Biochemical Pharmacology, Vol. 46, No. 10, pp. 1791–1796, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

FIBRATES MODIFY RAT HEPATIC FATTY ACID CHAIN ELONGATION AND DESATURATION *IN VITRO*

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(*Received* 5 *April* 1993; *accepted* 28 *July* 1993)

Abstract—Three fibric acid derivatives, clofibric acid (CFB), bezafibrate (BFB), and gemfibrozil (GFB), mainly used in the treatment of hypertriglyceridaemic or mixed hyperlipidaemic states, have been tested for their ability to modify fatty acid chain elongation and desaturation *in vitro*. Both endogenous and exogenous (saturated, monounsaturated and polyunsaturated) fatty acid elongations were inhibited by fibrates at concentrations well within the physiological range (IC_{50} values for GFB were between 0.1 and 0.3 mM). The potency order was GFB > BFB > CFB. Inhibition was not due to an impairment of the activation step from free fatty acids to acyl-CoAs, as palmitoyl-CoA synthetase was only slightly inhibited (IC_{50} value for GFB = 2.8 mM). Fibrates (GFB) appeared to behave as mixed non-competitive inhibitors with respect to malonyl-CoA when the rate limiting step of elongation, the condensing enzyme, is assayed. Further, Δ^6 and Δ^5 desaturates were inhibited by the three drugs (GFB > BFB > CFB), although not to the same extent as the elongation system. In contrast, Δ^9 desaturase activity was not affected by fibrates.

Fibrates are hypolipidaemic compounds widely used as first choice drugs for the treatment of hypertriglyceridaemic or mixed hiperlipidaemic states [1]. Despite their clear clinical effect on lipoprotein levels, reducing plasma very low density lipoprotein (VLDL) and increasing high density lipoprotein (HDL) concentrations [2], the underlying biochemical mechanism/s of action is not clearly established. Although they seem to act mainly by increasing VLDL catabolism [3] and by decreasing its hepatic production [4], the molecular basis of these phenomena is not defined.

As fatty acid composition of glycerolipids has an important role in controlling lipoprotein metabolism [5, 6], we have been interested in studying the effect of fibric acid derivatives on enzyme activities related to fatty acid synthesis [7, 8]. Given that mammalian cells are capable of modifying chain length and number of unsaturations of preformed fatty acids through concerted activities of elongation and desaturation enzymes [9], we now report the effect of three fibric acid derivatives, namely gemfibrozil (GFB†), bezafibrate (BFB) and clofibric acid (CFB), on those enzyme activities *in vitro*.

MATERIALS AND METHODS

Materials

Malonyl-CoA, palmitoyl-CoA (16:0 CoA), palmitoleoyl-CoA (9–16:1 CoA), γ-linolenic acid (6,9,12–18:3), stearic acid (18:0), linoleic acid (9,12–18:2), oleic acid (9–18:1), arachidonic acid (5,8,11,14–20:4), dihomo-γ-linolenic acid (8,11,14–20:3), NAD(P)H, fatty-acid free bovine serum albumin (BSA), rotenone, CoA, reduced glutathione (GSH), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), ATP, CFB and Trizma base were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [2-^{14}C]Malonyl-CoA (50 mCi/mmol), [1-^{14}C]stearic acid (58 mCi/mmol), [1-^{14}C]linoleic acid (50 mCi/mmol) and [1-^{14}C]dihomo-γ-linolenic acid (47 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Scharlau Co 136 and Co 130 liquid scintillation fluids were from Scharlau (Barcelona, Spain) and $AgNO_3$-silica gel plates were from Alltech (Deerfield, IL, U.S.A.). BFB and GFB were a generous gift from Boehringer-Mannheim (Barcelona, Spain) and Parke-Davis (Barcelona, Spain), respectively. General chemicals were obtained from commercial sources and were of analytical grade.

