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Inhibition of rat liver microsomal fatty acid chain elongation by gemfibrozil in vitro

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Gemfibrozil, a hypolipidemic drug mainly used in the treatment of hypertriglyceridemic states, strongly inhibits the rat hepatic microsomal fatty acid chain elongation system in vitro. The inhibition is independent on the reducing cofactor used in the assay. Furthermore, gemfibrozil seems to act by inhibiting the rate-limiting step of the elongation process, the condensing reaction, without discriminating among the proposed three different condensing enzymes, devoted to condensation of saturated, mono-unsaturated and polyunsaturated acyl-CoA substrates.

Gemfibrozil; Fatty acid elongation system; Condensing enzyme; Hypolipidemic drug

1. INTRODUCTION

The microsomal fatty acid chain elongation system, in concert with desaturases, leads to the formation of long-chain poly-unsaturated fatty acids. The general mechanism of microsomal fatty acid elongation involves the sequential activities of four enzymes: (1) the condensing enzyme, the rate-limiting step of the whole system; (2) β-ketoacyl-CoA reductase; (3) β-hydroxyacyl-CoA dehydrase; and (4) *trans*-2-enoyl-CoA reductase [1]. Although the biochemical properties of the hepatic microsomal elongation system have been studied extensively [2,3], little is known about the effects of xenobiotics on this system [4].

Gemfibrozil is a widely used hypolipidemic drug, mainly in hypertriglyceridemic states. Generally classified as a fibric acid derivative, it exhibits different pharmacological properties from other related drugs. Gemfibrozil stimulates triglyceride-rich lipoprotein clearance, decreases hepatic VLDL production and increases HDL-cholesterol levels [5]. Nevertheless, the fundamental mechanism of action of gemfibrozil is not well established. The quality of fatty acids (chain length and number of unsaturations), either in the free form or incorporated into glycerolipids, plays an important role controlling lipoprotein metabolism [6]. We thus aimed to test the effect of gemfibrozil on enzymes related to the hepatic fatty acids synthesis. As a part of this project, we have studied the in vitro effect of gemfibrozil on microsomal fatty acid chain elongation.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Malonyl-CoA, palmitoyl-CoA (16:0 CoA), palmitoleoyl-CoA (9-16:1 CoA), γ-linolenic acid (6,9,12-18:3), NAD(P)H, fatty acid free albumin, rotenone, CoA and Trizma were obtained from Sigma (St. Louis, MO, USA). [2-^{14}C]malonyl-CoA (50 mCi/mmol) was purchased from New England Nuclear (Boston, MA, USA). Scharlau Co 136 liquid scintillation fluid was from Scharlau Co. (Barcelona, Spain) and gemfibrozil was a generous gift from Lab. Parke-Davis (Barcelona, Spain). General chemicals were obtained from commercial sources and were of analytical grade.

2.2. *Isolation of microsomes*

Male Sprague–Dawley rats (180–200 g) were maintained in starvation for 24 h and then refed with a high carbohydrate fat-free diet (Fat Free Diet U.S. Biochemical Corporation, Cleveland, OH, USA) for 48 h. The animals were then killed by decapitation between 8 and 9 a.m. Livers were removed and perfused with ice-cold NaCl 0.9%, to remove contaminating haemoglobin. The tissue was homogenized in ice-cold 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 7.4, and microsomes were obtained as described previously [7]. Protein was determined by the method of Bradford, using BSA as standard [8].

2.3. *Synthesis of γ-linolenoyl-CoA*

The CoA derivative of γ-linolenic acid was prepared by the mixed anhydride procedure as described by Fung and Schulz [9], and purified as described by Al-Arif and Blecher [10]. The concentration of the synthesized γ-linolenoyl-CoA was measured by the method of Ellman [11], after cleavage of the thioester bond with hydroxylamine.

2.4. *Microsomal condensation and total elongation of fatty acids*

The assay of fatty acid microsomal endogenous elongation was performed by the measurement of the incorporation of 2-^{14}C] malonyl-CoA into endogenous acyl-CoAs, basically as described previously [4]. The assay medium contained, in final concentrations: 100 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 2.5 mM ATP, 500 μM NAD(P)H, and 250 μg/ml microsomal protein. Gemfibrozil was added from stock solutions adjusted to pH 8–8.5 with 0.1 N NaOH. The pH of the assay medium was not modified by this addition. After 5 min preincubation at 37°C, the reaction was started by adding 25 μM malonyl-CoA (containing 0.037 μCi [2-^{14}C] malonyl-CoA). When NADH was used

