.25 *M* sucrose, and opened longitudinally. The mucosa (50–60 mg) was scraped from the gut with a glass slide and homogenized in a 2-ml capacity (small) Ten Broeck homogenizer with 20 vol of the sucrose solution. Subsequent procedures with the tissue were identical to that outlined for the liver.

All the epididymal fat was used for the assay and the tissue weighed between 2 and 3 g. The tissue was pressed through a "C" type disc (pores 1-mm diameter) using the Thomas Delepine Press. The tissue was then homogenized with 3 ml of 0.25 *M* sucrose in a 4-ml capacity (medium) Ten Broeck homogenizer. The homogenate was centrifuged at 2000*g* for 20 min (4°), and the supernatant fluid containing the fat was discarded. The insoluble protein pellet was prepared in the same manner as for the liver.

The pellet remaining after the final centrifugation was used for the adenyl cyclase assay in all cases. Incubation was completed within 2 hr after the rat was killed, and the tissue extract was kept at 4° until incubation was begun. The volume of the incubation mixture was 0.4 ml and contained: Tris HCl, 4×10^{-2} *M*, pH 7.6; theophylline, 10^{-2} *M*; sodium fluoride, 10^{-2} *M*; magnesium sulfate, 3.3×10^{-3} *M*; ATP 1.2×10^{-3} *M*; and 8-^{14}C-ATP, 1.2 μCi (30–50 mCi/mmole, Schwarz Laboratories). Incubation was carried out for 15 min and stopped with the addition of 0.1 ml of 3′,5′-(cyclic) AMP (5 mg/ml) and immersion into boiling water for 5 min. Measurement of 3′,5′-(cyclic) AMP was done by the method of Krishna (14), and the tissue extract used for incubation was assayed for protein by the method of Lowry (15).

Hepatic phosphodiesterase activity of the particulate fraction was compared in four control and four clofibrate-fed animals. The particulate tissue fraction was prepared in the same manner as described for the adenyl cyclase assay. The volume of the incubation mixture was 0.4 ml and consisted of, magnesium sulfate 10^{-3} *M*; Tris HCl, 0.02 *M*, pH 7.6; 3′,5′-AMP 10^{-3} *M*; and tritiated 3′,5′-(cyclic) AMP 200 μCi (1 mCi/mmole, Schwarz Laboratories). Incubations were carried out for 5-, 10-, and 15-min periods at 30°. Incubation was stopped with the addition of 0.1 ml of theophylline, 0.05 *M*, and immersion into boiling water for 5 min. Separation and measurement of the 3′,5′-(cyclic) AMP was performed by the method of Krishna (14), using the reaction mixture without enzyme to determine the percentage of recovery.

The *in vitro* effect of clofibrate on adenyl cyclase activity was measured in the liver of four animals with each animal serving as its own control. Clofibrate was solubilized in propylene glycol and ethanol. The ethanol was evaporated with gentle heat under a stream of nitrogen. Equal amounts of propylene glycol were added to the control mixtures.

Results. The effect of clofibrate feeding on rat tissue adenyl cyclase is shown in Fig. 1. The clofibrate-treated rats showed approximately one third the adenyl cyclase activity as those rats receiving no treatment. Similar changes were found in the non-pair-fed animals. The apparent difference in adenyl cyclase activity might be accounted for by increased phosphodiesterase activity. However, there was no difference in phosphodiesterase activity of the 2000*g* fraction when the two groups were compared (Fig. 2).

There was no inhibition of the adenyl cyclase activity in control animal tissues when clofibrate ranging from 10^{-2} to 10^{-4} *M* was added to the incubation media (Table I).

Discussion. These findings are consistent with the hypothesis that the lipid-lowering action of clofibrate is mediated through the inhibition of adenyl cyclase, and would suggest that the site of action of clofibrate might be in the membrane structure at the locus of

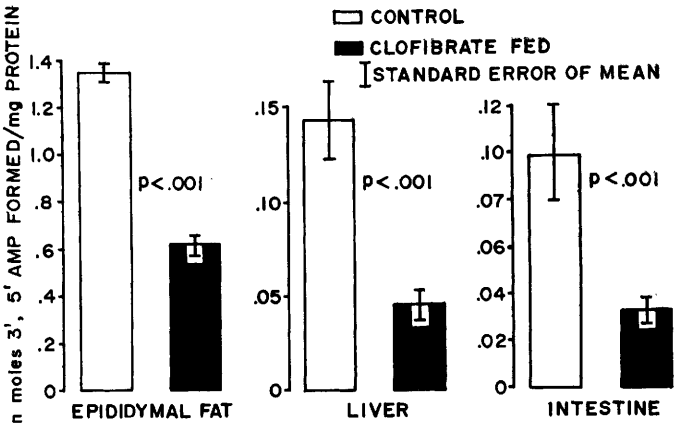

FIG. 1. The effect of clofibrate on the activity of adenyl cyclase in rat epididymal fat, liver, and jejunum. The height of the bar indicates the mean for 10 rats and the standard error of the mean is indicated by the brackets. Activity is expressed as nmoles of cyclic AMP formed per 15 min incubation per mg protein. The non-fluoride-stimulated adenyl cyclase activity was not statistically significantly different between the two groups.

adenyl cyclase. Although clofibrate does not enter the tissues to any appreciable degree (16), it was of interest that it had no effect on the *in vitro* adenyl cyclase activity. Conceivably the clofibrate might be transformed *in vivo* into another substance not measurable as clofibrate.