Methods

Isolation of microsomal and cytosolic fractions. Male Sprague–Dawley rats (160–200 g) from Letica (Barcelona, Spain), were either fed standard laboratory chow (Panlab, Barcelona, Spain), for measuring Δ^6 and Δ^5 desaturase activities, or starved for 24 hr, followed by 48-hr refeeding with a fat-free diet (U.S. Biochemical Corp., Cleveland, OH, U.S.A.). The animals were killed by decapitation between 8.00 and 9.00 a.m. Livers were removed, perfused with ice-cold 0.9% NaCl and homogenized in ice-cold 0.25 M sucrose, 50 mM Tris–HCl buffer, pH 7.4. Microsomal and cytosolic fractions were obtained as described previously [10]. Protein was

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† Abbreviations: CFB, clofibric acid; BFB, bezafibrate; GFB, gemfibrozil; GSH, reduced glutathione; BSA, bovine serum albumin; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid).

determined by the method of Bradford [11], using BSA as standard.

Synthesis of acyl-CoA derivatives. The CoA derivatives were prepared by the mixed anhydride procedure as described by Fong and Schulz [12] and purified as described by Al-Arif and Blecher [13]. The concentration of the synthesized acyl-CoA derivatives was measured by the method of Ellman [14], after cleavage of the thioester bond with hydroxylamine.

Microsomal condensation and total elongation of fatty acids. The assay of microsomal fatty acid condensation and total elongation was performed by the measurement of [2-^{14}C]malonyl-CoA incorporation into endogenous and exogenous acyl-CoAs, as described previously [15]. Under our assay conditions, the enzyme activities of condensation and endogenous and exogenous fatty acid elongation were proportional to the amount of microsomal protein added (up to 400 μg/mL) and were linear for at least 15 min. When needed, fibric acid derivatives were added 5 min before assaying the enzyme activities, 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.

Desaturation of fatty acids. Δ^9 desaturase activity was assayed basically as described previously [16]. In our standard assay conditions, the enzyme activity was proportional to the amount of microsomal protein added, up to 400 μg/mL and the reaction was linear for at least 10 min.

The assay mixture for Δ^6 and Δ^5 desaturation reactions contained, in final concentrations: 100 mM Tris–HCl, pH 7.4, 1.5 mM GSH, 0.5 mM NADH, 125 μg cytosolic protein, 0.5 mg microsomal protein and, in the case of Δ^5 desaturase, 5 mM ATP and 0.2 mM CoA in a total volume of 0.5 mL. After 5 min preincubation at 37°, the reaction was started by the addition of 50 μM linoleoyl-CoA (containing 0.035 μCi [1-^{14}C]linoleoyl-CoA), for assaying Δ^6 activity, or 50 μM dihomo-γ-linolenic acid (containing 0.05 μCi [1-^{14}C]dihomo-γ-linolenic acid), for assaying Δ^5 activity. After 5 min (for Δ^5 desaturase assay) or 10 min (for Δ^6 desaturase assay) incubation at 37°, the reaction was stopped with 0.5 mL 15% (w/v) KOH in methanol (containing 0.015 mg butylhydroxytoluene/mL as antioxidant), followed by the addition of 25 μL of an ethanolic solution containing 75 μg each of linoleic and γ-linolenic acids, as chromatographic carriers for Δ^6 desaturase assay, or 75 μg each of dihomo-γ-linolenic and arachidonic acids for Δ^5 desaturase assay. The lipids were saponified for 45 min at 65–70° and, after acidification with 0.5 mL 5 N HCl, the free fatty acids were extracted three times with 2 mL hexane. The pooled hexane fractions were evaporated to dryness under N_2. After methylation of the fatty acids with diazomethane, each sample was redissolved in 50 μL $CHCl_3$ and separated in an activated 10% $AgNO_3$ silica gel G plate, with diethyl ether/petroleum ether 1:1 (v/v) (Δ^5 desaturase assay) [17] or benzene/ethyl acetate 90:10 (v/v) (Δ^6 desaturase assay) [18] as solvent system. The spots were identified under UV light after spraying the plate with 0.2% dichlorofluorescein in ethanol, scraped into scintillation vials and, after the addition of 7 mL Scharlau Co 130 scintillation liquid, counted in a Beckman LS 1800 liquid scintillation counter. Desaturation activity was determined by dividing the radioactivity found in the product by the total radioactivity present in the product plus the substrate. The ratio obtained was then converted to nmol γ-linolenic acid/min/mg microsomal protein for Δ^6 desaturase activity and nmol arachidonic acid/min/mg microsomal protein for Δ^5 desaturase activity.