as cofactor. 2.0 μM rotenone was added to the assay medium. When measuring exogenous elongation, $MgCl_2$ and ATP were omitted from the assay medium, and 20 μM BSA was added. After 4 min preincubation at 37°C, 40 μM acyl-CoA was added, and 1 min later the reaction was started by adding 60 μM malonyl-CoA (containing 0.037 μCi [2-^{14}C] malonyl-CoA). Under these conditions, acyl-CoA hydrolase activity was not detectable (data not shown). The condensation activity was measured in the same manner, except that NAD(P)H, necessary for the reductive steps of the complete elongation process, was omitted from the assay medium. In all cases, after 5 min incubation at 37°C, the reaction was stopped by adding 1 ml 15% KOH in methanol, followed by saponification at 65°C for 45 min. After acidification (pH 1.5) with ice-cold 5 N HCl, the free fatty acids were extracted three times with 3 ml ice-cold hexane (9 ml total volume). The pooled hexane fractions were placed into scintillation vials, dried under N_2 and after addition of 7 ml of scintillation mixture, the radioactivity incorporated was counted in a Beckman LS 1800 liquid scintillation counter. The data are presented as nmol [2-^{14}C] malonyl-CoA incorporated/minute/mg of microsomal protein.

Under our assay conditions, the enzymatic activities of endogenous and exogenous fatty acid elongation and condensation were proportional to the amount of microsomal protein added (up to 400 μg/ml) and were linear for at least 15 min.

2.5. *Data analysis*

IC_{50} values and their 95% confidence limits were calculated by means of a 'Graded Dose-Response' computer program designed following Tallarida's instructions [12]. Statistical comparisons (ANOVA test) were performed by the EPISTAT computer program.

3. RESULTS

The activities for endogenous fatty acid elongation were 0.76 nmol/min/mg in the presence of NADH, and 1.07 nmol/min/mg in the presence of NADPH, showing the well-known preference of the elongation system for NADPH as reducing cofactor [1,3]. The preincubation of microsomal protein with gemfibrozil for 5 min caused inhibition of the endogenous activity. The inhibitory effect was dependent on the drug concentration added, but independent of the reducing cofactor used (Table I).No significant difference was found between the IC_{50} value obtained in the presence of NADH or NADPH.

The addition of exogenous acyl-CoAs further increased the NADPH elongation activities, thus obtaining values of 2.39 nmol/min/mg with palmitoyl-CoA as substrate, 2.18 nmol/min/mg with palmitoleoyl-CoA and 1.96 nmol/min/mg with γ-linolenoyl-CoA. Again, the preincubation of microsomal protein with gemfibrozil caused inhibition of the exogenous NADPH dependent elongation activity. The inhibitory effect was always dependent on the gemfibrozil concentration added, but the IC_{50} values obtained (Table I) were very similar, irrespective of the acyl-CoA used in the assay (no significant difference among the three IC_{50} values). Moreover, when endogenous and exogenous IC_{50} values were pooled, no significant difference was found.

When NADPH was omitted from the incubation medium, condensation activities were assayed. The values obtained: 1.47 nmol/min/mg with palmitoyl-CoA as substrate, 1.09 nmol/min/mg with palmitoleoyl-CoA and 1.64 nmol/min/mg with γ-linolenoyl-CoA, were always lower than the corresponding elongation values, given the feed-back inhibition of the condensing enzyme induced by the reaction product, the β-ketoacyl-CoA derivative [2]. Table I shows the IC_{50} values for gemfibrozil inhibition of the condensing enzymes activities. As in the case of elongation, the inhibitory effect was dependent on gemfibrozil concentration, but unrelated to the acyl-CoA used as substrate (no significant difference). Moreover, when IC_{50} values from condensation and NADPH exogenous elongation activities were analyzed together, no significant difference was obtained.

Table I

Effect of gemfibrozil on endogenous and exogenous fatty acid chain elongation and condensation activities. IC_{50} values were calculated as described in section 2. Gemfibrozil concentrations tested were from 0.01 to 1 mM. Each curve was obtained with six concentration points. Each point is the mean of three different experiments performed in duplicate, each time with microsomes from two pooled rat livers.

	Reducing cofactor	Acyl-CoA added	IC_{50} (μM)	95% confidence limits (μM)
Endogenous Elongation	NADH	—	0.30	0.27–0.33
	NADPH	—	0.26	0.23–0.32
Exogenous Elongation	NADPH	palmitoyl-CoA	0.13	0.12–0.14
	NADPH	palmitoleoyl-CoA	0.18	0.16–0.22
	NADPH	γ-linolenoyl-CoA	0.10	0.09–0.12
Condensation	—	palmitoyl-CoA	0.16	0.14–0.17
	—	palmitoleoyl-CoA	0.14	0.12–0.17
	—	γ-linolenoyl-CoA	0.20	0.17–0.25