The inhibition of adenyl cyclase seems to

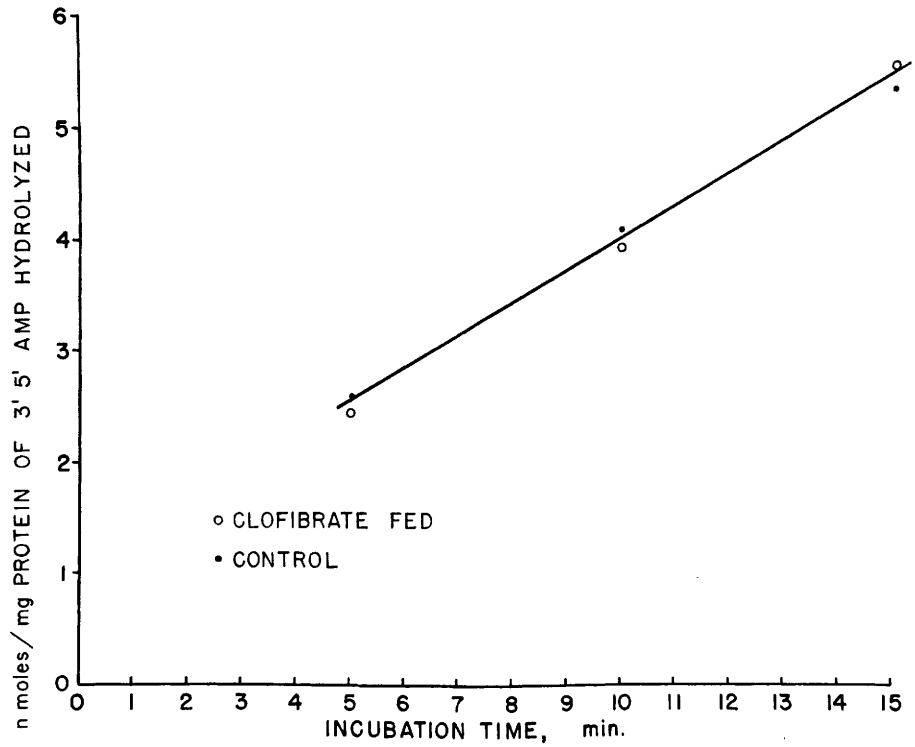

FIG. 2. The effect of clofibrate on the 2000*g* fraction of rat liver phosphodiesterase. Theophylline 10^{-2} *M*, completely inhibited phosphodiesterase activity in this fraction.

TABLE I. *In Vitro* Effect of Clofibrate on Liver Adenyl Cyclase Activity.[a]

	Control	Clofibrate added		
		$10^{-2}M$	$10^{-3}M$	$10^{-4}M$
Average nmole/mg protein	.124	.123	.127	.119
Standard error of mean	.037	.036	.043	.028

[a] Incubation was terminated at 12 min.

be specific since previous investigations have shown that only some enzymes are affected by clofibrate (3–5). In rats the action of clofibrate may be a dual one, inhibiting adenyl cyclase as well as glycolytic and lipogenic enzymes (2). At present we have no information as to the effect of clofibrate on human adenyl cyclase. However, this is currently under study in our laboratory.

Summary. The effect of clofibrate (ethyl-*p*-chlorophenoxyisobutyrate) on adenyl cyclase activity in rat liver, epididymal fat, and jejunum has been studied. Rats fed a diet high in fructose with 1% clofibrate had a statistically significant decrease in adenyl cyclase activity in all the tissues tested. There was no effect on the phosphodiesterase activity in the membrane preparation used for the adenyl cyclase assay. When clofibrate was added to the incubation media there was no inhibition of the adenyl cyclase activity in the control animal tissues.

This finding is consistent with the hypothesis that the lipid-lowering action of clofibrate may be through its inhibition of adenyl cyclase activity.

1. Oliver, M. F., Lancet **1**, 1321 (1962).

2. Thorp, J. M., J. Atheroscler. Res. **3**, 347 (1963).

3. Zakim, David, Herman, R. H., Rosensweig, N. S., and Stifel, F. B., Gastroenterology **56**, 496 (1969).

4. Zakim, D., Pardini, R. S., and Herman, R. H., Biochem. Pharmacol. **19**, 305 (1970).

5. Maragoudakis, M. E., J. Biol. Chem. **244**, 5005 (1969).

6. Wing, D. R., and Robinson, D. S., Biochem. J. **109**, 841 (1968).

7. Tolman, E. L., Tepperman, H. M., and Tepperman, J., Am. J. Physiol. **218**, 1313 (1970).

8. Mishkel, M. A., and Webb, W. F., Biochem. Pharmacol. **16**, 897 (1967).

9. Korn, E. D., J. Biol. Chem. **215**, 1 (1955).

10. Havel, R. J., Proc. 1968 Deuel Conf. Lipids, p. 119 (1968).

11. Sutherland, E. W., Oye, I., and Butcher, R. W., Recent Progr. Hormone Res. **21**, 623 (1965).

12. Weiss, B., Davies, J. I., and Brodie, B. B., Biochem. Pharmacol. **15**, 1533 (1966).

13. Hunninghake, D. B., and Azarnoff, D. L., Metabolism **17**, 588 (1968).

14. Krishna, G., Weiss, B., and Brodie, B. B., J. Pharmacol. Exp. Ther. **163**, 379 (1968).

15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

16. Barrett, A. M., Brit. J. Pharmacol. **26**, 363 (1966).

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