In our assay conditions, Δ^6 and Δ^6 desaturase activities increased in the presence of cytosolic protein, being maximal at 250 μg cytosolic protein/mL for both systems. Above this concentration, enzyme activity was not modified (Δ^6 desaturase), or slightly decreased (Δ^5 desaturase). The enzyme activities were proportional to the amount of microsomal protein added (up to 2 mg/mL) and were linear for 10 min.

Palmitoyl-CoA synthetase activity. The assay mixture contained, in final concentrations, 0.1 M Tris–HCl, pH 8.0, 15 mM $MgCl_2$, 150 mM KCl, 1 mM palmitic acid, 1.5 mM Triton X-100 and 300 μg/mL microsomal protein. After 5 min preincubation at 37°, the reaction was started by addition of 1 mM CoA and 10 mM ATP. Final assay volume was 0.5 mL. After 5 min incubation at 37°, the reaction was stopped by adding 25 μL 105% (w/v) trichloracetic acid, followed by centrifugation for 10 min at 22,000 *g*. Supernatant (0.1 mL) was added to 0.9 mL 0.1 M Tris–HCl buffer, pH 8.0, 2 mM DTNB. After a 10 min reaction, the absorbance at 412 nm was recorded in a Perkin–Elmer 550S UV-Vis spectrophotometer. For each assay, the absorbance at zero incubation time was measured, corresponding to the total amount of CoA added. No significant consumption of CoA was detected in the absence of ATP. The enzyme activity was obtained from the difference between the absorbance at zero and at 5 min incubation time, using an extinction coefficient of 13.6 $mM^{-1}\,cm^{-1}$ [14]. The results are expressed as nmol palmitoyl-CoA formed/min/mg microsomal protein. In the optimal assay conditions, palmitoyl-CoA synthetase activity was proportional to the amount of microsomal protein added (0–400 μg/mL) and was linear for 7 min.

Dihomo-γ-linolenoyl-CoA synthetase activity. The assay mixture and the incubation conditions were the same as those used for assaying Δ^5 desaturase activity. After 5 min incubation at 37°, the reaction was stopped with 0.5 mL 5 N HCl followed by the addition of 200 μL 0.5% Triton X-100. The free fatty acids were extracted four times with 4 mL hexane. The aqueous fraction was placed into scintillation vials and mixed with 10 mL Scharlau Co 136 scintillation liquid, and the incorporated radioactivity counted in a Beckman LS 1800 liquid scintillation counter. The data are presented as nmol dihomo-γ-linolenoyl-CoA formed/min/mg microsomal protein. To assay the effect of the drugs on enzyme activities, fibrates were added as described for the elongation assay.

Data analysis. IC_{50} values and their 95% confidence limits were calculated by means of a Graded Dose Response computer program designed following Tallarida and Murray's instructions [19]. Statistical comparisons (ANOVA test) were performed by the

