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REDUCED ACTIVITY OF HEPATIC MICROSOMAL FATTY ACID CHAIN ELONGATION SYNTHESIS IN CLOFIBRATE-FED RATS

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Abstract—Na-clofibrate dissolved in drinking water was administered at a dose of 12 mg per day per animal to adult male Wistar rats. After different periods of drug administration, measurement was made of the activity of hepatic acetyl-CoA carboxylase, fatty acid synthetase and both microsomal and mitochondrial fatty acid chain elongation systems. Data obtained indicate a significant reduction in all synthetic activities, with the exception of the mitochondrial, even after dialysis of the investigated subcellular fractions. This reduction was found to increase with increased drug-administration periods, reaching the maximum after 7–8 days. A similar effect was previously shown by clofibrate *in vitro* [7]. The present results indicate an increase of 66.4% in hepatic cyclic AMP level as well after seven days of drug feeding to rats. Similarly, both serum aspartate aminotransferase and alanine aminotransferase activities were found to increase by about 30% after 13 days. The hypothesis is advanced that *in vivo* clofibrate probably reduces the synthetic activities under investigation by firmly binding to active enzyme-protein sites. In addition, the possibility that also the altered cyclic AMP level induced by this drug is responsible for both the above reductions and other metabolic variations reported elsewhere should not be excluded.

Clofibrate has been widely used to lower human plasma lipid levels [1–3]. To date, however, its action mechanism as hypolipidaemic agent has not been completely clarified. Many reports have shown this drug to affect a number of lipogenic enzymes both *in vivo* and *in vitro* [4–6]. Recent evidence has been obtained in our laboratory that *in vitro* clofibrate strongly reduces fatty acid chain elongation synthesis by rat liver microsomes [7]. Other authors have shown this drug to produce a variety of further metabolic variations in addition to lipid metabolism [8–11]. Among several postulated mechanisms, Green *et al.* [12] have suggested the lipid-lowering action of clofibrate as due to its inhibition of adenylate cyclase activity.

The present paper indicates that in clofibrate-fed rats a notable reduction occurs not only in fatty acid synthetase and acetyl-CoA carboxylase, but in hepatic microsomal fatty acid chain elongation synthesis as well. In addition, a significant increase has been observed in the liver cyclic 3′,5′-adenosine monophosphate level of such animals. Discussion is made of the possible bimodal clofibrate action mechanism through direct action on enzyme-protein and impaired cyclic AMP concentration.

MATERIALS AND METHODS

Animals. Adult male Wistar rats weighing 200–250 g received normal diet with free access to food and water until killed. The treated animals received Na-clofibrate dissolved in drinking water (0.03% w/v). Since each rat drank an average of 40 ml water per day, the drug dosage was assumed to be 12 mg per day per animal. After the indicated period, the rats were killed by decapitation and liver subcellular fractions prepared as explained elsewhere [7]. Where indicated, the obtained subcellular fractions dissolved in 0.25 M sucrose were used either as such or after dialysis against a volume of 0.33 M sucrose 70 times higher, containing 3 mM dithiothreitol and 0.7 mg/ml bovine serum albumin. Duration of dialysis was 6 hr at 4°. Protein was determined according to Gornall *et al.* [13].

Incubation conditions. In order to test fatty acid synthetic capacity by cell sap or microsomes, incubation was carried out in air for 10 min at 37° in glass tube in a Dubnoff incubator. The reaction mixture (final vol., 1 ml) contained: 50 μM [1,3-^{14}C]malonyl-CoA (specific activity, 4 μCi/μmole), 20 μM acetyl-CoA, 1 mM NADH, 1 mM NADPH, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 1 mM ATP, 80 mM Tris–HCl buffer (pH 7.4) and 40 mM sucrose. In the presence of the soluble fraction, ATP and NADH were omitted from the reaction mixture.