4. DISCUSSION

The endoplasmic reticulum of mammalian liver is able to catalyze the elongation of fatty acids. The easiest way to assay this activity is to determine the so called endogenous elongation. In this situation, the system uses, as substrates, the fatty acids present in microsomal membranes, activated to acyl-CoAs by the microsomal acyl-CoA synthetase, provided that exogenous malonyl-CoA, ATP, and Mg are added. When the endogenous elongation activity was assayed in the presence of the hypolipidemic drug gemfibrozil, strong inhibition was found. As shown in Table I, the inhibitory potency of gemfibrozil was independent of the reducing cofactor used, NADPH or NADH. At this point, these results could be fitted to two hypotheses. (1) Gemfibrozil inhibits the endogenous elongation by acting on the rate-limiting step of the system, the condensing enzyme [2]. As the condensing reaction between the acyl-CoA and malonyl-CoA is the initial step in the elongation process, and is not dependent on reducing factors, this hypothesis could explain the similarity between the two IC_{50} values obtained (either with NADH or

NADPH). (2) Gemfibrozil could inhibit endogenous elongation indirectly, by affecting the previous activation of the fatty acids to the acyl-CoAs by the microsomal acyl-CoA synthetase. In this way it is also possible to obtain similar IC_{50} values with the two pyridin nucleotides.

To test the second hypothesis, we assayed the effect of gemfibrozil on the exogenous elongation activity. By adding acyl-CoAs directly to the incubation medium, we were able to bypass the synthetase reaction. Further, as Prasad et al. [13] have shown the existence of at least three different condensing enzymes for saturated, mono-unsaturated and poly-unsaturated acyl-CoAs, respectively, we used palmitoyl-CoA, palmitoleoyl-CoA, and τ-linolenoyl-CoA as substrates, in order to evaluate the effect of gemfibrozil on the three different systems. In this set of assays, we used only NADPH as cofactor so as not to increase the complexity of the results; Nagi et al. [14] have shown the existence of two β-keto acyl-CoA reductases, one dependent on NADPH and stimulated by the presence of BSA in the assay medium, and the other using NADH and being inhibited by the addition of BSA. From the results shown in TAble I, and comparing these with the IC_{50} value for NADPH-dependent endogenous elongation, we can see that: (1) there is no significant difference among the four IC_{50} values, thus ruling out a possible acyl-CoA synthetase inhibition by gemfibrozil. Furthermore, when the inhibitory potency of gemfibrozil was assayed on palmitoyl-CoA synthetase activity (data not shown), an IC_{50} of 2.8 mM was obtained, far from the IC_{50} values obtained for the elongation system; (2) gemfibrozil inhibits the three elongation activities with the same potency; this behaviour is different from that of Ebselen, so far the only other organic compound tested for in vitro inhibition of the three elongation systems, which showed a marked inhibitory preference for palmitoyl-CoA elongation [4].

Excluding the condensing enzyme, the other three enzymes of the elongation system have enough activity to fulfil the maximal rate of elongation, even at 50% of their maximal activity (e.g. in fat-free diet-fed animals, activities are around 15 nmol/min/mg for NADPH dependent β-ketoacyl-CoA reductase [14], 80 nmol/min/mg for β-hydroxyacyl-CoA dehydrase and 25 nmol/min/mg for *trans*-2-enoyl-CoA reductase [15]]. Thus, it seems possible to ascribe the inhibitory effect of gemfibrozil to an inhibition of the condensing enzyme. Nevertheless, to test this hypothesis we assayed the effect of gemfibrozil on the condensation activity, again using the three different acyl-CoAs (16:0, 16:1 and τ18:3 CoA). As shown in Table I, the IC_{50} values for condensation activity are very similar, irrespective of the acyl-CoA used as primer; moreover, there is no significant difference between IC_{50} values corresponding to elongation and condensation, confirming that gemfibrozil acts on elongation by inhibiting the condensing step.

The IC_{50} values of gemfibrozil obtained for endogenous and exogenous elongation are within the reported plasma concentration of the drug (0.1–0.8 mM) [16] after its administration to laboratory animals or humans. Further, knowning that it has a tendency to accumulate in liver tissue [16], an effect of gemfibrozil on elongation activity after its administration in vivo may be expected. To our knowledge, the in vitro or in vivo effects of gemfibrozil on elongation activity have not been tested; there are only some contradictory data referring to another fibric acid derivative, clofibric acid. Landriscina et al. [17] reported an inhibition of elongation activity by clofibric acid in vitro, although the inhibitory potency was about 20-fold lower than that reported here for gemfibrozil. After semi-chronic administration in vivo, either an induction [18] or an inhibition [19] of palmitoyl-CoA elongation have been reported, although it should be pointed out that the dosage and duration of treatment were not exactly the same in these two different studies. As gemfibrozil seems to be a more powerful drug than clofibric acid, either in its effects on enzymatic activities in vitro [20,21] or as a therapeutic hypolipemic agent [5], we are now conducting in vivo experiments to ascertain the effect of gemfibrozil on elongation activity after semi-chronic administration. In this respect, it is interesting to note that Prasad and Cinti [22] have shown that feeding rats with di (2-ethylhexyl) phthalate (DEHP), another peroxisomal inducer which is structurally unrelated to gemfibrozil, leads to induction of palmitoyl-CoA elongation activity, while those of palmitoleoyl-CoA and τ-linolenoyl-CoA decreased.

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