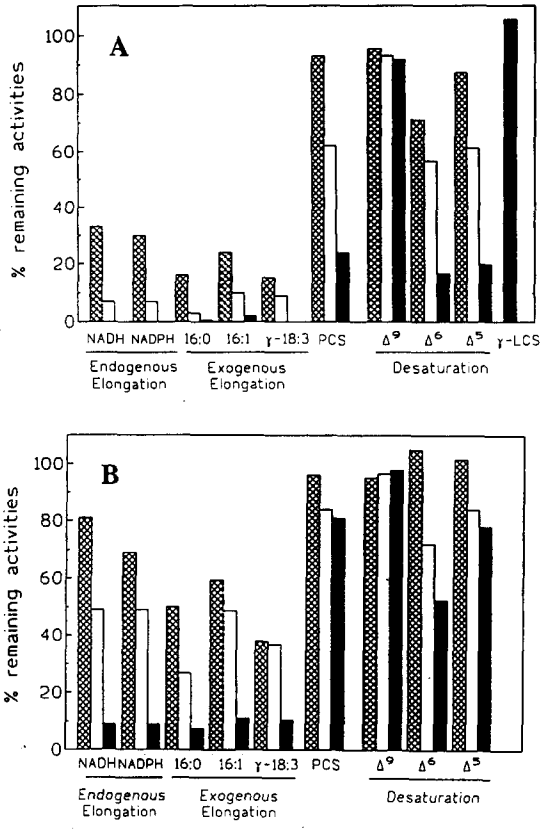

Fig. 1. Enzyme activities in the presence of fibrates: panel A, 5 mM; panel B, 1 mM. CFB (▩), BFB (□), GFB (■). Results are expressed as percentages with respect to control activities (100%) = NADH endogenous elongation, 0.85 nmol/min/mg microsomal protein; NADPH endogenous elongation, 1.21 nmol/min/mg; palmitoyl-CoA exogenous elongation, 2.39 nmol/min/mg; palmitoleoyl-CoA exogenous elongation, 2.18 nmol/min/mg; γ-linolenoyl-CoA exogenous elongation, 1.96 nmol/min/mg; palmitoyl-CoA synthetase (PCS), 248 nmol/min/mg; Δ^9 desaturase, 2.91 nmol/min/mg (N = 4); Δ^6 desaturase, 0.10 nmol/min/mg (N = 3); Δ^5 desaturase, 0.71 nmol/min/mg (N = 3) and dihomo-γ-linolenoyl-CoA synthetase (γ-LCS), 5.27 nmol/min/mg. Values are the mean of two or N experiments performed in duplicate, with microsomes from two pooled rat livers. Gemfibrozil (5 mM) inhibits nearly 100% elongation activities.

EPISTAT computer program. Apparent kinetic constants were calculated by means of a nonlinear regression program (ENZFITTER).

RESULTS

Effect of fibric acid derivatives on elongation and desaturation systems

Figure 1 shows the percentage variation of rat liver endogenous and exogenous elongation activities with respect to control values, assayed in the presence of two different concentrations (1 and 5 mM) of CFB, BFB and GFB. The three drugs produced a strong inhibition of both endogenous and exogenous elongation activity, even at 1 mM, concentration which can be attained in plasma in the therapeutic use of fibrates [20]. Further, as acyl-CoA synthetase provides the substrates for the endogenous elongation and desaturation activities, we assayed the effect of these fibrates on this activity. Palmitoyl-CoA synthetase activity was only slightly decreased by drug addition, the IC_{50} value for GFB, the strongest inhibitor, being 2.8 mM (Table 1). The IC_{50} values obtained in the presence of CFB, BFB and GFB for the inhibition of the endogenous and exogenous fatty acid elongation systems are shown in Table 1. No significant difference between the IC_{50} values obtained in the presence of NADH or NADPH for endogenous elongation was found; for this reason, all the exogenous elongation assays reported in Table 1 were performed by using only NADPH as reducing cofactor. The fibrate IC_{50} values obtained for the exogenous elongation activity using NADPH were similar, irrespective of the acyl-CoA used as substrate (no significant difference was found among the IC_{50} values for each substrate, Table 1). In contrast, when the IC_{50} values for endogenous and exogenous elongation were compared, significant differences were found for CFB and BFB, while the difference for GFB was near significance ($P = 0.08$).

Desaturase activities were also assayed in the presence of two different concentrations (1 and 5 mM) of CFB, BFB and GFB (Fig. 1). No inhibition was detected for Δ^9 desaturase activity, while Δ^6 and Δ^5 activities were inhibited. The maximal inhibition was obtained in the presence of GFB, while CFB was almost uneffective. Further, the effect of 5 mM GFB on dihomo-γ-linolenoyl-CoA synthetase activity is also shown in Fig. 1.