The incubation mixture for measuring the activity of mitochondrial chain elongation of fatty acids made up to 1 ml contained 45 μM [1-^{14}C]acetyl-CoA (specific activity, 2.2 μCi/μmole), 10 mM β-mercaptoethanol, 0.5 mM NADH, 0.5 mM NADPH, 30 mM phosphate buffer (pH 6.5), 2 mM $MnCl_2$, 4 mM ATP and 50 mM sucrose. The reaction was carried out under N_2 for 10 min at 37°. Mitochondria were disrupted by freezing and thawing prior to use. At the end of incubation, the reaction was stopped by the addition of 0.7 ml 10 N KOH. After saponification, fatty acids were extracted and total radioactivity was measured as previously reported [14].

The activity of acetyl-CoA carboxylase was measured as described in ref. 14.

Table 1. Hepatic acetyl-CoA carboxylase activity at different periods in clofibrate-fed rats

Animals	Duration of feeding (days)	Activity (nmoles [1-^{14}C]malonyl-CoA formed/min/mg protein)	Inhibition (%)
Crude cell sap			
Control	—	0.61 ± 0.08	—
Clofibrate-fed rats	1	0.55 ± 0.07	10
Clofibrate-fed rats	2	0.52 ± 0.07	15
Clofibrate-fed rats	7	0.27 ± 0.04	56
Clofibrate-fed rats	13	0.26 ± 0.03	57
Ammonium sulphate precipitated cell sap			
Control	—	2.10 ± 0.27	—
Clofibrate-fed rats	5	1.13 ± 0.11	46
Clofibrate-fed rats	7	0.95 ± 0.13	55
Clofibrate-fed rats	13	0.97 ± 0.12	54

Rats fed the stock diet with Na-clofibrate (0.03% w/v) administered in drinking water for the time indicated. Results relative to crude cell sap the average of 4 animals ± standard deviation (S.D.); those relative to ammonium sulphate precipitated cell sap (salt concentration, 0.4 stn.) the mean of 3 animals ± standard deviation (S.D.). In both cases protein concentration 0.5 mg.

Enzymatic analysis. The aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) activities in rat serum were assayed colorimetrically according to Bergmeyer and Bernt [15].

Cyclic AMP assay. Rat liver biopsies (50 to 100 mg), obtained under urethane anesthesia after 1 min liver perfusion with 10 ml of 0.9% (w/v) NaCl solution to eliminate impregnating blood and thus avoid interferences, were rapidly immersed in liquid nitrogen and cooled to −158°. Tissue samples were drawn by freezing *in situ* with clamps previously cooled in the same environment. The tissue was stored in liquid nitrogen until assayed, then powdered in a liquid nitrogen-cooled stainless steel mortar, weighed and transferred to a glass homogenizer tube. The above operation was performed at −20°. Homogenization was carried out in the presence of 1.5 ml of ice cold 10% (w/v) trichloroacetic acid. The homogenate was left at 0° for 10 min before being centrifuged for 15 min at 28,000 *g* at 5°. The supernatant solution was extracted four times with 5 vol. cold diethylether saturated with acidic H_2O and the extracts heated in boiling water for 3–5 min until the odor of ether was no longer discernible. Cyclic AMP was then determined in the extracts according to the Gilman protein binding method [16].

RESULTS AND DISCUSSION

Table 1 describes the activity of acetyl-CoA carboxylase as measured in liver cell sap from clofibrate-fed rats. It appears to be differently reduced depending on the length of time the animals received the drug. In fact, after 2 days the reduction is equal to 15% while after 7 days it reaches 56% and shows no further change after longer treatment periods. The same behaviour is exhibited by carboxylase when ammonium sulphate precipitated cell sap is used as the enzyme source. These data confirm previous *in vitro* observations [7] according to which some direct interference is made by clofibrate on acetyl-CoA carboxylase [17].

When the incorporation of [1,3-^{14}C]malonyl-CoA into fatty acids by liver cell sap from clofibrate-treated rats is studied, a significant inhibition is already evident after 1 day of drug administration (Table 2). This reduction remains at values of about 40 per cent from the fifth day onwards. As *in vitro* clofibrate has also been reported to reduce *de novo* fatty acid synthesis [6, 7, 18, 19] by about the same amount, cell sap was dialyzed prior to incubation with [1,3-^{14}C]malonyl-CoA. Since the dialyzed fraction exhibits essentially the same reduced activity as

Table 2. Effect of clofibrate feeding on soluble fatty acid synthetase in rat liver

Animals	Duration of feeding (days)	Activity (nmoles [1,3-^{14}C]malonyl-CoA incorporated into fatty acids/min/mg protein)		Inhibition (%)	
		Undialyzed	Dialyzed	Undialyzed	Dialyzed
Control	—	2.54 ± 0.31	2.08 ± 0.27	—	—
Clofibrate-fed rats	1	1.91 ± 0.24	N.M.	25	N.M.
Clofibrate-fed rats	2	1.78 ± 0.15	N.M.	30	N.M.
Clofibrate-fed rats	5	1.50 ± 0.16	1.19 ± 0.13	41	43
Clofibrate-fed rats	8	1.35 ± 0.16	1.06 ± 0.11	47	49
Clofibrate-fed rats	13	1.40 ± 0.12	0.87 ± 0.10	45	58

Rats treated as indicated in Table 1 were used. Results the average of 4 animals ± standard deviation (S.D.). Protein, 0.5 mg. N.M. = not measured. Cell sap dialyzed as described in Materials and Methods.

Table 3. Effect of clofibrate feeding on microsomal fatty acid chain elongation synthesis in rat liver

Animals	Duration of feeding (days)	Activity (nmoles [1,3-^{14}C]malonyl-CoA incorporated into fatty acids/min/mg protein)		Inhibition (%)	
		Undialyzed	Dialyzed	Undialyzed	Dialyzed
Control	—	0.53 ± 0.06	0.54 ± 0.05	—	—
Clofibrate-fed rats	1	0.50 ± 0.06	N.M.	6	N.M.
Clofibrate-fed rats	2	0.41 ± 0.05	N.M.	23	N.M.
Clofibrate-fed rats	5	0.37 ± 0.04	0.35 ± 0.03	30	35
Clofibrate-fed rats	8	0.31 ± 0.02	0.33 ± 0.02	41	39
Clofibrate-fed rats	14	0.28 ± 0.03	0.26 ± 0.02	47	52

Rats treated as described in Table 1 for the time indicated. Results the average of 5 animals ± standard deviation (S.D.). Protein, 1.0 mg. N.M. = not measured. Microsomes dialyzed as described in Materials and Methods.

the undialyzed one, we may affirm that this is not due to simple drug-binding with enzyme-protein but that probably some active site of fatty acid synthetase is firmly tied to the drug. That this is the case is shown by the results of Table 3, which compare the activity of the fatty acid chain elongation system of undialyzed and dialyzed microsomes from clofibrate-treated rats. It is evident that in both cases a similarly reduced incorporation is shown after different drug-feeding periods. Based on the previous observation of competitive inhibition by clofibrate with substrate malonyl-CoA by the rat liver microsomal chain elongation system [7], it is possible that this drug *in vivo* inhibits fatty acid synthesis both by altering the conformation of acetyl-CoA carboxylase (cf. refs 17 and 20) and by blocking active groups of soluble fatty acid synthetase and microsomal system. However as clofibrate has been shown to decrease fatty acid esterification into glycerolipid with a consequent increase of the former in free form [5, 21, 22], the above reduce synthetic capacity could also be due to a higher amount of free fatty acids, known inhibitors of several metabolic activities [23–25], in the subcellular fractions under investigation.

In line with the activity measured *in vitro* by the mitochondrial fatty acid chain elongation system in the presence of clofibrate [7], Table 4 shows that [1-^{14}C]acetyl-CoA incorporation into fatty acids remains unaffected in mitochondria from drug-treated animals as well.