Mechanism of inhibition of microsomal condensing enzymes by fibrates

The studies described here were performed by assaying the effect of two GFB concentrations on the palmitoyl-CoA condensation activity. In these conditions, the inhibitory effect of GFB was independent of the amount of microsomal protein present in the assay medium (50–350 μg/mL) and the preincubation time (up to 15 min) (data not shown).

In order to study the kinetics of the inhibition of microsomal condensing enzyme by GFB, condensation activity was assayed at different malonyl-CoA concentrations in the presence of 0.1 and 0.3 mM GFB, and a fixed palmitoyl-CoA concentration (40 μM). Lineweaver–Burk plots of the results (Fig. 2) provided the apparent kinetic constants, showing an increase in the apparent K_m value for malonyl-CoA and a slight decrease in the apparent V_{max} value in the presence of GFB.

When malonyl-CoA concentration was held constant (60 μM) and palmitoyl-CoA concentration was changed, control values fitted ($r^2 = 1.0$) to the Hill equation for cooperative enzymes (Fig. 3). The apparent kinetic constants thus obtained and their 95% confidence limits were: apparent V_{max} = 1.94 nmol/min/mg microsomal protein (1.57–2.31), apparent K_m = 7.69 μM (5.92–9.46) and a coefficient of cooperativity of 2.84 (2.26–3.41). When GFB was present in the assay medium, data did not fit to the known kinetic equations, showing a complex

Table 1. Fibrate IC_{50} values for endogenous and exogenous elongation and for palmitoyl-CoA synthetase activities

	CFB	BFB	GFB
Endogenous elongation			
NADH	3.47 (2.78–4.34)	1.12 (0.90–1.40)	0.30† (0.27–0.33)
NADPH	3.63* (3.14–4.28)	1.31* (1.11–1.55)	0.26† (0.23–0.32)
Exogenous elongation			
16:0 CoA	0.59 (0.34–0.65)	0.43 (0.28–0.66)	0.13† (0.12–0.14)
16:1 CoA	0.53 (0.31–0.99)	0.47 (0.26–0.84)	0.18† (0.16–0.22)
γ-18:3 CoA	1.06 (0.95–1.20)	0.48 (0.36–0.63)	0.10† (0.09–0.12)
Palmitoyl-CoA synthetase	>5	>5	2.8 (2.5–3.2)

The IC_{50} values and their respective 95% confidence limits (values in parentheses) are expressed as mM concentration. Each concentration–effect 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.

* Statistically significant difference ($P < 0.05$) versus exogenous values.

IC_{50} values marked with † have been previously reported in Ref. 15.

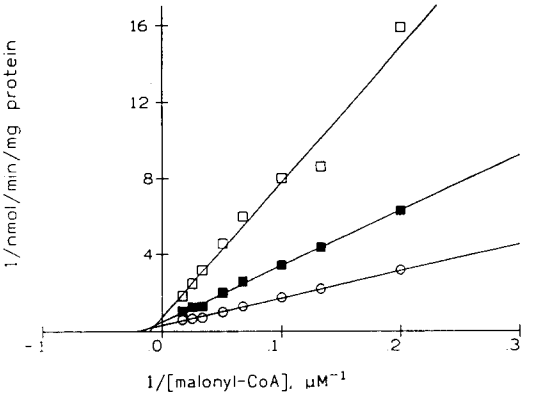

Fig. 2. Lineweaver–Burk plots of velocity versus malonyl-CoA concentration for the condensation reaction in the absence (○) and in the presence of 0.1 (■) and 0.3 mM (□) GFB. Values are the means of three experiments performed in duplicate, each time with microsomes obtained from two pooled rat livers. Apparent kinetic constants are V_{max} (95% confidence limits) = 3.0 (2.7–3.8), 1.8 (1.7–2.0), 1.7 (1.3–2.3) nmol/min/mg microsomal protein for control, 0.1 and 0.3 mM GFB, respectively; K_m = 44.0, 53.0, 129.7 μM malonyl-CoA for control, 0.1 and 0.3 mM GFB, respectively.