From Table 5 it is evident that following clofibrate administration periods of longer than 8 days, both serum aspartate aminotransferase and alanine aminotransferase are increased by about 30 per cent, thus indicating that this drug gives rise to some hepatic derangement as previously shown by a number of reports [26–28].

Clofibrate has been reported to produce a variety of hepatic metabolic alterations [4–6, 8–11, 29–31]. We have tried to verify whether these can be due to change in cyclic AMP level. Table 6 shows that after 7 days of drug-administration to rats, an increase of 66.4 per cent in this cyclic nucleotide concentration in liver is evident. A similar increase already occurs after 6 days of treatment with no change seen until the 14th day. Analogous behaviour was also in evidence for blood cyclic AMP level in clofibrate-treated rats. At the moment it is difficult to explain this discrepancy with the data of Green *et al.* [12]. Probably this is due to diet difference, since the animals they used were fed a high sucrose diet. In fact, it has been observed that while in these rats the basal insulin level was decreased by clofibrate [32], in normally fed humans the concentrations of this hormone are not changed by the drug [33]. In addition, our data are consistent with the observation that in liver an increased concentration of certain enzymes is induced by clofibrate [34, 35], thus accounting for both the increased oxidation [36, 37] and enhanced rat liver microsomal hydroxylation involved in bile acid formation [38]. Indeed, the increased concentration of hepatic cyclic AMP level caused by clofibrate could justify all these results, since it has recently been proposed that this cyclic nucleotide is capable of selectively stimulating the synthesis or induction of a number of enzymes.

Table 4. Rate of mitochondrial fatty acid chain elongation synthesis from liver of clofibrate-fed rats

Animals	Duration of feeding (days)	Activity (nmoles [1-^{14}C]acetyl-CoA incorporated into fatty acids/min/mg protein)	Inhibition (%)
Control	—	0.18 ± 0.03	—
Clofibrate-fed rats	1	0.18 ± 0.03	—
Clofibrate-fed rats	2	0.18 ± 0.02	—
Clofibrate-fed rats	5	0.17 ± 0.02	6
Clofibrate-fed rats	8	0.16 ± 0.01	11
Clofibrate-fed rats	13	0.16 ± 0.01	11

Rats treated as described in Table 1 for the time indicated. Results the average of 5 animals ± standard deviation (S.D.). Protein, 0.8 mg.

Table 5. Rat serum aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) activities after various time intervals of clofibrate feeding

Animals	Duration of feeding (days)	GOT (mU/ml)	Increase (%)	GPT (mU/ml)	Increase (%)
Control	—	135 ± 27	—	21 ± 5	—
Clofibrate-fed rats	1	134 ± 30	—	20 ± 4	—
Clofibrate-fed rats	2	136 ± 29	—	21 ± 5	—
Clofibrate-fed rats	3	142 ± 25	5	22 ± 6	6
Clofibrate-fed rats	9	166 ± 33	23	27 ± 5	28
Clofibrate-fed rats	13	174 ± 38	29	28 ± 8	31

Rats treated as described in Table 1 for the indicated time. Results the average of 8 animals ± standard deviation (S.D.). Enzymatic activity expressed in International Units.

Table 6. The effect of clofibrate administration on rat liver cyclic AMP level

Animals	Cyclic AMP (pmoles/g wet tissue)	Increase (%)
Control	715 ± 139	—
Clofibrate-fed rats	1190 ± 304	66.4

Rats received Na-clofibrate as described in Table 1 for 7 days. Results the mean of twelve rats ± standard deviation (S.D.).

Lastly, we wish to add that the suggestion that clofibrate *in vivo* could inhibit hepatic fatty acid synthesis by increasing cyclic AMP level is supported by previous reports according to which this nucleotide reduces lipogenesis by acting on several levels [39–41].

Alternatively the possibility that clofibrate could increase hepatic cyclic AMP level, thus accounting for many of its effects, is in full agreement with the recent proposal of Gamble [42] that this drug may act through the alteration of ATP level.

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