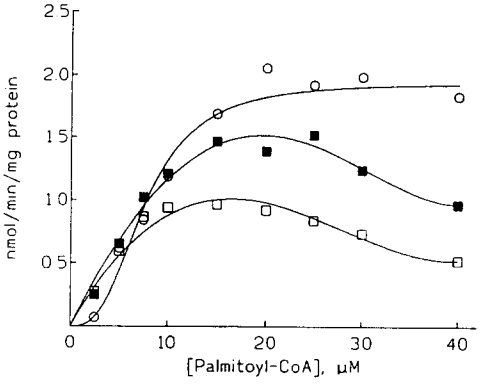

Fig. 3. Velocity versus palmitoyl-CoA concentration curves in the absence (○) and in the presence of 0.1 (■) and 0.3 mM (□) GFB. Values are the means of three experiments performed in duplicate, each time with microsomes obtained from two pooled rat livers.

behaviour. Thus, the inhibition of condensation activity was maximal at 40 μM palmitoyl-CoA, and decreased through all the range of substrate concentrations assayed. Careful observation of individual values shows that, for lower palmitoyl-CoA concentrations, GFB even induced a stimulation of condensation activity. For example, at 2.5 μM palmitoyl-CoA, activities of 0.072, 0.252 and 0.275 nmol/min/mg protein for control, 0.1 and 0.3 mM GFB were obtained.

DISCUSSION

Fibrates, at least BFB and GFB, strongly inhibited endogenous microsomal fatty acid elongation. This inhibition was dependent on the concentration of fibrate present in the assay medium and independent of the reductive cofactor (NADH or NADPH) used to carry on the reaction (Fig. 1 and Table 1).

The inhibition seems to affect the enzymes involved in chain elongation directly rather than the activation of fatty acids. When endogenous elongation is assayed, fatty acids present in the microsomal membranes are used as substrates, previously activated to acyl-CoA derivatives by the microsomal acyl-CoA synthetase, provided that

CoA, ATP and Mg^{2+} are present in the assay medium. As can be seen in Fig. 1, even at 5 mM GFB, there is enough palmitoyl-CoA synthetase activity left (around 60 nmol/min/mg) to provide excess of substrate to the elongation system. With the IC_{50} value calculated for GFB inhibition of palmitoyl-CoA synthetase activity (2.8 mM), this assumption is even more acceptable.

Prasad *et al.* [21] have reported at least three different elongation systems for saturated, monounsaturated and polyunsaturated acyl-CoAs. By using palmitoyl-CoA, palmitoleoyl-CoA and γ-linolenoyl-CoA as exogenous substrates for the elongation reaction, we have been able to show that fibrates equally inhibited the three elongation activities (Table 1). Further, these results confirm the assumption that the inhibition of endogenous elongation is not due to an inhibition of the activation step from free fatty acids to acyl-CoAs; although, when IC_{50} values for endogenous and exogenous NADPH-dependent elongation were compared, CFB and BFB showed a higher inhibitory potency in the exogenous assay. The order of potency for inhibition of endogenous and exogenous elongation was GFB > BFB > CFB similar to the one reported for other inhibitory effects on lipogenic enzymes mediated by fibrates *in vitro* [7, 8].

Recently, we have ben able to demonstrate that GFB inhibits fatty acid elongation by affecting the rate limiting step of the system, the condensing enzyme [22], without discriminating among the three condensing enzymes for saturated, mono and polyunsaturated substrates [15]. Given the similar behaviour of CFB, BFB and GFB on elongation activity, we can assume that the three fibrates tested act in a similar way. Thus, we have pursued the characterization of the inhibition effect studying the inhibition of palmitoyl-CoA condensation by GFB. From the kinetic constants reported in Fig. 2, it can be seen that GFB acts as a mixed non-competitive inhibitor [23] with respect to malonyl-CoA, impairing both enzyme–substrate interaction and product formation. Regarding palmitoyl-CoA, our data for control values fitted to the Hill equation, showing a coefficient of cooperativity of 2.84, which means that the condensing enzyme would have at least three binding sites for palmitoyl-CoA with relatively strong cooperativity. The discrepancy between our results and those reported by Prasad *et al.* [21], who fitted data to the Michaelis–Menten equation, is probably due to the fact that those authors used fewer points than us in the lower range of palmitoyl-CoA concentrations, and it is in this range where the cooperativity becomes really evident. When GFB was added to the medium it behaved as an activator at low substrate concentrations, reducing the sigmoidicity observed in the control. On the contrary, when palmitoyl-CoA concentration increased, inhibition of condensation activity was evident and velocity even decreased at the highest substrate concentrations. Thus, inhibition was maximal at 40 μM palmitoyl-CoA, the highest concentration tested. Assuming this, it is easier to explain the higher inhibition of exogenous versus endogenous elongation found for fibrates, if we take into account that the acyl-CoA concentration present in the endogenous elongation assay medium is lower than the 40 μM concentration of acyl-CoA used in the exogenous assay.

Elongation and desaturation systems act jointly to synthesize mono and polyunsaturated fatty acids in mammalian cells [9]. We have also studied the main desaturase activities in hepatic cells, namely Δ^9, Δ^6 and Δ^5 desaturases. As can be seen in Fig. 1, fibric acid derivatives had a differential effect on the three activities assayed. Thus, while Δ^9 desaturase was not affected by fibrates addition, Δ^6 and Δ^5 activities were inhibited in their presence, although not to the same extent as in the elongation system. It seems reasonable to assume that the inhibition of Δ^6 and Δ^5 reactions is due to a direct interaction between the fibrate and the terminal desaturase. As described in the Materials and Methods, Δ^5 desaturase was assayed by adding the free fatty acid as substrate. When dihomo-γ-linolenoyl-CoA synthetase activity was assayed in the same conditions as Δ^5 desaturase, the presence of GFB in the assay medium had little effect on this activity (Fig. 1). Thus, the decrease in the Δ^5 activity is not due to a blockade of the activation step by GFB.

The fibrate plasma concentrations obtained after their administration *in vivo* to experimental animals and humans (near 1 mM) [20], the selective accumulation in certain organs, such as the liver and the kidney described for GFB, where fibrate concentration is higher than in plasma [24] and the time span of hypolipidaemic treatments (usually lifetime), do not preclude an effect *in vivo*. The results obtained from laboratory animals treated with fibrates are inconsistent, at least with respect to the elongation system [17, 25, 26]. Moreover, the modification of the elongation–desaturation system (i.e. induction of Δ^9 desaturase and palmitoyl-CoA elongation) in rats has been related to the peroxisomal induction produced by fibrate administration to this species [17]. Nevertheless, we have been able to show that GFB, after 15 days administration to guinea-pigs, induces a change in the fatty acid composition of hepatic microsomal phospholipids, consistent with an inhibition of the elongation system and associated to a clear hypolipidemic effect [27]; it should be borne in mind that fibrates do not produce peroxisomal proliferation in guinea-pigs and in humans [28]. Moreover, Agheli and Jacotot [29] recently reported an increase in the relative content of saturated and monounsaturated fatty acids of cholesterol esters, phospholipids and tryglycerides in very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL) obtained from human patients affected by primary hypercholesterolaemia and treated with fenofibrate, another fibric acid derivative. The latter work and our results in guinea-pig are evidence which support the idea that modifications in elongation–desaturation activities *in vivo* are not related to peroxisomal proliferation.

Acknowledgements—This research was partly supported by grants 91/0147 from the Fondo de Investigaciones Sanitarias de la Seguridad Social. M. Alegret and M. Vázquez were recipients of F.P.I. grants from Generalitat de Catalunya. We also thank Mr R. Rycroft (Language

Advice Service of the University of Barcelona) for his helpful assistance. Further, we greatly appreciate the technical support provided by Ms T. Iglesias.

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